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ORIGINAL PAPER

Biostimulation of anaerobic BTEX biodegradation under fermentative methanogenic conditions at source-zone groundwater contaminated with a biodiesel blend (B20)

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Abstract Field experiments were conducted to assess the potential for anaerobic biostimulation to enhance BTEX biodegradation under fermentative methanogenic conditions in groundwater impacted by a biodiesel blend (B20, consisting of 20 % v/v biodiesel and 80 % v/v diesel). B20 (100 L) was released at each of two plots through an area of 1 m^2 that was excavated down to the water table, 1.6 m below ground surface. One release was biostimulated with ammonium acetate, which was added weekly through injection wells near the source zone over 15 months. The other release was not biostimulated and served as a baseline control simulating natural attenuation. Ammonium acetate addition stimulated the development of strongly anaerobic conditions, as indicated by near-saturation methane concentrations. BTEX removal began within 8 months in the biostimulated source zone, but not in the natural attenuation control, where BTEX concentrations were still increasing (due to source dissolution) 2 years after the release. Phylogenetic analysis using quantitative PCR indicated an increase in concentration and relative abundance of Archaea (Crenarchaeota and Euryarchaeota), *Geobacteraceae* (*Geobacter* and *Pelobacter* spp.) and sulfate-reducing bacteria (*Desulfovibrio*, *Desulfomicrobium*, *Desulfuromusa*, and *Desulfuromonas*) in the biostimulated plot relative to the control. Apparently, biostimulation fortuitously enhanced the growth of putative anaerobic BTEX degraders and associated commensal microorganisms that consume acetate and H_2, and enhance the thermodynamic feasibility of BTEX fermentation. This is the first field study to suggest that anaerobic-methanogenic biostimulation could enhance source zone bioremediation of groundwater aquifers impacted by biodiesel blends.

Keywords Acetate · Biodiesel · Biostimulation · BTEX · Methanogenic · Natural attenuation

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Introduction

Environmental concerns associated with fossil fuels have encouraged the use of renewable transportation fuels such as biodiesel, which was added to the Brazilian energy matrix in 2005 (Law 11.097). Since 2010 the content of biodiesel in diesel is 5 % on a

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

Reactions	$\Delta G^{\circ\prime}$ (kJ mol^{-1})
(1) β-Oxidation of LCFA (oleate $C_{18:1}$): $C_{18}H_{33}O_2 + 16H_2O \rightarrow 9CH_3COO^- + 8H^+ + 15H_2$	+390.8
(2) Benzene fermentation: $C_6H_6 + 6H_2O \rightarrow 3CH_3COO^- + 3H^+ + 3H_2$	+73.8
(3) Syntrophic acetate oxidation: $CH_3COO^- + 4H_2O \rightarrow 2HCO_3^- + 4H_2 + H^+$	+104.6
(4) Reductive acetogenesis: $4H_2 + 2HCO_3^- + H^+ \rightarrow CH_3COO^- + 4H_2O$	−104.6
(5) Hydrogenothrophic methanogenesis: $4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O$	−135.6
(6) Aceticlastic methanogenesis: $CH_3COO^- + H_2O \rightarrow HCO_3^- + CH_4$	−31.0

(1) Sousa et al., 2009; (2) Rakoczy et al., 2011; (3, 4, 5 and 6) Hattori et al., 2008 and Lee and Zinder, 1988. Standard Gibbs energies in aqueous solution, pH = 7, 25 °C, 1 M solute concentration and gas partial pressure of 1 atm

volumetric basis (Brazil 2009) and is likely to increase to 20 % in the near future. The increasing use of biodiesel could lead to a higher frequency of groundwater contamination by accidental and incidental releases during transportation, storage and use. While biodiesel itself does not pose significant hazards to public health and is readily biodegradable (National Contaminants Standard for Biodiesel 2003; Pasqualino et al. 2006), new biodiesel formulations (i.e., B5 and B20) contain BTEX (benzene, toluene, ethylbenzene and xylenes isomers) or PAHs (polycyclic aromatic hydrocarbons), which can become co-occurring contaminants that pose a health risk and require remedial action.

The high biochemical oxygen demand (BOD) exerted by biofuel blends in impacted aquifers generally results in the development of strongly anaerobic conditions (Mackay et al. 2006; Feris et al. 2008; Corseuil et al. 2011b). The theoretical BOD of biodiesel is twice that exerted by ethanol (Gomes 2008), which suggests a higher tendency to drive the system towards strongly anaerobic (methanogenic) conditions. Although BTEX biodegradation is known to occur under methanogenic conditions (Grbic-Galic and Vogel 1987; Kazumi et al. 1997; Weiner and Lovley 1998; Ulrich and Edwards 2003; Reinhard et al. 2005), such processes are generally much slower and offer a more narrow catabolic range than aerobic biodegradation. Consequently, limited attention has been given towards the stimulation of such anaerobic biodegradation processes for the cleanup of regular groundwater fuel spills. Nevertheless, aerobic biostimulation is not universally applicable and the high BOD exerted by biodiesel could make the delivery of sufficient oxygen highly unfeasible. Thus, the feasibility of anaerobic biostimulation strategies should be assessed for the cleanup of biodiesel blend releases, particularly near the source zone which has a high propensity to become anaerobic. Furthermore, 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. 2011b), which motivates a focus on source zone bioremediation.

Under methanogenic conditions, bioremediation is accomplished by complex microbial food webs involving syntrophic interactions. Volatile fatty acids such as acetic acid and H_2 produced during the fermentation of organic compounds can accumulate transiently in methanogenic environments and inhibit BTEX biodegradation (Ahring and Westermann 1988; Cozzarelli et al. 1994; Corseuil et al. 2011a). Syntrophic interactions such as interspecies H_2 transfer and commensal acetate consumption are important to enable thermodynamic feasibility (Cord-Ruwisch et al. 1998; McInerney et al. 2008; Stams and Plugge 2009), as the fermentation of biodiesel and BTEX are unfeasible under standard conditions (Table 1, reactions 1, 2) (McInerney et al. 1979). Commensal syntrophs oxidize acetate to hydrogen and carbon dioxide (reaction 3). The reverse reaction (reductive homoacetogenesis) may also occur at low acetate concentrations. Homoacetogenic bacteria can use carbon dioxide and hydrogen to produce acetate (reaction 4), which in turn may be converted by aceticlastic methanogens to methane (reaction 6). Other important syntrophs include hydrogenotrophic methanogens and iron- or sulfate-reducers that can consume hydrogen (reaction 5), making the overall

reaction exergonic (Hattori 2008; Lee and Zinder 1988). Nonetheless, whether broad stimulation of such microbial communities could exert beneficial effects on anaerobic BTEX degradation has not been demonstrated. In particular, it is unknown whether the positive effect of common carbon sources (e.g., acetate) on microbial growth, which is conducive to faster degradation rates, outweighs potential inhibitory effects on BTEX metabolic flux and biodegradation rate per unit cell (Lovanh and Alvarez 2004).

In this work, anaerobic biostimulation was assessed as an approach to accelerate BTEX biodegradation under fermentative-methanogenic conditions at source-zone groundwater impacted by biodiesel (B20). Ammonium acetate, which is a low-cost, readily available, soluble substrate that is amenable for easy injection, was chosen as the biostimulatory compound. A natural attenuation experiment was conducted in parallel to discern the effects of biostimulation on the growth of selected microbial populations and BTEX removal.

Materials and methods

Field experiments

Controlled biodiesel releases were conducted at the Ressacada Experimental Farm in Florianópolis, SC, Brazil (latitude 27°30′S, longitude 48°30′W). Regional geology is characterized by unconsolidated deposits of aeolian, alluvial, lacustrine and marine sands (Lage 2005). The subsurface layer is composed by 80 % of gray fine sand, 5 % silt and less than 5 % of clay. Soil organic carbon ranges between 0.16 and 0.68 %. The groundwater flow velocity is 6 m year^{-1} and effective porosity between 0.17 and 0.20. The climate is mesothermic humid with a 78 mean annual precipitation of 1,600 mm. Average groundwater temperature is 26 °C in the summer and 22 °C in the winter.

Two release experiments were conducted in parallel and in neighboring areas (Fig. 1). A natural attenuation (control) experiment started 2 years prior to the start of the biostimulation experiment. BTEX concentrations at the start of the experiment and in background wells were consistently below detection limits in each plot, indicating minimal potential for cross contamination. Source zones for both experiments were established by releasing 100 L of B20 (20 % biodiesel and 80 % diesel) into a source-zone area of 1 × 1 × 1.6 m deep down to the water table. Each experiment covered a monitored area of 330 m^2 with a total of 46 and 41 monitoring wells installed in the natural attenuation and biostimulation plot, respectively. Each well contained a bundle of 3/16″ ID polyethylene tubing to allow groundwater sampling at different depths [2, 3, 4, 5 and 6 m below ground surface (BGS)]. Biostimulation was performed by injecting ammonium acetate (300 mg L^{-1}) on a weekly basis. A total of 25 L was injected per week, by delivering 1 L to each of the sampling depths (2, 3, 4, 5 and 6 m BGS) to each of the five injection wells installed upstream of the source zone (Fig. 1). Whether biostimulation was evenly achieved throughout the source zone transect was not determined. Both natural attenuation and biostimulation plots were covered with gravel and tarp to minimize rainfall infiltration and potential confounding effects on NAPL dissolution and source zone mobilization.

Groundwater analyses

Samples were collected from monitoring wells at depths 2, 3, 4, 5 and 6 m below ground surface. A peristaltic pump and Teflon tubing was used to collect groundwater samples into capped sterile vials without headspace. BTEX and methane were analyzed by gas chromatography using a GC HP model 6890 II equipped with a flame ionization detector (FID), HP 1 capillary column (30 cm × 0.53 mm × 2.65 mm) and HP 7694 headspace auto sampler. Redox potential, pH, dissolved oxygen and temperature were measured on site using a Micropurge Flow Cell (MP20). Nitrite, nitrate, sulfate and acetate analyses were performed by ion chromatography using a Dionex ICS-1000 equipped with a conductivity detector and an AS14A column. Iron (Fe^{2+}) and sulfide (S^{2-}) were analyzed using a spectrophotometer (DR/2500, Hach), according to the 1.10 phenanthroline and colorimetric methylene blue method, respectively (American Public Health Association 1992). Detection limits were 1 μg L^{-1} for BTEX, 10 μg L^{-1} for methane, 5 μg L^{-1} for sulfide, 0.01 mg L^{-1} for Fe^{2+}, 0.1 mg L^{-1} for nitrite, nitrate, sulfate and acetate and 0.2 mg L^{-1} for dissolved oxygen.

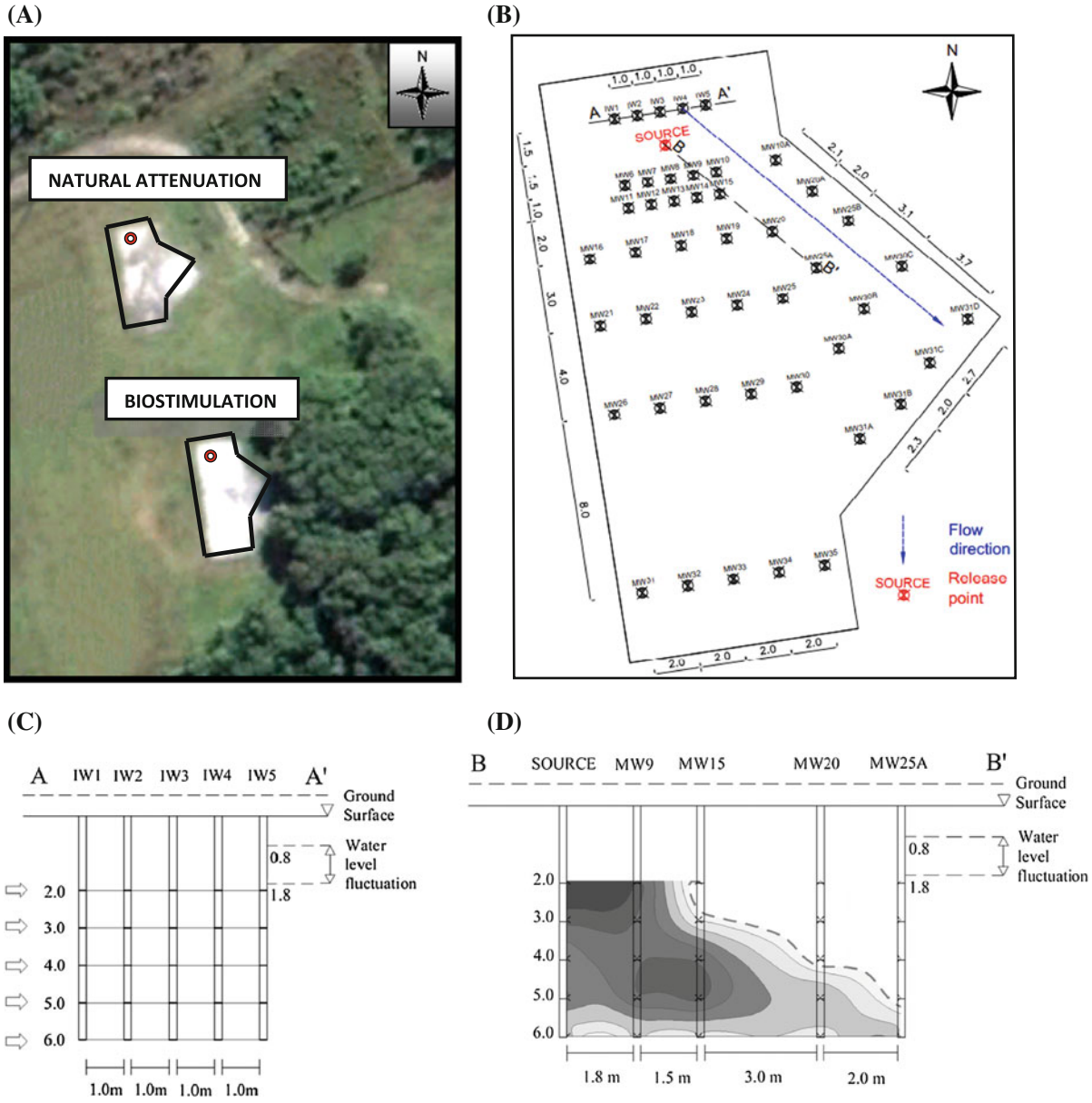

Fig. 1 Plan view depicting the location of natural attenuation and biostimulation field experiments (**a**), schematic view of experimental areas configuration (**b**), cross sections of transect *A*–*A*′ (**c**) and *B*–*B*′ (**d**) from biostimulation plot. *Arrows* in transect *A*–*A*′ (**c**) indicate the sampling depths where ammonium acetate was injected. The plume in transect *B*–*B*′ (**d**) illustrates the vertical distribution of dissolved BTEX in the biostimulated plot, after 1.6 years following the release. All distances are given in meters

Microbial analysis

Real-time quantitative polymerase chain reaction (qPCR) was used to estimate the concentration of total Bacteria to evaluate biomass growth, *Deltaproteobacteria*—including *Geobacter, Pelobacter, Desulfovibrio*, *Desulfomicrobium*, *Desulfuromusa* and *Desulfuromonas*—to assess the presence and contribution of iron and sulfate reducers and Archaea (groups Crenarchaeota and Euryarchaeota), which includes methanogens. These microorganisms were chosen based on previous studies that reported their association with anaerobic

Table 2 Primers and probe sequences used for qPCR

Target group	Forward primer	Reverse primer	Probe
Total bacteria[a]	5′CGGTGAATACGTTCYCGG3′ (BACT 1369F)	5′GGWTACCTTGTTACGACTT3′ (PROK1492R)	FAM-5′CTTGTACACACCGCC CGTC3′-BHQ-1 (TM1389F)
Geobacteraceae[b]	5′-GCG TGT AGG CGG TTT CTT AA-3′ (561F)	5′-TAC CCG CRA CAC CTA GTT CT-3′ (825R)	**Gbc2** 5′-/56-FAM/CTC AAC CCA GGA AGT GCA TTG GAT AC/36-TAMSp/-3′
Sulfate-reducing bacteria[b,c]	5′-AAG CCT GAC GCA SCA A -3′ (361F)	5′-ATC TAC GGA TTT CAC TCC TAC A -3′ (685R)	**EUB1** 5′/56-FAM/GTA TTA CCG CGG NTG CTG GC/36-TAMSp/-3′
Archaea[a]	5′CGGTGAATACGTCCCTGC3′ (ARCH1-1369F)	5′AAGGAGGTGATCCTGCCGCA3′ (PROK1541R)	FAM-5′CTTGTACACACCGCC CGTC3′-BHQ-1 (TM1389F)
	5′CGGTGAATATGCCCCTGC3′ (ARCH2-1369F)		

[a] Primers sequences based on the work of Silva and Alvarez (2004)

[b] Primers sequences based on the work of Stults et al. (2001)

[c] Sulfate-reducing bacteria (SRB) genes were quantified using the probe EUB1 with the primers 361F and 685R, capable of targeting ∂-Proteobacteria [complementary to many iron- and sulfate-reducing genera including *Geobacter*, *Pelobacter* (including fermentative species), *Desulfovibrio*, *Desulfomicrobium*, *Desulfuromusa*, and *Desulfuromonas* (including dissimilatory S reducers)]

hydrocarbons biodegradation (Ulrich and Edwards 2003; Caldwell and Suflita 2000; Lovley 1997; Muyzer and Stams 2008; Coates et al. 2001; Silva and Alvarez 2004). Primer sequences used for each group are given in Table 2. A total of 5 and 3 sampling events were conducted at the source zone to collect groundwater from the biostimulation and natural attenuation experiment, respectively.

Groundwater samples were filtered with Millipore membrane (polyethersulfone, hydrophilic), 0.22 μm pore size. DNA was extracted according to the MoBio Power Soil™ kit (Carlsbad, CA) protocol. PCR mixtures contained 1× Taqman PCR Master Mix or SYBR GREEN (Applied Biosystems, Foster City, CA, USA); 500 nM forward and reverse primers, 250 nM of the probe (for reactions using Taqman) and sterile DNAase-free water to make up a final volume of 25 μL. PCRs were performed using a Eppendorf (Model Mastercycler® ep realplex Thermal Cyclers, CA, USA) with the following temperature conditions: 50 °C for 2 min, followed by 95 °C for 10 min and 40 cycles at 95 °C for 15 s, and 58 °C for 1 min.

To estimate microbial communities concentration, standard curves were performed by serial dilutions with the standard DNA of the following microorganisms: *Pseudomonas aeruginosa* (10^1–10^6 gene copies, $r^2 = 0.99$), *Geobacter metallireducens* (10^1–10^6 gene copies, $r^2 = 0.99$), *Methanococcus maripaludis* (10^1–10^5 gene copies, $r^2 = 0.99$) for the quantification of total Bacteria, *Geobacteraceae* and sulfate-reducing bacteria (SRB), and Archaea, respectively. The detection limit of each assay was about 10^2 gene copies g^{-1}.

Results and discussion

The injection of ammonium acetate (as supplementary carbon and nitrogen source) into the source zone of the B20 release accelerated the development of fermentative methanogenic conditions, as intended. This is reflected by the rapid decrease in oxidation–reduction potential (ORP) (from +161 to −89 mV after 1 year) compared to natural attenuation control (from +430 to −20 mV after 2.4 years) (Fig. 2a). Furthermore, methane concentrations in the biostimulated plot reached near-saturation levels (≈22 mg L^{-1} at 24 °C) within 0.7 years, whereas the maximum concentration measured in the natural attenuation plot was 10 mg L^{-1}, 2.4 years after the release (Fig. 2b). Aceticlastic methanogens apparently played an important role in methane generation since methane levels tended to increase with decreasing acetate concentrations (Fig. 2c).

Ammonium acetate is a commonly used compound for stimulating fermentation processes (Monot et al. 1982; Ladisch 1991). Although discerning the contribution of acetate versus ammonium in the observed biostimulation was beyond the scope of this project, a previous study to enhance fermentation processes

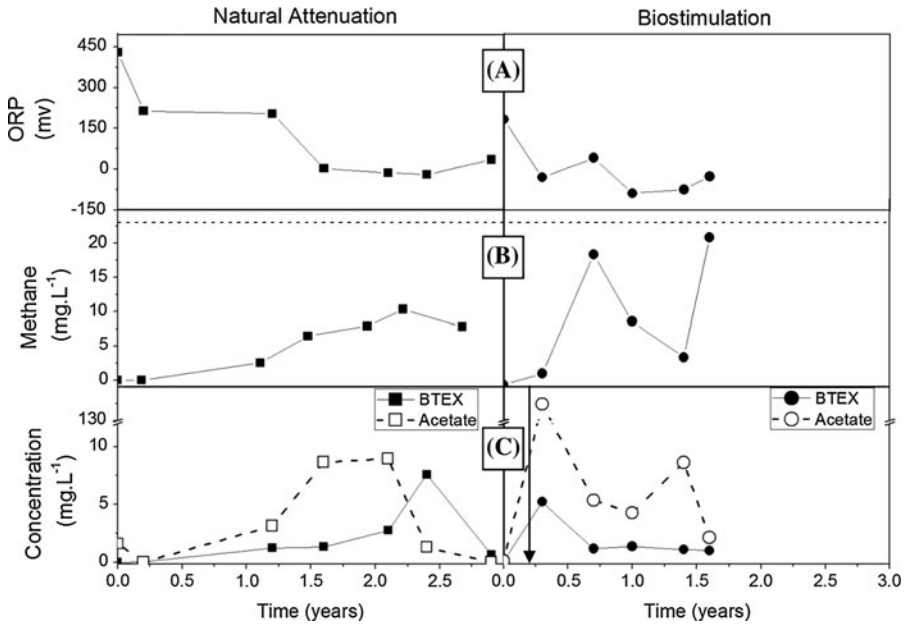

Fig. 2 Geochemical footprint of B20 releases at the source zones of the biostimulated and natural attenuation plots. The *figure* shows **a** redox potential; **b** methane concentrations [*dotted horizontal lines* represent methane saturation limit in groundwater ($\approx$22 mg L^{-1})]; and **c** BTEX and acetate concentrations, at 2 m below ground surface. *Arrow* indicates start of ammonium acetate injection

concluded that neither acetate nor ammonium alone was as effective as their concurrent addition (Gu et al. 2009).

BTEX biodegradation at the source zone occurred predominantly under fermentative methanogenic conditions. Background terminal electron acceptors (e.g., oxygen, nitrate, sulfate and iron) were rapidly depleted after the B20 release (i.e., from 0.5 to 0.2 mg L^{-1} for dissolved oxygen, from to 8.5 to 0.2 mg L^{-1} for nitrate, and from 3.4 to 2.7 mg L^{-1} for sulfate), which was corroborated by the low concentrations of reduced species that indicate the consumption of anaerobic electron acceptors (i.e., 0.1 mg L^{-1} for sulfide and 6 mg L^{-1} for ferrous iron).

BTEX concentrations in groundwater reflect the outcome of multiple processes such as source dissolution, migration (including dispersion and dilution), abiotic losses (e.g., sorption and volatilization) and biodegradation (Alvarez and Illman 2006). The latter process, which is of critical importance to remedial action, can be influenced positively or negatively by the presence of alternative carbon sources. At the individual cell level, common substrates such as acetate can hinder biodegradation by exerting metabolic flux dilution and catabolic repression (Lovanh et al. 2002). Furthermore, if the co-substrate is an intermediate in the degradation pathway of the target pollutant (as is also the case for acetate), thermodynamic inhibition could also occur (Bradley and

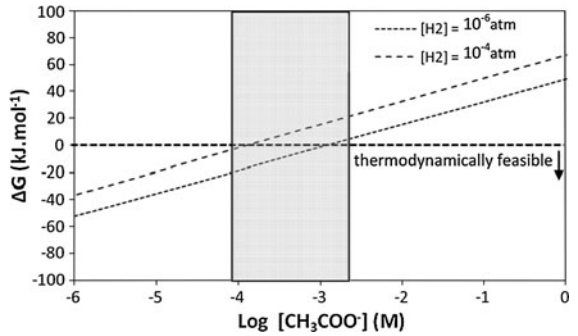

Fig. 3 Thermodynamic feasibility of benzene fermentation (0.9 mg L^{-1}) to acetate and hydrogen (reaction 2) for a range of likely H_2 concentrations (10^{-4}–10^{-6} atm). *Shaded area* indicates the range of acetate concentration (max: log−2.7 M and min: log−4.1 M) measured in the biostimulated source zone

McInerney 2002; Dolfing et al. 2008; Corseuil et al. 2011a; Rakoczy et al. 2011). Theoretical calculations, using the highest benzene concentration observed (900 μg L^{-1} at $t = 0.3$ years) and a range of plausible hydrogen concentrations in anaerobic aquifers (10^{-6} and 10^{-4} atm) (Kotelnikova and Pedersen 1997; Heimann et al. 2009), suggests that even at a H_2 partial pressure of 10^{-6} atm—which is the minimum needed to support hydrogenotrophic methanogens (Thauer et al. 1977)—acetate accumulation at 75 mg L^{-1} or higher would hinder BTEX biodegradation, as reaction 2 becomes endergonic (Fig. 3). This may explain the limited BTEX removal observed

when acetate concentrations were higher than this theoretical inhibitory threshold (Fig. 2c). Nevertheless, this negative effect can be eventually offset as acetate is degraded below inhibitory thresholds and its consumption promotes microbial growth (including the fortuitous growth of specific degraders), which is conducive to faster degradation rates (Ruiz-Aguilar et al. 2003; Lovanh and Alvarez 2004; Silva and Alvarez 2004).

Accordingly, acetate (either amended or produced as an anaerobic metabolite) was apparently biodegraded preferentially in both releases, leading to a temporary decrease in BTEX consumption and a (dissolution-related) increase in BTEX concentrations (Fig. 2c). Nevertheless, the consumption of acetate enhanced biomass growth, represented by total Bacteria, in the biostimulated plot (from 9.3×10^5 to 9.3×10^8 gene copies g^{-1} after 1.6 years) as well as sulfate-reducing bacteria and *Geobacter* spp. (Fig. 4) that have been associated with BTEX degradation (Coates et al. 1996; Anderson et al. 2003; Silva and Alvarez 2004). Many sulfate reducers can degrade BTEX fermentatively in the absence of sulfate (Bryant et al. 1977; Lengeler et al. 1999), and it is likely that such putative BTEX degraders (including perhaps some *Geobacter* spp.) contributed to the remediation process. The targeted sulfate reducers and *Geobacter* spp. represented only 0.63 and 1.15 % of the total Bacteria, respectively (Fig. 4), which is low in terms of relative abundance but nonetheless significant compared to the natural attenuation plot, where such putative BTEX degraders were not detected ($<10^2$ gene copies g^{-1}). Furthermore, Archaea (e.g., methanogens) likely participated commensally in BTEX degradation (Table 1, reactions 5, 6). Their proliferation after biostimulation (up to 3.7×10^8 gene copies g^{-1}) is in agreement with the observed BTEX removal and methane production (Figs. 2, 4). Although a clear etiology between specific bacterial species and biodegradation processes could not be established, the higher concentrations of putative BTEX degraders are chronologically consistent with the faster BTEX removal observed in the biostimulated plot.

A significant decrease in BTEX concentrations was noticeable 0.7 years after biostimulation, whereas significant BTEX removal in the natural attenuation plot started later, 2.9 years after the release (Fig. 2c). Peak dissolved BTEX concentrations at the source

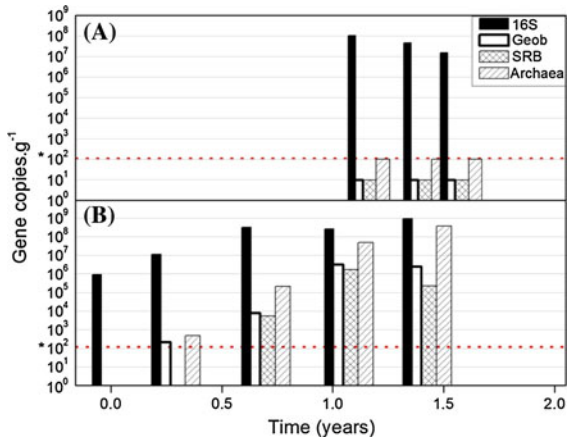

Fig. 4 Concentrations of total bacteria (16S), *Geobacteraceae*, sulfate-reducing bacteria (SRB) and Archaea, at the source zone of the natural attenuation (**a**) and biostimulation (**b**) plots. *Detection limit for the microbial analysis were 10^2 gene copies g^{-1} (*red dotted line*). (Color figure online)

zone were also lower in the biostimulated plot (5,178 μg L^{-1} at $t = 0.3$ years) than in the natural attenuation control (12,487 μg L^{-1} at $t = 2.4$ years). Among BTEX compounds, benzene typically determines the need for corrective actions due to its carcinogenic potential and stringent action levels (US EPA 1998). Although it is relatively recalcitrant under anaerobic conditions (Kazumi et al. 1997; Anderson et al. 1998; Chakraborty and Coates 2004), benzene concentrations decreased significantly in the anaerobic source zones of both releases, from 900 to 32 μg L^{-1} in the biostimulated plot after 1.6 years, and from and 975 to 135 μg L^{-1} in the natural attenuation control after 2.9 years. The faster benzene removal in the biostimulated plot corroborates the potential benefit of ammonium acetate addition. Hydraulic effects that could confound the influence of biostimulation on contaminant removal (e.g., dilution by rainfall) were dismissed because the plots were covered with gravel and tarp to avoid direct recharge. Moreover, the injection of 25 L per week of solution into the groundwater would have negligible effects on source zone dislodgement and groundwater flow characteristics. This amendment represents <1 % of the groundwater flowing through the plot area.

Overall, BTEX removal was faster in the biostimulated plot, which was attributed to the observed fortuitous growth of putative BTEX degraders as well as commensal populations that would consume acetate and other fermentation byproducts below thermodynamically and

metabolically inhibitory levels. This is the first field scale study to demonstrate the potential for stimulating anaerobic fermentative/methanogenic conditions to enhance the cleanup of groundwater contaminated with biodiesel blends, and suggests that this approach should be considered for source zone BTEX bioremediation when the high BOD encountered at these contaminated sites makes aerobic biostimulation unfeasible.

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