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ORIGINAL PAPER

Assessment of microbial communities associated with fermentative–methanogenic biodegradation of aromatic hydrocarbons in groundwater contaminated with a biodiesel blend (B20)

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Abstract A controlled field experiment was conducted to assess the potential for fermentative–methanogenic biostimulation (by ammonium-acetate injection) to enhance biodegradation of benzene, toluene, ethylbenzene and xylenes (BTEX) as well as polycyclic aromatic hydrocarbons (PAHs) in groundwater contaminated with biodiesel B20 (20:80 v/v soybean biodiesel and diesel). Changes in microbial community structure were assessed by pyrosequencing 16S rRNA analyses. BTEX and PAH removal began 0.7 year following the release, concomitantly with the increase in the relative abundance of *Desulfitobacterium* and *Geobacter* spp. (from 5 to 52.7 % and 15.8 to 37.3 % of total Bacteria 16S rRNA, respectively), which are known to anaerobically degrade hydrocarbons. The accumulation of anaerobic metabolites acetate and hydrogen that could hinder the thermodynamic feasibility of BTEX and PAH biotransformations under fermentative/methanogenic conditions was apparently alleviated by the growing predominance of *Methanosarcina*. This suggests the importance of microbial population shifts that enrich microorganisms capable of interacting syntrophically to enhance the feasibility of fermentative–methanogenic bioremediation of biodiesel blend releases.

Keywords Biodegradation · Biodiesel · BTEX · PAH · Pyrosequencing · Syntrophy

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Introduction

The use of biodiesel blends as an alternative renewable transportation fuel is increasing worldwide to alleviate dependence on fossil fuels and to minimize atmospheric emissions and greenhouse effects. The increasing biodiesel demand can, however, increase the probability of groundwater contamination as result of accidental and incidental spills during its production, transportation and storage. Although biodiesel is commonly referred to as a harmless and readily

Table 1 Main reactions involved in biodiesel, BTEX and PAH degradation and $\Delta G^{\circ\prime}$ values (kJ mol^{-1})

Reactions involved in linoleic acid (1, 2, 3 and 4), benzene (5, 6, 7 and 8) and naphthalene (9, 10, 11 and 12) degradation	$\Delta G^{\circ\prime}$ reaction[a] (kJ mol^{-1})
Linoleic acid ($C_{18:2}$)	
(1) $C_{18}H_{31}O_2^- + H^+ + 16H_2O \rightarrow 9CH_3COO^- + 9H^+ + 14H_2$	+272.33
(2) $9CH_3COO^- + 9H^+ + 18H_2O \rightarrow 36H_2 + 18CO_2$	+854.26
(3) $50H_2 + 12{,}5CO_2 \rightarrow 12{,}5CH_4 + 25H_2O$	−1634.34
(4) Sum (1) + (2) + (3): $C_{18}H_{31}O_2^- + H^+ + 9H_2O \rightarrow 12{,}5CH_4 + 5{,}5CO_2$	−507.75
Benzene	
(5) $C_6H_6 + 6H_2O \rightarrow 3CH_3COO^- + 3H^+ + 3H_2$	+70.73
(6) $3CH_3COO^- + 3H^+ + 6H_2O \rightarrow 12H_2 + 6CO_2$	+284.754
(7) $15H_2 + 3{,}75CO_2 \rightarrow 3{,}75CH_4 + 7{,}5H_2O$	−490.30
(8) Sum (5) + (6) + (7): $C_6H_6 + 4{,}5\ H_2O \rightarrow 3.75CH_4 + 2.25CO_2$	−134.82
Naphthalene	
(9) $C_{10}H_8 + 10H_2O \rightarrow 5CH_3COO^- + 5H^+ + 4H_2$	+101.12
(10) $5CH_3COO^- + 5H^+ + 10H_2O \rightarrow 10CO_2 + 20H_2$	+474.59
(11) $24H_2 + 6CO_2 \rightarrow 6CH_4 + 12H_2O$	−784.48
(12) Sum (9) + (10) + (11): $C_{10}H_8 + 8H_2O \rightarrow 6CH_4 + 4CO_2$	−291.75

$\Delta G_f^{\circ}(aq)$ values of linoleic acid and naphthalene were obtained in Lalman (2000) and Dolfing et al. (2009), respectively. All other compounds ΔG_f° (aq and g (for H_2)) values were obtained in Thauer et al. (1977)

[a] Standard Gibbs energies were calculated under standard conditions (1 M solute concentration, pH = 7, T = 298 K and gas partial pressure of 1 atm)

biodegradable biofuel (Zhang et al. 1998), it is usually blended with petroleum diesel fuel that contains priority pollutants such as benzene, toluene, ethylbenzene and xylenes (BTEX) and polycyclic aromatic hydrocarbons (PAH). These hydrocarbons include carcinogenic compounds (e.g., benzene and benzo[*a*]-pyrene) that are generally monitored to determine the need for corrective remedial action.

The high biochemical oxygen demand exerted by indigenous microorganisms during biodiesel biodegradation rapidly drives impacted aquifers towards methanogenic conditions. This phenomenon is particularly noticeable at the source zone region where higher concentration of organic compounds stimulates the consumption of terminal electron acceptors. Under methanogenic conditions where the energetic yield is close to the minimum needed for microbial sustenance (≈ -20 kJ mol^{-1} required for ATP formation) (Schink 1997), bioremediation is usually accomplished by syntrophic microorganisms (Morris et al. 2013). Syntrophic anaerobes can play a critical role in the biodegradation of long-chain fatty acids (Sousa et al. 2009), BTEX (Rakoczy et al. 2011) and PAH (Berdugo-Clavijo et al. 2012), since initial fermentative/methanogenic biotransformations are thermodynamically unfeasible (endergonic) (Table 1, reactions 1, 5 and 9) without consumption of degradation byproducts by commensal microorganisms. Long-chain fatty acids (represented by linoleic acid) derived from biodiesel esters hydrolysis can be further oxidized to acetate and hydrogen via β-oxidation (Sousa et al. 2009). Although this reaction is thermodynamically unfeasible (Table 1, reaction 1), it might proceed if syntrophic microorganisms consume metabolites that can impose thermodynamic constraints (reactions 2 and 3) thus making the overall reaction exergonic (reaction 4). Similarly, fermentative/methanogenic BTEX and PAH biodegradation (represented by benzene and naphthalene, respectively) is plausible (reactions 8 and 12) when syntrophic microorganisms consume the metabolites (reactions 6, 7, 10 and 11).

We previously demonstrated that anaerobic biostimulation by the addition of ammonium acetate into B20 contaminated groundwater induced fermentative–methanogenic conditions that enhanced BTEX removal (Ramos et al. 2013). This enhancement in BTEX anaerobic biodegradation was hypothesized to occur due to the proliferation of putative hydrocarbon

degraders thriving syntrophically with methanogenic archaea. Nonetheless, the microbial community structure was not characterized to identify potential syntrophic associations. Therefore, in this work, microbial 16S rRNA pyrosequencing analyses were conducted to assess temporal changes in microbial community structure during anaerobic biostimulation of groundwater contaminated with a biodiesel blend. Emphasis was placed on studying microbial populations putatively associated with aromatic hydrocarbon biodegradation. This information advances our current understanding of fermentative–methanogenic bioprocesses by identifying dominant microorganisms during anaerobic bioremediation of B20 releases.

Materials and methods

Controlled release field experiment

A controlled release field experiment was monitored over 2 years to investigate whether anaerobic biostimulation could enhance BTEX biodegradation under fermentative–methanogenic conditions. Detailed information on this experiment set up and groundwater monitoring was previously described (Ramos et al. 2013). Briefly, a source zone was established by releasing 100L of B20 (20 % v/v soybean biodiesel and 80 % v/v diesel) into an area of 1 m^2 × 1.6 m deep down to the water table. Fermentative–methanogenic biostimulation was performed by weekly injection of ammonium acetate (300 mg L^{-1}) into 5 wells installed 1.5 m upstream of the source zone.

Chemical analyses

Groundwater was monitored at the source zone since biodiesel blend releases are not readily miscible in groundwater and behave as a fixed, decaying, yet long-lived source with relatively small region of influence compared to soluble biofuels such as ethanol (Corseuil et al. 2011). Groundwater pH, redox potential, dissolved oxygen, nitrite, nitrate, sulfate, sulfide, ferrous iron, acetate, BTEX and methane were monitored over time (Ramos et al. 2013).

PAH were extracted from groundwater using solid phase SPE cartridges, according to EPA method 525.2, and measured by gas chromatography (HP model 6890 II with a flame ionization detector (FID) and HP-5 capillary column). Detection limits were (in parenthesis): naphthalene (7 μg L^{-1}), methylnaphthalene (5 μg L^{-1}), dimethylnaphthalene (7 μg L^{-1}), acenaphthylene (8 μg L^{-1}), acenaphthene (8 μg L^{-1}), fluorene (8 μg L^{-1}), phenanthrene (9 μg L^{-1}), anthracene (9 μg L^{-1}), fluorathene (10 μg L^{-1}), pyrene (9 μg L^{-1}), benzo[*a*]anthracene (9 μg L^{-1}), chrysene (10 μg L^{-1}), dibenzo[*a,h*]anthracene (12 μg L^{-1}), benzo[*b*]fluoranthene (12 μg L^{-1}), benzo[*k*]fluoranthene (31 μg L^{-1}), benzo[*a*] pyrene (36 μg L^{-1}), indeno[1,2,3-*cd*]pyrene (28 μg L^{-1}) and benzo[*g,h,i*]-pyrene (11 μg L^{-1}).

Dissolved hydrogen analyses were conducted 2.4 years following the B20 release using in situ passive samplers. These samplers were deployed for 5 days in groundwater, according to the methods developed by Spalding and Watson 2006 and Spalding and Watson 2008. Hydrogen was measured by a gas chromatography (model UC-13 Construmaq equipped with a 30-ft long × 1/8 in stainless-steel column packed with 100/120 mesh Haye Sep*D* solid phase and thermal conductivity detector). The detection limit was 10^{-9} M.

Microbial analysis

16S rRNA pyrosequencing analyses were conducted to identify, characterize and assess temporal changes in microbial community structure during anaerobic biostimulation of groundwater contaminated with a biodiesel blend. DNA samples were extracted from groundwater before the B20 release as background information on indigenous microorganisms and then after 0.3, 0.7, 1.0, 1.4 and 1.6 years following the fuel release.

To amplify the 16S rRNA region two primer pairs were used: one for Bacterial 16S and one for Archaeal 16S. Bacteria 16S rRNA was amplified within hyper variable region 3 and 4 (bases 347-803) using primers designed specifically for 454 sequencing (which included 454 adapter sequence, 10 base nucleotide barcode, and target primer). Target primers were: forward 5′-GGAGGCAGCAGTRRGGAAT-3′, reverse 5′-CTACCRGGGTATCTAATCC-3′ (Nossa et al. 2010). Archaea 16S was amplified between bases 571–1,204. Target primers were: forward 5′-GCYTAAAGSRICCGTAGC-3′, reverse 5′-TTMGGGGCATRCIKACCT-3′ (Gantner et al. 2011). PCR was carried out using Qiagen Top Taq

Table 2 Metabolic characteristics of the bacteria genera found in groundwater source-zone

Bacteria	Class	Respiration mode	Substrates	References
Janthinobacterium	β-Proteobacteria	Aerobic	PAH	Bodour et al. (2003), Lima et al. (2012)
Ralstonia	β-Proteobacteria	Aerobic or denitrifier	Fatty acids, alcohols, aromatic compounds	Cramm (2009), Bodour et al. (2003)
Burkholderia	β-Proteobacteria	Facultative	Methyl esters, acetate, alkanes, aromatic compounds and chlorinated compounds	Philippe et al. (2001), O' Sullivan and Mahenthiralingam (2005), Belova et al. (2006)
Geobacter	δ-Proteobacteria	Iron-reducer, fermentative	Short-chain fatty acids, alcohols and monoaromatic compounds	Lovley et al. (1993), Cord-Ruwisch et al. (1998)
Geobacillus	Bacilli	Facultative	Organic acids, alcohols, aromatic compounds and carbohydrates	Nazina et al. (2001)
Staphylococcus	Bacilli	Facultative	Sugars	Kloos (1980)
Desulfitobacterium	Clostridia	Sulfate-, thiosulfate-, sulfite-, or iron-reducer, fermentative	Halogenated organic compounds, aromatic compounds, organic acids	Villemur et al. (2006), Kunapuli et al. (2010)
Gp 1, 3, 13, 23	Acidobacteria Gp 1, 3, 13, 23	Facultative	Sugars, amino acids, alcohols, organic acids	Ward et al. (2009), Geissler et al. (2009)
Nitrospira	Nitrospira	Aerobic or nitrite oxidation	Nitrogenated compounds	Blackbourne et al. (2007)
Pseudomonas	γ-Proteobacteria	Facultative denitrifier	Aromatic compounds	Shim et al. (2005), Bruce et al. (2010), Lima et al. (2012)
Desulfovibrio	δ-Proteobacteria	Sulfate-, thiosulfate- or sulfite reducer, fermentative	Long-chain fatty acids, aromatic compounds, organic acids, alcohol	Allen et al. (2008), Sousa et al. (2007), Heidelberg et al. (2004), Bryant et al. (1977)
Desulfosporosinus	Clostridia	Sulfate-, thiosulfate-, sulfite-, or arsenate-reducer	Sugars, alcohols, monoaromatic compounds	Robertson et al. (2001), Liu et al. (2004), Ramamoorthy et al. (2006)
Clostridium III	Clostridia	Fermentative	Long-chain fatty acids, organic acids	Chauhan and Ogram (2006), Hatamono et al. (2007)
Geothrix	Holophagae	Iron-reducer or fermentative	Organic acids	Coates et al. (1999), Nevin and Lovley (2002)
Anaeromyxobacter	δ-Proteobacteria	Denitrifier or iron-reducer	Chlorinated organic compounds, organic acids	Treude et al. (2003), Sanford et al. (2002), He and Sanford (2004)
Desulfomonile	δ-Proteobacteria	Sulfate-reducer	Chlorinated organic compounds	Dolfing and Tiedje (1991)
Pelotomaculum	Clostridia	Fermentative	Organic acids, alcohols	Imachi et al. (2002)
Holophaga	Holophagae	Fermentative	Short-chain fatty acids, methylated aromatic compounds	Liesack et al. (1994), Chauhan and Ogram (2006)

Table 2 continued

Bacteria	Class	Respiration mode	Substrates	References
Peptococcaceae	Clostridia	Denitrifier, sulfate- or iron-reducer	Aromatic compounds	van der Zaan et al. (2012)
Microvirgula	β-Proteobacteria	Aerobic or denitrifier	Organic acids and alcohols	Patureau et al. (1998)
Curvibacter	β-Proteobacteria	Aerobic	Sugars, amino acids	Ding and Yokota (2010)
Nevskia	γ-Proteobacteria	Aerobic	Carbohydrates	Leandro et al. (2012)
Desulfovirga	δ-Proteobacteria	Sulfate-, thiosulfate-, or sulfite-reducer	Short-chain fatty acids, alcohols	Tanaka et al. (2000)

PCR kit as per manufacturer's specifications (Qiagen, Carlsbad, California). Thermal cycling was carried out at 94 °C for 3 min, followed by 30 cycles of (94 °C for 30 s, 55 °C for 30 s, 72 °C for 60 s) followed by 10 min at 72 °C and hold at 4 °C. PCR products were run on an agarose gel and the amplicons verified for size against DNA ladder and further excised for purification. Gel bands were purified using the Qiagen Qiaquick gel extraction kit as per manufacturer's specifications. Purified DNA was eluted with TE buffer, and quantified using Picogreen fluorescent DNA quantitation kit. 16S amplicons from different samples were pooled in equimolar concentrations prior to emulsion PCR and sequencing using Roche 454 Jr. Image data was processed using the Amplicon processing pipeline in the 454 Jr. (Roche, Branford, CT) suite of processing software included with the sequencer.

Raw sequencing data was initially processed using the Ribosomal Database Project (RDP) Pyrosequencing pipeline (http://pyro.cme.msu.edu) (Cole et al. 2009). Raw sequences were first sorted by barcode, and fusion primers were removed. Quality filter removed sequences with lengths less than 150 bases or having more than one ambiguity (*N*) or those with more than two changes in forward or reverse primers. Filtered, trimmed sequence data was then classified using RDP classifier at the genus level.

Results and discussion

Eight dominant phylogenetic classes constituted the groundwater microbial community: β-Proteobacteria, γ-Proteobacteria, δ-Proteobacteria, Bacilli, Clostridia, Acidobacteria, Nitrospira and Holophagae (Table 2). Prior to B20 fuel release, microaerophilic conditions prevailed with groundwater dissolved oxygen concentration of 0.6 mg L^{-1} and redox potential of +104 mV (Table 3). Such microaerophilic conditions likely favored the growth of aerobic facultative hydrocarbon degrading bacteria, including some *Janthinobacterium*, *Ralstonia*, *Pseudomonas* and *Geobacillus* species (Fig. 1). After 0.3 year following the fuel release, *Burkholderia* was the main bacterial genus detected. This bacterium is generally capable of degrading a variety of organic compounds (Philippe et al. 2001; Belova et al. 2006) (Table 2). Nonetheless, negligible BTEX and PAH removal was observed

Table 3 Temporal shifts in groundwater geochemical conditions at the source zone (2 m below ground surface)

Time (years)	Temperature (°C)	pH	Redox potential (mV)	DO (mg L^{-1})	NO_3^- (mg L^{-1})	NO_2^- (mg L^{-1})	Fe II (mg L^{-1})	SO_4^{2-} (mg L^{-1})	S^{2-} (mg L^{-1})	Acetate (mg L^{-1})	CH_4 (mg L^{-1})
0	21.8	4.3	+104	0.62	16.8	<0.1	6.1	2.2	0.01	<0.1	<0.005
0.3	21.1	3.9	+31	0.35	<0.1	<0.1	174.3	1.1	0.04	131	1.5
0.7	25.8	4.5	−36	0.77	<0.1	<0.1	13.9	0.8	0.01	5.3	17.5
1.0	27.2	4.8	−131	0.40	<0.1	<0.1	4.1	5.2	0.02	4.2	8.5
1.4	23.1	4.6	−77	0.26	<0.1	<0.1	4.5	0.9	0.01	16.8	3.7
2.0	26.6	4.9	−140	0.08	<0.1	<0.1	6.5	0.5	0.03	8.9	7.2
2.4	24.6	4.5	−73	0.4	<0.1	<0.1	9.2	0.5	0.30	3.9	6.2

during the time frame in which *Burkholderia* was predominant (Figs. 1, 2). Therefore, it is plausible that the proliferation of *Burkholderia* was primarily associated with the consumption of acetate or biodiesel methyl-esters.

Noticeable BTEX removal occurred at 0.7 year, considerably faster than under natural attenuation conditions (with a noticeable onset of BTEX biodegradation after 2.5 years) (Ramos et al. 2013). BTEX removal was accompanied by an increase in the relative abundance of *Desulfitobacterium* and *Geobacter* (from 5 to 52.7 and 15.8 to 37.3 % of total Bacteria 16S rRNA, respectively) (Fig. 1). These bacteria have been widely reported to participate in the anaerobic biodegradation of aromatic hydrocarbons under iron-reducing conditions (Lovley et al. 1993; Coates et al. 1995; Kunapuli et al. 2010), which is consistent with the observed accumulation of iron (II) (Table 3). *Geobacter* and *Desulfitobacterium* remained abundant even after the establishment of fermentative conditions (Fig. 2), suggesting that these organisms may have relied on fermentative metabolism as well (Cord-Ruwisch et al. 1998; Kunapuli et al. 2010). It should be noted that bacteria belonging to δ-Proteobacteria (iron and sulfate-reducing genera including *Geobacter*, *Pelobacter*, *Desulfovibrio*, *Desulfomicrobium*, *Desulfuromusa* and *Desulfuromonas*) were below detection limit (10^2 gene copies g^{-1}) in the experimental control plot not biostimulated (Ramos et al. 2013).

The predominance of methanogenic archaeal communities coincided with the establishment of methanogenic conditions in groundwater (i.e., 17.5 mg CH_4 L^{-1} measured at 0.7 year following the fuel release (Table 3)). Archaea community analysis revealed the presence of 7 different genera: *Thermoprotei*, *Methanosarcinaceae*, *Thermogymnomonas*, *Methanosaeta*, *Methanosarcina*, *Methanospirillum* and *Methanoregula* (Fig. 3). *Thermoprotei*, which can thrive using iron (III) as a terminal electron acceptor (Slobodkin 2005; Wagner and Wiegel 2008) were first detected after 0.3 year following the release (Fig. 1) and concomitantly with the prevalence of iron-reducing conditions (Fig. 2; Table 3). Both *Methanosaeta* and *Methanosarcina* are commonly found in acetate-rich methanogenic environments (Liu and Whitman 2008). The increased abundance of *Methanosarcina* compared to aceticlastic *Methanosaeta* (Liu and Whitman 2008) (Fig. 3) could be

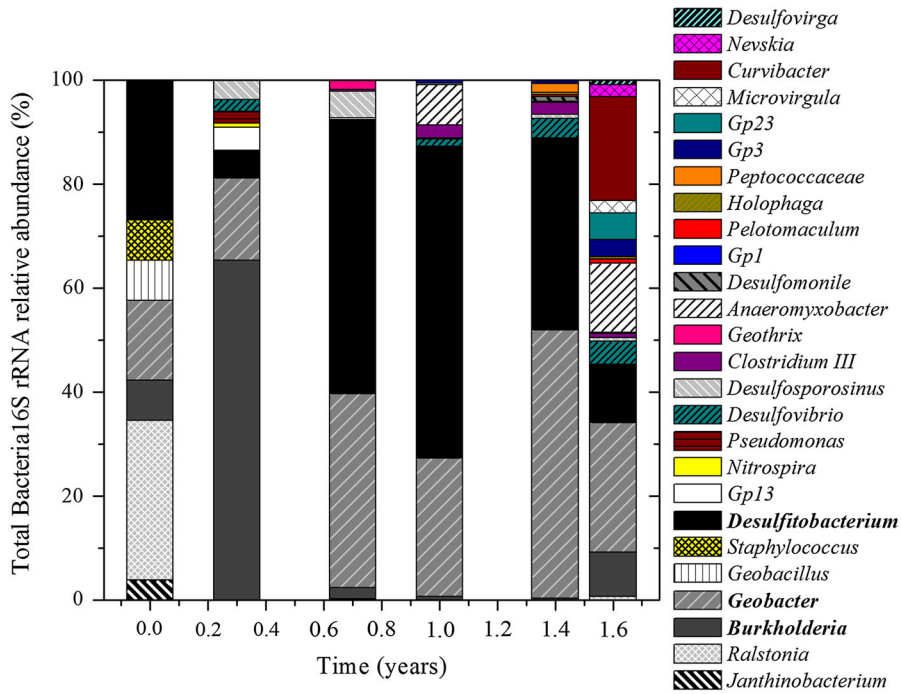

Fig. 1 Temporal changes in 16S rRNA relative abundance (%) of bacteria communities in groundwater samples from the B20 source zone

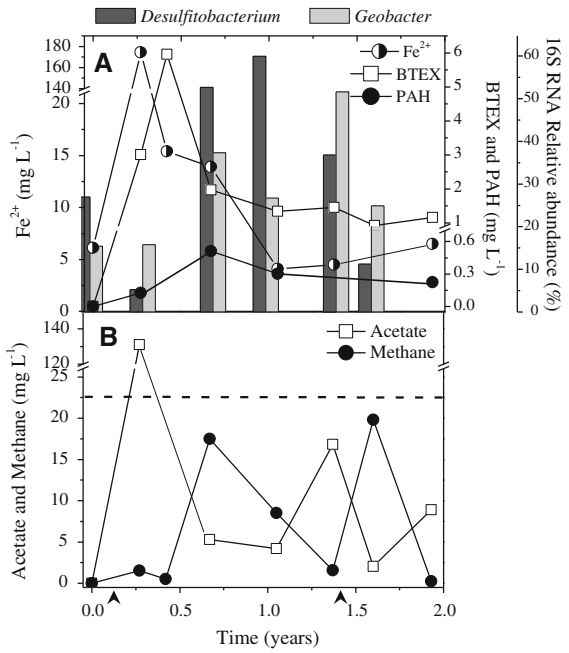

Fig. 2 **a** Iron (II), BTEX and total PAH concentrations and associated changes in the abundance of the putative aromatic hydrocarbon degraders *Geobacter* and *Desulfitobacterium,* expressed as a % of the total Bacteria 16S rRNA. **b** Acetate and methane profiles. The *dashed horizontal line* is the methane solubility limit (22 mg L^{-1} at 24 °C and 1 atm). *Arrows* indicate the start and the end of biostimulation with ammonium acetate

attributable to their faster growth rates when acetate is not limiting (Smith and Ingram-Smith 2007) and versatile metabolism (e.g., hydrogenotrophic, aceticlastic or methylotrophic while *Methanosaeta* is exclusively aceticlastic) (Galagan et al. 2002; Liu and Whitman 2008).

One year after the release, *Methanoregula* and *Methanospirillum* were detected in groundwater (Fig. 3). The presence of these hydrogenotrophic microorganisms (Worm et al. 2011; Bräuer et al. 2011) suggests their role as H_2 consumers that could alleviate the thermodynamic constraints caused by H_2 accumulation (Table 1, reactions 3, 7 and 11).

Syntrophic cooperation between aromatic hydrocarbons degraders and methanogens

Syntrophic relationships allow microorganisms to thrive in energetically constrained systems (e.g., methanogenic environments). To discern whether a biological process meets the requirements for being considered syntrophy, one may consider the reactions involved and discern whether they are feasible without microbial cooperation (Morris et al. 2013). Table 1 summarizes the need for syntrophic cooperation to enhance the thermodynamic feasibility of biodiesel, BTEX and PAH biodegradation.

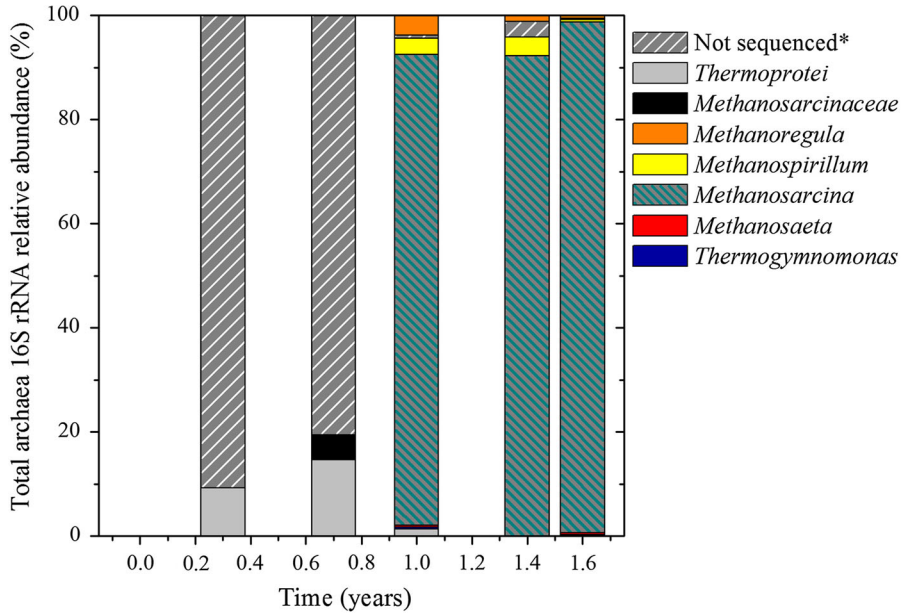

Fig. 3 Temporal changes in 16S rRNA relative abundance (%) of Archaea communities in groundwater samples from the B20 source zone

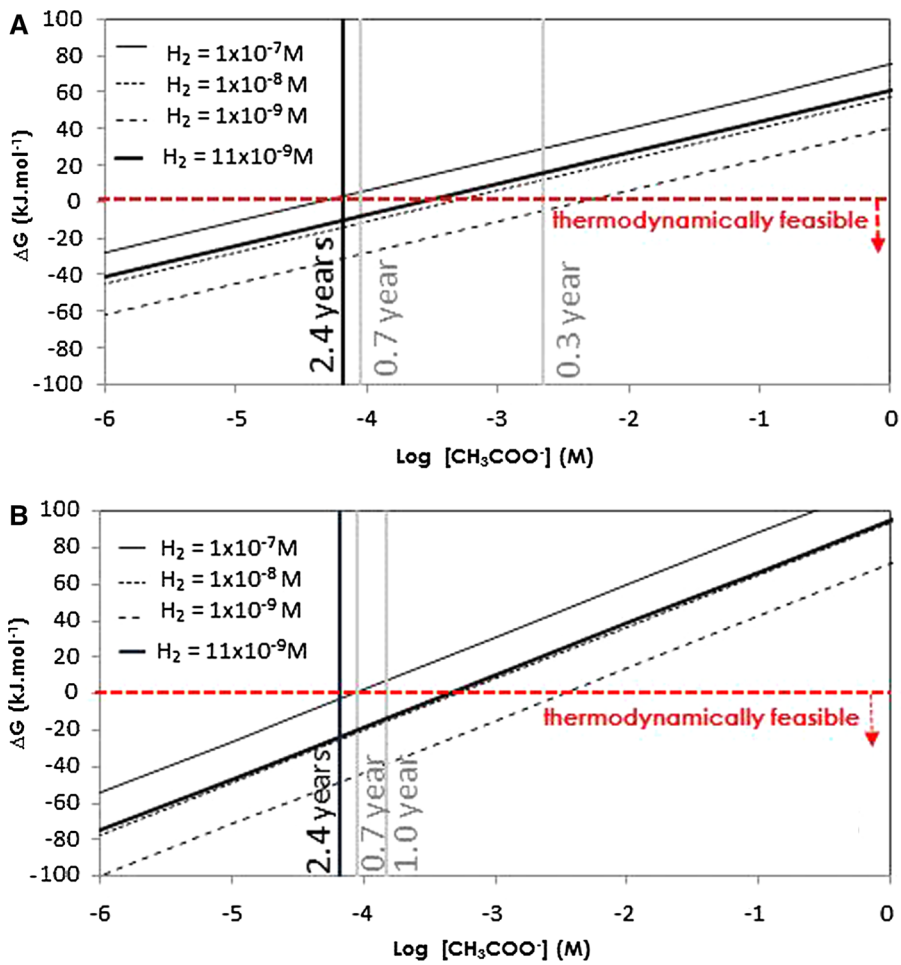

Fig. 4 Thermodynamic feasibility of benzene (0.9 mg L^{-1}) (**a**) and naphthalene (0.16 mg L^{-1}) (**b**) fermentation to acetate and hydrogen (Table 1, reactions 5 and 9) for typical H_2 concentrations of 10^{-7} to 10^{-9} M (*grey diagonal lines*), dissolved H_2 concentration detected in groundwater at 2.4 years (*black diagonal lines*) and pH = 4.5. *Grey vertical lines* represent acetate concentration at 0.3 (131 mg L^{-1}), 0.7 (5 mg L^{-1}) and 1.0 year (8.9 mg L^{-1}) after the release. *Black vertical lines* correspond to acetate concentration at 2.4 years (3.9 mg L^{-1})

Thermodynamic calculations considered the highest contaminant concentrations (0.9 mg L^{-1} for benzene and 0.16 mg L^{-1} for naphthalene) and typical dissolved H_2 concentrations found in anaerobic aquifers (10^{-7} to 10^{-9} M) (Heimann et al. 2009), as well as dissolved H_2 concentrations detected in groundwater 2.4 years after the release. As shown in Fig. 4a, the presence of 131 mg L^{-1} of acetate (at 0.3 year) hindered fermentative/methanogenic BTEX biodegradation (represented by benzene) for dissolved H_2 concentrations $\geq 0.2 \times 10^{-8}$ M. With a decrease in acetate concentrations from 131 to 5 mg L^{-1} (measured at 0.7 year) benzene biodegradation became thermodynamically feasible. These predictions were in agreement with the observed in situ BTEX removal pattern shown in Fig. 2. Biodegradation of PAH (represented by naphthalene, 0.16 mg L^{-1}) was thermodynamically feasible for almost all H_2 concentrations except 10^{-7} M (at 1.0 year) (Fig. 4b).

Conclusions

This assessment of microbial community structure over time advances our understanding of the syntrophic metabolic niches that evolve during fermentative–methanogenic bioremediation of aromatic hydrocarbons. Microbial population shifts were consistent with observed geochemical changes and dominance shifted towards the putative anaerobic hydrocarbon degraders *Geobacter* and *Desulfitobacterium*. The accumulation of anaerobic metabolites acetate and H_2 that could thermodynamically hinder BTEX and PAH biodegradation was likely alleviated by the proliferation of *Geobacter*, *Desulfitobacterium* and hydrogenotrophic or aceticlastic *Methanosarcina*. Overall, this study suggests that fermentative–methanogenic biostimulation can promote favorable microbial population shifts that enhance the natural attenuation of B20 blend releases.

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