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Microbial consortia involved in the anaerobic degradation of hydrocarbons

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Abstract

In this review, we examine the energetics of well-characterized biodegradation pathways and explore the possibilities for these to support growth of multiple organisms interacting in consortia. The relevant phenotypic and/or phylogenetic characteristics of isolates and consortia mediating hydrocarbon degradation coupled with different terminal electron-accepting processes (TEAP) are also reviewed. While the information on metabolic pathways has been gained from the analysis of individual isolates, the energetic framework presented here demonstrates that microbial consortia could be readily postulated for hydrocarbon degradation coupled to any TEAP. Several specialized reactions occur within these pathways, and the organisms mediating these are likely to play a key role in defining the hydrocarbon degradation characteristics of the community under a given TEAP. Comparing these processes within and between TEAPs reveals biological unity in that divergent phylotypes display similar degradation mechanisms and biological diversity in that hydrocarbon-degraders closely related as phylotypes differ in the type and variety of hydrocarbon degradation pathways they possess. Analysis of microcosms and of field samples suggests that we have only begun to reveal the diversity of organisms mediating anaerobic hydrocarbon degradation. Advancements in the understanding of how hydrocarbon-degrading communities function will be significantly affected by the extent to which organisms mediating specialized reactions can be identified, and tools developed to allow their study *in situ.*

Introduction

The structure and activity of microbial communities reflects a dynamic interaction between microbes and the environment they inhabit. Prior to entry of hydrocarbon pollutants, shallow aquifers are often aerobic with relatively low levels of dissolved organic carbon (Anderson & Lovley 1997). Indigenous aerobic bacteria readily metabolize hydrocarbon pollutants (such as fuels) entering these systems. These bacteria use molecular oxygen to initiate substrate transformation via the activity of oxygenases and to serve as the terminal electron acceptor (TEA) in respiratory energy generation. Collectively, the use of oxygenases and aerobic respiration allow aerobic bacteria to effectively degrade a wide spectrum of compounds present in fuels and other petroleum- and coal-products. However, levels of hydrocarbons introduced by spills are typically far in excess of the dissolved oxygen levels needed to support their degradation. Thus, aerobic organisms deplete oxygen and thereby transform the aquifer environment from aerobic to anaerobic (Vroblesky & Chapell 1994; Anderson & Lovley 1997; Lovley 1997). For hydrocarbon degradation to continue under anaerobic conditions, microbes must be able to 1) initiate substrate oxidation without the benefit of oxygenases and, 2) generate energy by fermentation or by coupling substrate oxidation to respiratory processes linked to reduction of TEAs other than oxygen.

Compounds serving as TEAs may be either organic or inorganic and are used in sequence such that the majority of the electron flow is channeled through the process with the greatest relative energy yield. Table

1 summarizes major electron-accepting reactions that may occur under anaerobic conditions, use of which would initiate from the most favorable nitrate reduction reactions listed at the bottom. Depletion of one electron acceptor allows the use of those yielding less energy, and results in stratification of aquifers into regions where a given terminal electron-accepting process (TEAP) is considered predominant. TEAP stratification provides a link between microbiological and geochemical characteristics of an aquifer and is discussed in detail elsewhere (Zehnder & Stumm 1988; Chapelle & Lovley 1992; Lovley et al. 1994a; Anderson & Lovley 1997; Lovley 1997, 1998). The issue for the present review is how the composition and activity of microbial communities involved in hydrocarbon biodegradation may vary within and/or between TEAP zones. A number of dynamic interactions between microbes and their environment likely affect these communities, and the diversity of organisms involved directly or indirectly in hydrocarbon degradation is yet unknown. However, two factors that undoubtedly affect the diversity and abundance of organisms comprising these communities are the variety of biodegradation reactions they mediate and the spectrum of TEAs they can use.

In the following sections, we examine the energetics of well-characterized degradation pathways and explore the possibilities for these to support one or more organisms that are operative in degrading specific hydrocarbons alone or as part of a consortium. We then examine the diversity of organisms (individual isolates and consortia) characterized from laboratory tests that carry out these biodegradation processes coupled with a given TEAP. In the last section, the physiological and molecular analysis of microbial communities in microcosm and field studies are examined for trends that may be consistent with expectations developed from theoretical energetic considerations and experimental analysis of laboratory cultures. A reference database of energetic parameters and a mechanistic framework for interpreting experimental data from syntrophic consortia are available at http://www.soils.wisc.edu/∼hickey/microbial.html.

Energetics of hydrocarbon degradation: electron donor and TEAP pathways

The anaerobic degradation pathways of organic compounds have been studied for some time, but those occurring with hydrocarbons that are major constituents of fuels and other environmental pollutants have only recently been determined. These pathways are reviewed elsewhere (Heider & Fuchs 1997; Harwood et al. 1999; Heider et al. 1999), and examples of those that are currently the most thoroughly characterized are presented in Table 2 as a series of redox-balanced, half and full reactions. The mechanistic nature, substrates and products are identified for each pathway step, together with an estimate of the Eh^{07} (half reactions) or ΔG^{07} (full reactions). For comparative energetics, the energy of coupling each pathway half reaction with methanogenesis ($Eh^{07} = -0.2447$ V) and denitrification ($Eh^{07} = 0.7470$ V) as boundary TEAPs are shown. In some cases, the energy of a process is known because the ΔG^0_f of all reaction components is known (e.g., Eq. 1.1). For these reactions, a comparison of the coupled energetics of the process with the sum of the estimated energies of each pathway step provides an integrated measure of the validity of each estimate (cf., Eqs. 1.1 and 1.1.7).

These pathways are not the only ones that anaerobes have developed to metabolize these compounds. Nevertheless, these pathways can be used to illustrate how degradation processes could be carried out by organisms operating as individuals or as part of a metabolically linked consortium of organisms. An interesting feature of these degradation pathways is the pervasiveness of β-oxidation type processes: 1) oxidation of saturated to unsaturated (Table 2, Eq. 1.1.2; $Eh^{07} \cong 0$ V); 2) hydrolytic hydroxylation of unsaturated to hydroxy (Table 2, Eq. 1.1.3; $\Delta G^{07} \cong 0$ kJ); oxidation of hydroxy to keto (Table 2, Eq. 1.1.4; $Eh^{07} \cong -0.2$ V); 3) hydrolytic keto cleavage to a carboxy and a saturated C product, which for classic β-oxidation is acetate (Table 2, Eq. 1.1.5; $\Delta G^{07} \cong -50$ kJ). It should be recognized that, in practice, energy transduction via acyl-CoA mechanisms is integrally involved in β-oxidation and related biochemical pathway reactions (Thauer et al. 1977; Harris 1982). Less common reactions occurring in the pathways include the fumarate condensation/recycling mechanism for anaerobic methyl oxidation (Eq. 1.1.1; $\Delta G^{07} \cong -50$ kJ), dearomatization (Eq. 1.2.5; $Eh^{07} \cong -0.5$ V), and carboxylation to initiate or allow progression of anaerobic hydrocarbon oxidation (Eq. 2.1.3, 4.1.1; $\Delta G^{07} \cong 30$ kJ). These pathways have been determined from the study of pure cultures, but in principle, it could be hypothesized that various parts of a multi-step pathway could be mediated by a different microbial component of a hydrocarbon-degrading consortium. The points at which pathways

Table 1. Metabolites consumed and produced by microbial communities growing anaerobically on hydrocarbons: Dissimilatory electron acceptor reactions[1]

Process	Biochemical half reaction or reaction			
	Eq.	Basic components	Eh^{07} (V)	ΔG^{07} (kJ) with −0.3 V e^- donor
Oxidized C reduction: −0.2 to −0.3V Eh^{07}				
CO_2 reduction to acetate	1.1	$8e^- + 2CO_2(0e^-) \rightarrow CH_3CO_2^-(8e^-)$	−0.2910	−7
CO_2 reduction to methanol	1.1.1	$6e^- + CO_2(0e^-) \rightarrow CH_3OH(6e^-)$	−0.3830	48
CO_2 reduction to CO	1.1.2	$2e^- + CO_2(0e^-) \rightarrow CO(2e^-)$	−0.5179	42
Condensation of CO and methanol to acetate	1.1.3	$CO(2e^-) + CH_3OH(6e^-) \rightarrow CH_3CO_2^-(8e^-)$		−97
CO_2 reduction to methane	1.2	$8e^- + CO_2(0e^-) \rightarrow CH_4(8e^-)$	−0.2447	−43
CO_2 reduction to methanol	1.2.1	$6e^- + CO_2(0e^-) \rightarrow CH_3OH(6e^-)$	−0.3830	48
Methanol reduction to methane	1.2.2	$2e^- + CH_3OH(6e^-) \rightarrow CH_4(8e^-)$	0.1690	−91
Oxidized S reduction: −0.2 to −0.3 V Eh^{07}				
Sulfate reduction to sulfide	2.1	$8e^- + SO_4^=(0e^-) \rightarrow H_2S(8e^-)$	−0.2168	−64
Sulfate reduction to sulfite	2.1.1	$2e^- + SO_4^=(0e^-) \rightarrow HSO_3^-(2e^-)$	−0.5168	42
Sulfite reduction to sulfide	2.1.2	$6e^- + HSO_3^-(2e^-) \rightarrow H_2S(8e^-)$	−0.1168	−106
Sulfate reduction to sulfur	2.2	$6e^- + SO_4^=(0e^-) \rightarrow S(6e^-)$	−0.1992	−58
Sulfur reduction to sulfide	2.3	$2e^- + S(6e^-) \rightarrow H_2S(8e^-)$	−0.2696	−6
Oxidized Fe reduction: 0.4 to −0.3 V Eh^{07}				
Ferric hydroxide reduction to ferrous (direct)	3.1	$e^- + Fe(OH)_3(0e^-) \rightarrow Fe^{2+}(1e^-)$	−0.2614	−4
Ferric hydroxide reduction to 10^{-4} M ferrous	3.1a	$e^- + Fe(OH)_3(0e^-) \rightarrow Fe^{2+}(1e^-)[10^{-4}\ M]$	−0.0246	−27
Ferric hydroxide reduction to ferrous (indirect)	3.2	$3e^- + Fe(OH)_3(0e^-) \rightarrow 3Fe^{2+}(1e^-)$	−0.2614	−11
Ferric hydroxide reduction to magnetite	3.2.1	$e^- + 3Fe(OH)_3(0e^-) \rightarrow Fe_3O_4\ (1e^-)$	0.4172	−69
Magnetite reduction to ferrous	3.2.2	$2e^- + Fe_3O_4(1e^-) \rightarrow 3Fe^{2+}(1e^-)$	−0.6007	58
Magnetite reduction to 10^{-4} M ferrous	3.2.2a	$2e^- + Fe_3O_4(1e^-) \rightarrow 3Fe^{2+}(1e^-)[10^{-4}\ M]$	−0.3639	6
Oxidized Mn reduction: 0.5 to 0.4 V Eh^{07}				
Manganese dioxide reduction to manganous	4.1	$2e^- + MnO_2(0e^-) \rightarrow Mn^{2+}(2e^-)$	0.4019	−135
Manganese dioxide reduction to 10^{-4} M manganous	4.1a	$2e^- + MnO_2(0e^-) \rightarrow Mn^{2+}(2e^-)[10^{-4}\ M]$	0.5203	−158
Oxidized N reduction: 0.9 to 0.3 V Eh^{07}				
Nitrate reduction to ammonium	5.1	$8e^- + NO_3^-(0e^-) \rightarrow NH_4^+(8e^-)$	0.3628	−512
Nitrate reduction to nitrite	5.1.1	$2e^- + NO_3^-(0e^-) \rightarrow NO_2^-(2e^-)$	0.4328	−141
Nitrite reduction to ammonium	5.1.2	$6e^- + NO_2^-(2e^-) \rightarrow NH_4^+(8e^-)$	0.3395	−370
Nitrate reduction to dinitrogen	5.2	$10e^- + 2NO_3^-(0e^-) \rightarrow N_2(10e^-)$	0.7470	−1010
Nitrate reduction to nitrite	5.2.1	$4e^- + 2NO_3^-(0e^-) \rightarrow 2NO_2^-(2e^-)$	0.4328	−283
Nitrite reduction to dinitrogen	5.2.2	$6e^- + 2NO_2^-(2e^-) \rightarrow N_2(10e^-)$	0.9565	−727
Nitrous oxide reduction to dinitrogen	5.2.2.1	$2e^- + N_2O(8e^-) \rightarrow N_2(10e^-)$	1.3550	−319

[1] The reactions consist only of redox active components; to facilitate redox balancing, the number of available e^-, n_c, contained by each component is included in parentheses. A table containing additional TEAP reactions, the associate energetic variables, and an explanation of the Eh^{07} and ΔG^7 values are available at http://www.soils.wisc.edu/∼hickey/microbial.html

could support various members may be determined by examining the energetics of a transformation or series of transformations. For example, it is unlikely that an organism will release metabolites in which it has a significant energy investment. Toluene degradation (Eq. 1) may be used to illustrate how various steps in the pathway could support multiple organisms in a consortium. The pathway can be subdivided into three phases: 1) toluene oxidation to benzoate (Eq. 1.1); 2) benzoate oxidation to acetate (Eq. 1.2); 3) acetate oxidation to carbon dioxide (Eq. 1.3). The comparative energetics are such that release of benzoate as an intermediate is possible, but is much more likely under denitrifying conditions than it is under

Table 2. Metabolites consumed and produced by microbial communities growing anaerobically on hydrocarbons: dissimilatory electron donor pathway for toluene, ethylbenzene, xylenes and hexadecane[1]

Process	Biochemical half reaction or reaction				
			Eh^{07}	ΔG^{07} (kJ) with e^- acceptor Eh^{07}	
	Eq	Basic components	(V)	−0.2447 V	0.7470 V
Toluene oxidation to CO_2	1	$C_6H_5CH_3(36e^-) \rightarrow 7CO_2(0e^-) + 36e^-$	−0.2894	−155	−3.600
Toluene oxidation to benzoate	1.1	$C_6H_5CH_3(36e^-) \rightarrow C_6H_5CO_2^-(30e^-) + 6e^-$	−0.2484	−2	−576
Condensation of toluene and fumarate with intramolecular oxidation of the methyl and reduction of the unsaturated C components, to benzylsuccinate	1.1.1	$C_6H_5CH_3(36e^-) + {}^-O_2CCH{=}CHCO_2^-(12e^-) \rightarrow C_6H_5CH_2CH(CO_2^-)CH_2CO_2^-(48e^-)$		$(-52 \pm 2)^{2a}$	$(-52 \pm 2)^{2a}$
Oxidation of saturated methyl C of benzylsuccinate, to unsaturated C analog phenyl itaconate	1.1.2	$C_6H_5CH_2CH(CO_2^-)CH_2CO_2^-(48e^-) \rightarrow C_6H_5CH{=}C(CO_2^-)CH_2CO_2^-(46e^-) + 2e^-$	$(0.0)^{2b}$	(47)	(−144)
Hydrolytic hydroxylation of unsaturated C of phenyl itaconate, to hydroxy C analog hydroxymethyl phenyl succinate	1.1.3	$C_6H_5CH{=}_2C(CO_2^-)CH_2CO_2^-(46e^-) \rightarrow C_6H_5CHOHCH(CO_2^-)CH_2CO_2^-(46e^-)$		$(0 \pm 5)^{2c}$	$(0 \pm 5)^{2c}$
Oxidation of hydroxy C of hydroxymethylphenyl succinate, to the keto analog ketomethylphenyl succinate	1.1.4	$C_6H_5CHOHCH(CO_2^-)CH_2CO_2^-(46e^-) \rightarrow C_6H_5COCH(CO_2^-)CH_2CO_2^-(44e^-) + 2e^-$	$(-0.22)^{2d}$	(5)	(−187)
Hydrolytic keto cleavage of ketomethylphenyl succinate, to benzoate and succinate	1.1.5	$C_6H_5CHOHCH(CO_2^-)CH_2CO_2^-(44e^-) \rightarrow C_6H_5COCH(CO_2^-)CH_2CO_2^-(44e^-)$		$(-50 \pm 5)^{2e}$	$(-50 \pm 5)^{2e}$
Oxidation of saturated C of succinate to unsaturated C of fumarate	1.1.6	${}^-O_2CCH_2CH_2CO_2^-(14e^-) \rightarrow {}^-O_2CCH{=}CHCO_2^-(12e^-) + 2e^-$	0.0300	53	−138
Sum of pathway for toluene oxidation to benzoate	1.1.7	$C_6H_5CH_3(36e^-) \rightarrow C_6H_5CO_2^-(30e^-) + 6e^-$		(3 ± 12)	(−571 ± 12)
Benzoate oxidation to acetate	1.2	$C_7H_5O_2^-(30e^-) \rightarrow 3C_2H_3O_2^-(8e^-) + CO_2(0e^-) + 6e^-$	−0.3240	−46	−620
Reductive dearomatization of benzoate to cyclohex-1,5-ene-1-ate	1.2.1	$2e^- + C_6H_5(CO_2)^-(30e^-) \rightarrow C_6H_7(CO_2)^-(32e^-)$	$(-0.5)^{2f}$	(49)	(241)
Reduction of unsaturated C of cyclohex-1,5-ene-1-ate to cyclohex-1-ene-ate	1.2.2	$2e^- + C_6H_7(CO_2)^-(32e^-) \rightarrow C_6H_9(CO_2)^-(34e^-)$	$(0.0)^{2g}$	(−47)	(144)
Hydrolysis of unsaturated C of cyclohex-1-ene-1-ate to 2-hydroxy-cyclohexane-1-ate	1.2.3	$C_6H_9(CO_2)^-(34e^-) \rightarrow C_6H_9OH(CO_2^-)(34e^-)$		$(0 \pm 5)^{2c}$	$(0 \pm 5)^{2c}$
Oxidation of hydroxy C of 2-hydroxy-cyclohexane-1-ate to 2-keto-cyclohexane-1-ate	1.2.4	$C_6H_9OH(CO_2^-)(34e^-) \rightarrow C_6H_8O(CO_2)^-(32e^-) + 2e^-$	$(-0.22)^{2d}$	(5)	(−187)
Hydrolytic keto cleavage of 2-keto-cyclohexane-1-ate to pimelate	1.2.5	$C_6H_8O(CO_2)^-(32e^-) \rightarrow {}^-O_2C(CH_2)_3\ CH_2CH_2CO_2^-(32e^-)$		$(-50 \pm 5)^{2e}$	$(-50 \pm 5)^{2e}$
Oxidation of saturated β-C of pimelate, to unsaturated (-C of 2,3-dehydropimelate	1.2.6	${}^-O_2C(CH_2)_3CH_2CH_2CO_2^-(32e^-) \rightarrow {}^-O_2C(CH_2)_3CH{=}CHCO_2^-(30e^-) + 2e^-$	$(0.0)^{2b}$	(47)	(−144)
Hydrolytic hydroxylation of unsaturated β C of 2,3 dehydropimelate, to 3-hydroxy pimelate	1.2.7	${}^-O_2C(CH_2)_3CH{=}CHCO_2^-(30e^-) \rightarrow {}^-O_2C(CH_2)_3CHOHCH_2CO_2^-(30e^-)$		$(0 \pm 5)^{2c}$	$(0 \pm 5)^{2c}$

Table 2. Continued

Process	Biochemical half reaction or reaction				
			Eh^{07}	ΔG^{07} (kJ) with e^- acceptor Eh^{07}	
	Eq	Basic components	(V)	−0.2447 V	0.7470 V
Oxidation of hydroxy β-C of 3 hydroxypimelate, to 3-ketopimelate	1.2.8	$^-O_2C(CH_2)3CHOHCH_2CO_2^-(30e^-) \rightarrow {}^-O_2C(CH_2)3COCH_2CO_2^-(28e^-) + 2e^-$	$(-0.22)^{2d}$	(5)	(-187)
Hydrolytic keto cleavage of 3 ketopimelate, to glutarate and acetate	1.2.9	$^-O_2C(CH_2)_3COCH_2CO_2^-(28e^-) \rightarrow {}^-O_2C(CH_2)_3CO_2^-(20e^-) + CH_3CO_2^-(8e^-)$		$(-50 \pm 5)^{2e}$	$(-50 \pm 5)^{2e}$
Oxidation of saturated β C of glutarate, to glutaconate	1.2.10	$^-O_2CCH_2CH_2CH_2CO_2^-(20e^-) \rightarrow {}^-O_2CCH{=}CHCH_2CO_2^-(18e^-) + 2e^-$	$(0.0)^{2b}$	(47)	(-144)
Decarboxylation of glutaconate, to crotonate and CO_2	1.2.11	$^-O_2CCH{=}CHCH_2CO_2^-(18e^-) \rightarrow {}^-O_2CCH{=}CH_2CH_3(18e^-) + CO_2(0e^-)$		$(-30 \pm 5)^{2f}$	$(-30 \pm 5)^{2f}$
Hydrolytic hydroxylation of unsaturated β C of crotonate to β hydroxy butyrate	1.2.12	$CH_3CH{=}CHCO_2^-(18e^-) \rightarrow CH_3CHOHCH_2CO_2^-(18e^-)$		$(0 \pm 5)^{2c}$	$(0 \pm 5)^{2c}$
Oxidation of β hydroxy butyrate to β keto butyrate (acetoacetate)	1.2.13	$CH_3CHOHCH_2CO_2^-(18e^-) \rightarrow CH_3COCH_2CO_2^-(16e^-) + 2e^-$	-0.2618	-3	-195
Hydrolytic keto cleavage of acetoacetate to 2 acetate	1.2.14	$CH_3COCH_2CO_2^-(16e^-) \rightarrow 2CH_3CO_2^-(8e^-)$		-56	-56
Sum of pathway for benzoate oxidation to acetate	1.2.15	$C_7H_5O_2^-(30e^-) \rightarrow 3C_2H_3O_2^-(8e^-) + CO_2(0e^-) + 6e^-$		(-83 ± 30)	(-657 ± 30)
Acetate oxidation to CO_2	1.3	$CH_3CO_2^-(8e^-) \rightarrow 2CO_2(0e^-) + 8e^-$	-0.2910	-36	-801
Reversed acetyl CoA pathway					
Disproportionation of acetate to CO and methanol	1.3.1	$CH_3CO_2^-(8e^-) \rightarrow CO(2e^-) + CH_3OH(6e^-)$		98	98
CO oxidation to CO_2		$CO(2e^-) \rightarrow CO_2(0e^-) + 2e^-$	-0.5179	-53	-244
Methanol oxidation to CO_2	1.3.2	$CH_3OH(6e^-) \rightarrow CO_2(0e^-) + 6e^-$	-0.3830	-80	-654
Methanol oxidation to formaldehyde	1.3.3	$CH_3OH(6e^-) \rightarrow HCHO(4e^-) + 2e^-$	-0.1816	12	-179
Formaldehyde oxidation to formate	1.3.4	$HCHO(4e^-) \rightarrow HCO_2^-(2e^-) + 2e^-$	-0.5346	-56	-247
Formate oxidation to CO_2	1.3.5	$HCO_2^-(2e^-) \rightarrow CO_2(0e^-) + 2e^-$	-0.4316	-36	-227
Ethylbenzene oxidation to carbon dioxide	2	$C_6H_5CH_2CH_3(42e^-) \rightarrow 8CO_2^-(0e^-) + 42e^-$	$(-0.29)^{2i}$	(-184)	(-4.203)
Ethylbenzene oxidation to benzoate and acetate	2.1	$C_6H_5CH_2CH_3(42e^-) + CO_2(0e^-) \rightarrow C_6H_5CO_2^-(30e^-) + CH_3CO_2^-(8e^-) + 4e^-$			
Oxidation of saturated 2C of ethyl benzene to hydroxy 2C of phenyl ethanol	2.1.1	$C_6H_5CH_2CH_3(42e^-) \rightarrow C_6H_5CHOHCH_3(40e^-) + 2e^-$	$(0.01)^{2j}$	(49)	(-142)
Oxidation of hydroxy 2C of phenyl ethanol to keto 2C of acetophenone	2.1.2	$C_6H_5CHOHCH_3(40e^-) \rightarrow C_6H_5COCH_3(38e^-) + 2e^-$	$(-0.22)^{2d}$	(5)	(-185)
Carboxylation of 1C of acetophenone to benzoyl acetate	2.1.3	$C_6H_5COCH_3(38e^-) + CO_2(0e^-) \rightarrow C_6H_5COCH_3CO_2^-(38e^-)$		$(30 \pm 5)^{2k}$	$(30 \pm 5)^{2k}$
Hydrolytic keto cleavage of benzoyl acetate to benzoate and acetate	2.1.4	$C_6H_5COCH_3CO_2^-(38e^-) \rightarrow C_6H_5CO_2^-(30e^-) + CH_3CO_2^-(8e^-)$		$(-50 \pm 5)^{2e}$	$(-50 \pm 5)^{2e}$
Sum of pathway for ethylbenzene oxidation to benzoate and acetate	2.1.5	$C_6H_5CH_2CH_3(42e^-) \rightarrow 8CO_2^-(0e^-) + 42e^-$		(29 ± 5)	(-354 ± 5)
Xylene oxidation to carbon dioxide	3	$C_6H4(CH_3)2(42e^-) \rightarrow 8CO_2^-(0e^-) + 42e^-$	$(-0.29)^{2i}$	(-184)	(-4.203)
Xylene oxidation to methyl benzoate (via methyl pheny succinate)	3.1	$C_6H4(CH_3)2(42e^-) \rightarrow C_6H4(CH_3)CO_2^-(36e^-) + CO_2^-(0e^-) + 6e^-$		$(3 \pm 7)^{2l}$	$(-571 \pm 7)^{2l}$
Hexadecane oxidation to carbon dioxide	4	$CH_3(CH_2)12CH_2CH_3(98e^-) \rightarrow 16CO_2(0e^-) + 98e^-$	$(-0.29)^{2m}$	(-428)	(-9.807)
Hexadecane oxidation to tetradecanoate and propionate	4.1	$CH_3(CH_2)_{12}CH_2CH_2CH_3(98e^-) + \text{“}CO_2\text{”}(0e^-) \rightarrow$ $CH_3(CH_2)_{12}CO_2^-(80e^-) + CH_3CH_2CO_2^-(14e^-) + 4e^-$			

Table 2. Continued

Process	Biochemical half reaction or reaction				
			Eh^{07}	ΔG^{07} (kJ) with e^- acceptor Eh^{07}	
	Eq	Basic components	(V)	−0.2447 V	0.7470 V
Carboxylation of saturated 2-C of hexadecane to 2-methyl hexadecanoate	4.1.1	$CH_3(CH_2)_{12}CH_2CH_3(98e^-) + "CO_2"(0e^-) \rightarrow CH_3(CH_2)_{12}CH_2CH(CH_3)CO_2^-(98e^-)$		$(30 \pm 5)^{2k}$	$(30 \pm 5)^{2k}$
Oxidation of saturated β-C of 2-methyldecanoate to unsaturated β-C analog	4.1.2	$CH_3(CH_2)_nCH_2CH(CH_3)CO_2^-(98e^-) \rightarrow CH_3(CH_2)_{12}CH{=}CH(CH_3)CO_2^-(96e^-) + 2e^-$	$(0.0)^{2b}$	(47)	(−144)
Hydrolytic hydroxylation of unsaturated β-C to β-hydroxy 2-methyldecanoate	4.1.3	$CH_3(CH_2)_{12}CH{=}CH(CH_3)CO_2^-(96e^-) \rightarrow CH_3(CH_2)_{12}CH(OH)CH(CH_3)CO_2^-(96e^-)$		$(0 \pm 5)^{2c}$	$(0 \pm 5)^{2c}$
Oxidation of β-hydroxy 2-methyldecanoate to β-keto 2-methyldecanoate	4.1.4	$CH_3(CH_2)_{12}CHOHCH(CH_3)CO_2^-(96e^-) \rightarrow CH_3(CH_2)_{12}COCH(CH_3)CO_2^-(94e^-) + 2e^-$	$(-0.22)^{2d}$	(5)	(−187)
Hydrolytic keto cleavage of β-keto 2-methyldecanoate to tetradecanoate & propionate	4.1.5	$CH_3(CH_2)_{12}COCHCO_2^-(94e^-) \rightarrow CH_3(CH_2)_{12}CO_2^-(80e^-) + CH_2CO_2^-(14e^-)$		$(-50 \pm 5)^{2e}$	$(-50 \pm 5)^{2e}$
Sum of pathway for hexadecane oxidation to tetradecanoate and propionate	4.1.6	$C_{16}H_{34}(98e^-) + CO_2(0e^-) \rightarrow C_{14}H_{27}O_2^-(80e^-) + C_3H_5O_2^-(14e^-) + 4e^-$		(27 ± 10)	(-365 ± 10)
Tetradecanoate oxidation to didecanoate and acetate	4.2	$CH_3(CH_2)_{12}CO_2^-(80e^-) \rightarrow CH_3(CH_2)10CO_2^-(68e^-) + CH_3CO_2^-(8e^-) + 4e^-$			
Oxidation of saturated β-C of tetradecanoate to unsaturated β-C analog	4.2.1	$CH_3(CH_2)_{10}CH_2CH_2CO_2^-(80e^-) \rightarrow CH_3(CH_2)_{10}CH{=}CHCO_2^-(78e^-) + 2e^-$	$(0.0)^{2b}$	(47)	(−144)
Hydrolytic hydroxylation of unsaturated β-C to β-hydroxy decanoate	4.2.2	$CH_3(CH_2)_{10}CH{=}CHCO_2^-(78e^-) \rightarrow CH_3(CH_2)_{10}CHOHCH_2CO_2^-(78e^-)$		$(0 \pm 5)^{2c}$	$(0 \pm 5)^{2c}$
Oxidation of β-hydroxy decanoate to β-keto decanoate	4.2.3	$CH_3(CH_2)_{10}CHOHCH_2CO_2^-(78e^-) \rightarrow CH_3(CH_2)_{10}COCH_2CO_2^-(76e^-) + 2e^-$	$(-0.22)^{2d}$	(5)	(−187)
Hydrolytic keto cleavage of β-ketodecanoate to didecanoate & acetate	4.2.4	$CH_3(CH_2)_{10}COCH_2CO_2^-(76e^-) \rightarrow CH_3(CH_2)_{10}CO_2^-(68e^-) + CH_3CO_2^-(8e^-)$		$(-55)^{2n}$	$(-55)^{2n}$
Sum of pathway for tetradecanoate oxidation to didecanoate and acetate	4.2.5	$C_{14}H_{27}(80e^-) \rightarrow C_{12}H_{23}O_2^-(68e^-) + C_2H_3O_2^-(8e^-) + 4e^-$		(-3 ± 5)	(-386 ± 5)

[1] See Table 1.

[2] Eh^{07} and DG^{07} numbers in parentheses are approximations obtained from the literature and/or analogous pathway energetics.

[2a] From Heider and Fuchs (1997); and from analogous condensations such as: (1) ethylene($12e^-$) with acetate($8e^-$) to form butyrate($20e^-$), −51 kJ; and (2) acrylate($12e^-$) with formate($2e^-$) to form succinate($14e^-$), −53 kJ.

[2b] Based on saturated to unsaturated oxidation of: (1) butyrate($20e^-$) to crotonate($18e^-$), −0.03V; (2) propionate($14e^-$) to acrylate($12e^-$), −0.03 V; (3) succinate ($14e^-$) to fumarate($12e^-$), 0.03V.

[2c] Based on hydrolysis of unsaturated to hydroxy β- or 3-C compounds, such as: (1) fumarate($12e^-$) to malate($12e^-$), −4 KJ; (2) cis-aconitate($18e^-$) to isocitrate($18e^-$), −2 kJ; (3) crotonate($16e^-$) to β hydroxybutyrate($16e^-$), 0 kJ; and (4) acrylate($12e^-$) to lactate($12e^-$), 6 kJ.

[2d] Based on hydroxy to keto oxidations of: (1) isopropanol($18e^-$) to acetone($16e^-$), −0.28 V; (2) β-hydroxybutyrate($18e^-$) to acetoacetate($16e^-$), −0.26 V; (3) lactate($12e^-$) to pyruvate($10e^-$), −0.19 V; malate ($12e^-$) to oxaloacetate($10e^-$), −0.17 V.

[2e] Based on hydrolytic cleavage of: (1) acetone($16e^-$) to acetate($8e^-$) and methane($8e^-$), −62 kJ; (2) acetoacetate($16e^-$) to 2 acetate($8e^-$), −56 kJ; (3) oxaloacetate($10e^-$) to oxalate($2e^-$) and acetate($8e^-$), −49 kJ; and (4) pyruvate($10e^-$) to acetate($8e^-$) and formate($2e^-$), −49 kJ.

[2f] From Boll and Fuchs (1998), consistent with the need for 2 ATP to accomplish the reaction, where 1 ATP is worth about 0.2 V in redox terms for a $2e^-$ system (Thauer et al, 1977; Harris, 1982).

[2g] Based on unsaturated to saturated reduction versions of 4b.

[2i] Based on decarboxylation of: (1) fumarate($12e^-$) to acrylate($12e^-$), −36 kJ; (2) oxaloacetate($10e^-$) to pyruvate($10e^-$), −32 kJ; (3) acetoacetate ($16e^-$) to acetone($16e^-$), −30 kJ; (4) oxalosuccinate($16e^-$) to a ketoglutarate($16e^-$), −28 kJ; (5) malate ($12e^-$) to lactate($12e^-$), −27 kJ; succinate($14e^-$) to propionate($14e^-$), −25 kJ.

[2i] Approximated from Eh^{07} of benzene($30e^-$), −0.294 V, and methyl benzene, toluene($36e^-$), −0.289 V.

[2j] Based on saturated to oxidative hydroxylation of: (1) propionate($14e^-$) to lactate($12e^-$), 0.00 V; (2) propionate($14e^-$) to β-hydroxypropionate($12e^-$), 0.01 V; (3) succinate($14e^-$) to malate($12e^-$), 0.01V; and (4) butyrate($20e^-$) to β-hydroxybutyrate($18e^-$), 0.02 V.

[2k] Based on carboxylation reversal of 4h. [2l] Analogous to oxidation of toluene to benzoate via phenyl succinate, Eq 1.1.1 to 1.1.6.

[2m] Approximated from Eh^{07} of acetate($8e^-$), −0.291 V, propionate($14e^-$), −0.291 V, and butyrate($20e^-$), −0.291 V.

methanogenic conditions (Eq. 1.1, 1.1.7). For denitrifiers, the high energetic cost of the initial reductive reactions involved in benzoate metabolism could also contribute to benzoate being released as a product (Eqs. 1.2.1, 1.2.2). Denitrifiers metabolizing benzoate to acetate would realize a net ΔG^{07} of -620 kJ (Eq. 1.2). A variety of other intermediates could also be released; the first of these could be 3-ketopimelate (Eq. 1.2). Organisms mediating the initial transformations in a methanogenic consortium would likely metabolize toluene at least as far as pimelate (Eq. 1.2.5) with a net ΔG^{07} of -43 kJ; pimelate has been detected in at least one benzoate-degrading methanogenic consortium (Shlomi et al. 1978). After pimelate, the next intermediate energetically feasible to release would be acetate. The degradation pathways for ethylbenzene and xylene (Table 2) show a pattern similar to toluene, in that the release of benzoate and methylbenzoate, respectively (Eqs. 2.1, 3.1) would be possible under denitrifying but not methanogenic conditions. Alkanes are another major class of hydrocarbon contaminants and the degradation pathway for hexadecane (Eq. 4.1.1 to 4.2.5) illustrates that, similar to the aromatics, it is possible to infer intermediates that may be released and used as substrates by other consortium organisms (e.g., tetradecanoate and propionate).

Table 1 presents similar pathway and reaction energy information for anaerobic TEAPs, grouped according to redox-active element and listed in sequence of increasing electron-accepting potential. These reactions consist only of redox-active components, but the n_c property of each component is included to facilitate tracking of the redox balance. An expanded table of TEAPs and the associated energetic values is available at http://www.soils.wisc.edu/∼hickey/microbial.html. The energetics of coupling each TEAP pathway step with H_2 (10^{-4} atm)/$2H^+$ as electron donor ($Eh^{07} = -0.3$ V) is highly relevant to the ecophysiology of syntrophic, anaerobic degradation of hydrocarbons and is provided as a comparative reference. As indicated above for multi-step electron donor pathways, each component of a multi-step TEAP could in principle be considered as a candidate for a different microbial component of an anaerobic, hydrocarbon-degrading consortium. However, again on energy grounds it is unlikely that a metabolite that requires a highly endergonic formation reaction (such as sulfite, Eq. 2.1.1) would be released as a substrate for another organism. Possible endpoints of the electron acceptor pathways can also be identified from Table 1. For example, in the iron(III) reduction pathway, reduction of ferric hydroxide to magnetite is highly favorable (Eq. 3.2.1, Eh^{07} = 0.4 V) whereas subsequent reduction of magnetite to iron(II) is not (Eq. 3.2.2a, $Eh^{07} = -0.6$ V). The information from Tables 1 and 2 can be combined to evaluate biodegradation processes coupled to TEAPs occurring between denitrification and methanogenesis (Table 3). Integrating this information illustrates that the energetic value of each electron donor step increases as the TEAP redox potential increases. One implication is that, since all pathway steps are worth more at higher redox potentials, a consortium organism mediating the initial attack on a hydrocarbon could better afford to release an early stage product, providing multiple substrates which could support increased consortium membership. Such a process would be consistent with the 'proliferation hypothesis' formulated by Caldwell et al. (1997), which holds that biological systems may be more successful if they proliferate in association with other biological systems, and as such environmental selection may occur at levels beyond the individual. Thus, while consortial degradation processes are typical under methanogenic conditions, these could be expected under other TEAPs as well.

Organisms and consortia mediating hydrocarbon degradation: Phenotypic and phylogenetic characteristics

In this section, organisms are grouped according to the TEAP under which they mediate hydrocarbon degradation, and their relevant phenotypic and phylogenetic characteristics are examined. To gain a broader perspective of the diversity of processes and organisms some examples of consortia that carryout hydrocarbon degradation under various TEAPs, but which have not been characterized as extensively, are also included.

Nitrate reduction

Nitrate is used as a terminal electron acceptor in two respiratory processes, dissimilatory nitrate reduction to ammonium (DNRA) and reduction of nitrate to nitrous oxide and ultimately dinitrogen (denitrification). DNRA (Table 1, Eq. 5.1) is mediated by strict- and facultative-anaerobes, which may also grow by fermentation or use of iron(III), sulfate, or carbon dioxide as TEAs (Stouthamer 1988; Tiedje 1988). Hydrocarbon oxidation coupled to DNRA would be most likely to occur after conditions progressed well into

Table 3. Selected dissimilatory reactions for microbial communities growing on hydrocarbons as e^- donors with e^- acceptor systems of increasing e^- accepting potential

Process	Biochemical half reaction or reaction			ΔG^{07} (kJ) of 1 mole e^- donor coupling with selected terminal e^- acceptors[1]						
				$8e^- + CO_2 \rightarrow CH_4$	$8e^- + SO_4^+ \rightarrow H^2S$	$4e^- + C_4H_5O_3^- \rightarrow C_4H_7O^2_-$	$8e^- + NO_3^- \rightarrow NH_4^+$	$2e^- + MnO^2 \rightarrow Mn^{2+}$	$3e^- + 3Fe(OH)\ 3\gamma\ Fe_3O_4$	$5e^- + NO_3^- \rightarrow 1/2N^2$
	Eq	Basic components	Eh^{07} (V)	Eh^{07} (V) = −0.2447	Eh^{07} (V) = −0.2168	Eh^{07} (V) = −0.1435	Eh^{07} (V) = 0.3628	Eh^{07} (V) = 0.4019	Eh^{07} (V) = 0.4172	Eh^{07} (V) = 0.7470
Acetate	1									
Acetate oxidation to CO^2	1.1	$C^2H_3O_2^-(8e^-) \rightarrow CO^2(0e^-) + 8e^-$	−0.2910	−36	−57	−114	−505	−535	−547	−801
Acetate dismutation to CO^2 and methane	1.2	$C^2H_3O_2^-(8e^-) \rightarrow CO^2(0e^-) + CH_4(8e^-)$, −36 kJ								
Benzoate	2									
Benzoate oxidation to acetate & CO^2	2.1	$C_7H_5O_2^-(30e^-) \rightarrow 3C^2H_3O_2^-(8e^-) + CO^2(0e^-) + 6e^-$	−0.3240	−46	−62	−105	−398	−420	−429	−620
Benzoate oxidation to CO^2	2.2	$C_7H_5O_2^-(30e^-) \rightarrow 7CO^2(0e^-) + 30e^-$	−0.2976	−153	−234	−446	−1,912	−2,025	−2,069	−3,024
Ethyl benzene	3									
Ethylbenzene oxidation to acetate	3.1	$C_8H_{10}(42e^-) \rightarrow 4C^2H_3O_2^-(8e^-) + 10e^-$	(−0.29)	−44	−71	−141	−630	−668	−682	−1,001
Ethylbenzene oxidation to CO^2	3.2	$C_8H_{10}(42e^-) \rightarrow 8CO^2(0e^-) + 42e^-$	(−0.29)	−184	−297	−594	−2,646	−2,804	−2,866	−4,203
Hexadecane	4									
Hexadecane oxidation to propionate and acetate	4.1	$C_{16}H_{34}(98e^-) + CO^2(0e^-) \rightarrow C_3H_5O_2^-(14e^-) + 7C^2H_3O_2^-(8e^-) + 28e^-$	(−0.29)	−122	−198	−396	−1,764	−1,870	−1,911	−2,802
Hexadecane oxidation to CO^2	4.2	$C_{16}H_{34}(98e^-) \rightarrow 16CO_2^-(0e^-) + 98e^-$	(−0.29)	−428	−692	−1,385	−6,174	−6,543	−6,688	−9,807
Toluene	5									
Toluene oxidation to benzoate	5.1	$C_7H_8(36e^-) \rightarrow C_7H_5O_2^-(30e^-) + 6e^-$	−0.2484	−15	−128	−425	−2,477	−2,636	−2,698	−4,034
Toluene oxidation to acetate and CO^2	5.2	$C_7H_8(36e^-) \rightarrow 3C^2H_3O_2^-(8e^-) + CO^2(0e^-) + 12e^-$	−0.2862	−48	−80	−165	−752	−797	−815	−1,196
Toluene oxidation to CO^2	5.3	$C_7H_8(36e^-) + \rightarrow 7CO^2(0e^-) + 36e^-$	−0.2894	−155	−252	−507	−2,266	−2,402	−2,455	−3,600
Xylene	6									
Xylene oxidation to acetate and CO^2	6.1	$C_8H_1\,0(42e^-) \rightarrow 3C^2H3O_2^-(8e^-) + 2CO^2(0e^-) + 18e^-$	(−0.29)	−79	−127	−254	−1,134	−1,202	−1,228	−1,801
Xylene oxidation to CO^2	6.2	$C_8H_{10}(42e^-) \rightarrow 8CO^2(0e^-) + 42e^-$	(−0.29)	−184	−297	−594	−2,646	−2,804	−2,866	−4,203

[1]See Table 2 for details of the selected and alternative e^- donor processes, and Table 1 for the selected and alternative e^- acceptor processes. To derive a combined redox reaction that is e^- – and mass-balanced in all respects (except for ionic charge, O and H) the coefficient for the e^- accepting half of the reaction is n_d/n_a, where n_d and n_a are the number of e^- in the e^- donor and e^- acceptor half reactions, respectively.

anaerobisis (e.g., to iron/manganese-reducing conditions or further) with well-established communities of anaerobes. Under natural conditions, nitrate would be depleted before DNRA could become a significant TEAP. However, in cases where large amounts of nitrate are added to an anaerobic aquifer (e.g., as a stimulant for bioremediation), hydrocarbon degradation by DNRA and/or denitrification could be important. Denitrification is mediated by a diverse assortment of bacteria (Zumft 1992), most of which are facultative anaerobes in which the expression and/or activity of reductases involved in denitrification is repressed by oxygen. Because the Eh^{07} of 0.747 V for denitrification (Table 1, Eq. 5.2) is almost as positive as that of 0.8150 V for O_2 reduction, (Harris & Arnold 1995) the energy yields of hydrocarbon oxidation coupled to denitrification approach those obtained by aerobic respiration and exceed that of other TEAPs (Table 3). Thus, nitrate reduction reactions should dominate over the other anaerobic TEAPs during hydrocarbon degradation as long as nitrate is available (Anderson & Lovley 1997). The favorable energetics of denitrification and high water solubility of nitrate have been two factors driving the use of nitrate-supplementation as a strategy for in situ remediation of contaminated aquifers (Reinhard et al. 1997; Thomas et al. 1997; Hutchins et al. 1998). The effectiveness of this approach is perhaps most significantly affected by the capability of the targeted organisms to oxidize hydrocarbons by mechanisms that do not involve oxygenases such as those summarized in Table 2.

Nitrate reduction is mediated by a diversity of phylotypes, and includes representatives from almost all taxa comprising the *Bacteria* domain (Zumft 1992). But, to date, most of the organisms isolated from denitrifying, alkylbenzene-degrading enrichment cultures have been assigned to the beta-2 subgroup (*Rhodocyclus* group) of the *β-Proteobacteria*, as *Thaurea*, *Azoarcus* or a closely affiliated phylotype. Toluene-degrading *Azoarcus* include *A. tolulyticus*, *A. toluvorans*, and *A. toluclasticus* as well as several isolates that have not yet been classified to the species level (Zhou et al. 1995; Heider & Fuchs 1997; Song et al. 1999). The toluene-degrading species of *Azoarcus* identified so far were isolated from a variety of environments including hydrocarbon-contaminated groundwater, hydrocarbon-contaminated soil, non-contaminated soil and non-contaminated compost (Fries et al. 1994; Song et al. 1999). Thus, these organisms may be common constituents of microbial communities and their distribution may not necessarily be affected by prior exposure to contamination.

While the alkylbenzene-degrading nitrate-reducers isolated thus far form a relatively tight phylogenetic cluster, they also present a diversity of hydrocarbon degradation abilities. Toluene is mineralized by almost all of the isolates, the exceptions being some strains that were enriched using other alkylbenzenes. For example, strain PbN1 (Rabus & Widdel 1995) degrades propylbenzene and ethylbenzene, strain EB1 (Ball et al. 1996) degrades ethylbenzene, and strain pCyN2 (Harms et al. 1999a) degrades *p*-cymene. The isolates from toluene-amended enrichments differ in the pattern of alkylbenzenes that they degrade; strain EB1 and EbN1 (Rabus & Widdel 1995) metabolize toluene and ethylbenzene while *A. tolulyticus* strain Td-15 and *Azoarcus* sp. strains T and mXyN1 degrade both toluene and *m*-xylene (Dolfing et al. 1990; Schocher et al. 1991; Fries et al. 1994; Rabus & Widdel 1995; Beller & Spormann 1997a; Krieger et al. 1999). For most of the isolates, the alkylbenzenes that support growth under denitrifying conditions are also degraded aerobically. The exceptions are strains EbN1, PbN1, ToN1, and mXyN1, which couple oxidation of some substrates to nitrate reduction only. In pure culture, the nitrate reducing, alkylbenzene-degraders differ in their accumulation of, and sensitivity to, nitrite (Dolfing et al. 1990; Schocher et al. 1991; Rabus & Widdel 1995; Harms et al. 1999a). Thus, in heterogeneous communities these organisms may benefit from associations with organisms having a greater affinity for nitrite-reduction.

Iron reduction

Geobacter metallireducens couples toluene (as well as phenol and *p*-cresol) mineralization to iron reduction or to DNRA (Lovely & Phillips 1988; Lovley & Lonergan 1990; Lovley et al. 1993). The toluene degradation pathway of *G. metallireducens* has not been established. *Geobacter* are commonly isolated from pristine and contaminated anaerobic environments in which iron reduction is an important TEAP (Coates et al. 1996). There are several characterized species of *Geobacter*, which is classified within the *Geobacteraceae* family of the *δ-Proteobacteria*. Other genera included in the *Geobacteraceae* are *Desulfuromonas*, *Desulfuromusa*, and *Pelobacter*; all are strict anaerobes that have in common the ability to use iron(III) and/or elemental sulfur as a TEA. Other than *G. metallireducens* and a phylogenetically similar strain

TACP-5 (Coates et al. 1996), no other *Geobacteraceae* have as yet been demonstrated to grow by coupling alkylbenzene oxidation to iron reduction (Lonergan et al. 1996). Thus, although evidence from field and microcosm studies indicates that hydrocarbon degradation coupled to iron reduction is a major process, knowledge of the organisms mediating these reactions is quite limited.

Iron (III) is the most abundant TEA in most anaerobic aquifers (Lovley 1991). In principle, organisms carrying out iron reduction obtain relatively good energy yields compared to those of sulfate-reducers and methanogens (Table 3), and on this basis should be competitive in anaerobic environments (Chapelle & Lovley 1992). In practice, however, their growth and activity may be limited by the bioavailability of iron(III). For example, *G. metallireducens* grows more rapidly with soluble iron-chelates than with amorphous $Fe(OH)_3$ (Lovley et al. 1994b). Also, addition of a variety of iron-chelates to microcosms can enhance rates of benzene and alkylbenzene mineralization (Lovley et al. 1994b; Lovley & Woodward 1996; Lovley et al. 1996).The role of iron-reducing organisms in anaerobic, hydrocarbon-degrading consortia could be either as a hydrogen and acetate consumer or as the initiator of hydrocarbon catabolism (Lovely & Phillips 1988; Lovley 1991; Coates et al. 1996). *G. metallireducens*, as discussed above, can couple the complete oxidation of toluene to iron- reduction in pure culture. However, it can also initiate toluene degradation in co-culture with *Wolinella succinogenes* when fumarate is the TEA (Meckenstock 1999). *G. metallireducens* alone cannot use fumarate as an electron acceptor. Instead, the electrons are transferred to *W. succinogenes*, which uses fumarate as a TEA, via interspecies electron transfer. Lovley et al. (1999) demonstrated that humic-like substances could facilitate this transfer of electrons between *G. metallireducens* and *W. succinogenes*. Iron-reducing organisms can also consume metabolites (Lovely & Phillips 1988; Lovley & Phillips 1989; Lovley 1991; Coates et al. 1996) produced by a hydrocarbon-fermenting organisms (e.g., acetate and hydrogen). In environments with high levels of bioavailable iron(III), iron-reducers may inhibit sulfate reduction and methanogenesis by out-competing the sulfate-reducers and methanogens for hydrogen, acetate, or other fermentation products (Lovley 1991; Chapelle & Lovley 1992; Anderson & Lovley 1997). Increasing hydrogen concentrations in anaerobic aquifers as the TEAP gradient approaches methanogenesis reflect this idea (Chapelle et al. 1995). This postulated role for iron reducers has not yet been demonstrated in a defined hydrocarbon-degrading, co-culture, but because of the high affinity of known iron-reducers for hydrogen and acetate (Anderson & Lovley 1997) it is a potentially important relationship.

Sulfate reduction

Organisms defined physiologically as sulfate-reducers encompass a diversity of bacteria that span the domains *Archaea* and *Bacteria*. Within the *Bacteria*, sulfate-reducing bacteria (SRB) occur in the Gram-positive and *Proteobacteria* divisions, and in the latter comprise eight distinct phylogenetic lineages within the δ-subdivision (Holt et al. 1994). As a group, the SRB are metabolically versatile and able to grow either by using sulfate or sulfur oxyanions (Table 1, Eqs. 2.1–2.3) as electron acceptors or by fermentation (Widdel 1988). This characteristic of SRB makes them potentially important members of degradative consortia.

Pure cultures of SRB have been isolated that grow on alkylbenzenes and alkanes. Alkylbenzene metabolism has been characterized with *Desulfobacula toluolica* strain Tol2 (Rabus & Heider 1998) and strain PRTOL1 (Beller & Spormann, 1997b), both couple oxidation of toluene to sulfate reduction. Two other alkylbenzene-degrading SRB are strains oXyS1 and mXyS1, which were isolated from oil-contaminated marine sediment (Harms et al., 1999b). Both grew on crude oil and degraded toluene and benzoate but differed in the ability to metabolize isomers of alkyl-benzenes and alkyl-benzoates: oXyS1 preferentially attacked those that were *o*-substituted (*o*-xylene, *o*-ethyltoluene and *o*-methylbenzoate) while mXyS1 only degraded *m*-substituted compounds (*m*-xylene, *m*-ethyltoluene, *m*-isopropyltoluene, and *m*-methylbenzoate). The strains were also of different phylogenetic lineage; based on 16S rDNA sequence similarities the closest relatives to oXyS1 were *Desulfobacterium cetonicum* (98.4%) and *Desulfosarcina variabilis* (98.7%), while that of mXyS1 was *Desulfococcus multivorans* (86.9%).

At least four pure cultures of alkane-degrading SRB have been isolated: strain TD3 from the Guaymas Basin, (Rueter et al. 1994); strain Hxd3 from an oil-water separator in Hamburg, Germany (Aeckersberg et al. 1991); strain AK-01 from the contaminated intertidal sediment in Aurther Kill, N.Y., (So & Young 1999); strain Pnd3, a mesophile, from marine mud (Aeckersberg 1998). Phylogenetic analysis showed

that thermophilic strain TD3 was deeply branched within the *δ-Proteobacteria*, but was most closely related to *Desulfovibrio* (Rueter et al. 1994; So & Young 1999), while the mesophile strains Hxd3, Pnd3, and AK-01 were more closely related to each other and to the genera *Desulfosarcina*, *Desulfonema*, and *Desulfococcus* (Aeckersberg et al. 1998). Aside from the ability to reduce sulfate and metabolize fatty acids and alkanes of approximately the same length (So & Young 1999), these organisms were metabolically and phylogenetically distinct.

In the absence of sulfate, the SRB continue to play a central role in biodegradation processes by establishing metabolic links with other organisms. The SRB are commonly associated with methanogenic consortia (see discussion in the following section) and may have a variety of functions, including acting as a homoacetogen forming acetate from hydrogen and carbon dioxide (Widdel 1988). This is a pivotal role given that hydrogen and acetate are the main substrates consumed by methanogens. It is also possible for SRB to affiliate with consortia in which the prevailing TEAP occurs at a redox level more favorable than sulfate reduction. For example, strain TRM1 (a toluene degrading, sulfate-reducer) cannot, as a pure culture, couple toluene oxidation to nitrate- or fumarate-reduction. But, strain TRM1, like *G. metallireducens*, can degrade toluene when incubated in co-culture with the hydrogen consuming, nitrate- and fumarate-reducing *W. succinogenes* (Meckenstock 1999). The environmental significance of SRB in consortial interactions such as these is unknown. However, there are at least two possibilities where this might occur: 1) when nitrate is added to anaerobic aquifers as a biostimulant for bioremediation, 2) during the initial transition to anaerobisis when nitrate is still present. Either of these would likely involve competition between SRB and a variety of other nitrate-reducers for the hydrocarbons, acetate, or hydrogen.

Methanogenesis

The endpoint of temporal and spatial progression in redox stratification in anaerobic aquifers is establishment of methanogenesis as the predominant TEAP. All known methanogens occur within the *Euryarchaeota* family of the domain *Archaea* (Garcia 1990). These organisms are strict anaerobes that produce methane as a product of catabolic metabolism. Methane production occurs via reduction of carbon dioxide with a limited number of electron donors (i.e., hydrogen, acetate, formate, methyl compounds, and alcohols), and/or by fermentation of acetate (aceticlastic methanogenesis). Methanogens are unable to transform hydrocarbons or other complex organics to these relatively simple substrates and as such are dependent on degradative activities of other organisms to supply their electron donors. Thus, methanogenic degradation processes characteristically involve consortia (Oremland 1988; Kafkewitz & Togna 1998).

In natural environments, the composition of methanogenic consortia carrying out biodegradation processes is typically unknown, but based on analysis of model laboratory systems, these are likely to be a diverse and dynamic mixture of organisms. For example, Fernandez et al. (1999) examined the microbial community in a methanogenic digester that had been fed glucose for 1505 days. The reactor was then sampled over a 575-day interval, during which time the microbial activity (as indicated by methane and volatile fatty acid production, pH, and chemical oxygen demand reduction) was relatively stable. Selected samples were used to construct *Bacteria* and *Archaea* 16S rDNA clone libraries, and the diversity of organisms comprising these was determined by using restriction enzyme analysis to generate operational taxon units (OTUs). Collectively, at least 75 *Bacteria* and 21 *Archaea* OTUs were identified, the occurrence and abundance of which varied over time. Methanogenic aquifers contaminated with heterogeneous mixtures of hydrocarbons might support development of similarly complex communities.

Methanogenic consortia mediate degradation of a variety of alkanes and aromatics (Edwards & Grbić-Galić 1994; Phelps et al. 1998; Weiner & Lovley 1998a; Ficker et al. 1999; Zengler et al. 1999). One of the most extensively characterized and relevant to hydrocarbon pollutant degradation is a toluene-degrading consortium (Edwards & Grbić-Galić 1994; Ficker et al. 1999). This consortium is used as a model in Figure 1 and is composed of four physiological groups defined as: syntroph 1, oxidizing toluene and releasing one or more oxidative or fermentation pathway intermediates (in this example specified as toluene oxidation to acetate coupled to proton reduction to H_2); syntroph 2, a homoacetogen catalyzing the reversible oxidation of acetate coupled to the reduction of protons to H_2 (depending on relative activities of acetate and H_2); syntroph 3, a methanogen fermenting acetate and producing CO_2 and methane; and syntroph 4, a methanogen using H_2 to reduce CO_2 to methane. Like other methanogenic consortia, the activity of this

model toluene-degrading consortium is dependent on the control of H_2 and acetate concentrations, which is effected by the collective activity of the syntrophs. For example, a relatively small change in H_2 could reverse the acetyl CoA pathway syntroph 2 uses for energy generation. The model is also similar to other consortia in illustrating the possibility for different groups of methanogens (hydrogen vs. acetate consuming) to carryout parallel reactions simultaneously. A defining feature of the toluene-degrading consortium is the activity of the organism(s) that initiate degradation, which would likely involve specialized transformations (see Table 2).

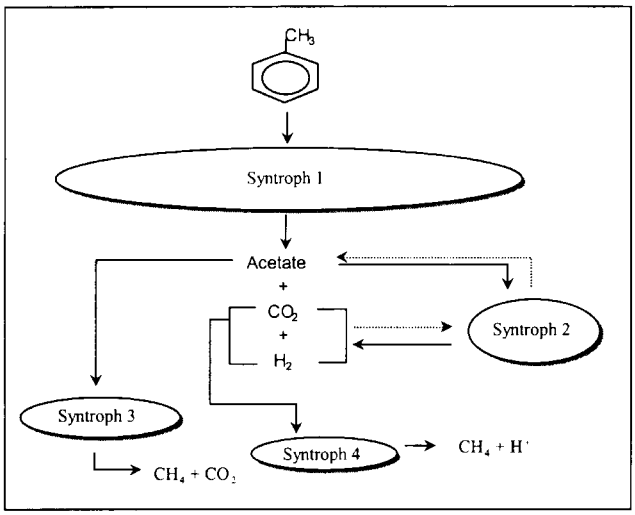
Ficker et al. (1999) applied a battery of phylogenetic analyses to determine the composition of the toluene-degrading consortium. Six major *Bacteria* OTUs and two *Archaea* OTUs were identified in a clone library of the consortium. A series of probes were developed from these sequences and used to quantify organism abundance by hybridization. The two archaeal sequence types collectively accounted for about 29% of the cells hybridizing to an *Archaea* domain probe, and two bacterial sequences represented 87% of all cells hybridizing to a *Bacteria* domain probe. The *Archaea* sequences were determined to be *Methanosaeta* and *Methanospirillum*; one of the *Bacteria* sequences detected by hybridization was a *Desulfotomaculum*-like SRB while the other had no similarity to a known genus. The investigators developed a hypothetical assignment of function to phylotype by working backward from the terminal processes. Methane production by acetate fermentation (Figure 1, syntroph 3) was assigned to the Methanosaeta sequence-type, as this is the only known growth mechanism for this genus (Archer & Harris 1986; Garcia, 1990). *Methanospirillum* was postulated to mediate a parallel path of methanogenesis (Figure 1, syntroph 4) using hydrogen to reduce carbon dioxide. The *Desulfotomaculum*-like SRB was presumed not to mediate the initial attack on toluene as addition of excess sulfate inhibited degradation, instead of enhancing it (i.e., toluene oxidation was not directly coupled to sulfate reduction). Thus, by the process of elimination, the hypothesized roles of the SRB and unknown bacterial sequence types were homoacetogen (Figure 1, syntroph 2) and toluene-oxidizer (Figure 1, syntroph 1), respectively. The phylogenetic analysis provided one of the first insights into the composition of an alkylbenzene-degrading consortium. However, the occurrence of an organism can not be used as direct evidence for any particular activity. Clearly, identification and characterization of the organisms mediating the initial transformation of alkylbenzenes, and the other consortium activities, is needed to advance our understanding of these important processes.

Impacts of hydrocarbon contamination on microbial community structure in anaerobic aquifers: Microcosm and field studies

The issue for the present review is how the composition and activity of microbial communities involved in hydrocarbon biodegradation may vary within and/or between TEAP zones. Explorations of microbial community structure alterations that accompany variation in TEAP stratification have been relatively recent undertakings. The issue has been addressed by integrating geochemical analyses with molecular phylogenetic and/or phenotypic examination of microbial communities. This approach has been applied for the direct analysis of samples from the field and of samples taken from microcosms. The former provides the most accurate insights into community structure *in situ*, but because of the complexity of communities and chemical characteristics of the environment, cause and effect relationships are difficult to discern. Microcosms allow establishment of controlled conditions needed to correlate microbial community dynamics with environmental variables, but extrapolation of findings to field studies is difficult. The two approaches are thus complementary and continuing studies integrating these approaches have the potential to improve our understanding of these environments.

Microcosm studies

A number of studies have included microcosms to examine hydrocarbon degradation under a variety of TEAP conditions (Hutchins 1991; Edwards & Grbić-Galić 1992; Edwards et al. 1992; Ball & Reinhard 1996; Borden et al. 1997; Hess et al. 1997; Hutchins 1997; Kazumi et al. 1997; Salanitro et al. 1997; Nales et al. 1998; Weiner et al. 1998; Weiner & Lovley 1998a, b; Phelps & Young 1999; Shi et al. 1999). The present review is restricted to those that included a detailed analysis of the microbial community. Microcosm studies examining effects of nitrate supplementation on microbial community structure have been prompted in part because of the interest in using this approach as a bioremediation strategy. It is



Syntroph	Phylogenetic match	Growth Reaction	$\Delta G^{o'}$ (kJ)	$\Delta G'$ (kJ)
1	unknown	$C_7H_8 + 11.28H^+ \rightarrow 2.82C_2H_3O_2^- + 0.82CO_2 + 5.64H_2 + 0.54$ Cell-C	154	-54
2	*Desulfotomaculum*	$1.41C_2H_3O_2^- + 11.12H^+ \rightarrow 2.78CO_2 + 5.56H_2 + 0.04$ Cell-C	133	-3
3	*Methanosaeta*	$1.41C_2H_3O_2^- \rightarrow 1.39CO_2 + 1.39CH_4 + 0.04$ Cell-C	-49	-52
4	*Methanospirillum*	$11.20H_2 + 2.82CO_2 \rightarrow 22.40H^+ + 2.78CH_4 + 0.04$ Cell-C	-364	-97
	Overall Consortium Reaction	$C_7H_8 \rightarrow 2.17CO_2 + 4.17CH_4 + 0.66$ Cell-C	-126	-207

Figure 1. Proposed metabolic interactions in the model toluene-degrading consortium based on Edwards and Grbić-Galić (1994) and Ficker et al. (1999). Growth reactions are derived from the energetics of the dissimilatory and assimilatory half-reactions (see footnotes Table 1). This is information is available at http://www.soils.wisc.edu/~hickey/microbial.html.

well established that, in most cases, nitrate amendment stimulates alkylbenzene degradation (Heider & Fuchs 1997; Hutchins et al. 1998; Nales et al. 1998). However, the impacts of these treatments on microbial community structure are unknown. Column microcosm studies by Hess et al. (1997) examined the distribution of the denitrifying alkylbenzene-degrader *A. tolulyticus* and overall population composition in diesel fuel-contaminated aquifer material. The column influent contained hydrocarbons and nitrate, and a redox gradient was established between the inlet (oxic conditions) and outlet (denitrifying conditions). Phylogenetic probes targeting *A. tolulyticus* and divisions and subdivisions within the *Bacteria* were used in fluorescent *in situ* hybridization tests to determine organism abundance along the redox gradient. In the oxic zone, *A. tolulyticus* was detected at an abundance of ca. 2% of the bacterial community, but outside this region dropped to $\leq$ 1% of the community. The occurrence of *A. tolulyticus in situ* was thus confirmed, but its role in anaerobic degradation of the hydrocarbons was difficult to assess based on its distribution relative to the TEAP. The oxic zone also had the greatest abundance of α-*Proteobacteria* (10% of *Bacteria*) and outside this region these organisms dropped to < 2% of the community. The α-*Proteobacteria* where most abundant across the redox gradient and ranged from 80 to 87% of the *Bacteria* in the aerobic zone, 42% in the anoxic zone and 66% in the anaerobic zone. The γ-*Proteobacteria* accounted for 10–16% of the community along the entire column. Shi et al. (1999) also found that the β- and γ-*Proteobacteria* were more abundant than the α-*Proteobacteria* in nitrate-supplemented, fuel-contaminated, anaerobic aquifer sediment. However, these investigators also noted that when toluene was added a significant fraction of the bacterial DNA (55–65%) did not hybridize to the α-, β-, γ-, or δ-*Proteobacteria* or high G + C probes used.

Collectively, these studies suggest that of the *Proteobacteria*, the γ- and/or β-groups may compose a large fraction of hydrocarbon-degrading, nitrate-reducing community but that the diversity of organisms active in this process is as yet unknown.

Field studies

Relating microbial community composition to biodegradation processes occurring in contaminated aquifers is a challenging undertaking, and has been approached by using culture-based and/or molecular methods. For the former, the most probable number technique has been used to enumerate physiological groups of organisms that are defined by their ability to grow by fermenting selected organics, or by coupling oxidation of a selected electron donor to a given TEA (TEA-MPN). Data from TEA-MPN analyses provide a quantitative estimate of abundance and distribution for different physiological groups. However, a limitation of TEA-MPN is that culturing probably recovers only a subset of the targeted organisms, which may give an underestimate of abundance. Also, some organisms may be able to grow with multiple TEAs. This may be one factor (along with spatial heterogeneity) that may complicate interpretation of TEA-MPN data in the context of the apparent prevailing TEAP in the location sampled. Molecular phylogenetic analyses provide qualitative insights into community diversity. Techniques commonly employed include those mentioned above (restriction enzyme and sequence analysis of 16S rDNA clone libraries, hybridization) and denaturing gradient gel electrophoresis (DGGE). For DGGE analysis, PCR is first used to amplify a segment of the 16S rDNA using community DNA extracts as template. The PCR products generated have a uniform size, but differ in their internal nucleotide sequence and consequently have variable melting behavior. The latter characteristic can be exploited to separate individual PCR products into bands based on electrophoretic migration in a gel containing a concentration gradient of a denaturing agent (Muyzer et al. 1993; Muyzer et al. 1996; Muyzer & Smalla 1998). Each band represents at least one microbial phylotype, and comparisons between samples in the number of bands and/or banding patterns provide a qualitative indicator of differences in microbial diversity. Bands can also be recovered from gels for sequencing and the phylotype assignment (Muyzer & Smalla 1998).

A study illustrating the use of TEA-MPN to determine the spatial distribution of bacterial physiological types was that of Ludvisgen et al. (1999). These investigators were studying an aquifer contaminated by landfill leachate (located in Grinsted, Denmark) and used TEA-MPN to determine the distribution and abundance of nitrate-reducers, iron-reducers, manganese- reducers, sulfate-reducers, and methanogens. The microbial community diversity and biomass was largest in the methanogenic zone, with the abundance of physiological groups decreasing in the order (approximate maximum density) iron-reducers (10^7 g^{-1}) > nitrate-reducers manganese-reducers methanogens (10^5 g^{-1}) > sulfate-reducers (10^4 g^{-1}). Outside the methanogenic region, numbers of methanogens and sulfate-reducers dropped to $< 10^2$ g^{-1} while iron-reducers decreased to levels similar to those of nitrate- and manganese-reducers (ca. 10^4 g^{-1}). Other than the enrichment of methanogens in the methanogenic region, there was no apparent correlation between changes in physiological group abundance and changes in TEAP stratification. These results could reflect the above-mentioned limitation of MPN-TEA, namely the possibility for some organisms to grow by multiple mechanisms. For example, in the methanogenic zone organisms enumerated as iron-reducers might be growing by some form of fermentation. Also, organisms could employ survival strategies allowing them to persist in the absence of a suitable TEA (Richardson 2000). The spatial heterogeneity of these systems is another factor that complicates correlating microbiological and physical-chemical analyses.

Bekins et al. (1999) examined a petroleum-contaminated aquifer in Bemidji, Minnesota by TEA-MPN. The groups of organisms enumerated were similar to those examined in the Grinsted aquifer, except these tests included fermenters and excluded manganese-reducers. Iron-reduction and methanogenesis were the predominant TEAPs within the plume, while the surrounding non- contaminated regions were aerobic. Within the plume, iron-reducers and aerobes were predominant (10^4 to 10^6 g^{-1}) at all locations; fermenters were generally less abundant and numbered 10^0 to 10^5 g^{-1}. Nitrate- and sulfate-reducers were detected throughout the plume and in non-contaminated regions, but were present in the majority of samples at $\leq 10^2$ g^{-1}. Methanogens ranged from nondetectable to 10^2 g^{-1}, and their peak numbers coincided with reduced numbers of iron-reducers. The negative correlation between methanogen and iron-reducer numbers was statistically significant, and the invest-

igators suggested that this alteration was indicative of regions that either were methanogenic or progressing from iron reducing to methanogenesis.

The Bemidji aquifer was further characterized by Rooney-Varga et al. (1999), who used PCR (*Bacteria* and *Geobacteraceae* primers) and DGGE. The *Bacteria* PCR products generated from the non-contaminated, oxic zone were separated into four to five bands while those generated from the iron-reducing and methanogenic zones contained up to 25 bands. The increased banding pattern complexity in samples from the contaminated zone would be consistent with the physiological analysis of Bekins et al. (1999) in suggesting the development of more diverse microbial communities. One of the iron-reducing zones was shown to be a location in which benzene mineralization was occurring, and selected DGGE bands apparent only in this sample were sequenced to identify the organisms that appeared to be enriched in this zone. The majority of these additional bands recovered were determined to be sequence types belonging to the *Geobacteraceae.* The oxic and iron- reducing zones also had different DGGE patterns for the *Geobacteraceae* PCR products. Subsequent sequencing of the bands confirmed that different sequence types were amplified from the oxic and iron-reducing zones. Those from the latter were closely related to *Geobacter* spp., which as yet is the only organism group that has demonstrated mineralization of aromatic compounds coupled to dissimilatory iron reduction. The *Geobacter* spp. sequence types were also identified in a benzene-degrading enrichment culture. Collectively, these findings indicated that one of the major shifts in community structure involved general proliferation of *Geobacteraceae* in the iron-reducing zones and specific establishment of *Geobacter* spp.-like organisms in areas in which benzene degradation was occurring.

Sequence analysis of 16S rDNA clone libraries has also been used to study shifts in microbial community structure associated with pollution and changes in TEAP stratification. Dojka et al. (1998) reported phylogenetic analysis of a hydrocarbon- and chlorinated solvent- contaminated aquifer in Michigan. Clone libraries of 16S rDNA sequences were established from zones in which the predominant TEAP was considered to be iron-reduction, sulfate- reduction or methanogenesis. Sixty-four clones were categorized into ten, well-recognized divisions (e.g., *Proteobacteria*), 21 into four of the 'OP' candidate divisions that currently lack cultured representatives, and ten apparently novel sequences formed six groups of a new candidate division. A variety of OTUs were recovered from all zones, but the number of different types increased in the order iron- reducing (six OTU), < sulfate-reducing (ten OTU), < methanogenic (37 OTU). Thus, microbial biodiversity appeared to be greatest in the methanogenic zone, which would be consistent with the studies of Bekins et al. (1999) and Ludvisgen et al. (1999) that suggest microbial diversity increases as the predominant TEAP progresses to methanogenesis. In some cases, insights into potential function could be inferred from sequence information. For example, the predominant archaeal sequence type recovered by Dojka et al. (1998) from the methanogenic zone was 97% similar to *Methanosaeta concillii.* As mentioned above, *Methanosaeta* are only known to grow only by acetoclastic methanogenesis and as such the investigators postulated the occurrence of this activity *in situ.* Complementary activities, like organic acid and hydrogen production, were hypothesized to be mediated by sequence types identified as *Syntrophus gentianae.* Other archaeal sequences matched *Methanospirillum*, which might mediate methane formation by carbon dioxide reduction. This collection of activities was similar to that proposed for the methanogenic toluene- degrading consortium described above. Studies of the Michigan aquifer illustrate the difficulty in identifying organisms mediating the critical initial transformations of the hydrocarbons. None of the sequence types retrieved from the iron- or sulfate-reducing zones showed similarities to organisms known to link growth to these TEAPs. Which could indicate that these organisms were present but not detected, that unknown organisms mediated these processes, and/or that the predominant TEAP in these zones was not correctly assigned.

Conclusions

While hydrocarbon degradation has been documented in a variety of anaerobic aquifers, the nature of the microbial communities mediating these processes is still largely unknown. Insights into the types of organisms mediating hydrocarbon degradation and the biochemical pathways by which hydrocarbons are metabolized have been gained from the study of individual isolates and consortia. While still rather limited, the data suggest unity as well as diversity in the microbiology and biochemistry underlying these processes. Unity in that similar biochemical mechanisms have been

documented in widely divergent phylotypes, and diversity in that hydrocarbon-degraders closely related as phylotypes may differ in the type and variety of hydrocarbon degradation pathways they possess. This situation is similar to what is known for the more thoroughly researched area of aerobic degradation of hydrocarbons. While most of the information on biochemical pathways has been gained from the analysis of individual isolates, the energetic framework presented here demonstrates how microbial consortia could be involved in hydrocarbon degradation regardless of the predominant TEAP. Certain organisms within such consortia are likely to mediate specialized biochemical transformations, which play a key role in defining the hydrocarbon degradation characteristics of the community under a given TEAP. Analyses of microcosms and field samples suggest that the diversity of organisms mediating these processes have not yet been adequately characterized. Advancements in the understanding of how hydrocarbon-degrading communities function will be significantly affected by the extent to which organisms mediating specialized reactions can be identified and tools developed to allow their study *in situ.*

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