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Groundwater microbial analysis to assess enhanced BTEX biodegradation by nitrate injection at a gasohol-contaminated site

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ABSTRACT

Groundwater samples from a gasohol contaminated aquifer were analyzed to investigate the effects of nitrate injection on microbial communities associated with benzene, toluene, ethylbenzene and xylenes (BTEX) biodegradation. Real-time quantitative PCR was used to quantify total bacteria (16S rDNA), nitrate-, iron-, sulfate-reducing bacteria and methanogens. Anaerobic BTEX degradation potential was assessed by targeting the *bssA* gene which encodes for benzylsuccinate synthase (BSS), an enzyme that initiates the biodegradation of toluene and xylenes. Aerobic BTEX biodegradation potential was assessed by targeting the catabolic genes: toluene dioxygenase (TOD), naphthalene dioxygenase (NAH), ring hydroxylating monooxygenase (RMO), phenol hydroxylase (PHE), and/or biphenyl dioxygenase (BPH). 16S rDNA gene copies were higher ($\sim 4 \times 10^5$ cells ml^{-1}) at the plume centerline coinciding with the highest concentrations of BTEX (~ 26 mg-total l^{-1}) and ethanol (~ 3 mg l^{-1}). Regions with high nitrate consumption coincided with the increased nitrate-reducing bacteria population. The establishment of Fe(III)-reducing zones were unlikely associated with bacteria belonging to *Geobacter* genus. Sulfate-reducing bacteria and methanogens were not detected corroborating with the geochemical footprints. Nitrate did not stimulate the fortuitous growth of anaerobic BTEX degraders as indicated by the absence of bssA amplification. Nitrate alleviated the high biological oxygen demand (BOD) associated with BTEX and ethanol biodegradation thus maintaining microaerophilic niche that supported the growth of aerobic BTEX degraders as indicated by the presence of PHE gene copy numbers ($\sim 2 \times 10^5$ PHE gene copies ml^{-1}). Among the genes tested, the non-functional 16S rDNA showed significant correlation ($r^2 = 0.94$; $p < 0.05$) with BTEX (but not ethanol) first-order (k') biodegradation rates. Further investigations are required, however, to validate qPCR accuracy and reliability to estimate contaminants removal rates for a wide range of contaminated sites.

1. Introduction

Bioremediation of benzene, toluene, ethylbenzene and xylene isomers (BTEX) plumes often relies on the addition of oxygen and nutrients in the groundwater to stimulate aerobic biodegradation (National Research Council, 2000). In gasohol plumes however, BTEX aerobic biostimulation approaches could be thermodynamically unfavorable due to the higher ethanol concentration relative to BTEX and the associated consumption of the aerobic electron acceptors required for the preferential ethanol biodegradation (Da Silva and Alvarez, 2002, 2004). In such cases, anaerobic bioremediation strategies should be considered for the cleanup of gasohol releases, especially near the source zone, which are invariably anaerobic. Although slower than aerobic biodegradation, anaerobic microbial metabolism of toluene, ethylbenzene and xylenes has been demonstrated (Morgan et al., 1993; Ball and Reinhard, 1996). Benzene, which is the most toxic of the BTEX compounds and the most recalcitrant in the absence of oxygen, can also be degraded anaerobically under nitrate- (Burland and Edwards, 1999; Coates et al., 2001), iron- (Lovley et al., 1996), sulfate-reducing (Coates et al., 1996a; Anderson and Lovley, 2000), methanogenic conditions (Ficker et al., 1999), or when coupled to an electrode as source of electron acceptor (Zhang et al., 2010). Among these anaerobic electron acceptors, nitrate has been widely used to enhance anaerobic BTEX biodegradation (Reinhard et al., 1997; Burland and Edwards, 1999; Cunningham et al., 2001; Coates et al., 2001; Mancini et al., 2003; Ulrich and Edwards, 2003; Chakraborty and Coates, 2005; Ulrich et al., 2005; Kasai et al., 2006) even in the presence of ethanol (Da silva et al., 2005;

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Costa et al., 2009) due to its high solubility (>900 g l^{-1} at 25 °C), high oxidation potential [$E^0_h = +0.42$ for NO_3^- to N_2; (Schwarzenbach et al., 1993)], high dissimilative capacity (5 e^- equivalents accepted per mol of NO_3^- reduced to N_2) and potential to be used as a nutrient. Nonetheless, the effects of nitrate injection on the groundwater microbial communities during the bioremediation of BTEX and ethanol plumes have not been previously demonstrated.

Molecular microbial ecology techniques and specific biomarkers have been used to obtain supporting evidence of bioremediation or monitored natural attenuation (MNA) (Suzuki et al., 2000; Scow and Hicks, 2005; Weiss and Cozzarelli, 2008). Aerobic BTEX biodegradation has been assessed by targeting genes coding for toluene dioxygenase (TOD), naphthalene dioxygenase (NAH), ring hydroxylating monooxygenase (RMO), phenol hydroxylase (PHE), and biphenyl dioxygenase (BPH3) (Futumata et al., 2001; Alfreider et al., 2002; Beller et al., 2002; Baldwin et al., 2003; Wenderoth et al., 2003; Balcke et al., 2004; Abraham et al., 2005; Capiro et al., 2008; Nebe et al., 2009; Kao et al., 2010). Anaerobic BTEX biodegradation potential has been determined by targeting the *bssA* gene that codes for benzylsuccinate synthase, an ubiquitous enzyme that initiates the anaerobic degradation of toluene and xylenes by catalyzing the addition of fumarate to the methyl group (Biegert et al., 1996; Rabus and Heider, 1998; Beller and Spormann, 1999; Krieger et al., 1999; Achong et al., 2001; Winderl et al., 2008). This enzyme has been detected in a variety of anaerobic environments including phototrophic (Zengler et al., 1999), nitrate- (Beller and Spormann, 1997a), sulfate- (Beller and Spormann, 1997b), iron-reducing (Kane et al., 2002), and methanogenic conditions (Beller and Edwards, 2000; Da Silva and Alvarez, 2004; Kazy et al., 2010). Besides providing supporting evidence of contaminant biodegradation, previous studies have demonstrated that the quantification of specific biomarkers were useful to predict methanogenic benzene (Da Silva et al., 2007) and toluene (Kazy et al., 2010) biodegradation rates.

In this work, the effect of nitrate biostimulation on groundwater microbial community was investigated. Samples collected from a controlled gasohol release field experiment were analyzed for bacteria populations based on their functional groups, including iron-, nitrate-, sulfate-reducing, methanogens, and catabolic potential towards BTEX and ethanol biodegradation. Gene copies numbers were utilized as biomarkers to estimate the in situ BTEX and ethanol removal rates (k') within the contamination plume.

2. Materials and methods

2.1. Site information

A controlled gasohol release experiment was conducted at field scale in southern of Brazil [Florianópolis, Santa Catarina, Ressacada's farm (27°40′49.00″S and 48°31′56.94″W)] to assess the effectiveness of nitrate injection on the natural attenuation of BTEX and ethanol mixtures (Costa et al., 2009). Briefly, 36 monitoring wells were distributed in a grid consisting of five rows installed downgradient of the source (Fig. 1). These wells locations were arranged according to the preferential groundwater flow as determined by tracer studies. The physical–chemical characteristics of the sandy aquifer prior to contamination are shown in Table 1. To establish the source zone, one hundred liters of commercial grade Brazilian gasoline containing 25% anhydrous ethanol (v/v) was released into an area of 1 m^2 and 1 m below soil surface. Nitrate delivery was initiated 2 months after the gasohol release. Five liters of a concentrated nitrate stock solution (4 g l^{-1}) was injected three times a week into 6 injection wells for 300 days

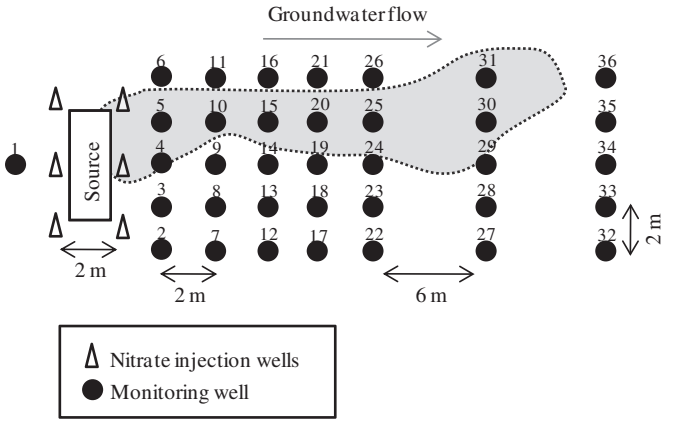

Fig. 1. Schematic representation of the monitoring wells (MW) and nitrate injection points. Dashed line delineates the plume contours following 42 months after the controlled release.

by using a peristaltic pump (Millipore Easy-Load) (Fig. 1). A total of 39 kg of nitrate was delivered over the duration of the experiment.

2.2. Analytical procedures

Groundwater samples were collected over time after the controlled release. Samples were collected at 4.8 m below soil surface from the monitoring wells (MW) MW-1, MW-5, MW-10, MW-15, MW-20 and MW-30. Low-flow samples were collected using a peristaltic pump (Millipore Easy-Load) and Masterflex® Tygon tubing. Groundwater samples were transferred directly into 40 ml flasks for chromatographic analyses, 200 ml flasks for physical-chemical analyses, and 1 L flasks for microbial analyses. Samples for the physical–chemical analyses were immediately preserved at pH < 2 using hydrochloric solution, stored on ice and transported to the laboratory.

Ethanol, methane, BTEX and phenol were analyzed by gas chromatography [Hewlett–Packard (HP) 6890, Palo Alto, CA] as previously described (Costa et al., 2009). Nitrate, sulfate and acetate were analyzed by ion chromatography (IC 1000, DIONEX) equipped with an ion conductivity detector and an AS14A-4 mm column (Standard Methods, 1998). Carbonate and bicarbonate were used as carrier solutions. Iron (II) and sulfide were analyzed using a spectrophotometer (DR/2500, Hach) according to the 1,10 phenanthroline and the methylene blue methods, respectively (Standard Methods, 1998). pH, dissolved oxygen (DO) and oxidation–reduction potential (ORP) were measured in situ using the

Table 1
Background groundwater physical–chemical characteristics.

Parameter	Value
Total Organic Carbon (TOC)	0.2–1.4%
Temperature	24 °C
Oxidation–reduction potential (ORP)	+520 mV
pH	4.2
Dissolved oxygen (DO)	3–5 mg l^{-1}
Acidity	6–10 mg l^{-1} as $CaCO_3$
Iron (II)	<0.01 mg l^{-1}
Nitrate	1 mg l^{-1}
Sulfate	2–6 mg l^{-1}
Methane	<0.01 mg l^{-1}
Phosphate	<0.01 mg l^{-1}
Effective porosity (η_e)	0.2
Groundwater flow velocity (V)	6 m yr^{-1}
Water table fluctuation	0.7–2 m

Micropurge® Flow Cell (model MP 20). All chemicals used for standards analysis were analytical grade with purity ≥99.9%. Detection limits (μg l^{-1}) were: BTEX (5), ethanol (1000), methane (10), nitrate (50), iron (II) (8), sulfate (2), sulfide (15) and acetate (5).

2.3. DNA extraction

Groundwater microbial characterization was conducted 42 months after the controlled release. 1-L groundwater samples were vacuum-filtered using a 0.22 μm filter (Osmonics Inc., Minnetonka, MN). The filter was used as a matrix for DNA extraction using the MoBio Power Soil™ kit (Carlsbad, CA) according to the manufacturer's protocol. DNA concentration and purity was measured based on the wavelength absorbance ratio (A260/A280) (absorbance of 260 nm for DNA and 280 nm for protein) using a spectrophotometer (NanoDrop, Thermo Scientific, Wilmington, DE). A 2 μL aliquot of bacteriophage-λ DNA (500 bp) (Sigma–Aldrich, St. Louis, MO) was spiked in each sample prior to DNA extraction and used as internal standard for the determination of DNA recovery efficiency (Beller et al., 2002).

2.4. Real-time quantitative PCR (qPCR)

qPCR was used to quantify total Bacteria 16S rDNA, nitrate-, iron-, sulfate-reducing bacteria and methanogens. The concentration of total bacteria 16S rDNA genes was measured using the universal bacterial primers BACT1369F and PROK1492R (Beller et al., 2002). Nitrate-reducing bacteria genes were quantified using the primers nirK1F and nirK5R as well as the nirS1F and nirS6R (Braker et al., 1998). Dissimilatory iron-reducing bacteria (DIRB) genes were targeted using the *Geobacter*-specific primers 361F, and 685R with the GBC1 probe (Stults et al., 2001). Sulfate-reducing bacteria (SRB) genes were analyzed using the probe EUB1 with the primers 361F and 685R capable of targeting δ-*Proteobacteria* [complementary to many iron- and sulfate-reducing genera including *Geobacter*, *Pelobacter* (including fermentative species), *Desulfovibrio, Desulfomicrobium, Desulfuromusa,* and *Desulfuromonas* (including dissimilatory S reducers) (Stults et al., 2001)]. The concentration of methanogens genes was measured using the primers ME1F and ME2R (Hales et al., 1996). *bssA* gene was used as a biomarker to assess the potential stimulatory effects of nitrate injection on specific BTEX denitrifying bacteria. These primers were designed based on *bssA* gene sequences from specific denitrifying toluene and xylene-degrading bacteria (Beller et al., 2002). Aerobic BTEX biodegradation potential was assessed by targeting the catabolic genes coding for toluene dioxygenase (TOD), naphthalene dioxygenase (NAH), ring hydroxylating monooxygenase (RMO), phenol hydroxylase (PHE), and biphenyl dioxygenase (BPH3) (Baldwin et al., 2003).

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. PCR reactions were performed using an Eppendorf (Model Mastercycler® ep realplex Thermal Cycler, CA, USA) with the following temperature conditions: 50 °C for two min, followed by 95 °C for 10 min and 40 cycles at 95 °C for 15 s, and 60 °C for one min. The genomic DNA sequences of various reference strains were utilized to prepare calibration curves for the targeted genes. *Geobacter metallireducens* was used for iron-reducing (GBC1), *Thauera aromatica* for bssA, *Pseudomonas aeruginosa* for 16S rDNA and nirS, *Rhizobium radiobacter* for nirK, *Desulfobacterium autotrophicum* for EUB1, *Metanococcus maripaludis* for methanogens, *P. putida* F1 for toluene dioxygenase, *P. putida* G7 for naphthalene dioxygenase, *P. pseudoalcaligenes* KF707 for biphenyl dioxygenase, *Ralstonia picketti* PK01 for ring hydroxylating monooxygenase, and *P. putida* CF600 for phenol hydroxylase. Dilutions (10^1–10^8 gene copies ml^{-1}) were prepared for all calibration curves, yielding r^2 values ≥0.99.

The gene copy numbers in each sample was estimated based on the following equation:

$$\text{Gene copy numbers}\,\mu L^{-1} = \left(\mu g\,\text{DNA}\,\mu L^{-1}\right) \Big/ \left(\text{bp genome}^{-1}\right) \times \text{bp}\,\mu g\,\text{DNA}^{-1} \times \text{genes genome}^{-1}$$

This equation assumes the size of the bacterial genome base pair (bp) used as the standard in the calibration curves (bp) (http://www.genomesonline.org) and there are approximately (9.1×10^{14} bp μg^{-1} of DNA). Gene copy numbers per genome varied according to the strain used in the calibration curve (http://rrndb.cme.msu.edu). All primers and probes were obtained from Integrated DNA Technologies (Coralville, IA). The detection limit of each assay was 100 gene copies ml^{-1}.

2.5. Correlation between gene copy numbers and contaminant removal rates

BTEX and ethanol biodegradation rates were estimated using first-order equation (Alvarez and Illman, 2006) :

$$dC/dt = -k'C \quad (1)$$

Where: k' = first-order degradation rate, C = total BTEX or ethanol concentrations, and t = time. The coefficient k' was determined as the slope of the linearized plot of Ln(C) versus time. The k' values obtained for both BTEX and ethanol were correlated with gene copy numbers using best-fitting linear regression analyses.

3. Results and discussion

3.1. In situ BTEX and ethanol biostimulation

Groundwater monitoring was conducted at this site for over 4 years to assess the effects of nitrate injection on the enhancement of BTEX and ethanol natural attenuation. Detailed information on contamination plume dynamics, remediation time frame, hydrogeology and geochemical footprints were previously reported (Costa et al., 2009). Similar controlled released field experiment was conducted in parallel to discern the effects of nitrate injection on BTEX and ethanol biodegradation compared to natural attenuation alone (Corseuil et al., 2011). After 2.7 years, total BTEX mass in the nitrate-amended groundwater was 33% lower compared to the natural attenuation alone indicating that nitrate contributed to faster contaminant removal.

3.2. Groundwater microbial analysis

Groundwater microbial analysis were conducted at this site in attempt to better understand the effects of nitrate injection on microbial community structure involved with BTEX and ethanol bioremediation or MNA. Samples were collected 42 months following the controlled release when BTEX and ethanol plumes had migrated approximately 16 m downgradient from the source zone (MW-30) (Fig. 1). BTEX (1–13 mg l^{-1}) and ethanol (12 mg l^{-1}) concentrations were highest at the plume centerline located at 6–10 m from the source (MW-15 and MW-25) (Fig. 1 and Fig. 2A). The increased bacteria population (Fig. 2D) observed at these locations associated with the high electron acceptor consumption (Fig. 2A–C) suggested that intense

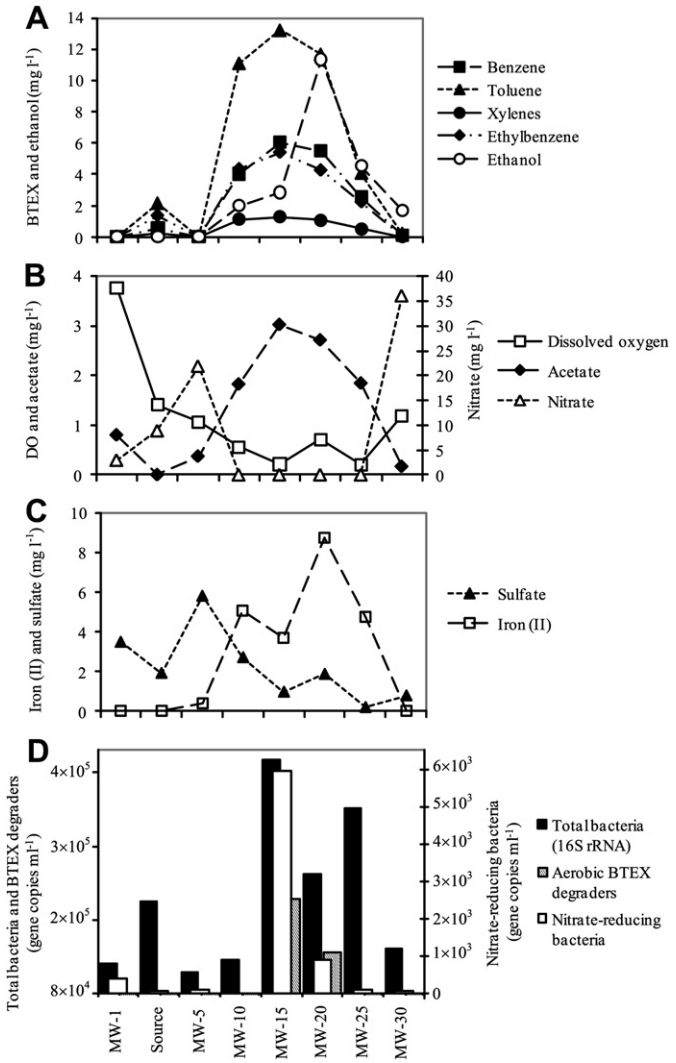

Fig. 2. Groundwater concentrations profile of BTEX and ethanol (A); dissolved oxygen (DO), acetate and nitrate (B); sulfate and iron II (C); and bacterial genes distribution (D). Analytical error inherent to sampling processing and instrument was <8%.

microbial activity towards BTEX, ethanol and/or their metabolites degradation was occuring.

Dissolved oxygen (DO) concentrations ranged from 3.8 mg l^{-1} in the vicinity of the plume down to 0.2 mg l^{-1} in the plume centerline (Fig. 2B). At these microaerophilic conditions, benzene is commonly partially oxidized into phenol as the most stable and the major metabolite which is latter biotransformed into catechol and benzoate (Yerushalmi et al., 2001, 2002). Moreover, under the stimulatory effects of nitrate injection, benzene oxidation could also lead to accumulation of phenol or benzoate as intermediate metabolites (Dou et al., 2010). Although not quantified, phenol was detected in groundwater samples collected at the MW-10, MW-15, MW-20 and MW-25 but not at MW-1, MW-5 and MW-30 (data not shown). Circumstantial evidence of aerobic BTEX biodegradation at these MW was further evidenced by the presence of phenol hydroxylase (PHE) gene (Fig. 2D), thus corroborating with the anticipated metabolic pathway. The broad substrate specificity of phenol hydroxylase that also includes catechol (Kukor and Olsen, 1990) (a key intermediate formed during BTEX oxidation) (Tao et al., 2004) suggests its usefulness as biomarker to successfully demonstrate aerobic BTEX biodegradation potential at different contaminated sites (Nebe et al., 2009; Kao et al., 2010).

The increased nitrate consumption coincided with zones with higher concentrations of nitrate-reducing bacteria (Fig. 2B and D), although denitrifying bacteria concentrations were unexpectedly low representing only ≈1.4% of the total bacteria present. The absence of nitrite as intermediate of nitrate reduction to nitrogen also served to indicate that denitrification activity was minor. Therefore, whether nitrate served as nutrient rather than electron acceptor to support the growth of overall bacteria at this site cannot be ruled out. *BssA* gene was not detected in groundwater samples. The low nitrate-reducing bacteria population could explain the lack of detectable *bssA* amplification by the PCR method specifically developed to target *bssA* sequences in toluene + *o*-xylene denitrifying bacteria.

The establishment of Fe(III)-reducing zones was demonstrated by the geochemical analysis through the increased concentrations of dissolved Fe(II) (Fig. 2C). Fe(III)-reducing zones coincided with regions with higher concentrations of acetate as potential electron donor to support the growth of DIRB (Fig. 2B and C). Members of the genus *Geobacter* that constitute the majority of DIRB frequently associated with oxidation of hydrocarbons present at contaminated sites (Coates et al., 1996b; Kunapuli et al., 2010) were not detected in groundwater samples (detection limit 10^2 gene copies ml^{-1}). Elucidating the phylogenetic diversity of DIRB present at this site was beyond the scope of this study. However, it is plausible that others DIRB not belonging to Geobacteraceae family thrived at this site (e.g. *Geothrix* spp.) (Anderson et al., 1998; Coates et al., 1999). *Geotrhix* spp. have been found in hydrocarbon contaminated sites and are known to grow at the expense of various simple organic acids as electron donor (e.g., acetate, propionate, lactate and fumarate) and Fe(III) or nitrate as alternative electron acceptors (Coates et al., 1999).

The relatively small decrease in sulfate concentrations along the plume length (Fig. 2C) was not accompanied by detectable concentrations of sulfide or the presence of SRB, thus suggesting that biological sulfate-reducing conditions were negligible. The absence of methane production was confirmed by the lack of methanogens. Thus, the injection of nitrate most likely inhibited the formation of strongly anaerobic conditions by out-competing SRB and methanogens for hydrogen or other fermentation products that could be utilized as substrates by these microorganisms (Anderson and Lovley, 1997).

We recognize that the low gene copy numbers measured in this study were likely underestimated because most subsurface microorganisms are attached to surfaces rather than suspended in groundwater samples (Lehman et al., 2001). Nonetheless, previous studies demonstrated that groundwater microbial analysis can provide acceptable level of information regarding to the most relevant processes carried out by microbial communities present at contaminated sites (including DIRB and SRB) (Gu et al., 2002; Da Silva et al., 2007; Beller et al., 2008).

3.3. Correlation between 16S rDNA gene copy numbers and BTEX removal rates (k′)

Groundwater BTEX and ethanol removal rates within the contamination plume ranged from $k' = 0.001$ to 0.04 d^{-1} (Fig. 3). BTEX removal was neglected in MW-5 and MW-10. To assess the potential use of bacterial genes as biomarker to infer on BTEX removal rates, the obtained k' values were correlated with gene copy numbers. Among the genes assessed by qPCR in this study, a significant correlation ($r^2 = 0.94$, $p < 0.05$) was obtained for BTEX (but not ethanol) k' with the universal 16S rDNA bacterial gene (Fig. 4). We recognize that the presence of bacteria does not warrant

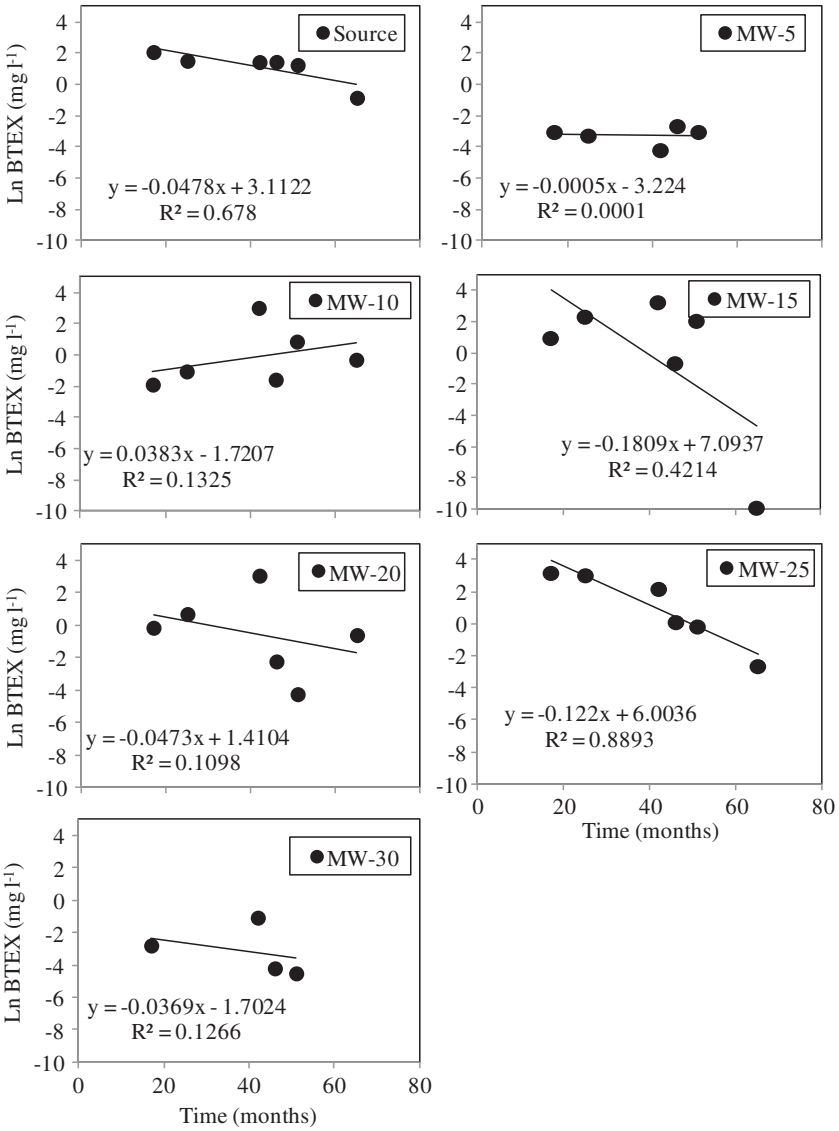

Fig. 3. First-order removal rates (k') were estimated based on BTEX concentrations measured between the 17 and 65 months after the controlled released and at different locations along the plume length.

their activities towards contaminant degradation. However, it is noteworthy to consider that the observed increased in bacteria concentration was likely stimulated by the availability of BTEX (at much higher concentrations than ethanol and acetate) as carbon source to promote their growth. This could unequivocally explain the significant correlation observed for BTEX removal rates k' and the universal 16S rDNA gene copy numbers. Significant correlation between trichloroethylene dechlorination rates were also demonstrated with the non-functional gene 16S rDNA but not with catabolic *tce*A genes or its associated mRNA (Da Silva and Alvarez, 2008). Contradicting these findings, however, anaerobic toluene degradation rates were only significantly correlated with the functional bssA genes but not with the 16S rDNA (Kazy et al., 2010). It is important to consider that the primers and probes developed to target functional genes such as oxygenases and/or bssA are unlikely capable to encompass all natural microbial genetic diversity encountered at the field scale. Consequently, limitations on the use of these functional genes may exist as result of amplification mismatches. The use of corresponding catabolic genes transcripts to predict bacterial activity also present its limitations due to the instability of mRNA and lower reverse transcriptase efficiency recovery.

Overall, the added nitrate did not contribute to stimulate the proliferation of *bssA*-harboring denitrifying bacteria capable of toluene or *o*-xylene anaerobic biodegradation. However, nitrate served to alleviate the high BOD exacerbated during ethanol and BTEX consumption and the accompanying formation of strongly anaerobic zones. Consequently, microaerophilic niche were established within the plume and supported the growth of aerobic BTEX degrading bacteria. The quantification of the non-functional 16S rDNA gene served as biomarker to predict in situ BTEX removal rate at this field experiment. This is the first line of evidence that demonstrate the usefulness of qPCR to determine BTEX removal rates in groundwater plumes containing ethanol at field scale and where transient hydrogeologic and geochemical conditions are encountered. However, further investigations are needed to investigate how site-specific conditions may interfere with the method accuracy and consistency to reliably infer on contaminants removal rates for a wide range of groundwater contaminated sites.

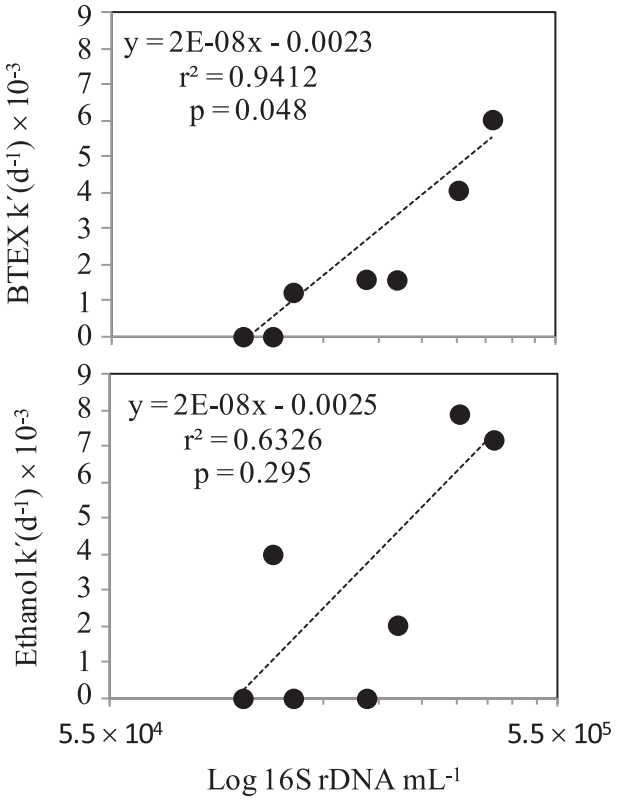

Fig. 4. Correlation between 16S rDNA gene copy numbers and BTEX and ethanol removal rates (k').

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