



Adaptive microbial population shifts in response to a continuous ethanol blend release increases biodegradation potential



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ABSTRACT

The fate of fuel releases largely depends on the poorly-understood response in microbial community structure and function. Here, we evaluate the impacts to the microbial community resulting from a pilot-scale continuous release (10 months) of a 10% v:v ethanol solution mixed with benzene and toluene (50 mg/L each). Microbial population shifts were characterized by pyrosequencing-based 16S rRNA analysis and by quantitative PCR targeting *Bacteria*, *Archaea*, and functional genes for methanogenesis (*mcrA*), acetogenesis (*fhs*) and aerobic degradation of aromatic hydrocarbons (PHE), which could occur in hypoxic micro-environments. The release stimulated microbial growth, increased species richness and diversity, and selected for genotypes involved in fermentative degradation (the relative abundance of *mcrA* and *fhs* increased 18- and 6-fold, respectively). The growth of putative hydrocarbon degraders and commensal anaerobes, and increases in microbial diversity and in degradation rates suggest an adaptive response that increases the potential for natural attenuation of ethanol blend releases.

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1. Introduction

Renewable transportation fuels such as ethanol are increasingly being used to decrease reliance on imported oil, mitigate air pollution and reduce greenhouse gas emissions (ITRC, 2011; Ma et al., 2013). The growing production and consumption of fuel ethanol has increased the potential for accidental or incidental releases that impact the subsurface. Microorganisms play a key role in the biodegradation of ethanol-blended fuel releases, often determining their region of influence and fate (Feris et al., 2008; Loffler and Edwards, 2006; Ma et al., 2013; Powers et al., 2001). Thus, it is important to understand how microbial communities respond to such releases under the influence of seasonal changes in groundwater temperature.

Previous studies investigating the microbial response to fuel ethanol releases focused on individual (phylogenetic or catabolic) genotypes associated with aromatic hydrocarbon degradation (Beller et al., 2008; Capiro et al., 2008; Da Silva and Alvarez, 2004; Feris et al., 2008; Mackay et al., 2006; Nelson et al., 2010). However, such approaches generally detect only predominant or well

characterized genotypes whereas contaminant biodegradation is usually carried out by a complex microbial food web (de Lorenzo, 2008), and smaller populations that fill important niches may remain undetected (Osborn and Smith, 2005). Therefore, the characterization of microbial population shifts in response to ethanol blend releases would benefit from using metagenomic tools that enable high sensitivity and resolution analysis of the community structure and its functional diversity. Recently, pyrosequencing technologies have enhanced the study of microbial communities with unprecedented coverage and resolution (Cardenas and Tiedje, 2008). However, the application of pyrosequencing to study the natural attenuation of ethanol-blended fuel releases has not been reported.

Pyrosequencing and quantitative real-time PCR (qPCR) analyses were conducted simultaneously in this work to characterize changes in microbial community structure, function and diversity in response to a 10-month continuous (pilot-scale) release of an ethanol-blended solution. Metabolic byproducts (e.g., methane and acetate) and several environmental variables (temperature, pH, redox potential and dissolved oxygen) were monitored to enhance our understanding of the relationship between bioremediation processes, biogeochemical indicators, and microbial population shifts.

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2. Material and methods

2.1. Pilot-scale aquifer system

An 8 m³ (3.7 m × 1.8 m × 1.2 m) pilot-scale continuous-flow tank packed with fine grain sand was used in this study (Fig. 1). Although this system was used for previous fuel release studies (Capiro et al., 2008, 2007; Stafford et al., 2009), it was filled with new sand for this experiment to avoid confounding by previous exposure history. Tap water was added at 170 L/day (average seepage velocity of 2.5 ft/day) to obtain a water table elevation of about 70 cm from the bottom of the tank. The total aquifer thickness was 115 cm and the depth of the water table was 45 cm below ground surface (BGS). Tap water amended with 10% (v/v) ethanol, 50 mg/L benzene, 50 mg/L toluene (E/B/T) and 24,000 mg/L of sodium bromide (NaBr) was continuously injected into the channel at 22.5 cm below the water table at a rate of 0.4 L/day for 10 months. NaBr was added as a conservative tracer, and to maintain a solution density to reach neutral buoyancy with the flowing groundwater (Ma et al., 2011). The added NaBr was diluted by the tank flow to less than 2000 mg/L (measured at groundwater sampling port, Fig. 1), which is within the typical tolerance range of soil bacteria (Atlas and Bartha, 1997). The experimental period covered winter and summer months (in Houston, TX), allowing for consideration of the effect of seasonal changes in groundwater temperature. The groundwater sampling port was at the same depth as the E/B/T injection port (22.5 cm below the water table). Details on the tank construction and packing methods can be found in Capiro et al. (2008); Ma et al. (2012), (2011).

2.2. Chemical and geochemical analyses

Groundwater geochemical parameters including temperature, pH, redox potential, and dissolved oxygen were monitored using a YSI 600XLM groundwater quality probe (YSI, inc. Yellow Springs, Ohio). Groundwater samples were collected from a groundwater sampling port 150 cm downgradient from the source. Ethanol, methane and volatile fatty acids (VFAs) including acetic, propionic, and butyric acid in the groundwater were measured by GC-FID. The detection limit was 1 mg/L for ethanol, acetic acid and propionic acid, 2 mg/L for butyric acid and 0.1 mg/L for methane. Benzene and toluene were measured by GC-MS with a detection limit of 1.0 µg/L. The sodium bromide tracer was measured with a bromide ion selective electrode. The detection limit was 1 mg/L. Detailed information on groundwater sampling and chemical measurement can be found in Capiro et al. (2008) and Ma et al. (2011), with the exception that we used a different sampling port for this

work (Fig. 1). The concentrations of ethanol, benzene and toluene were normalized to the respective influent concentrations $(C/C_0)_i$ and divided by the normalized bromide tracer concentrations $(C/C_0)_{Br}$ to factor out dilution effects and discern removal by natural attenuation processes (e.g., biodegradation and some possible volatilization).

2.3. Sand sample collection

Sand samples were collected from a depth of 50–75 cm below ground surface (Fig. 1) for pyrosequencing and qPCR analyses. Vertical differences in oxygen and substrate availability may affect microbial community structure. To avoid this confounding factor, we collected all samples at the same depth, within the anaerobic zone of the plume. Sampling was performed using a 1.2-m (diameter: 1.25 cm) stainless steel pipe. The pipe was hammered into the sand using a rubber mallet. When the pipe reached the target depth, the top of the pipe was sealed with a rubber septa and the pipe was then extracted by hand. Sand samples were dried in weighing boats at room temperature (24 °C) overnight and stored in a –80 °C freezer before DNA extraction.

2.4. qPCR analysis

DNA was extracted in triplicate from 0.25 g of sand using a PowerSoil DNA Kit (MOBIO Inc., Carlsbad, CA). Bacterio-phage λ DNA was used as the internal standard to calculate the recovery rate of DNA extraction (Beller et al., 2002). Absolute quantification was used to enumerate the gene copy number. Genomic DNA was used as the standard for the calibration curve. Detailed information about qPCR method, target genes, primer sequences, reaction reagents, and DNA standards can be found in Table S1.

2.5. Pyrosequencing

Four sand samples ($t = 0$ day, $t = 123$ days, $t = 184$ days, and $t = 318$ days) were used for 16S rRNA amplicon pyrosequencing analysis. DNA was extracted from 5 g sand using a modified Qiagen DNeasy kit (Qiagen Inc., Valencia, CA): three frozen (–80 °C) and thaw (60 °C) cycles and a lysozyme treatment (incubated the sand sample in 20 mg/L lysozyme (EMD Biosciences, Inc., San Diego, CA) solutions at 37 °C for 30 min) were added before kit extraction to increase DNA yield. The V4 region of bacterial 16S rRNA and archaeal 16S rRNA were amplified by PCR reaction. Detailed information on the PCR method can be found in the supporting information section.

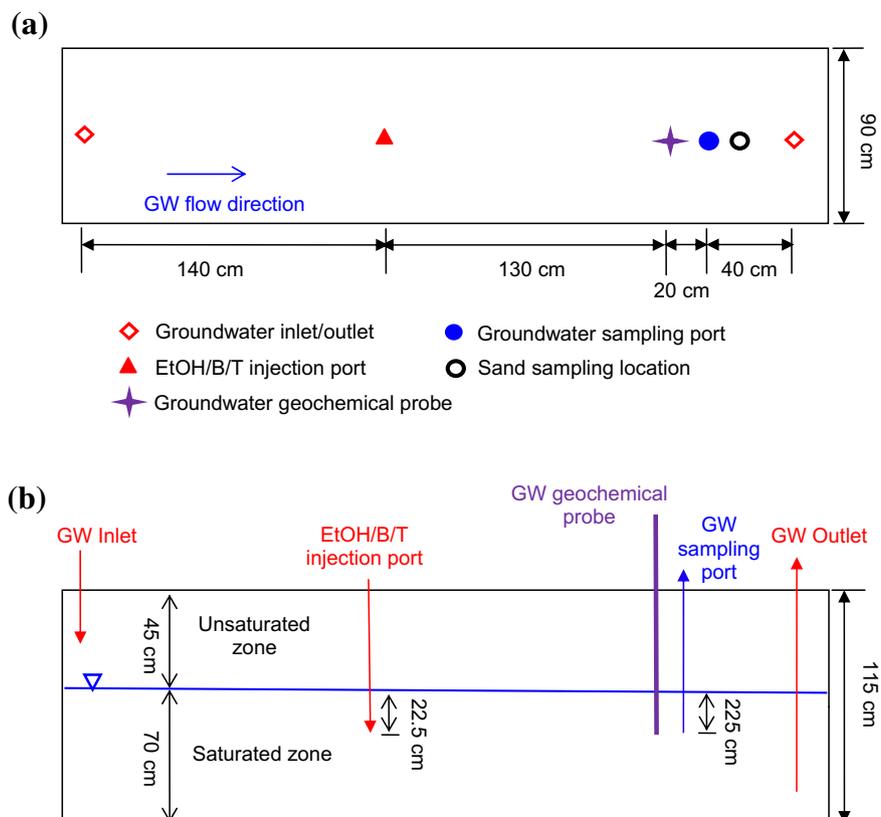


Fig. 1. Plan view (a) and profile view (b) of the pilot-scale aquifer system.

PCR products were separated in a 1% (wt/vol) gel. The bands between 270 and 300 bp were excised and DNA was recovered using a QIAquick gel extraction kit (Qiagen Inc., Valencia, CA). DNA samples were quantified using PicoGreen (Invitrogen, Eugene, OR) and pooled in normalized quantities for direct sequencing by a Genome Sequencer Junior System (454 Life Sciences, Branford, CT).

2.6. Pyrosequencing data analysis

A total of 51,924 raw sequences was obtained, deposited in the NIBI Sequence Read Archive (BioSample accession ID: SAMN01889068, SAMN01889069, SAMN01889070, SAMN01889071) and processed by the Ribosomal Database Project (RDP) Pyrosequencing Pipeline (<http://pyro.cme.msu.edu/>) (Cole et al., 2009). Raw sequences were sorted by barcode, and fusion primers were removed. The quality filter removed low quality sequences with lengths less than 150 bases or having any ambiguity (N) or those with any change in forward or reverse primers. After removing low quality sequences, 1139–1814 bacterial sequences per sample and 2024–11,570 archaeal sequences per sample were obtained (Table S2). Although deeper sequence efforts can yield more information on microbial diversity, several studies have shown that similar sequencing efforts (even of lower depth, with only a few hundred sequences per sample) can reliably discriminate microbial communities from different environments (e.g. saline versus nonsaline environments (Lozupone and Knight, 2007), soil samples from Florida versus Hawaii (Lemos et al., 2011), and gastrointestinal microflora in individuals with inflammatory bowel disease versus healthy individuals (Frank et al., 2007). Since our goal was to compare the microbial community in aquifer material before and after continued exposure to an ethanol-blend release, we deemed our level of sequencing analysis appropriate. Sequences were aligned by MUSCLE 3.5 (Multiple sequence comparison by log-expectation) (<http://www.drive5.com/muscle/>) (Edgar, 2004) and were classified using RDP II classifier with a 50% bootstrap confidence (Wang et al., 2007). To calculate richness and diversity indices of the microbial community, 1100 aligned sequences were randomly selected from each sample and clustered by RDP's complete-linkage clustering tool (Cole et al., 2009). Clustered files were processed by RDP's Estimate S Input File Formatter, and Estimate S 8.2.0 (<http://viceroy.eeb.uconn.edu/estimates>) was used to calculate species richness estimators (Chao 1, Jackknife and ACE) and diversity indices (Shannon and Simpson) (Colwell, 2009).

3. Results and discussion

3.1. Impacts of ethanol-blend release on the abundance of selected functional genes

To discern the effect of the release, two aquifer material samples were collected from the same location (Fig. 1) at different times: 1) a baseline (pre-release) sample ($t = 0$ day); and 2) a sample exposed to the ethanol blend for 10 months ($t = 318$ days). The corresponding groundwater temperatures during sample collection were very similar (29.9 °C for $t = 0$ and 28.6 °C for $t = 318$ days, Fig. S1) so that the temperature was not a confounding factor in comparing the microbial communities in these samples. As a readily degradable compound, ethanol represents a favourable carbon and energy source that stimulates microbial growth. Accordingly, the ethanol-blend release increased total *Bacteria* and *Archaea* populations by 14- and 110-fold, respectively (Fig. 2).

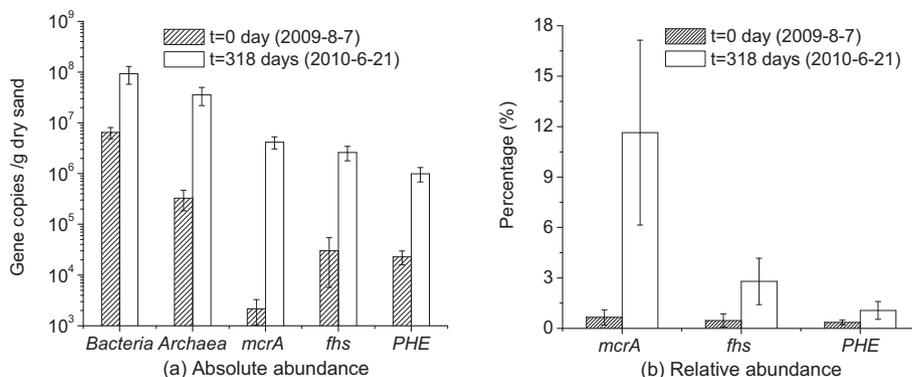


Fig. 2. A comparison of the absolute (a) and relative (b) abundance of selected genes between baseline sample ($t = 0$ day) and the sample following 10 months of continuous ethanol-blend release ($t = 318$ days). The relative abundance is expressed as a ratio of target functional genes to the corresponding 16S rRNA genes. Specifically, *fhs* and PHE were normalized to bacterial 16S rRNA and *mcrA* was normalized to archaeal 16S rRNA. Both samples were collected during the summer season.

The ethanol-blend release also increased the abundance of functional genotypes involved in the (syntrophic) anaerobic degradation of ethanol-blended fuel, such as the formyltetrahydrofolate synthetase gene (*fhs*) involved in acetogenesis (Xu et al., 2009) and the methyl coenzyme-M reductase gene (*mcrA*) that is critical for methanogenesis (Luton et al., 2002) (Fig. 2). The absolute abundance of *fhs* and *mcrA* increased 87- and 1939-fold respectively, and the relative abundance (normalized to 16S rRNA genes) increased 18- and 6-fold, respectively. The *fhs* and *mcrA* sequences are highly conserved, and are widely used as functional biomarkers for acetogenesis and methanogenesis respectively (Friedrich, 2005; Leaphart and Lovell, 2001; Luton et al., 2002; Xu et al., 2009). The high abundance of *mcrA* and *fhs* after 318 days is consistent with the high CH₄ and acetate groundwater concentrations measured in the summer (Fig. 4), reflecting the strong methanogenic and acetogenic activity stimulated by the release. The accumulation of ethanol fermentation byproducts (e.g., H₂ and acetate) could thermodynamically inhibit (i.e., make endergonic) the anaerobic biodegradation of aromatic hydrocarbons such as benzene (Corseuil et al., 2011). Therefore the enrichment of methanogens that consume H₂ and acetate is conducive to enhanced anaerobic bioremediation of ethanol-blended fuel.

The abundance of gene PHE, which codes for phenol hydroxylase (an enzyme that catalyzes the aerobic oxidation of hydroxylated metabolites of monoaromatic hydrocarbons (Baldwin et al., 2003), also increased (43-fold, Fig. 2). This gene is often detected under hypoxic or fluctuating dissolved oxygen conditions (Baldwin et al., 2009, 2008; Capiro et al., 2008; Da Silva and Corseuil, 2012; Duetz et al., 1994; Leahy and Olsen, 1997; Nebe et al., 2009). The presence of both PHE and *mcrA* at the same location reflects the common co-occurrence of aerobic and anaerobic microorganisms in different micro-niches (Kato et al., 2005; Liao et al., 2011; Wang et al., 2012). The increase in PHE abundance is likely due not only to microbial growth on benzene and toluene, but also to the fortuitous growth on ethanol or its byproduct acetate (Capiro et al., 2008). The growth of aromatic hydrocarbon degraders on ethanol would potentially enhance the rate of aerobic bioremediation in shallow aquifers after ethanol and its degradation by-products are degraded or flushed out.

3.2. Impacts of ethanol-blend release on microbial community structure

The ethanol blend release changed the indigenous bacterial community. Before the release, the bacterial community was mainly composed of aerobes such as *Acinetobacter*, *Pseudomonas*, *Acidovorax*, *Turneriella*, *Leptospira* and *Comamonas*. Following 10

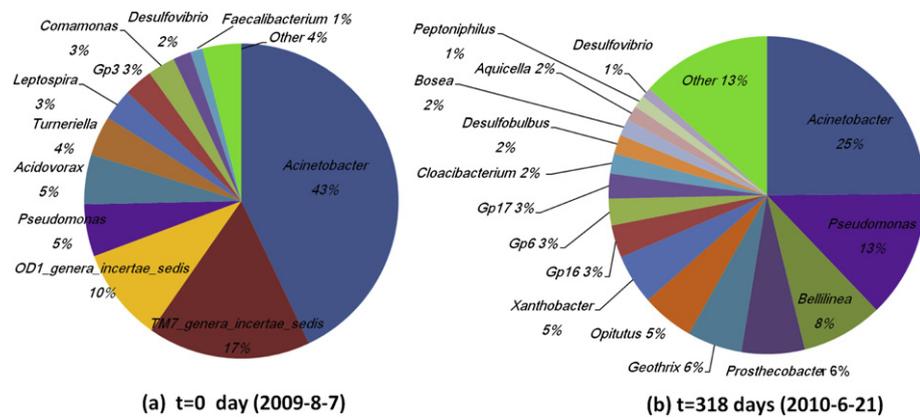


Fig. 3. Changes in bacterial communities (genus level) before (a) and after (b) 10 months exposure to the release. The category “other” contains all genera with relative abundance less than 1%. Both samples were collected during the summer season.

months of release, several genera that are likely involved in the degradation of ethanol blends were enriched, including *Pseudomonas* (from 5.4 to 13.0% of the total number of classified sequences), *Bellilinea* (from non-detected (ND) to 8.3%), *Prostheco bacter* (from ND to 6.5%), *Geothrix* (from ND to 5.6%), *Opatitus* (from ND to 5.3%) and *Xanthobacter* (from ND to 5.1%)

(Fig. 3). The genus *Pseudomonas* includes many species that are commonly associated with the aerobic biodegradation of benzene and toluene (Cantwell et al., 1978; Grimberg et al., 1996; Gupta et al., 2008; Lee et al., 2010; Reardon et al., 2000; Whyte et al., 1997). Similarly, *Xanthobacter* is associated with the degradation of a wide range of organic compounds (Torz et al., 2007; Zhou et al., 1999), including benzene and toluene at low oxygen concentrations (<0.2 ppm) (Hirano et al., 2004). Although ethanol-blended fuels rapidly induce anaerobic conditions (Capiro et al., 2008), oxygen entrainment in such open systems and aerobic micro-niches (Brune et al., 2000) may facilitate the aerobic biotransformation. *Opatitus* is a group of obligate anaerobes that produce acetate and propionate (Chin et al., 2001). *Bellilinea* is another group of obligate anaerobic-fermenters that produce volatile fatty acids (VFAs) (Yamada et al., 2007). Thus, *Opatitus* and *Bellilinea* may be predominant fermenters that initiate the anaerobic degradation of ethanol blends and interact syntrophically with methanogens. *Geothrix* is a group of strict anaerobes that grows on various simple organic acids such as acetate, propionate, and lactate (Coates et al., 1999). Therefore, *Geothrix* may have participated in the consumption of volatile fatty acids (mainly acetate and butyrate (Ma et al., 2011)) generated during the anaerobic degradation of the release.

Compared to the bacterial community, the archaeal community was much less diverse. In this aquifer system, only four archaeal genera (i.e., *Methanobacterium*, *Methanosarcina*, *Thermogymnomonas*, and *Halalkalicoccus*) were detected and methanogens were the predominant groups (Fig. S2).

3.3. Impact of ethanol-blend release on species richness and diversity

The release increased both species richness and taxonomic diversity of the bacterial community. The Chao1 (species richness) index increased from 70.3 ($t = 0$ day) to 177.0 ($t = 318$ days), while the Shannon (taxonomic diversity) index increased from 3.09 ($t = 0$ day) to 4.04 ($t = 318$ days) (Table 1). Other richness estimators (Jackknife and ACE) and diversity indices (Simpson) corroborate this trend (Table 1).

Environmental pollution is generally believed to decrease microbial diversity due to the selective proliferation of fast-growing r-strategists that feed on the contaminants (Atlas and Bartha, 1997). However, contrary to toxic or recalcitrant organic contaminants, ethanol is a readily degradable compound that can be metabolized by a wide variety of microorganisms. Thus, ethanol is likely to serve as a broadly stimulatory substrate. Uncontaminated aquifers usually have very low initial biomass due to scarcity of carbon and

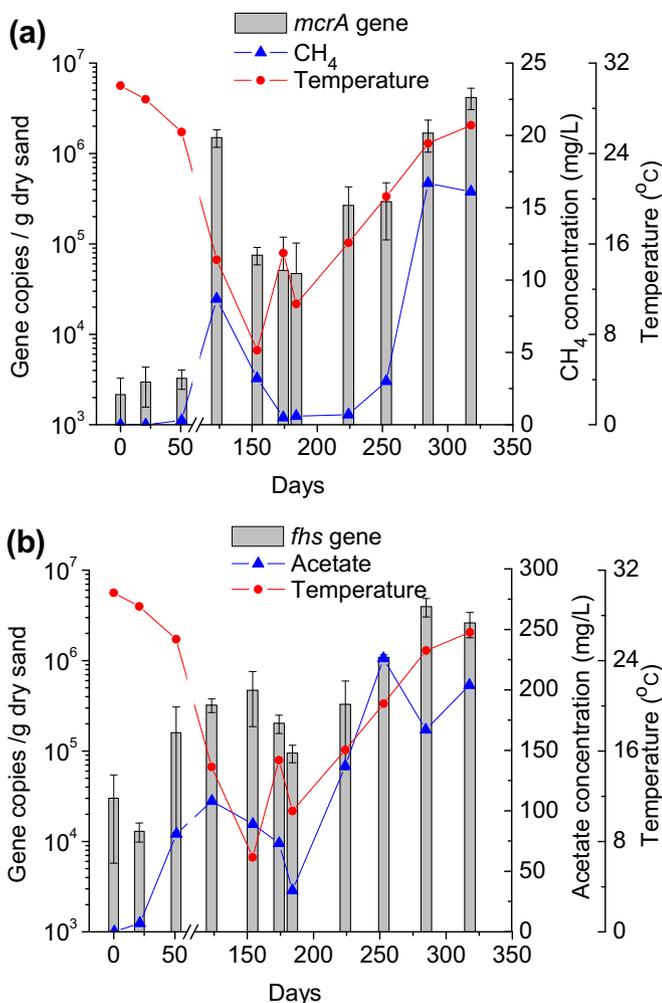


Fig. 4. Seasonal variations of the abundance of functional genes *mcrA* (a) and *fhs* (b) with groundwater temperature fluctuations, and the corresponding CH_4 and acetate groundwater concentrations. The release started at $t = 10$ days on August 17, 2009.

Table 1
Richness estimators and diversity indices.^a

Samples	OTUs ^b	Richness estimators			Diversity indices	
		Chao1	ACE	Jackknife	Shannon	Simpson
<i>t</i> = 0 day	49	70.3	68.3	60	3.09	12.0
<i>t</i> = 123 days	93	177.0	169.5	100	3.26	12.9
<i>t</i> = 184 days	120	143.6	142.1	124	3.87	21.1
<i>t</i> = 318 days	153	177.0	174.7	140	4.04	30.2

^a The richness estimators and diversity indices were calculated based on equal number of sequences (1100 sequences) which were randomly selected from each sample.

^b Operational taxonomic units (OTUs) were calculated based on 97% sequence similarity.

nutrient sources (Griebler and Lueders, 2009). The ethanol-blend release would increase available metabolic niches in the aquifer, likely stimulating the growth of diverse species and increasing richness and diversity. Although species richness and ecological resilience have been positively correlated (Botton et al., 2006), further research is needed to determine whether ethanol-driven increases in richness and diversity would enhance the resilience of aquifers to additional releases or to hydrocarbons remaining after the ethanol is consumed.

3.4. Effect of seasonal fluctuations in groundwater temperature

We previously reported that the biodegradation of ethanol, benzene and toluene proceed faster in this system during the summer than in the winter (Ma et al., 2011). Here, we show a corroborating impact of temperature on functional gene abundance and groundwater geochemical footprints (i.e., pH, redox potential and dissolved oxygen). In winter, low temperature inhibited microbial fermenting activities. Correspondingly, the abundance of methanogenesis (*mcrA*) and acetogenesis (*fts*) functional genes decreased to the lowest level (Fig. 4). Significant correlations ($p < 0.05$) between CH_4 concentrations and *mcrA* gene abundance (Fig. S3) and between acetate concentrations and *fts* gene abundance (Fig. S4) were observed. Slower microbial activities in the winter also resulted in less oxygen consumption and less organic acid production, which led to the rebound of dissolved oxygen concentration, redox potential and pH (Fig. S1). Higher dissolved oxygen solubility at lower temperatures also likely contributed to the rebound of dissolved oxygen concentrations.

Microbial community structure also changed in different seasons (Fig. 3, Figs S5 and S6), likely reflecting changes in dissolved oxygen concentration, redox potential, pH and temperature. The phylum *Proteobacteria* predominated in three samples: “*t* = 0 day” (August), “*t* = 123 days” (December) and “*t* = 318 days” (June), but the phylum *Verrucomicrobia* (specifically *Subdivision3_genera_incertae_sedis*) predominated in “*t* = 184 days” (February) during the coldest month of the year (Fig. S1). *Verrucomicrobia* is relatively abundant in a wide range of aquatic and terrestrial systems (Sangwan et al., 2005; Wagner and Horn, 2006), and has also been found in hydrocarbon-, pesticide-, uranium-, and mercury-contaminated sites (Cebon et al., 2009; Michalsen et al., 2007; Rastogi et al., 2010; Vishnivetskaya et al., 2011). However, the broader phylogenetic relationships and physiologic characteristics of *Verrucomicrobia* are still unknown, which precludes the discussion about its potential roles and why this taxon predominated in the winter.

3.5. Overall effects on biodegradation of the continuous ethanol-blend release

Whether microbial adaptation and associated changes in microbial community structure resulted in increased rate of

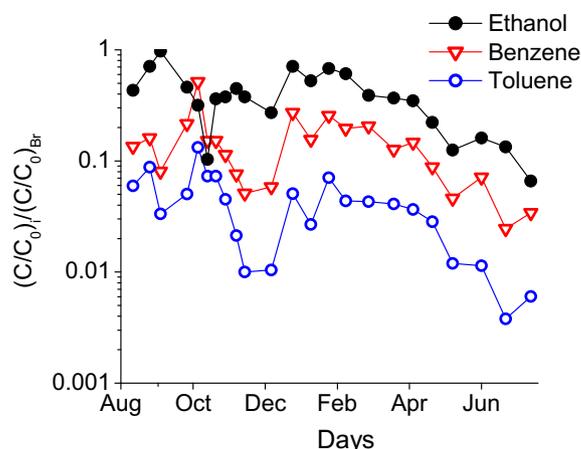


Fig. 5. Bio-attenuation of ethanol, benzene and toluene during the continuous release of ethanol-blended solution.

attenuation was determined by linear regression of normalized concentration versus time data (Figs S7–9). This normalization approach factors out dilution effects and discerns removal processes (mainly biodegradation) (Alvarez and Illman, 2005). Despite temperature fluctuations that are known to affect biodegradation rates, a statistically significant trend ($p < 0.05$) of decreasing normalized concentrations was observed for ethanol, benzene and toluene (Fig. 5). Since influent concentrations remained relatively constant, this suggests that biodegradation rates increased over the 10-month experiment. This observation is consistent with the adaptive shift in microbial community structure (indicated by higher microbial diversity and greater abundance of related functional genes), which are conducive to enhanced bioremediation of an ethanol-blended fuel.

4. Conclusion

The enrichment of acetogens and methanogens following an ethanol-blend release reflects an adaptive response for anaerobic bioremediation. Unlike recalcitrant contaminants, the release of ethanol increased the species richness and diversity. By influencing functional gene abundance and microbial community structure, groundwater temperature can significantly affect biodegradation activities and their geochemical footprints (e.g., pH, redox potential, and dissolved oxygen). Overall, this study demonstrates adaptive microbial population shifts that enhance biodegradation of an ethanol-blended fuel, and underscores the importance to consider seasonal changes in groundwater temperature for assessing and predicting the performance of natural attenuation.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.envpol.2013.03.057>.

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