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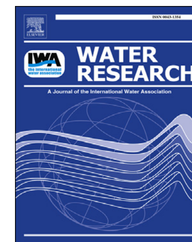

Groundwater ecosystem resilience to organic contaminations: microbial and geochemical dynamics throughout the 5-year life cycle of a surrogate ethanol blend fuel plume

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ARTICLE INFO

Article history:
Received 13 January 2015
Received in revised form
31 March 2015
Accepted 4 May 2015
Available online 9 May 2015

Keywords:
Phylogenetic
Taxonomic
Biofuel
Resilience
Metagenomic
Diversity

ABSTRACT

The capacity of groundwater ecosystem to recover from contamination by organic chemicals is a vital concern for environmental scientists. A pilot-scale aquifer system was used to investigate the long-term dynamics of contaminants, groundwater geochemistry, and microbial community structure (by 16S rRNA gene pyrosequencing and quantitative real-time PCR) throughout the 5-year life cycle of a surrogate ethanol blend fuel plume (10% ethanol + 50 mg/L benzene + 50 mg/L toluene). Two-year continuous ethanol-blended release significantly changed the groundwater geochemistry (resulted in anaerobic, low pH, and organotrophic conditions) and increased bacterial and archaeal populations by 82- and 314-fold respectively. Various anaerobic heterotrophs (fermenters, acetogens, methanogens, and hydrocarbon degraders) were enriched. Two years after the release was shut off, all contaminants and their degradation byproducts disappeared and groundwater geochemistry completely restored to the pre-release states (aerobic, neutral pH, and oligotrophic). Bacterial and archaeal populations declined by 18- and 45-fold respectively (relative to the time of shut off). Microbial community structure reverted towards the pre-release states and alpha diversity indices rebounded, suggesting the resilience of microbial community to ethanol blend releases. We also found shifts from O_2-sensitive methanogens (e.g., *Methanobacterium*) to methanogens that are not so sensitive to O_2 (e.g., *Methanosarcina* and *Methanocella*), which is likely to contribute to the persistence of methanogens and methane generation following the source removal. Overall, the rapid disappearance of contaminants and their metabolites, rebound of geochemical footprints, and resilience of microbial community unequivocally document the natural capacity of groundwater ecosystem to attenuate and recover from a large volume of catastrophic spill of ethanol-based biofuel.

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http://dx.doi.org/10.1016/j.watres.2015.05.003
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1. Introduction

Groundwater constitutes the largest terrestrial freshwater ecosystem, but still belongs to the least explored one on earth (Griebler et al., 2014). The capability of groundwater ecosystems to recover from pollution (such as fuel spills) is a vital concern for environmental scientists. As the most important mechanism (sometimes the only available one) for contaminant elimination in groundwater, biodegradation plays a key role for the resilience of groundwater ecosystems to contaminations (Loffler and Edwards, 2006). However, few studies have comprehensively investigated the temporal variability in microbial community structure and the long-term evidence for contaminant elimination and groundwater geochemistry evolution throughout the whole life cycle of a contaminant release, from initial plume expansion through its stabilization and eventual disappearance (Yagi et al., 2010). Improved understanding on dynamics of microbial community (supported by contaminants and geochemical monitoring data) throughout the entire life cycle of a contaminant plume would undoubtedly improve our understanding of the susceptibility and recovery of groundwater ecosystem to organic contaminations.

The growing use of ethanol as transportation biofuel is increasing the likelihood of encountering ethanol in current and future fuel releases (Ma et al., 2013b). Previous studies focused on microbial responses following the start of the ethanol blend fuel releases (Capiro et al., 2008; da Silva and Corseuil, 2012; Elazhari-Ali et al., 2013; Feris et al., 2008; Ma et al., 2013a; Nelson et al., 2010). However, few studies investigated microbial responses after the contaminant source was removed, which is usually the first and the most important step to remediate a contaminated site. Ma et al. used GeoChip to characterize the changes in functional gene structure in response to 2-year continuous releases of ethanol blend release and complete shut off of the release for 4 months (Ma et al., 2015a). That study showed that most functional genes returned to pre-release abundance levels, but the final functional structure still significantly differed from pre-release conditions (Ma et al., 2015a). As it acknowledged, 4 months of recovery time in that study was too short to fully understand the microbial response following source removal. It is still not known whether microbial community is able to completely restore to pre-release conditions following a longer recovery period.

Moreover, most of previous taxonomic studies rely on finger printing tools such as denaturing gradient gel electrophoresis (DGGE) (Capiro et al., 2008; Elazhari-Ali et al., 2013), automated ribosomal intergenic space analysis (ARISA) (Nelson et al., 2010), and quantitative real-time PCR (qPCR) (Beller et al., 2008; Capiro et al., 2008; da Silva and Corseuil, 2012; Feris et al., 2008; Nelson et al., 2010). These approaches generally detect only predominant microbial groups 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). Ma et al. used 454 pyrosequencing to characterize microbial community in impacted aquifer, however, this study only detected 1000–2000 16S rRNA gene sequences per sample which was too low to have complete coverage (Ma et al., 2013a). A more in-depth characterization (e.g., >10,000 16S rRNA gene sequences/sample) of microbial community would undoubtedly reveal previously unrecognizable level of biodiversity, thus providing a more complete and accurate picture of microbial ecology in impacted sites.

In a pilot-scale model aquifer system, 16S rRNA gene pyrosequencing and qPCR was used to characterize microbial successions throughout the 5-year life cycle of a surrogate ethanol blend fuel plume. The pilot-scale experiments are unique in that they are of sufficient scale such that more realistic three-dimensional contaminant plumes can be established, but at a small enough scale to provide sufficiently controlled experimental conditions. Contaminant concentrations (ethanol, benzene, toluene, methane, acetate, and butyrate) and groundwater geochemical parameters (temperature, pH, redox potential, and dissolved oxygen) were monitored throughout the plume life cycle to provide a comprehensive perspective of impacts and dynamics of the blend release.

2. Materials and methods

2.1. Pilot-scale tank aquifer

An 11-m^3 (3.7 m × 1.8 m × 1.2 m) pilot-scale continuous-flow tank packed with fine grain southeast Texas sand (Circle Sand; Houston, Texas) was used in this study (Fig. S1 in the supporting information). Tap water was added from the "inlet" (Fig. S1) at 170 L/day (average seepage velocity of 0.76 m/day) to obtain a water table elevation of about 70 cm from the bottom of the tank. The influent water contained around 5.5 mg/L of dissolved oxygen (DO). The groundwater retention time in this model tank was around 4 days. The total aquifer thickness was 115 cm and the depth of the water table was 45 cm below ground surface. The surrogate ethanol blend solution was a water solution containing 10% (v/v) ethanol, 50 mg/L benzene, 50 mg/L toluene and 24,000 mg/L of sodium bromide (NaBr). The blend solution was continuously injected into the tank from the ethanol blend injection well (22.5 cm below the water table) at a rate of 0.4 L/day. NaBr was added as a conservative tracer, and to maintain a solution density to reach neutral buoyancy with the flowing groundwater. The added NaBr was diluted by the tank flow to less than 2000 mg/L (measured at groundwater sampling port, see Fig. 1), which was within the typical tolerance range of soil bacteria (Atlas and Bartha, 1997). The groundwater sampling well was at the same depth as the ethanol blend injection well (22.5 cm below the water table). Details on the tank construction and packing methods can be found in Ma et al. (2011) and Ma et al. (2012).

2.2. Release stages and plume life cycle

This pilot-scale release experiment lasted for 5 years, which can be divided into 4 experimental stages (6 time points) (Fig. 1). General information for each stage can be found in Table 1. Stage 1 was the pre-release baseline. Stage 2 began with the continuous ethanol blend release (10% ethanol + 50 mg/L benzene + 50 mg/L toluene) and lasted 2

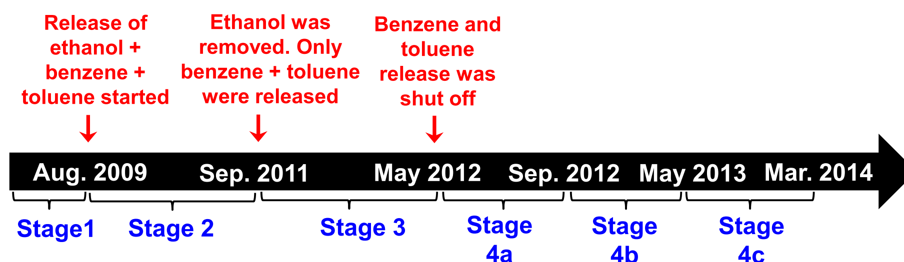


Fig. 1 – Timeline of the release experiment.

years. Stage 3 followed the removal of ethanol from the release, resulting in continuous exposure to 50 mg/L benzene and 50 mg/L toluene for 8 months. This stage, which mimicked the earlier removal of ethanol than hydrocarbons (Corseuil et al., 2011), investigated how the disappearance of ethanol affects the fate of benzene and toluene. Stage 4 was the return to initial conditions (benzene and toluene releases were shut off), when clean water continuously flowed through the aquifer material. Stage 4 can be divided into 3 subsections: Stage 4a, Stage 4b, and Stage 4c, which were at 4 months, 1 year, and 2 years after benzene and toluene releases were shut off. At the end of each experimental stage (Stage 1, 2, 3, 4a, 4b, and 4c), sand samples were collected for prokaryotic DNA pyrosequencing and soil property analysis, and groundwater samples were collected for chemical and geochemical analysis. The sampling date can be found in Table 1.

2.3. *Analysis of groundwater pollutants and geochemical parameters*

For chemical analysis, four replicate groundwater samples were collected from the groundwater sampling well using 50 mL syringes at the end of each experimental stage. Ethanol, methane, acetate, propionate, butyrate, and butanol were measured by GC-FID (Hewlett Packard, Palo Alto, CA, USA). Ethanol, acetate, propionate, butyrate, and butanol were measured by liquid injections while methane was measured by headspace injections. The detection limits (aqueous concentration) were 1 mg/L for ethanol, acetate, and propionate, 2 mg/L for butyrate and butanol, and 0.1 mg/L for methane. Benzene and toluene were pre-concentrated by Purge and Trap System (Tekmar, Vernon, BC, Canada) and measured by GC-MS (Agilent, Santa Clara, CA, USA) with a detection limit of 10 μg/L (aqueous concentration). Details on chemical measurement methods can be found in Ma et al. (2011).

Groundwater geochemical parameters during Stage 1 and 2 (including pH, dissolved O_2, temperature, redox potential and conductivity) were monitored by a YSI 600XLM groundwater geochemical probe (YSI, Yellow Springs, Ohio, USA) which was installed 15 cm upstream from the groundwater sampling well (Fig. S1). No redox and conductivity data was available 855 days after the release began since the probe broke. So after that, the temperature was measured by a Pen-Style Thermometer (Taylor Precision Products, Oak Brook, IL, USA); dissolved O_2 were measured by a Dissolved Oxygen AccuVac® Ampules kit (Hach, Loveland, CO, USA); pH was measured by a Pocket pH Tester (Davis Instruments, Vernon Hills, IL, USA).

2.4. *Sand sampling and analysis*

For genetic analysis (pyrosequencing and qPCR) and sand chemical analysis, 5 replicate sand samples were collected from a depth of 5–30 cm below water table (50–75 cm below the sand surface, Fig. S1). Details on sand sampling method can be found in Ma et al. (2013a). Dry sand samples were sent to the Soil, Water and Forage Testing Laboratory at Texas A&M University for the measurement of soil pH, total organic carbon content, conductivity, nitrate-nitrogen, P, K, Ca, Mg, S, and Na (Table S1). Details on soil analytical methods can be found in the supporting information.

2.5. *qPCR analysis*

DNA was extracted from 0.25 g sand in 4 replicates (collected from the same location) using a PowerSoil DNA Isolation Kit (MOBIO, Carlsbad, CA, US). Absolute quantification (Taqman PCR reactions) was used to enumerate the gene copy number for 16S rRNA gene for total Bacteria and total Archaea. The reaction mixtures contained 12.5 μL TaqMan® Environmental Master Mix 2.0 (Applied Biosystems; Foster City, CA, USA), 500 nM each primer, 200 nM probe and 2 μL template DNA in a total volume of 25 μL. An ABI 7500 Sequence Detector (Applied Biosystems, Foster City, CA, USA) was used to perform qPCR reactions with the following temperature program: 50 °C for 2 min, followed by 95 °C for 10 min and 40 cycles at 95 °C for 15s, and 30 s at annealing temperature for each primer set (Table S2), 40 s at 72 °C for extension. The practical quantification limits for bacterial and archaeal 16S rRNA gene 1000 copies/g dry sand. Details on target genes, primer sequences, and DNA standards can be found in Table S2.

2.6. *Pyrosequencing*

DNA was extracted from 10 g sand (triplicates for each time point × 6 time points, collected from the same location) using a modified (see supporting information) PowerMax Soil DNA isolation kit (MOBIO, Carlsbad, CA, US). For library preparation, the bacterial 16S rRNA gene was amplified using bacterial primers 347F and 803R and the archaeal 16S rRNA gene was amplified using primers A571F and UA1204R. Sequencing was conducted on a Genome Sequencer Junior System (454 Life

Table 1 – General information for each experimental stage.

Stage	Stage 1	Stage 2	Stage 3	Stage 4a	Stage 4b	Stage 4c
Sampling date	8/7/2009	9/5/2011	5/4/2012	9/2/2012	5/23/2013	3/25/2014
Days after the release stated	−10	749	991	1112	1375	1681
Action	Pre-release baseline	Exposed to ethanol, benzene and toluene mixture for 2 years	Exposed to benzene and toluene mixture for 8 months	Exposed to clean water for 4 months after Stage 3	Exposed to clean water for 1 year after Stage 3	Exposed to clean water for 2 years after Stage 3
Prevailing electron accepting conditions	Aerobic	Methanogenic	Aerobic	Aerobic	Aerobic	Aerobic

Sciences, Branford, CT, US). Details on amplicon library preparation (including primer set sequences) and pyrosequencing can be found in the supporting information.

2.7. *Sequence data processing and statistical analysis*

A total of 585, 585 bacterial raw sequences and 285, 036 archaeal raw sequences were obtained. The sequencing data were processed using the QIIME pipeline (Caporaso et al., 2010) and details can be found in the supporting information. After removing low quality and chimeric sequences, an average of 22,630 bacterial sequences per sample and 12,194 archaeal sequences per sample were obtained (Table S3). Since sample heterogeneity would bias the comparison of samples with different sequence numbers, all bacterial samples were rarefied to 12,000 sequences/sample and all archaeal samples were rarefied to 8000 sequences/sample. Rarefaction curves of both bacterial and archaeal libraries were provided in Fig. S2. Alpha diversity measures the diversity within a particular area or ecosystem, and beta diversity is a comparison of diversity between ecosystems (Whittaker, 1972). A variety of alpha diversity indices, that describe species richness (Chao1), evenness (Equitability), and diversity (Shannon), were calculated for both bacterial and archaeal libraries. Principal Coordinate Analysis (PCoA) and hierarchy clustering analysis were conducted to assess beta diversity (dissimilarity) of microbial communities among different stages based on weighted Unifrac distances.

3. Results and discussion

3.1. *Contaminant concentrations and geochemical footprints of the plume*

Biodegradation of ethanol blend rapidly consumed all dissolved oxygen (DO) and created a strongly anaerobic condition with low pH, and groundwater geochemical conditions completely reverted to the pre-release condition after the release was shut off. Changes in geochemical footprints throughout the plume life cycle can be found in Fig. 2 (DO and pH), Fig. S3 (temperature), and Fig. S4 (redox potential and conductivity). Changes in contaminant concentrations can be found in Fig. 3 (ethanol, benzene, and toluene) and Fig. S5 (methane, acetate, and butyrate). The blend release started on August 17th 2009 ($t = 0$ day). As the pre-release baseline sample, Stage 1 ($t = -10$ days) represented aerobic (5.1 mg/L of DO and +110 mV of redox potential) and oligotrophic (no pollutants) conditions. As the sample exposed to ethanol blend release for 2 years, Stage 2 ($t = 749$ days) represented highly reducing (0 mg/L of DO and −402 mV of redox potential) and organotrophic conditions. High concentrations of contaminants (ethanol, benzene, and toluene) and their anaerobic degradation byproducts (methane, acetate, and butyrate) were detected and fermentative degradation resulted in low pH (6.4) at Stage 2. Within Stage 2, DO and pH rebounded during Day 150–200 and Day 470–550 (Fig. 2). This was due to the low groundwater temperature in winter that inhibited the fermentative biodegradation activity, thus leading to less oxygen consumption and volatile fatty acids production (Fig. S5).

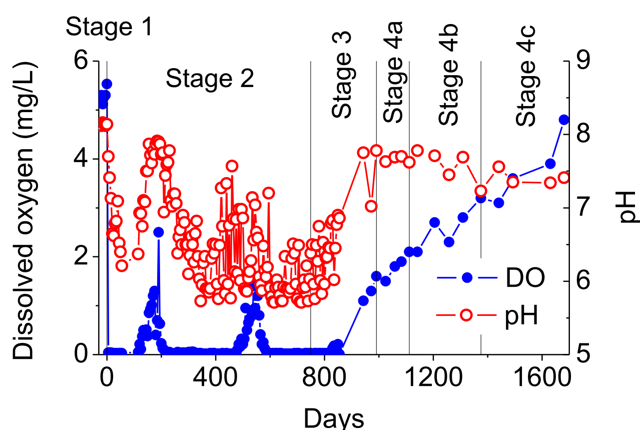


Fig. 2 – Changes in dissolved oxygen concentration (DO) and pH throughout the 5-year life cycle of ethanol blend plume. The release started at t = 0 day on August 17, 2009. DO and pH between day −30 and day 855 was measured by a groundwater geochemical probe. DO between day 855 and day 1681 was measured by Dissolved Oxygen AccuVac® Ampules kit. pH between day 855 and day 1681 was measured by a Pocket pH Tester. Other groundwater geochemical parameters such as temperature, redox potential, and conductivity can be found in supporting information.

More information about impacts of seasonal variation in temperature on contaminant biodegradation and groundwater geochemistry in this tank can be found in Ma et al. (2011). Stage 3 followed the removal of ethanol from the release solution and continuous exposure to 50 mg/L benzene and 50 mg/L toluene for 8 months. Since ethanol was the main carbon source in this model aquifer, removal of ethanol caused significant changes in groundwater geochemistry. At the end of Stage 3 (t = 991 days), DO increased to 1.6 mg/L and pH rebounded to near-neutral levels. Stage 4 was the return to initial conditions (benzene and toluene removed from the tank influent), when clean water continuously flowed through the aquifer material. At the end of Stage 4c (t = 1681 days), both DO and pH returned to the same level as Stage 1 and no contaminant (or their degradation byproducts) was detected, indicating complete attenuation of all contaminants and complete recovery of groundwater geochemical conditions. More information on contaminant plume and groundwater geochemical responses to 1) the start of the release and 2) the shut-off of such release can be found in Ma et al. (2011) and Ma et al. (2015b) respectively.

3.2. *Changes in total population of bacteria and archaea by qPCR*

As a readily degradable compound, ethanol represented a favorable carbon and energy source that stimulates microbial growth. Accordingly, 2-year continuous ethanol-blended release increased the abundance of bacterial and archaeal 16S rRNA gene by 82- and 314-fold respectively (Stage 2 VS Stage 1, Fig. 4). Following contaminant source removal, carbon source (including both dissolved contaminants in groundwater and organic matter attached on soil particles) gradually depleted (Fig. 3, S5, and Table S1). Accordingly, the abundance of bacterial and archaeal 16S rRNA gene decreased by 18- and 45-fold respectively relative to the time of shut off (Fig. 4).

3.3. *Overall dissimilarity for samples at different time points*

Ordination analysis for pyrosequencing data shows that 1) ethanol blend release significantly changed the structure of bacterial and archaeal communities; 2) after the release was shut off, the structure of both bacterial and archaeal communities shifted back towards the pre-release conditions. PCoA analysis (Fig. 5) and hierarchy clustering analysis (Fig. S6) shows that Stage 1 and Stage 2 had distinct microbial community structure. This corroborates our previous functional gene microarray (GeoChip) data (Ma et al., 2015a), suggesting that ethanol blend release led to dramatic changes in microbial structure. Fig. 5 and S6 also show that the dissimilarity of microbial community between Stage 1 and Stage 4c was much smaller than that between Stage 1 and Stage 2, suggesting that microbial communities reverted towards the pre-release states following the source removal. Note that

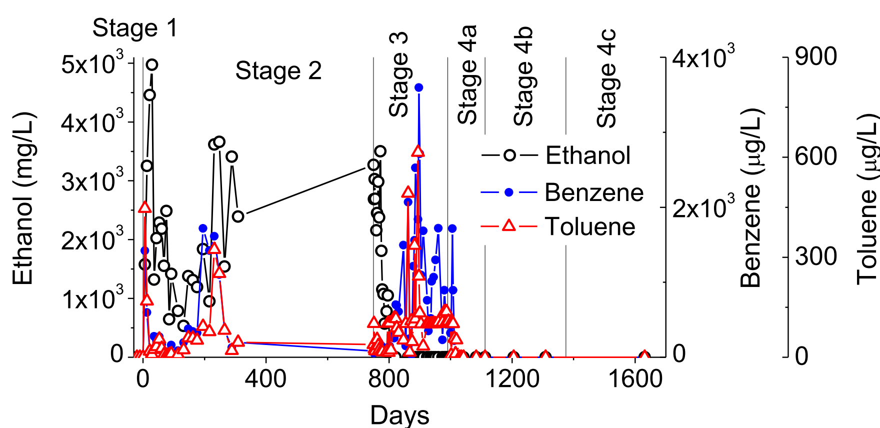


Fig. 3 – Changes in groundwater concentrations of ethanol, benzene, and toluene throughout the 5-year life cycle of ethanol blend plume. The release started at t = 0 day. The concentrations of dissolved CH_4, acetate, and butyrate can be found in supporting information.

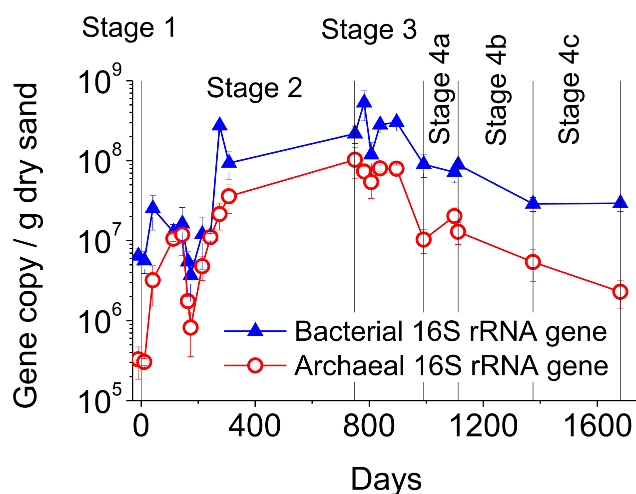
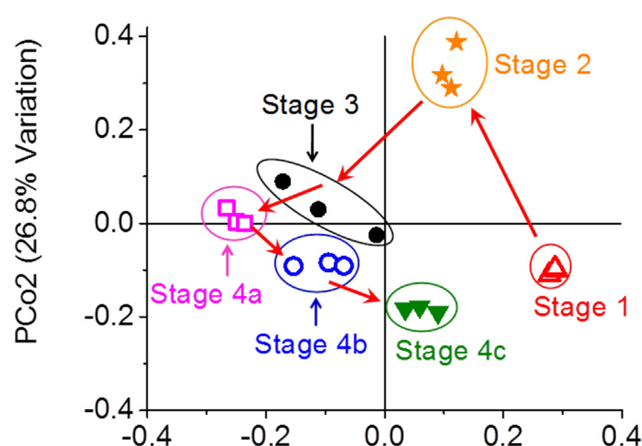


Fig. 4 – Changes in the abundance of bacterial and archaeal 16S rRNA gene throughout the 5-year life cycle of ethanol blend plume. The release started at t = 0 days.



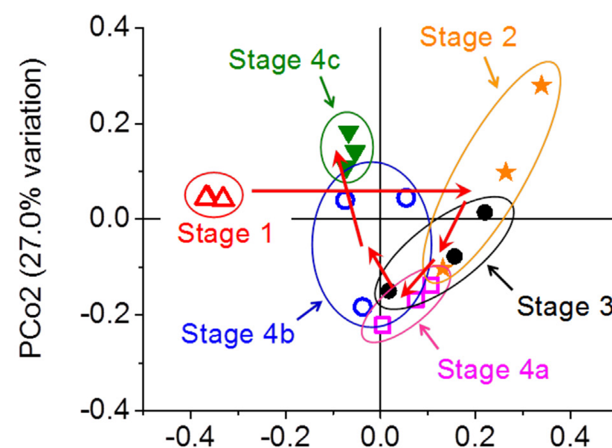


Fig. 5 – Principal coordinate analysis (PCoA) plot based on weighted UniFrac distance matrix showing successional trajectories of bacterial (a) and archaeal (b) communities throughout the 5-year plume life cycle. The weighted UniFrac distance matrix was calculated from 12,000 sequence even rarefactions for Bacteria and 8000 sequence even rarefactions for Archaea.

there were still some differences between Stage 4c and Stage 1. This indicated that microbial restoration process was slower than the recovery of groundwater geochemistry and such process had not been completed yet even two years after the source removal.

3.4. *Bacterial community shifts throughout the 5-year life cycle of the plume*

At Stage 1, the top 15 dominant bacterial genus were Gp6 (33.62%), Gp3 (13.5%), Gp16 (10.9%), *Nitrospira* (6.6%), *Sphingomonas* (4.9%), Gp2 (4.3%), Gp1 (3.5%), Gp12 (3.2%), Gp7 (2.6%), *Aquicella* (2%), Gp14 (1.4%), *Gemmatimonas* (1.2%), Gp22 (1.1%), Gp13 (1%), and *Ohtaekwangia* (1%) (Table 2). GP 6, GP3, Gp16, Gp2. Gp12, Gp7, Gp14, Gp22, and Gp13 all belong to the phylum Acidobacteria which is abundant in many soil and sediment environments. Most members in the phylum Acidobacteria are very difficult to isolate, so little is known about their physiology and ecological functions (Liles et al., 2010). *Nitrospira* is a group of nitrite-oxidizing chemolithoautotrophs (Ehrich et al., 1995; Luecker et al., 2010). As a group of strictly aerobic heterotrophs (Krieg, 2010), *Sphingomonas* are able to survive in low concentrations of nutrients (Stolz, 2009). Both *Aquicella* and *Ohtaekwangia* are strictly aerobic (Santos et al., 2003; Yoon et al., 2011). *Gemmatimonas* are aerobic or microanaerobic (Zhang et al., 2003).

The ethanol blend release drove the system to strongly anaerobic conditions and enriched a variety of anaerobic heterotrophs, fermenters, acetogens, and syntrophs at Stage 2 (Table 2). The enriched bacterial genera included *Clostridium* (26.3%), *Syntrophomonas* (12.3%), *Desulfovibrio* (11.8%), *Desulfuromonas* (8%), *Geobacter* (5%), *Propionispora* (3.8%), *Longilinea* (2.8%), Gp16 (2.7%), *Methylosarcina* (2.5%), *Desulfobulbus* (2.5%), *Pseudomonas* (2%), *Desulfocapsa* (1.7%), Gp6 (1.2%), *Methylocystis* (1.2%), and *Sporomusa* (1.1%). *Syntrophomonas* is a group of syntrophic volatile fatty acid degraders (Harms et al., 2003; Luecker et al., 2010). Some species of *Desulfovibrio* are also able to syntrophically metabolize ethanol and lactate (Sieber et al., 2012). *Desulfuromonas* are anaerobes that could degrade volatile fatty acids (Coates et al., 1995; Vandieken et al., 2006). *Propionispora* are anaerobic fermenters (usually produce acetate and propionate) (Abou-Zeid et al., 2004; Biebl et al., 2000). *Longilinea* are anaerobic fermenters that produce volatile fatty acids (Yamada et al., 2007). *Sporomusa* are homoacetogens (Hattori et al., 2013). *Geobacter* (Lovley et al., 2011), *Desulfobulbus* (Pagani et al., 2011), and *Desulfocapsa* (Finster et al., 1998) are all anaerobes. Although a variety of sulfate reducing bacteria (SRB) were found at Stage 2, this did not necessarily reflect the occurrence of sulfate reduction in the tank. Many studies showed that SRB can use many other electron acceptors (e.g., NO_3^-, NO_2^-, Fe(III), U(VI), Cr(VI), and As (VI)) for growth and can even ferment organic compounds in

Table 2 – The 15 most abundant bacterial genera at Stage 1, Stage 2, and Stage 4c.[a]

Stage 1		Stage 2		Stage 4c	
Genus	Percentage (%)[b]	Genus	Percentage (%)	Genus	Percentage (%)
Gp6	33.6	*Clostridium*	26.3	*Nitrospira*	16.0
Gp3	13.5	*Syntrophomonas*	12.3	*Pseudomonas*	15.4
Gp16	10.9	*Desulfovibrio*	11.8	*Bellilinea*	12.3
Nitrospira	6.6	*Desulfuromonas*	8.0	Gp6	10.2
Sphingomonas	4.9	*Geobacter*	5.0	*Ohtaekwangia*	8.2
Gp2	4.4	*Propionispora*	3.7	Gp3	5.4
Gp1	3.5	*Longilinea*	2.8	*Desulfuromonas*	3.2
Gp12	3.2	Gp16	2.7	*Geobacter*	2.6
Gp7	2.6	*Methylosarcina*	2.5	TM7	2.3
Aquicella	2.0	*Desulfobulbus*	2.1	Gp13	1.6
Gp17	1.4	*Pseudomonas*	2.0	*Methylosarcina*	1.5
Gemmatimonas	1.2	*Desulfocapsa*	1.7	*Azorhizophilus*	1.4
Gp22	1.1	Gp6	1.2	Gp2	1.0
Gp13	1.0	*Methylocystis*	1.2	Gp16	0.9
Ohtaekwangia	1.0	*Sporomusa*	1.1	*Bdellovibrio*	0.9

[a] Due to space limitation, the 15 most abundant bacterial genera at Stage 3, Stage 4a and Stage 4b were provided in the supporting information (Table S4).
[b] The percentage of each genus was the average value of triplicate samples at each stage.

the absence of inorganic electron acceptors (Muyzer and Stams, 2008). It was not known what types of terminal electron acceptors these SRB used for survival in the tank.

At Stage 4c, some dominant taxa of Stage 1 rebounded, including *Nitrospira* (16.0%), Gp6 (10.1%), *Ohtaekwangia* (8.2%), Gp3 (5.4%), Gp12 (1.7%), Gp2 (1%) and Gp16 (0.9%) (Table 2). In contrast, the relative abundance of anaerobic heterotrophs, fermenters and syntrophs (enriched at Stage 2) had a drastic decline at Stage 4c. Such changes in community structure corroborates ordination analysis (PCA and clustering), indicating the recovery of bacterial community towards the pre-release states. *Nitrospira* grow chemolithoautotrophically on NO_2^- (nitrite oxidation) and CO_2 (carbon fixation) (Ehrich et al., 1995; Luecker et al., 2010). Stage 4 represented aerobic (5.1 mg/L of DO) and oligotrophic (no pollutants) conditions, thus facilitating the enrichment of *Nitrospira*.

3.5. *Archaeal community shifts throughout the 5-year life cycle of the plume*

At Stage 1, the archaeal community was dominated by the class Thermoprotei (Table 3). Thermoprotei has been reported to be abundant in desert sands, and most of the species in this class are chemolithotrophs (Andrew et al., 2012). The pre-release Stage 1 had very low organic carbon content (0.063% ± 0.011%, Table S1) and other nutrients, thus is likely to facilitate the establishment of chemolithrophic Thermoprotei.

At Stage 2, the ethanol blend release enriched two acidiphilic methanogens (*Methanobacterium* and *Methanosarcina*) and significantly changed the archaeal community structure (Table 3). Although some studies showed that low pH may inhibit methanogenic activity, methanogens have been detected and isolated from acidic environments (e.g., pH < 4.5–5.5) (Braeuer et al., 2011; Brauer et al., 2006; Cadillo-Quiroz et al., 2009). The most acidiphilic hydrogenotrophic methanogen described to date is *Methanobacterium espanolae*, with a pH optimum between 5.5 and 6.0 and a minimum near 4.7 (Patel et al., 1990). Some strains of *Methanosarcina* spp. (acetoclastic methanogen) can grow near pH4.5 (Maestrojuan and Boone, 1991). At Stage 2, the fermentation of ethanol blend created an acidic environment with pH of 4.7–5.5 (Fig. 2). Interestingly, the dominant archaeal groups at Stage 2 were *Methanobacterium* and *Methanosarcina*. Apparently, the tolerance of *Methanobacterium* and *Methanosarcina* to low pH facilitated their enrichment at Stage 2. Enrichment of methanogens at Stage 2 was corroborated by the presence of high concentration of methane in groundwater (Fig. S5) and the enrichment of methanogenic functional gene (*mcrA*) detected by qPCR (Ma et al., 2013a, 2015b) and Geochip (Ma et al., 2015a).

At Stage 4c, some dominant taxa of Stage 1 (e.g., Thermoprotei) rebounded while methanogen populations (enriched at Stage 2) had a drastic decline (Table 3 and Fig. S7). Such changed in community structure corroborates ordination analysis (PCA and clustering), indicating the recovery of archaeal community towards the pre-release states.

3.6. *Methanogen structure shifts following source removal*

Methanogens and methanogenic activities persisted following the shut-off of the ethanol blend release. At Stage 4a, dissolved O_2 concentrations rebounded to 2.1 ± 0.3 mg/L (Fig. 2), but methanogens still occupied >85% of archaeal 16S rRNA gene abundance (Table 3). The unexpected persistence of methanogens was corroborated by qPCR (Ma et al., 2015b) and GeoChip analysis (Ma et al., 2015a) targeting methanogenic functional gene (*mcrA*). We also found that methane generation persisted in groundwater (>1.5 mg/L of dissolved methane) after the disappearance of ethanol and its metabolites (i.e., acetate, butyrate, and butanol), despite clean air-saturated water flowing continuously through the tank at a relative high seepage velocity (0.76 m/day) (Ma et al., 2015b).

Table 3 – Percentage of the most abundant archaeal taxa.[a]

Phylum	Class	Order	Family	Genus	Stage 1	Stage 2	Stage 3	Stage 4a	Stage 4b	Stage 4c
Euryarchaeota	Methanomicrobia	Methanosarcinales	Methanosarcinaceae	*Methanosarcina*	1.8	50.2	64.7	75.6	45.0	19.7
Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	*Methanobacterium*	0.3	45.3	23.7	15.2	15.4	1.6
Crenarchaeota	Thermoprotei	–[b]	–	–	57.5	0.2	1.8	1.2	4.7	32.3
Euryarchaeota	–	–	–	–	0.5	0.7	1.2	0.4	1.3	22.2
Euryarchaeota	Methanomicrobia	Methanocellales	Methanocellaceae	*Methanocella*	0.0	0.0	0.3	4.2	0.6	0.0
Euryarchaeota	Methanomicrobia	–	–	–	0.0	0.0	0.2	0.3	0.1	1.7
Euryarchaeota	Methanomicrobia	Methanosarcinales	Methanosarcinaceae	–	0.0	0.1	0.3	0.1	0.3	1.0
Euryarchaeota	Thermoplasmata	Thermoplasmatales	–	–	0.0	0.1	0.5	0.2	0.2	0.2
Euryarchaeota	Methanomicrobia	Methanosarcinales	–	–	0.0	0.0	0.0	0.1	0.0	0.7

[a] The archaeal 16S rRNA sequence that can not be classified in any phylum was not showed in this table.
[b] "–" indicates that 16S rRNA sequence can not be classified in any group at the corresponding phylogenetic level.

Such phenomena may owe to shifts in the community structure of methanogens following source removal. Compared to Stage 2, Stage 4a had a higher proportion of *Methanosarcina* and *Methanocella* and a lower proportion of *Methanobacterium* (Table 3 and Fig. S7). Although methanogenesis is generally thought to occur only in highly reducing anoxic environments, recent studies found that viable acetoclastic genera *Methanosarcina* and *Methanocella* are present in oxic terrestrial (Angel et al., 2012, 2011) and freshwater (Bogard et al., 2014; Grossart et al., 2011; Paganin et al., 2013) environments. Genomic studies show that these two genera possess several (6 or 7) genes encoding enzymes that detoxify reactive oxygen species (Angel et al., 2011; Erkel et al., 2006). The capability of *Methanosarcina* and *Methanocella* to survive in aerobic environments facilitated the enrichment of these two methanogenic taxa at Stage 4a (2.1 ± 0.3 mg/L of DO was present) and contributed to the temporary persistence of methanogens following the source removal.

Recently, there have been increasing concerns over the methane vapor intrusion risk associated with ethanol blend fuel releases (Freitas et al., 2010; Jewell and Wilson, 2011; Ma et al., 2014, 2012; Sihota et al., 2013). In addition to our pilot-scale study (Ma et al., 2015b), several field studies also reported persistence of dissolved methane following source removal (Corseuil et al., 2011; Sihota et al., 2013; Spalding et al., 2011). It is not known whether indigenous methanogen communities at these sites also had similar adaptive shifts from O_2-sensitive methanogenic taxa to O_2-insensitive ones following source removal.

3.7. *Species richness, evenness, and diversity*

Temporal changes in alpha-diversity indices also reflected the recovery of microbial communities following the source removal. Bacterial evenness (indicated by "Equitability") and diversity (indicated by "Shannon") decreased following the ethanol blend releases (Stage 2 VS Stage 1) and recovered to the pre-release level at Stage 4c (Fig. 6). The release enriched certain groups that were involved in the anaerobic degradation process, thus reducing species evenness at Stage 2. Decreased evenness further decreased bacterial diversity at Stage 2. At Stage 4c, "Equitability" and "Shannon" indices rebounded to the same level as Stage 1, providing further evidence for the restoration of the bacterial community. There were no significant changes ($p > 0.05$) in bacterial richness (index "Chao 1") among Stage 1, 2 and 4c. Archaeal richness (index "Chao 1") was significantly decreased following the release of ethanol blend solution (Stage 2) and recovered to the pre-release level at Stage 4c (Fig. 6). The rebound of richness indices provided another evidence for the restoration of the archaeal community following the source removal. There were no significant changes ($p > 0.05$) in archaeal evenness (index "Equitability") and diversity (index "Shannon") among Stage 1, 2 and 4c.

4. Conclusions

Ethanol blend releases significantly changed the groundwater geochemistry (resulted in anaerobic, low pH, and

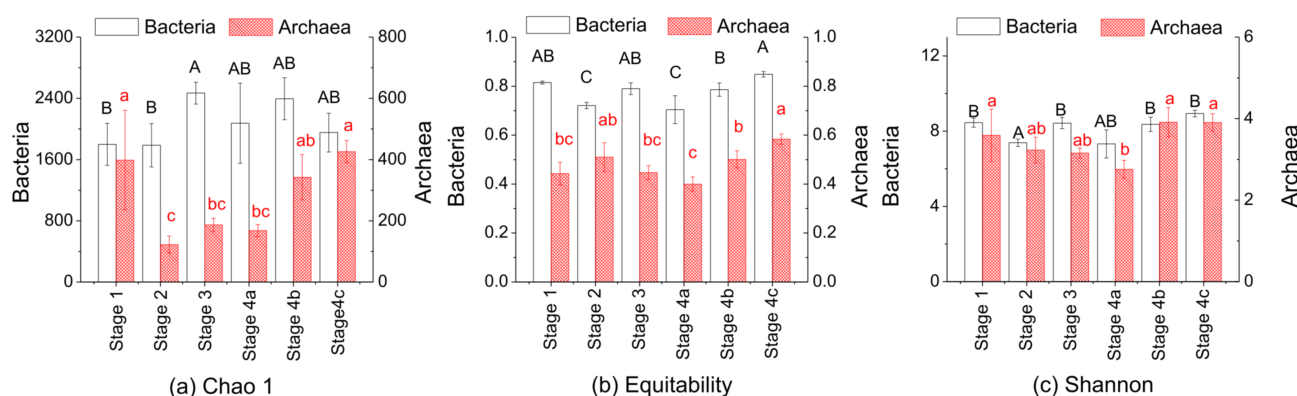


Fig. 6 – Alpha diversity indices of bacterial and archaeal communities. "Chao 1" is a species richness index. "Equitability" is an evenness index. "Shannon" is a diversity index. Letters above each column indicate statistical differences between different stages (at a *p* value of <0.05) by Fisher's least-significant-difference (LSD) test. Statistical difference existed between two stages that do not have the same letter. Capital letters describe bacterial communities and small letters describe archaeal communities.

organotrophic conditions) and microbial community structure (enriched fermenters, acetogens, methanogens, and anaerobic hydrocarbon degraders). After the contaminant source was removed, both groundwater geochemistry and microbial community structure restored towards the pre-release status (aerobic, neural pH, and oligotrophic). However, the restoration process of microbial community was slower than that of groundwater geochemistry. Overall, the rapid disappearance of contaminants, rebound of geochemical footprints, and resilience of indigenous microbial populations unequivocally document the natural capacity of groundwater ecosystem to attenuate and recovery from an accident spill of ethanol-based biofuel. Another interesting phenomena we found was the shift from O_2-sensitive methanogens (e.g., *Methanobacterium*) to the methanogens that were not so sensitive to O_2 (e.g., *Methanosarcina* and *Methanocella*) after the release was shut off. Such changes in methanogenic structure were likely to contribute to the persistence of methanogens and generation of methane (an important vapor intrusion risk driver) following source removal.

Acknowledgments

This work was funded by the American Petroleum Institute (No. 2012-106835), National Natural Science Foundation of China (No. 21407180), Science Foundation of China University of Petroleum-Beijing (No. 2462014YJRC016). The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2015.05.003.

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