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Anaerobic biodegradation of natural gas condensate can be stimulated by the addition of gasoline

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Abstract Biodegradation of a broad range of linear and branched alkanes, parent and alkyl alicyclic hydrocarbons, and benzene and alkyl-substituted benzenes was observed when sediment and groundwater samples collected from a gas condensate-contaminated aquifer were incubated under methanogenic and especially under sulfate-reducing conditions, even though no exogenous nitrogen or phosphorus was added. This finding expands the range of hydrocarbon molecules known to undergo anaerobic decay and confirms that natural attenuation is an important process at this site. The addition of 1 μl of gasoline to the samples (~10 ppm) had minimal impact on the biodegradation of saturated compounds, but substantially increased the diversity and extent of aromatic compounds undergoing transformation. We attribute this to the promotion or induction of biodegradation pathways in the indigenous microflora following the addition of the gasoline components. The promoting compounds are not precisely known, but may have been present in the initial condensate and reduced in concentration by various mechanisms (dissolution, biodegradation, etc.) such that their concentration in the aquifer fell below necessary levels. A variety of aromatic hydrocarbons would appear to be likely candidates.

Keywords Anaerobic biodegradation · Hydrocarbons · Sulfate-Reduction · Methanogenesis · Metabolic Fate

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Introduction

Petroleum hydrocarbons are a rich source of carbon and energy for a diversity of aerobic and anaerobic microbes (Prince 2005), and thus are readily biodegraded when released into the biosphere. Biodegradation under aerobic conditions has been extensively studied, and the pathways associated with the metabolism of many hydrocarbons are well established (Prince 1998; University of Minnesota 2005). Hydrocarbon biodegradation under anaerobic conditions is still an emerging field, but it is now clear, from both field and laboratory investigations, that at least some alkanes, cycloalkanes, and aromatic compounds can be metabolized under a variety of redox conditions (Cozzarelli et al. 1990, 1994; Eganhouse et al. 1996; Díaz and Prieto 2000; Achong et al. 2001; Chakraborty and Coates 2004;

Suflita et al. 2004; Townsend et al. 2004; Rabus 2005) Nevertheless, our understanding of anaerobic hydrocarbon biodegradation is mainly limited to studies with isolated microorganisms on pure compounds, and the fate of the suite of hydrocarbons in most seeps and spills remains under explored. Here we use samples collected from a shallow anoxic aquifer that overlies a natural gas field in the Denver Basin near Fort Lupton, CO, USA. The aquifer has been chronically contaminated by gas condensate (96% w/w C_5–C_{15} compounds), a by-product of the natural gas recovery process (Barker et al. 1996; Gieg et al. 1999). Condensate contains many of the hydrocarbons found in automobile gasolines, but in different relative proportions. We examine the biodegradation of condensate, with and without a small amount of gasoline, under sulfate-reducing and methanogenic conditions. A broad range of compounds were biodegraded under both conditions, but perhaps surprisingly some aromatic compounds in the condensate were only metabolized after the addition of a trace amount (1 µl per 50 g sediment) of gasoline.

Methods

Anoxic sediments and ground water used as inocula were obtained from a shallow contaminated aquifer that overlies a natural gas field in the Denver Basin near Fort Lupton, CO, USA as previously described (Barker et al. 1996; Gieg et al. 1999). Sediments were placed into 1-l mason jars, topped with groundwater, sealed without a headspace, and stored at 4°C until use. Anaerobic incubations containing 50 g (±0.5 g) sediment and 75 ml (±0.5 ml) groundwater in 160 ml serum bottles were as described previously (Townsend et al. 2004). No additional nutrients were added to the incubations and bacterial metabolism relied on endogenous nutrients in the incubations. The headspace in the bottles was N_2/CO_2 (80/20) at 15 kPa overpressure. A 1-month pre-incubation preceded the experiment so that endogenous sulfate in methanogenic incubations would be reduced to below 100 µM (Townsend et al. 2004). Some incubations received 1 µl of a standard gasoline (API 91–01), provided by the American Petroleum Institute (Fig. 1). Sulfate-reducing incubations and sulfate-amended sterile controls were amended with 20 mM Na_2SO_4, while methanogenic incubations received no external electron acceptor. Samples were incubated for various times up to 100 days, at room temperature, in the dark, inverted, and without agitation (Townsend et al. 2004). All treatments were performed in triplicate. Levels of sulfate reduction and methanogenesis were monitored throughout the experimental period by ion- and gas-chromatography, respectively, on separate replicate incubations as previously described (Townsend et al. 2003). The data for the gasoline-amended incubations presented here are from the 100 days incubations, but the 80-day incubations were very similar. Background samples without added gasoline were incubated for 209 days, in duplicate. Sterile control incubations were autoclaved for 20 min on three consecutive days.

Incubations were frozen until analysis, then thawed and 10 g of the solid contents placed in 40 ml sample vials and analyzed by purge and trap extraction (OI Analytical 4552 Water/soil autosampler). Gas chromatography used a Supelco Petrocol DH octyl column (100 m × 0.25 mm ID fused silica) in a Hewlett Packard 6890 chromatography system with a Hewlett Packard 5973 mass selective detector. Spectral tuning with decafluorotriphenylphosphine followed USEPA method 8270C. All samples from each experiment, with and without added gasoline, were analyzed together. Analytes were identified by reference to standard mixtures (Supelco, Bellefonte, PA, USA) and the HewlettPackard (Palo Alto, CA, USA) mass spectral libraries.

Primary biodegradation was identified as the preferential loss of some compounds at the expense of others (Prince et al. 1994; Prince and Douglas 2005): here we used 1,1,3-trimethylcyclohexane as the conserved internal marker (Townsend et al. 2004). We calculate the percent depletion of other analytes within the condensate using the equation:

$$\%\ \text{Loss} = [((A_0/C_0) - (A_S/C_S))/(A_0/C_0)] \times 100,$$

where A_s and C_s are the concentrations of the target analyte and conserved compound in the

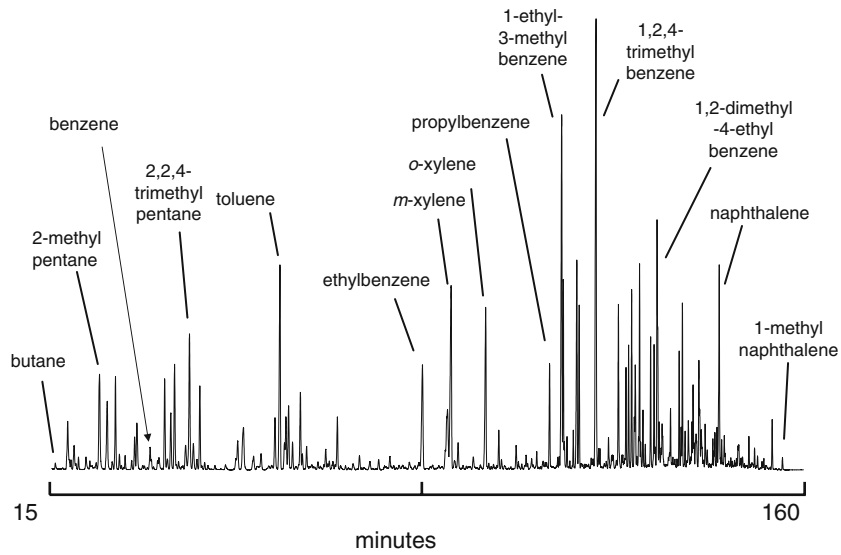

Fig. 1 Total ion chromatogram of the hydrocarbons in the gasoline used here. More than 100 compounds can be identified from their diagnostic ions and elution patterns (Uhler et al. 2003). 1,1,3-trimethylcyclohexane elutes at 86.4 min under our conditions, and can be identified by its characteristic m/z = 111 ion. This compound is not visible in the total ion chromatogram shown here because it is present at only about 280 ppm in the gasoline

sample, respectively, and A_0 and C_0 are the concentrations in the sterile controls. Treating data in this way compensates for the problems associated with essentially insoluble hydrocarbons that are principally absorbed to the sediment in our incubations (Prince and Douglas 2005), but

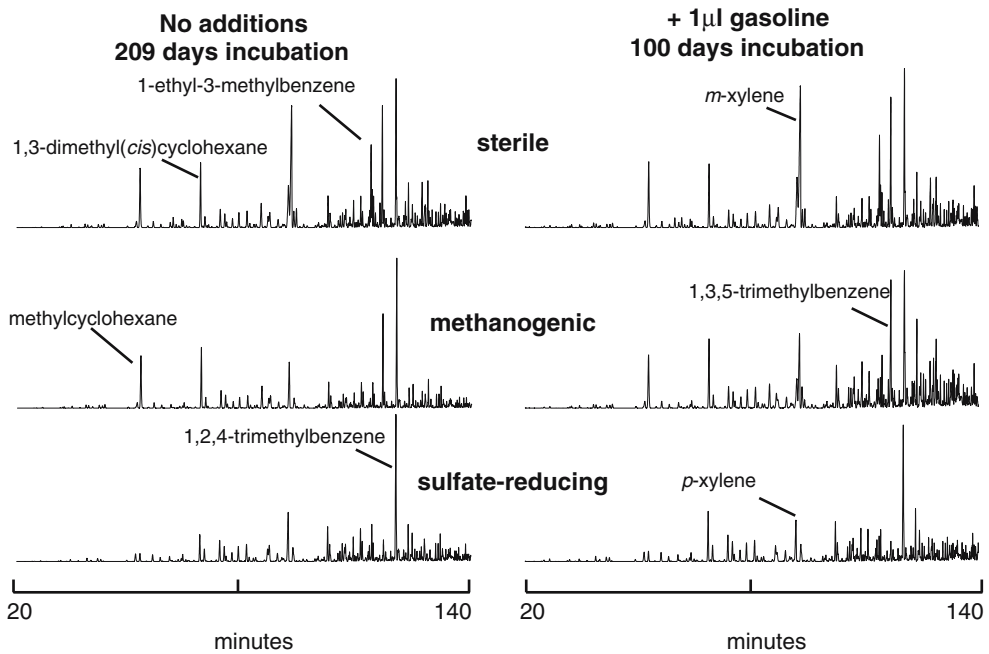

Fig. 2 Total ion chromatograms of hydrocarbons in sterile and non-sterile condensate-contaminated aquifer incubations. The chomatograms on the right resulted from incubations that were amended with 1 μl of gasoline. Non-sterile incubations were held under methanogenic and sulfate-reducing conditions. The spectra have been normalized to equal amounts of 1,1,3-trimethylcyclohexane, and are dominated by cyclic compounds

does not provide absolute quantitation of the individual analytes. We have rounded the numbers to the nearest 10%, focusing mainly on compounds where there is substantial (at least 50%) loss under at least some conditions of electron acceptor or gasoline addition.

Results

The condensate-contaminated sediment used in this work contains enough condensate that the added gasoline made up ≈5% of the hydrocarbons in the incubations. This can be seen in Fig. 2, where the total ion chromatograms of condensate and gasoline amended-condensate samples are compared. The smallest molecules detected in the condensate samples were methyl pentanes, while the largest reliably quantified by the purge-and-trap methodology used were dodecane and the tetramethylbenzenes. Gasoline contains molecules as small as butane, which were detected by our analyses (Uhler et al. 2003). The small amendment of gasoline to the hydrocarbon-contaminated samples had no statistically significant impact on either the background rate of sulfate reduction or methanogenesis.

The most abundant individual compounds in the condensate were cyclic compounds (Fig. 2), but the condensate contains *n*- and *iso*-alkanes, as does the gasoline amendment. No *n*-alkanes smaller than heptane were detected in the condensate, but, as shown in Table 1, heptane, octane, nonane, undecane, and dodecane were completely degraded under both methanogenic and sulfate-reducing conditions, regardless of the trace amount of gasoline amendment. Decane was probably present, and degraded, but could not be reliably analyzed because it coelutes with 1,2,4-trimethylbenzene under the chromatographic conditions, and this aromatic compound was present in much higher

Table 1 The percent biodegradation of representative alkanes and branched alkanes in incubations held under methanogenic and sulfate-reducing conditions, with (+) and without (–) a 1 µl amendment of gasoline

Hydrocarbon	Methanogenic		Sulfate-reducing	
	–	+	–	+
Butane	nd	10	Nd	30
Pentane	nd	60	Nd	60
Hexane	nd	90	Nd	100
Heptane	100	100	100	100
Octane	100	100	100	100
Nonane	100	100	100	100
Undecane	100	100	100	100
Dodecane	100	100	100	100
2-methylpentane	nd	60	Nd	40
3-methylpentane	10	50	20	30
2-methylhexane	100	90	100	90
3-methylhexane	100	90	100	90
2-methylheptane	90	100	100	100
3-methylheptane	70	100	100	100
3-methylnonane	80	70	100	100
2,2-dimethylpentane	10	40	10	20
2,3-dimethylpentane	10	30	10	10
2,4-dimethylpentane	10	40	10	20
2,5-dimethylhexane	10	20	10	20
2,5-dimethylheptane	10	0	10	20

The numbers have been rounded to the nearest 10%

nd not detected

Table 2 The percent biodegradation of representative alicyclic compounds in incubations held under methanogenic and sulfate-reducing conditions, with (+) and without (–) an amendment of 1 µl gasoline

Hydrocarbon	Methanogenic		Sulfate-reducing	
	–	+	–	+
Cyclohexane	0	40	100	100
Methylcyclohexane	10	40	100	100
Ethylcyclohexane	0	20	100	100
Propylcyclohexane	0	10	10	30
Butylcyclohexane	10	0	60	50
Pentylcyclohexane	30	0	40	30
cis-1,3-dimethylcyclohexane	0	0	80	50
1,1-dimethylcyclohexane	0	0	0	0
trans-1,2-dimethylcyclohexane	0	0	0	0
trans-1,3-dimethylcyclohexane	0	0	0	0
cis-1,2-dimethylcyclohexane	0	0	0	0
Cyclopentane	nd	nd	nd	nd
Methylcyclopentane	10	50	100	100
Ethylcyclopentane	0	20	100	100
1,1-dimethylcyclopentane	0	0	20	20
cis-1,3-dimethylcyclopentane	0	0	0	0
trans-1,3-dimethylcyclopentane	0	0	10	10
trans-1,2-dimethylcyclopentane	0	0	100	100
Cyclopentene	nd	90	nd	100
Methylcyclopentene	nd	90	nd	100

The numbers have been rounded to the nearest 10%

nd not detected

concentrations in the condensate. Butane, pentane, and hexane were present in the gasoline-amended samples, and a fraction of each substrate was degraded under both methanogenic and sulfate-reducing conditions although the smaller molecules were less readily metabolized by the resident microflora.

As expected, *n*-alkanes were more readily metabolized than the branched alkanes (Table 1), but singly methylated hexanes, heptanes, and octanes were essentially completely degraded in the incubations, and even dimethyl pentanes, hexanes, and heptanes were partially degraded. Just as the smaller *n*-alkanes were less-readily degraded substrates, so the smaller branched alkanes were more resistant to degradation; less than 60% of the 2- and 3-methylpentanes were degraded by the end of the experiment, compared to >90% degradation of 2- and 3-methylhexanes.

Several cycloalkane hydrocarbons were extensively degraded under anaerobic conditions, especially under sulfate-reducing conditions (Townsend et al. 2004). Table 2 shows that there was little difference in the fractional extent of degradation of these compounds, or its scope, when 1 μl of gasoline was added.

In contrast, the addition of a trace amount of gasoline had a significant effect on the biodegradation of some of the aromatic compounds present in the condensate. Benzene, toluene, and *o*-xylene were below detection limits in our samples of condensate, and the addition of 1 μl of gasoline added ~40 ppb benzene, 400 ppb toluene, and 90 ppb *o*-xylene, which could then be readily analyzed. *m*-Xylene was extensively degraded regardless of the presence or absence of gasoline (Fig. 3) but very little biodegradation of ethylbenzene or *p*-xylene occurred in the absence of gasoline. Yet, these compounds were extensively degraded in the rather shorter incubation in the presence of gasoline. Most of the benzene and toluene present in the gasoline, but not the *o*-xylene, was removed (Table 3).

Similarly, propyl, *iso*-propyl, and 1-ethyl-4-methylbenzenes were essentially completely consumed in the gasoline-amended incubations, but untouched in the condensate alone (Fig. 4, Table 3), as were the two *iso*-butyl isomers,

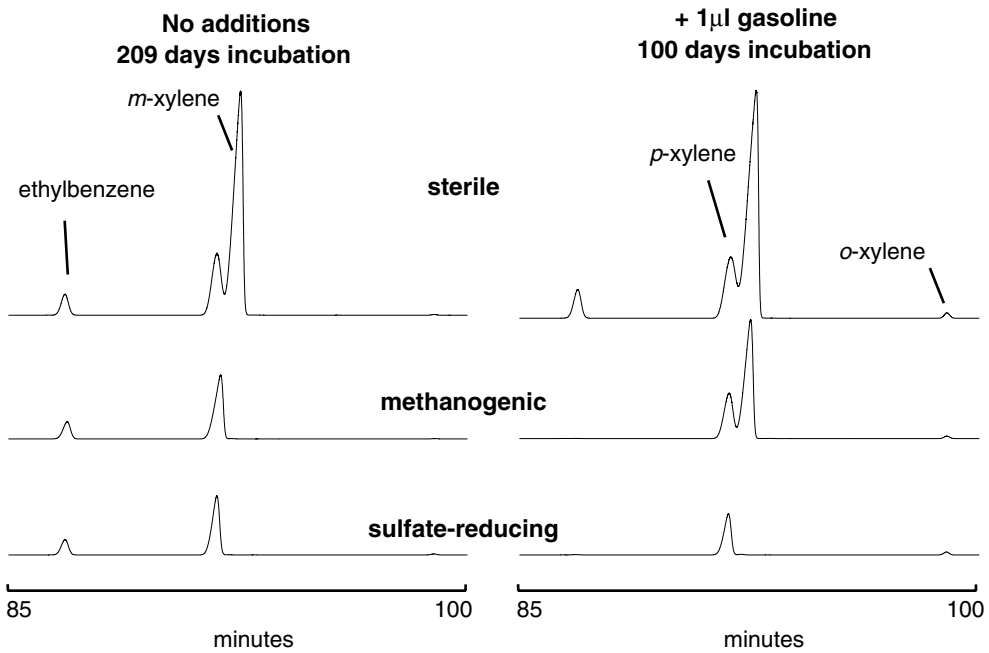

Fig. 3 Ethylbenzene and the xylenes in condensate and condensate amended with gasoline from sterile incubations, and samples incubated under methanogenic and sulfate-reducing conditions. The chromatograms are of the m/z = 91 ion, and have been normalized to equal amounts of 1,1,3-trimethylcyclohexane

Table 3. The percent biodegradation of representative aromatic compounds in incubations held under methanogenic and sulfate-reducing conditions, with (+) and without (-) an amendment of 1 μl gasoline

Hydrocarbon	Methanogenic		Sulfate-reducing	
	–	+	–	+
Benzene	nd	80	nd	80
Methylbenzene (toluene)	nd	100	nd	100
Ethylbenzene	0	100	10	100
Propylbenzene	0	100	0	100
Isopropylbenzene	0	100	0	100
Butylbenzene	30	100	100	100
(1-methylpropyl)benzene	0	100	0	100
(2-methylpropyl)benzene	0	100	0	100
1,2-dimethylbenzene (*o*-xylene)	nd	50	nd	20
1-ethyl-2-methylbenzene	0	20	0	30
1-methyl-2-propylbenzene	0	0	0	30
1-methyl-2-isopropylbenzene	0	0	0	0
1,3-dimethylbenzene (*m*-xylene)	100	70	100	100
1-ethyl-3-methylbenzene	100	100	100	100
1-methyl-3-propylbenzene	100	80	100	100
1-methyl-3-isopropylbenzene	100	50	100	100
1,4-dimethylbenzene (*p*-xylene)	0	50	0	50
1-ethyl-4-methylbenzene	30	80	10	80
1-methyl-4-propylbenzene	30	80	0	70
1-methyl-4-isopropylbenzene	0	80	0	70
1,4-diethylbenzene	0	50	0	50
1,2,3-trimethylbenzene	0	0	0	10
2-ethyl-1,3-dimethylbenzene	0	0	0	0
1,2,4-trimethylbenzene	0	20	0	20
1,4-dimethyl-2-ethylbenzene	0	0	0	0
2,4-dimethyl-1-ethylbenzene	0	0	0	0
1,2-dimethyl-4-ethylbenzene	0	0	0	0
1,3,5-trimethylbenzene	0	0	100	100
1-ethyl-3,5-dimethylbenzene	40	0	100	100
1,2,3,4-tetramethylbenzene	nd	nd	nd	nd
1,2,3,5-tetramethylbenzene	0	0	0	0
1,2,4,5-tetramethylbenzene	0	0	0	0

The numbers have been rounded to the nearest 10%
nd not detected

1-methyl-4-(1-methylethyl), and 1-methyl-4-propyl benzenes (Fig. 5, Table 3).

Discussion

Substantial biodegradation of the hydrocarbons in the condensate (and added gasoline) occurred under both methanogenic and sulfate-reducing conditions. Under both conditions the degradation was more extensive after the addition of the trace amount of gasoline; total losses were 32 and 37% in the absence and presence of gasoline under methanogenic conditions, and 43 and 51% under sulfate-reducing conditions. Note that the incubations relied on the indigenous organisms in the Fort Lupton, CO sediment, and that no additional nutrients such as biologically available nitrogen or phosphorus were added. While we did amend the sulfate-reducing incubations with additional sulfate, the experiments were fundamentally designed to assess the natural attenuation occurring at the site, and should not be interpreted as indicating the potential total extent of biodegradation.

Our results lead us to several conclusions. First they confirm that the indigenous microflora have a substantial ability to degrade a broad array of hydrocarbons in the condensate contaminant under both methanogenic and sulfate-reducing conditions (Gieg et al. 1999; Townsend et al. 2003, 2004;). These are the conditions that predominate at the Fort Lupton, CO site. Further, this metabolism has some very distinctive signatures. Some are reasonably expected, such as the preferential degradation of the *n*-alkanes before the singly methylated alkanes, and these before the doubly methylated ones. This effect has been recognized for decades (Miget et al. 1969; Pirnik et al. 1974) and represents a consistent metabolic feature associated with both aerobic and anaerobic metabolism. Other signatures are more subtle, for example only removing cis-1,3-dimethylcyclohexane of all the detectable dimethylcyclohexane isomers and trans-1,2-dimethylcyclopentane of all the dimethylcyclopentanes, and then only under sulfate-reducing conditions (Townsend et al. 2004).

Perhaps the most provocative finding is the substantial increase in the types of aromatic molecules undergoing biodegradation upon the addition of a trace amount of gasoline. The gasoline amendment was only a small fraction, some 5%, of the total hydrocarbon present in the condensate, although it did add enough of the small *n*-alkanes, benzene, toluene, and *o*-xylene that these were now above detection limit concentrations (Tables 1, 2, 3).

The addition had little effect on the biodegradation of the alkanes and cycloalkanes (Tables 1, 2), but substantially broadened the range of

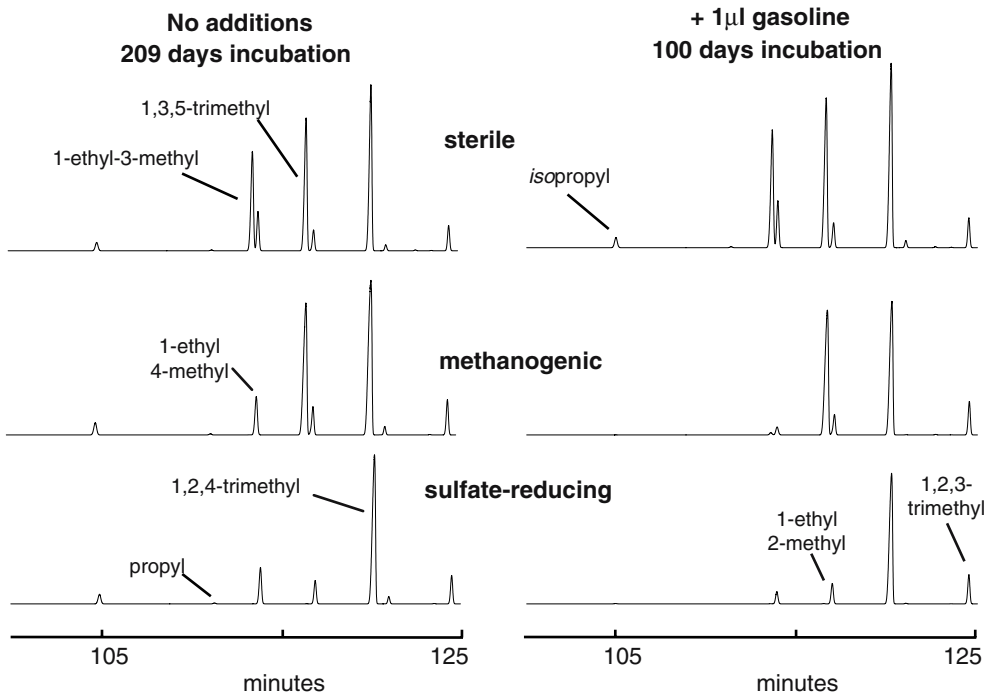

Fig. 4 Propyl, *iso*-propyl, methyl-ethyl, and trimethylbenzenes in condensate and condensate amended with gasoline from sterile incubations, and samples incubated under methanogenic and sulfate-reducing conditions. The chromatograms are of the m/z = 105 ion, and have been normalized to equal amounts of 1,1,3-trimethylcyclohexane

aromatic compounds degraded. In the absence of the gasoline, degradation of the singly substituted benzenes was essentially limited to butylbenzene. Degradation of the disubstituted forms in the absence of gasoline was effectively restricted to 1,3-substituted forms, with much less degradation of a few 1,2- and 1,4-isomers. In the absence of gasoline, only 1,3,5-trisubstituted forms were degraded, albeit mainly under sulfate-reducing conditions (Table 3).

The addition of gasoline substantially broadened the biodegradation, especially of the singly substituted and 1,4-disubstitued forms. Yet 1,2-disubstituted forms, the 1,2,3- and 1,2,4-trisubstituted forms, and the tetrasubstituted forms were only partially degraded and remained relatively resistant to biodegradation under the incubation conditions. Simple methylated or dimethylated aromatic hydrocarbons are well known to be amenable to anaerobic biodegradation and are the subject of numerous reviews (e.g., Chakraborty and Coates 2004; Suflita et al. 2004; Rabus 2005). However, more complicated substitution patterns undoubtedly influence the susceptibility of the resulting aromatic hydrocarbons to anaerobic metabolism. Eganhouse et al. (1996) noted relative differences in the concentration of a variety of hydrocarbons including a homologous series of alkylbenzenes along a transect of a crude oil spill that contaminated groundwater in a Bemidji, MN aquifer. They surmised that structure specific anaerobic biodegradation was the only process to account for the removal patterns observed in the field.

The stimulatory effect that we observe in our experiments seems most likely due to some derepression or promotion of biodegradation by one or more of the constituents of the added gasoline. There were no additional inorganic nutrients in the gasoline-amended incubations, so the effect was not due to the relief of some nutritional limitation. The effect was not a function of time for biodegradation, since the gasoline-amended samples were incubated for only about half the time of the unamended samples. Díaz and Prieto (Díaz and Prieto 2000) and Tropel and van der

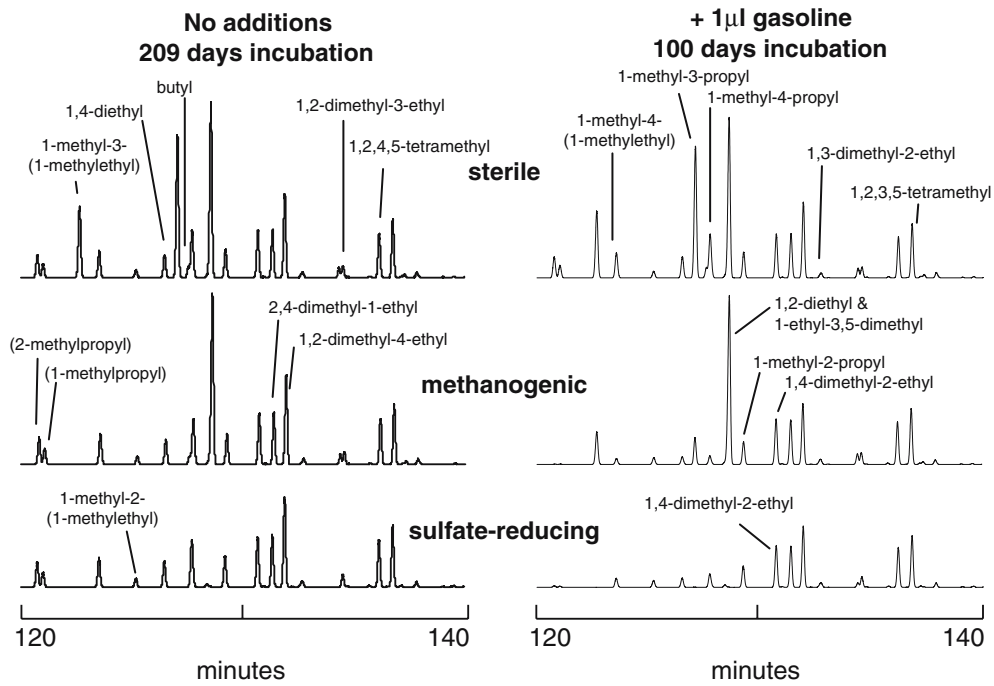

Fig. 5 Butyl, *iso*-butyl, methylpropyl, methylisopropyl, diethyl, and tetramethylbenzenes in condensate and condensate amended with gasoline from sterile incubations, and samples incubated under methanogenic and sulfate-reducing conditions. The chromatograms are of the m/z = 134 ion, and have been normalized to equal amounts of 1,1,3-trimethylcyclohexane

Meer (2004) have reviewed the various mechanisms of promotion of aromatic biodegradation. Most of the work has been done on aerobic organisms, but Leuthner and Heider (1998) have studied a two-component regulatory system of the denitrifying toluene-degrading bacterium *Thauera aromatica*. This system, named tdiSR (toluene degradation inducing sensor and regulator), is immediately upstream of the genes for benzylsuccinate synthase (bssDCAB). Benzylsuccinate synthase is a glycyl radical enzyme that catalyzes the addition of fumarate to the methyl group of toluene to form benzylsuccinate (Achong et al. 2001), and would seem to be a reasonable paradigm for much anaerobic hydrocarbon biodegradation (Suflita et al. 2004).

Our data suggest that at least one compound present in the gasoline, but not the condensate, up-regulates the synthesis of new biodegradative enzymes in the indigenous microbial communities. Tempting candidate hydrocarbons include benzene, toluene, and *o*-xylene, which have been implicated before (Díaz and Prieto 2000; Morasch et al. 2004). Most likely these compounds were present in the initial condensate, but were reduced in effective concentration by dissolution, biodegradation or by other mechanisms. Interestingly, addition of these hydrocarbons causes the expression of metabolic activity in the resident microflora with apparent broad specificity; ethylbenzene, for example, is untouched in the absence of gasoline, but completely consumed in its presence.

Our results suggest the perhaps counter-intuitive notion that the biodegradation of complex hydrocarbon mixtures in contaminated aquifers may be limited, at least in part, by the absence of small water-soluble components. These compounds might act as inducing substrates that de-repress important catalytic functions.

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