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Combined iron and sulfate reduction biostimulation as a novel approach to enhance BTEX and PAH source-zone biodegradation in biodiesel blend-contaminated groundwater

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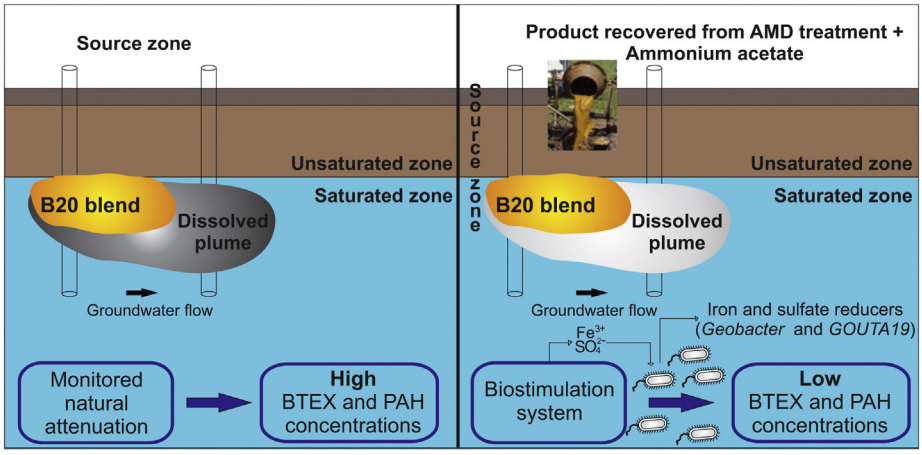
HIGHLIGHTS

- Two long-term controlled biodiesel blend field experiments were monitored.
- Combined iron and sulfate reduction processes were stimulated in one experiment.
- BTEX and PAH source-zone degradation was enhanced relative to natural attenuation.
- *Geobacter* spp. and *GOUTA19* spp. played a key role in B20 source-zone biodegradation.
- Biostimulation with AMD-derived product can cleanup B20-contaminated groundwater.

GRAPHICAL ABSTRACT



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ABSTRACT

The use of biodiesel as a transportation fuel and its growing mandatory blending percentage in diesel increase the likelihood of contaminating groundwater with diesel/biodiesel blends. A 100L-field experiment with B20 (20% biodiesel and 80% diesel, v/v) was conducted to assess the potential for the combined biostimulation of iron and sulfate reducing bacteria to enhance BTEX and PAH biodegradation in a diesel/biodiesel blend-contaminated groundwater. A B20 field experiment under monitored natural attenuation (MNA) was used as a baseline control. Ammonium acetate and a low-cost and sustainable product recovered from acid mine drainage treatment were used to stimulate iron and sulfate-reducing conditions. As a result, benzene and naphthalene concentrations (maximum concentrations were 28.1 $\mu g\,L^{-1}$ and 10.0 $\mu g\,L^{-1}$, respectively) remained lower than the MNA experiment (maximum concentrations were 974.7 $\mu g\,L^{-1}$ and 121.3 $\mu g\,L^{-1}$, respectively) over the whole experiment.

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Geochemical changes were chronologically consistent with the temporal change of the predominance of *Geobacter* and *GOUTA19* which might be the key players responsible for the rapid attenuation of benzene and naphthalene. To the best of our knowledge, this is the first field experiment to demonstrate the potential for the combined iron and sulfate biostimulation to enhance B20 source-zone biodegradation.

1. Introduction

The growing use of biodiesel as a transportation fuel and its current mandatory blending percentage of 8% in diesel (predicted to reach 15% by 2020 [1]) will increase the likelihood of groundwater contamination by diesel/biodiesel blends through accidental or incidental spills. Although biodiesel is biodegradable [2,3], it can contain BTEX (benzene, toluene, ethyl-benzene and xylenes) and PAH (polycyclic aromatic hydrocarbons) when blended with diesel, which are priority contaminants that include recalcitrant and carcinogenic compounds (*i.e.* benzene and benzo[a]pyrene) and require remedial action when released into the environment. Furthermore, when biodiesel is blended with diesel, biodiesel can be preferentially biodegraded [4,5] and thus, hinder BTEX degradation [5–8]. Therefore, remediation strategies need to be developed for the cleanup of diesel spills when amended with biodiesel. The high biochemical oxygen demand exerted by biodiesel blends typically leads to anoxic zones [6,9,10] that favor anaerobic strategies for treating biodiesel-diesel releases to groundwater.

Anaerobic approaches have been applied for the remediation of diesel-contaminated aquifers [11–14]. Field experiments with diesel/biodiesel blends amended with ammonium acetate to promote methanogenic conditions demonstrated an enhancement of BTEX biodegradation compared to monitored natural attenuation (MNA) [10]. Nevertheless, methanogenic processes are energetically less favorable and lead to slower biodegradation rates relative to other anaerobic approaches such as iron or sulfate reduction [15]. BTEX and PAHs biodegradation have been widely reported under iron [14,16–19] and sulfate reduction [11,20–26], while the combined application of both these terminal electron-accepting processes (TEAP) to cleanup BTEX and PAHs-contaminated groundwater has not yet been demonstrated.

Laboratory studies have shown that biodiesel fatty acid methyl esters (FAMEs) were efficiently biodegraded under nitrate-reduction [27], sulfate-reduction [28] and methanogenesis [29]. Biodiesel hydrolyzed metabolites, such as long-chain fatty acids (LCFA), were oxidized by obligate syntrophs [30,31], iron (III) [32] and sulfate reducers [33,34]. Furthermore, a microcosm study indicated that methanogens, sulfate and nitrate-reducers could all be implicated in diesel/biodiesel biodegradation [9]. Although iron and sulfate reducers demonstrated potential for metabolizing biodiesel and diesel compounds, the combined application of iron and sulfate reduction have yet to be shown to speed up bioremediation of BTEX and PAH in source-zone of diesel/biodiesel blend-contaminated groundwater.

In this study, a field experiment was conducted to assess the combined biostimulation of iron and sulfate reduction as a novel approach to enhance BTEX and PAH source-zone biodegradation of a B20 blend. Since biodiesel is not readily miscible and dissolves slowly into the groundwater, it exerts long-term effects over a relatively small region of influence [6] that thereby supports our focus on source-zone biodegradation. A low-cost and sustainable product recovered from acid mine drainage was used to stimulate both iron and sulfate reducing conditions. Microbial communities were assessed to discern key players associated with B20 anaerobic (iron and sulfate reduction) biodegradation. To the best of our knowledge, this is the first field experiment to demonstrate the potential of combined iron and sulfate reduction TEAP to enhance the cleanup of BTEX and PAH in a biodiesel blend (B20)-contaminated groundwater.

2. Materials and methods

2.1. Site description

The field experiment was conducted at Ressacada Experimental Farm, in Florianópolis, SC, Brazil (Latitude: 27°30′S, Longitude: 48°30′W). Regional geology is characterized by unconsolidated deposits of eolian, alluvial, lacustrine and marine sands and the subsurface layer is composed of 89.3% sand, 2.4% silt and 8.4% clay. The climate is mesothermic humid with an average groundwater temperature of 22° C and annual average precipitation of 1780 mm. Average soil organic carbon is 0.33%, effective average porosity is 0.28 and groundwater flow velocity is 6.5 m year^{-1}. The biostimulation experiment was established in an area of 180 m^2, containing 30 multilevel sampling wells (SW) installed perpendicular to groundwater flow direction (Fig. 1). 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)).

100 L of B20 (20% palm biodiesel and 80% commercial diesel, v/v) and a tracer solution (3 kg of potassium bromide dissolved in 6 L of groundwater pumped from an uncontaminated aquifer in a neighboring area) were released into a source-zone area of 2 × 1.5 × 1.8 m at the surface of the water table. Although the simulated release was conducted with commercial diesel, this bioremediation technology aimed at removing recalcitrant compounds (i.e., benzene and naphthalene) that are likely present in either commercial or weathered diesel [35]. Nevertheless, initial peak concentrations of recalcitrant compounds and their degradation rates over time would be difficult to assess in a weathered diesel-contaminated site, thus justifying the release with commercial diesel. In order to establish iron and sulfate-reducing conditions, 100 kg of a low-cost and sustainable product recovered from acid mine drainage (AMD) treatment were added to the source-zone. The solid AMD product was obtained through a sequential precipitation method [36] and was used as a supplementary source of iron oxyhydroxide particles (goethite 88.3% w/w) and sulfate (1.7% w/w) to stimulate iron and sulfate reduction processes. The characteristics of the AMD product is given in Table 1, based on the methods described previously [36]. AMD product produced a pH of 4.96 when added to water. Moreover, 2 kg of ammonium acetate were added to accelerate the initial growth of iron reducing microorganisms associated with anaerobic aromatic hydrocarbon degradation, such as *Geobacter* spp. [17,19,37], as previously demonstrated in other studies that added acetate as a biostimulatory compound [38,39]. Both AMD product and ammonium acetate were added on top of the water table, immediately after B20 was released. A past adjacent 100-L B20 field experiment under monitored natural attenuation was used as a baseline control [10]. The experimental areas were covered with gravel and tarp to minimize rainfall infiltration effects on NAPL dissolution.

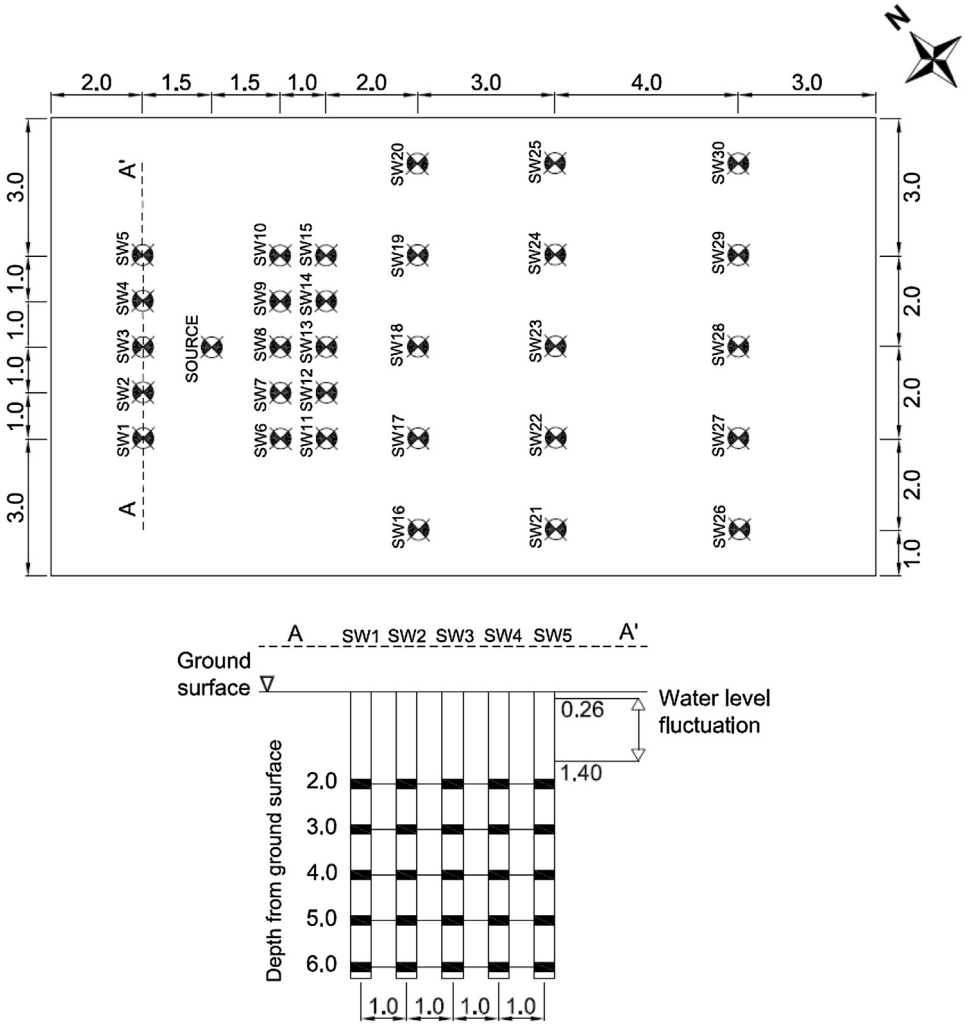

Fig. 1. Schematic (A) plan view and (B) cross section from A' to A of the experimental area. All distances are given in meters. Well cluster screens are shown as black shaded areas in the cross section.

Table 1
Characteristics of the AMD product.

Elements	Content (%)
Total sulfates	1.73
Iron oxide (Goethite)	88.30
Aluminum oxide	0.69
Silicon dioxide	0.97
Manganese oxide	0.10
Magnesium oxide	0.11
Calcium oxide	3.64
Sodium oxide	0.34
Barium oxide	0.25
Zinc oxide	0.31
Hydroxides[a]	3.56

[a] Ignition loss during thermogravimetric analyses.

2.2. Physicochemical analyses

A peristaltic pump and Teflon tubing were used to collect groundwater samples into capped sterile vials without headspace. Dissolved oxygen was measured on site using a QED Micropurge Flow Cell (MP20). Benzene (detection limit 1 $\mu g\,L^{-1}$) was analyzed by gas chromatography using a GC HP model 6890 II equipped with a flame ionization detector (FID), HP 1 capillary column (30 m × 0.53 mm × 2.65 mm) and HP 7694 headspace auto sampler. Naphthalene (detection limit 7 $\mu g\,L^{-1}$) was 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). Acetate, bromide and sulfate were analyzed by ion chromatography using a Dionex ICS-3000 equipped with a conductivity detector and an AS22 column (detection limit 0.1 $mg\,L^{-1}$). Ferrous iron (Fe^{2+}) (detection limit 0.01 $mg\,L^{-1}$) and sulfide (S^{2-}) (detection limit 5 $\mu g\,L^{-1}$) analyses were conducted using a spectrophotometer (DR/2500, HACH), with the 1.10 phenanthroline and colorimetric methylene blue method, respectively [40]. According to Gilbert, 1987 [41], when there is a value below the detection limit, one half of it may be used rather than reporting it as zero thus, we adopted half of detection limit for such cases.

2.3. Microbial analyses

Groundwater samples were filtered with a 0.22 μm pore size Millipore membrane (polyethersulfone, hydrophilic) and DNA was extracted using the MoBio Power Soil™ kit (Carlsbad, CA). Biomass (total bacteria), iron *(Geobacteraceae)* and sulfate reducers and the gene encoding benzylsuccinate synthase α-subunit (*bssA*), a catabolic gene biomarker used to assess the presence of anaerobic aromatic hydrocarbon degraders [42] were quantified by real-time quantitative polymerase chain reaction (qPCR). Primer sequences used for each analysis are given in Table 2. The quantification assays

Table 2
Primers sequences used for qPCR and sequences with adapters used for 16S *rRNA* sequencing.

Target gene/group	Forward primer	Reverse primer	Reference
Total bacteria (qPCR)	(341f) 5′CTACGGGAGGCAGCAG3′	(534r) 5′ATTACCGCGGCTGCTGGCA3′	[71]
bssA (qPCR)	(7772f) 5′GACATGACCGACGCSATYCT3′	(8546r) 5′TCGTCGTCRTTGCCCCAYTT3′	[42]
Geobacteraceae (qPCR)	(561f) 5′GCGTGTAGGCGGTTTCTTAA3′	(825r) 5′TACCCGCRACACCTAGTTCT3′	[72]
SRB* (qPCR)	(361f) 5′AAGCCTGACGCASCAA3′	(685r) 5′ATCTACGGATTTCACTCCTACA3′	[72]
16S amplicon PCR (Illumina)	5′TCGTCGGCAGCGTCAGATGTGTATAAGA GACAGCCTACGGGNGGCWGCAG	5′GTCTCGTGGGCTCGGAGATGTGTATAAG AGACAGGACTACHVGGGTATCTAATCC	[44]

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

for total bacteria and *bssA* gene were performed by Rotor-Gene® Q (QIAGEN). Each 20 μL PCR reaction mixture contained 10 μL 2x SensiFAST SYBR No-ROX Mix, 400 nM of each primer, 6.4 μL sterile DNAase-free water and 2 μL DNA template. Iron and sulfate reducer analysis was performed in an Eppendorf (Model Mastercycler® ep realplex Thermal Cyclers, CA, USA) and PCR reaction mixture contained 1x SYBR GREEN (Applied Biosystems, Foster City, CA, USA), 500 nM forward and reverse primers, 2 μL DNA template and sterile DNAase-free water (final volume of 25 μL).

Temperature settings for total bacteria followed the SensiFAST™ SYBR No-ROX kit protocol: 95 °C for 2 min followed by 30 cycles (95 °C for 5 s, 65 °C for 10 s and 72 °C for 15 s). Melting curves were constructed from 50 to 99 °C, read every 1 °C for 5 s. Temperature conditions for *bssA* gene were: 95 °C for 15 min, followed by 40 cycles (95 °C for 15 s, 55 °C for 20 s, 72 °C for 20 s) and 72 °C for 2 min. Melting curves were constructed from 55 to 95 °C and read every 0.6 °C for 2 s [43]. Temperature conditions for iron and sulfate reducers: 50 °C for 2 min, 95 °C for 10 min and 40 cycles (95 °C for 15 s and 58 °C for 1 min), followed by 95 °C for 15 s, 60 °C for 15 s, and 95 °C for 15 s for the melting curve. To estimate microbial community concentrations, standard curves were performed by serial dilutions with the DNA of the following microorganisms: *Pseudomonas aeruginosa* (10^1–10^7 gene copies, $r^2 = 0.97$) for the quantification of total bacteria and *Geobacter metallireducens* (10^1–10^7 gene copies, $r^2 = 0.99$), to quantify *bssA* gene, *Geobacteraceae* and sulfate-reducing bacteria (SRB). Detection limit of each assay was about 10^2 gene copies g^{-1}.

16S ribosomal RNA *(rRNA)* sequencing was performed to assess the shifts in microbial community structure and to discern the key players in B20 anaerobic biodegradation. The V3 and V4 regions of the 16S rRNA gene (*rrs*) were amplified by PCR [44]. PCR assays were conducted in a Biometra® Tpersonal Thermal Cycler using the primer sets described in Table 2. Each 25 μL PCR reaction mixture contained 2.5 μL Taq Buffer 10x, 0.5 μL Titanium Taq50x, 0.5 μL dNTP 10 mM, 10 μM of each amplicon PCR primer, 18 μL ultra-pure water and 2.5 μL DNA template. The following cycling conditions used were: 95 °C for 3 min, followed by 25 cycles (95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s) and 72 °C for 5 min. Triplicate reactions for each sample were pooled, visualized on 1.5% agarose gels, purified with illustra™ GFX™ PCR DNA and Gel Band Purification kit (GE), eluted in 20 μL of Tris-Cl 10 mM pH 8.5 and DNA concentration was determined with a Qubit 2.0 fluorometer (Invitrogen, Carlsbad, CA). Sequencing runs were performed on the Illumina MiSeq platform. Amplicons were demultiplexed and trimmed by the MiSeq System and forward and reverse reads, containing the target sequence with its corresponding primer, were compiled as fastq files.

The first 20 nucleotides of the fastq files obtained from Illumina sequencing were trimmed using FastX-Toolkit. Sequence quality was verified with a quality score graphic and a Phred score of 20 using the Quantitative Insights Into Microbial Ecology – QIIME (v1.9.0, http://qiime.org/index.html) software package. Illumina paired-end reads were assembled by PANDAseq [45], using the default quality scores. Merged sequences were clustered in operational taxonomic units (OTUs) and blasted against the most recent Greengenes database (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi), with 97% similarity, using QIIME pick_otus.py script and uclust [46].

3. Results and discussion

Injection of ammonium acetate and the product recovered from acid mine drainage (AMD) treatment established iron and sulfate-reducing conditions in groundwater as intended. This was indicated by the decrease in dissolved oxygen concentrations (from 2.7 to 0.5 mg L^{-1}) and the rapid increase in iron (II) concentrations (from 0 to 36.5 mg L^{-1}) after 3 months followed by the decrease in sulfate concentrations (from 38.8 to 20.6 mg L^{-1}) 7.4 months after the release (Fig. 2), while in the MNA experiment, iron (II) and sulfate concentrations were lower over the whole experimental time frame (average concentrations were 8.0 mg L^{-1} and 2.1 mg L^{-1}, respectively) after B20 release.

The increase in the electron acceptor pool (by adding a supplementary source of iron (III) and sulfate) was hypothesized to accelerate BTEX and PAH source-zone biodegradation. In the MNA site, dissolved concentrations of benzene and naphthalene were consistently high over the 4.4 years of monitoring (maximum concentrations were 974.7 μg L^{-1} and 121.3 μg L^{-1}, respectively), while in the biostimulated plot, benzene and naphthalene were lower (maximum concentrations were 28.1 μg L^{-1} and 10.0 μg L^{-1}, respectively) over the whole experimental time frame (Fig. 3). Benzene concentrations dropped below the maximum contaminant level (MCL) for benzene in drinking water (5 μg L^{-1}) [47] and were below detection limit (1 μg L^{-1}) after 1.1 years, while benzene concentrations in the MNA site were above the MCL even after 4.4 years following the release (Fig. 3). The same pattern was observed for naphthalene concentrations that were kept below MCL (60 μg L^{-1}) [48] during the whole biostimulated plot experiment (maximum concentration detected was 10 μg L^{-1}), whereas in MNA, naphthalene remediation goals were only achieved after 4.4 years (34.8 μg L^{-1}) (Fig. 3). A B20 methanogenic biostimulated field experiment conducted by Ramos et al. (2013) [10] also observed an enhanced BTEX biodegradation as compared to the MNA site, but the dissolved benzene concentrations were higher (maximum concentration of 900 μg L^{-1}) than those observed in the present experiment (28.15 μg L^{-1}) and did not meet environmental guidelines (32 μg L^{-1}) even after 1.6 years. Thus, these findings suggest that combined iron and sulfate reduction biostimulation could be a more suitable approach to BTEX and PAH cleanup in a B20-contaminated source-zone groundwater.

Biostimulation with ammonium acetate and AMD recovered product led to an enhanced growth of total biomass and aro-

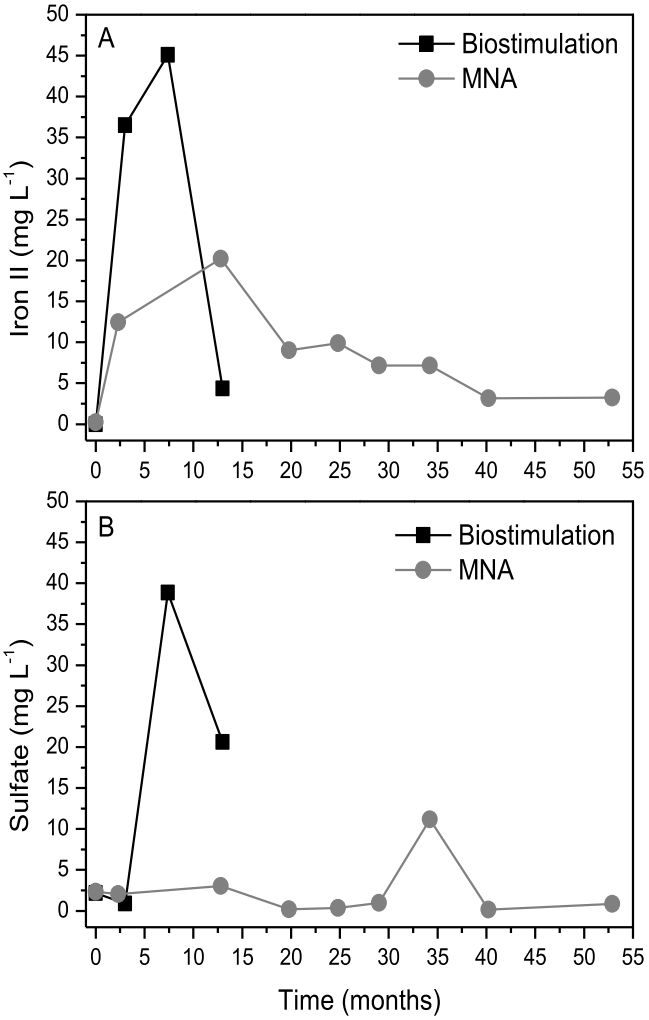

Fig. 2. Iron (II) (A) and sulfate (B) concentrations (mg L^{-1}) as a function of time at 3 m below ground surface (bgs) at the source zone of both the monitored natural attenuation and biostimulation experiments.

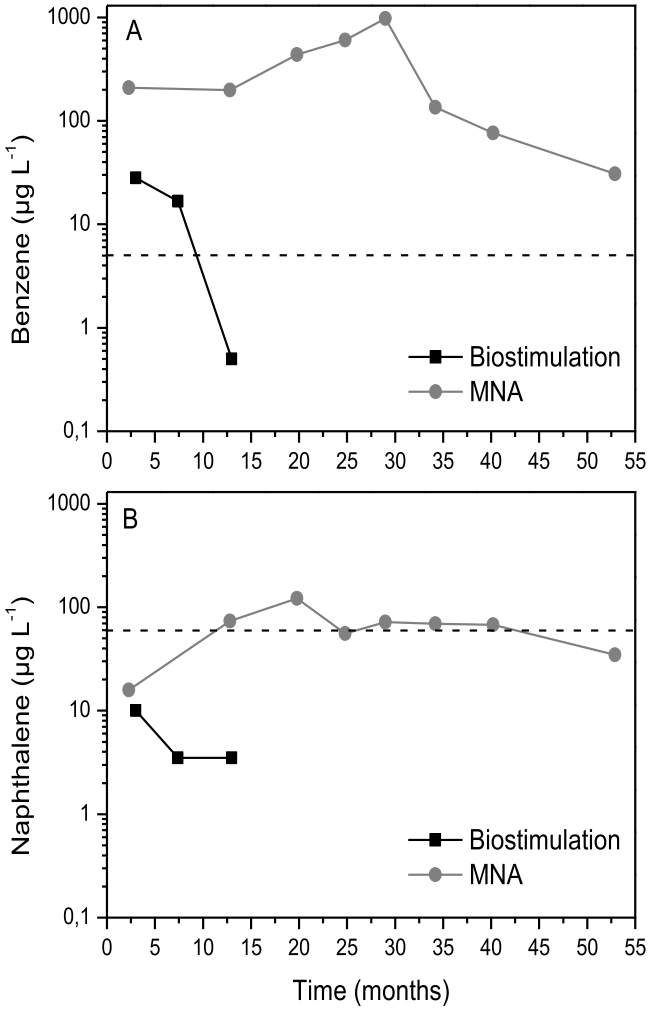

Fig. 3. Benzene (A) and naphthalene (B) dissolved concentrations (µg L^{-1}) as a function of time at 3 m below ground surface (bgs) at the source zone. Dashed lines represent maximum contaminant level (MCLs) for benzene (A) and naphthalene (B).

matic hydrocarbon degraders. Among the multiple mechanisms that can potentially influence aromatic compound concentrations in groundwater, biodegradation apparently prevailed over abiotic processes (*i.e.* dissolution, dispersion, sorption, dilution, *etc.*). Overall biomass based on 16S rRNA gene copies (*rrs* gene copies) was shown to increase (from 6.2×10^6 to 7.6×10^8 gene copies g^{-1} total suspended solids) 3 months after B20 release. The enhanced biomass growth and the continuous low dissolved concentrations of benzene and naphthalene (approx. 2 and 1 orders of magnitude lower than for the MNA site, respectively) observed over the entire experimental time frame in the biostimulated site was consistent with the biodegradation of these contaminants. An increase in gene copy numbers of iron and sulfate reducers and a gene marker associated with anaerobic aromatic hydrocarbon biodegradation (*bssA* gene) was observed. After 3 months following B20 release, *bssA* increased from 3.3×10^4 to 1.3×10^7 gene copies g^{-1}, and remained at similar levels over the entire experiment. An increase in *Geobacteraceae* (from 2.2×10^3 to 4.1×10^7 gene copies g^{-1}) and other sulfate reducers (10^1 to 3.8×10^7 gene copies g^{-1}) (Fig. 4) was also observed after 3 and 7.4 months following the release, and their presence was chronologically correlated with the geochemical changes mentioned previously. Furthermore, potential inhibitory effects of biodiesel or acetate on BTEX and PAH attenuation might have been alleviated due to the presence of these specific hydrocarbon degraders, since they have previously been shown to be involved in FAME, LCFA and acetate biodegradation [28,32–34,49,50]. Ramos et al. [10], showed no BTEX biodegradation inhibition due to acetate after 0.7 years, and yet did not observe degradation as rapidly as in the present study. In the MNA site, these specific degraders were not detected (Fig. 4) and BTEX and PAH persisted over the 4.4 years monitored. Thus, the combined stimulation of iron and sulfate reduction seemed to have accelerated BTEX and PAH biodegradation in diesel/biodiesel blends and was likely to have enhanced overall B20 compound source-zone biodegradation.

A beneficial response of microbial networks involved in B20 compounds anaerobic biodegradation was observed and to gain insight into the shifts in microbial communities over time and the key players involved in BTEX and PAH biodegradation, 16S rRNA gene sequencing was carried out. Background samples revealed a more diverse microbial community (as compared to the samples after B20 release) containing mainly aerobes (*Fimbriimonas* spp., *Planctomyces* spp., *Salinispora* spp.) [51–53], facultative aerobes (*Rhodoplanes* spp., *Staphylococcus* spp., *Cupriavidus* spp., *Pseudomonas* spp.) [54–57] and nitrate/nitrite reducers (*Candidatus koribacter* spp., *Alicyclobacillus* spp., *Candidatus solibacter* spp., *Planctomyces* spp.) [52,58–61] (Fig. 5). This is consistent with the groundwater microaerophilic conditions (1.2 mg L^{-1} of dissolved oxygen) encountered prior to the controlled release.

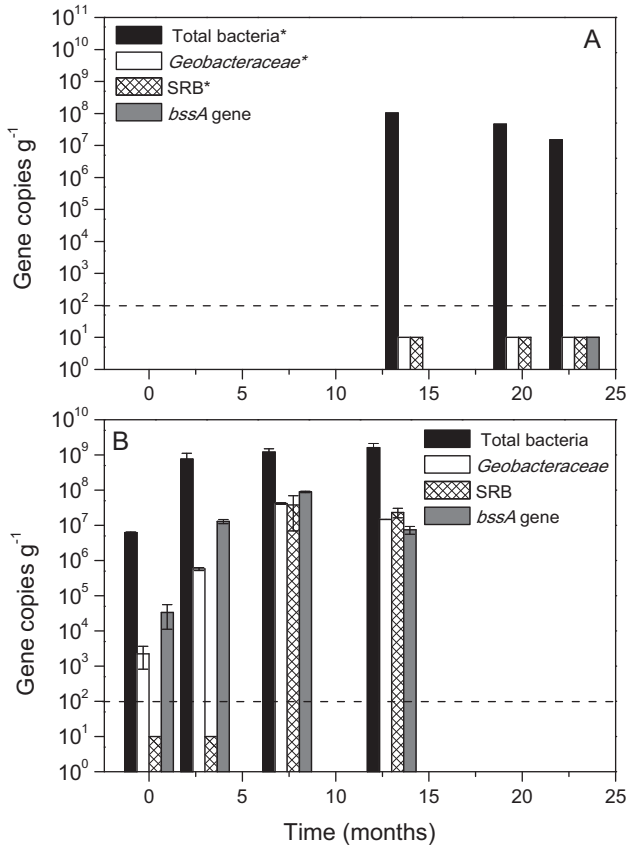

Fig. 4. Concentrations of total bacteria, *Geobacteraceae*, sulfate-reducing bacteria (SRB) (16S rRNA gene copies g^{-1}) and *bssA* gene (implicated in anaerobic hydrocarbon degradation) as a function of time at 3 m below ground surface at the source zone of the monitored natural attenuation (A) and biostimulation (B) experiments. Dashed lines represent the detection limit for the qPCR analysis.

*Data obtained from Ramos et al. [10].

A sharp increase in the relative abundance of the *Geobacter* genus (from 0.6 to 90.6% of the total Bacteria 16S rRNA gene) was observed 3 months after the release, as hypothesized to occur following the amendment of ammonium acetate and the AMD product (Fig. 5). Members of *Geobacteraceae* have previously been described as dominant members of microbial communities in iron-rich subsurface environments [17,37] and are able to degrade organic/aromatic compounds coupled to dissimilatory iron(III) reduction [12,13,17,19,62]. Therefore, the predominance of *Geobacter* up to 7.4 months (Fig. 5), is consistent with acetate depletion (from 67.8 mg L^{-1} to below detection limit (0.1 mg L^{-1}) at 7.4 months) and the increased iron (II) production (from 0 to 45 mg L^{-1}, at the same time frame). In addition, the observed low aromatic hydrocarbon concentrations are consistent with *Geobacter* genus playing a key role in benzene and naphthalene biodegradation under iron-reducing conditions.

Sulfate-reducing conditions were established after iron reduction ebbed as reflected by the decrease in both iron (II) concentration and *Geobacter* spp. abundance. Sulfate consumption was observed after 7.4 months (Fig. 2) and community dominance shifted towards the genus *GOUTA19* (family *Thermodesulfovibrionaceae*), which represented 59.6% of total Bacteria 16S rRNA gene copies after 13 months following the B20 release (Fig. 5). Although *GOUTA19* has been previously observed in rice paddy soils irrigated by acid mine drainage-contaminated water [63], alfalfa-rice rotation system [64], oil-storage cavities [65] and in reactors amended with monochlorobenzene-contaminated groundwater [66], this is the first field experiment to implicate *GOUTA19* as a potential key player in the anaerobic degradation of diesel mono and polyaromatic hydrocarbons in groundwater. The presence of *Thermodesulfovibrionaceae*-related bacteria has also been observed in sulfate reduction processes [33,63,67]. This is consistent with the observed decrease in sulfate concentrations and with sulfate reduction being the main TEAP at this point in time. Although hydrogen sulfide is generally produced during sulfate reduction [68], sulfide was not detected over the 13 months of groundwater monitoring. The absence of measureable sulfide production could be attributed to iron sulfide precipitation that was consistent with the decrease in iron (II) (Fig. 2) as well as the apparent ubiquity of this reaction in anaerobic environments [26,33,69,70]. Since hydrogen sulfide can inhibit microbial activity [26], the combined use of iron and sulfate reduction TEAPs can potentially offset the toxicity effects exerted on microbial communities as iron sulfide precipitates and reduces the occurrence of hydrogen sulfide in groundwater. The predominance of *GOUTA19* genus, the consumption of sulfate and the very low dissolved concentrations of benzene and naphthalene after 13 months following the release supports the involvement

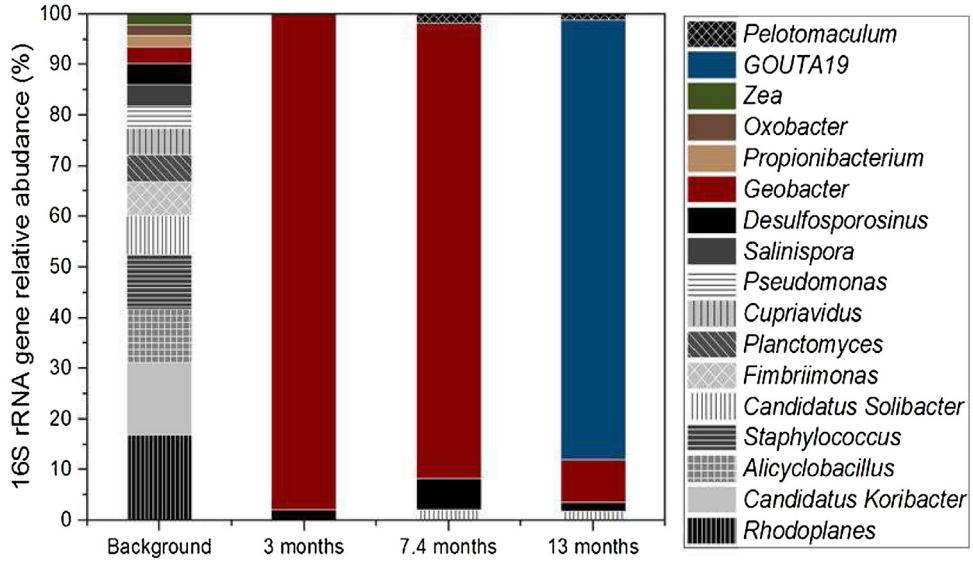

Fig. 5. Temporal changes in 16S rRNA gene relative abundance (%) of bacteria communities at 3 m below ground surface (bgs) at the source zone of the biostimulation experiment.

of *GOUTA19* in benzene and naphthalene biodegradation under sulfate-reducing conditions.

4. Conclusions

- The low-cost and sustainable product recovered from AMD treatment proved to be an efficient supplementary source of iron oxyhydroxide and sulfate that increased the electron acceptor pool and enhanced BTEX and PAH source-zone biodegradation. Application of this product represents a sustainable strategy for the removal of priority pollutants in groundwater.
- Combined biostimulation of iron and sulfate-reduction accelerated BTEX and PAH source-zone biodegradation in diesel/biodiesel blends and maintained low dissolved concentrations of benzene and naphthalene over the entire experiment as compared to the baseline control experiment under monitored natural attenuation.
- The application of this bioremediation strategy promoted a beneficial response of microbial community networks and a shift in dominance towards *Geobacter* spp. and *GOUTA19* spp., both of which might be key players in the anaerobic biodegradation of B20 compounds under iron and sulfate reduction.
- To the best of our knowledge, this is the first field experiment to study BTEX and PAH biodegradation in diesel/biodiesel blends under combined iron and sulfate reduction and to demonstrate the potential of added iron and sulfate for the cleanup of B20-contaminated source-zone groundwater.

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