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Title

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Impacts of Ethanol on Anaerobic Production of Tert-Butyl Alcohol (TBA) from Methyl Tert-Butyl Ether (MTBE) in Groundwater

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Abstract

Methyl tert-butyl ether (MTBE) is a contaminant of concern to groundwater resources due to its persistence in subsurface environments. MTBE appears to be degraded readily in the presence of oxygen but is recalcitrant under the anaerobic conditions prevalent in the subsurface, and can be converted into the more toxic compound tert-butanol (TBA). As ethanol is being promoted as a renewable fuel and a replacement for MTBE in gasoline formulations, its potential impact on the biodegradation of preexisting contaminants and on other components of petroleum must be examined. The purpose of this study is to investigate the effect of ethanol release on existing MTBE plumes and the fate of TBA under sulfate-reducing conditions. Our results suggest that TBA, MTBE and ethanol-induced methane concentrations are strong determinants of the composition of the indigenous microbial community that develops during MTBE transformation. Some of the changes in microbial communities induced by ethanol may be long lasting, thus potentially altering the natural attenuation capacity of the impacted aquifer.

Introduction and Problem Statement

Five years after being banned in California, methyl tert-butyl ether (MTBE) continues to threaten groundwater resources due to its persistence in the subsurface. MTBE was introduced into gasoline reformulations as an oxygenate and octane enhancer, and enters the environment primarily through leaking underground storage tanks (LUSTs). Ethanol, both as an oxygenate replacing MTBE and as a new fuel alternative, is expected to interact with existing subsurface MTBE plumes when it leaks from LUSTs previously used for MTBE-containing fuels. Previous studies have shown that ethanol may reduce biodegradation of benzene, toluene, ethyl-benzene and xylene (BTEX) and other gasoline contaminants. Moreover, ethanol may stimulate the partial degradation of MTBE to tert-butanol (TBA). TBA is a more problematic groundwater pollutant than MTBE due to its higher toxicity and mobility and because it is difficult to treat using conventional remediation methods. In this study, we focus on the role of microbial processes in the conversion of MTBE and production of TBA, and how these processes are affected by the presence of ethanol.

TBA is a serious emerging ground water threat, especially as ethanol usage in reformulated gasoline is rapidly increasing. Little is known about mechanisms responsible for the transformation of MTBE to TBA in the field and the microbial processes involved. Field data on the degradation potential of TBA under anaerobic conditions is inconclusive, and no microorganisms capable of degrading TBA without oxygen have been isolated in pure culture. In our study, we are investigating changes in the composition of microbial communities to better understand the impacts of MTBE and its degradation products on native microbial communities.

Objectives

Our goal is to understand anaerobic MTBE transformation at an environmentally relevant scale, both chemically and at the microbial community

level, by means of field experiments and controlled microcosms. Our established research site at the Vandenberg Air Force Base (VAFB) is dedicated to investigating the natural attenuation of gasoline and fuel additives in anaerobic aquifers. In the current study, controlled releases were carried out, of either MTBE combined with ethanol or of TBA alone. Groundwater samples were collected for enumeration of both bacterial and archaeal populations using quantitative polymerase chain reaction (qPCR), and analysis of community diversity using a DNA fingerprinting method, terminal restriction fragment length polymorphism (TRFLP). TRFLP profiles were analyzed using multivariate statistical methods, including canonical correspondence analysis (CCA), to determine how microbial communities are affected by the contaminants and environmental variables. A numerical model describing the VAFB field site is being assembled, coupling hydrogeological, chemical, and biological data, to investigate the role of biological degradation in MTBE transformation.

Procedure

The field site is located at the Vandenberg Air Force Base at a site where a leaking underground storage tank released MTBE, BTEX and other compounds into the shallow aquifer (Fig. 1). The original contaminated source area was excavated and back-filled in the mid 1990s. Following monitoring of the original plume, controlled release experiments were carried out by injecting contaminants of interest at known concentrations into the aquifer. Concentrations downstream of injection sites are monitored by sampling a dense network of monitoring wells (eight major transects 10 - 20 ft apart, each transect consisting of between 20 and 30 monitoring wells at ~3 ft apart; see Fig. 1).

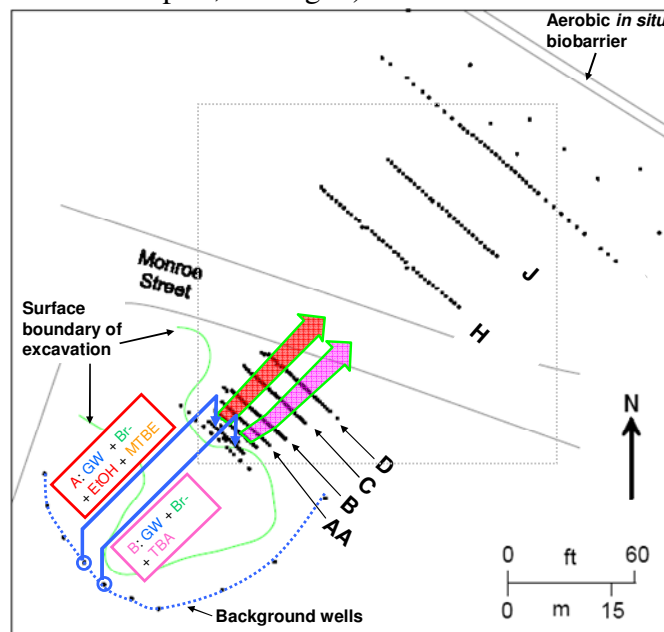


Figure 1. Layout of field experiment. Groundwater up gradient of the originally contaminated area (designated in green) was mixed with bromide, ethanol and MTBE in Lane A, and bromide and TBA in Lane B, and injected into upstream of AA transect. Concentrations of MTBE, TBA and bromide were monitored in the wells arranged in transects normal to flow. Relevant monitoring well transects are designated AA – J. The biobarrier prevented spread of injected compounds beyond test area. Dashed line square is the area sampled for microbial analyses.

The current experiment involved the release of a mixture of MTBE and ethanol (Lane A) and release of TBA (Lane B), to investigate 1) the effect of ethanol on the fate of MTBE and 2) the fate of TBA, both injected and produced (Fig. 2), under the strongly sulfate reducing conditions of the aquifer. Sulfate reduction is crucial to microbial metabolism of xenobiotic contaminants in many contaminated aquifers. Bromide was injected, as a conservative tracer, starting approximately two months before and ending two months after the injection period for MTBE, TBA and ethanol (Fig. 2). To characterize the contaminant plume over time, groundwater samples were collected regularly for gas chromatograph analysis of VOCs, and to measure levels of sulfate. Bromide was measured using HPLC. Descriptions of these analyses are described in Mackay et al (2006).

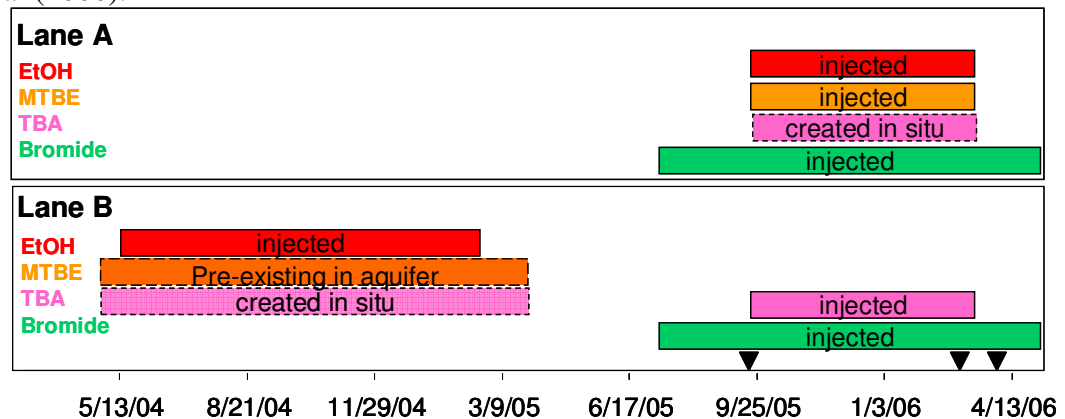


Figure 2 Concentrations of contaminants and tracer in the field in lanes A and B over the course of the current and previous experiment. Samples for microbial community analyses were taken on 9/20/2005, 2/22/2006, 4/06/2006 (▼), and post-experiment samples 9/22/2006 and 9/23/2007(unmarked).

In addition to VOC and bromide analysis, 500 ml groundwater samples were collected for investigations of microbial communities. Groundwater samples for microbial analysis were shipped to UC Davis in coolers with icepacks within 48 hours of sampling, and filtered using 0.22 µm nitrocellulose filters. Biomass-containing filters were stored at -20°C, and DNA was extracted using Bio101 FastDNA® SPIN Kit for Soil. DNA was quantified by measuring absorbance at 260 nm. Each 75 µl PCR contained 3 µl of 12.5 mM universal bacterial primer 27F, labelled with 6-FAM; 3 µl of 12.5 mM universal bacterial primer 907R; 7.5 µl of 2.5 mM dNTP; 7.5 µl of 10X Takara buffer; 0.9 µl of 5 U/µl Takara Ex Taq Polymerase. Amplification was performed in a TC-412 thermocycler, consisting of an initial 3 min denaturation at 95°C, followed by 32 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min, followed by a final extension at 72°C for 5 min. PCR samples were run on a 1 % agarose gel and the ~900 bp band, as determined by comparing to a Hi-Lo DNA mass ladder, was excised and extracted using the Qiagen Gel Extraction Kit, and the clean PCR product was eluted in 30 µl of pH 8.0 sterile water.

Restriction digests were performed using the restriction enzyme, MspI, in 10 µl volumes, containing 1 µl of 10x Buffer, 1 µl of 10x BSA, 0.25 µl of MspI, and 7.25 µl cleaned PCR product, at 37 °C for 4 h. Fragment analysis was performed by capillary electrophoresis using a ABI PRISM® 3100 Genetic

Analyzer, with a GS-500 ROX size standard. ABI PRISM® 3100 data files were imported into STRand software, and exported as labeled fragment sizes and intensities into Microsoft Excel. Following normalization of peak intensities and reformatting of data, bacterial types and abundances were exported into CANOCO statistical software and analyzed with CCA.

Quantitative PCR was performed using TaqMan PCR MasterMix, with the forward primer 1369F, reverse primer 1492R, and probe 1389 for bacteria; and forward primer 806F, reverse primer 349R, and probe 516 for archaea. An initial 2 min activation at 50 °C and 10 min denaturation at 96 °C were followed by 40 cycles of denaturing for 25 s at 96°C, annealing for 6 min at 59 °C for archaea and for 1 min at 56 °C for bacteria. Data from qPCR analysis was used to generate microbial concentration contours and as environmental variable input for CCA.

CCA was used to identify environmental variables that were correlated with changes in microbial communities spatially and over time. The CCA analysis presented here is based mainly on samples taken in February 2006, due to technical difficulties with obtaining complete sets of microbial and environmental variables for other sample sets.

Results

Fate of tracer and VOCs

Release of the conservative tracer, bromide, lead to formation of thin plumes (Fig. 3) over the entire period of release, maintained due to the low level of mixing between the plumes. The bromide release also confirmed that flow was restricted to a shallow permeable layer of the aquifer, sandwiched between layers of impermeable media, as close to 95 % of injected mass was recovered at the H transect, ~ 40 m from injection wells.

Ethanol was not detected in the monitoring wells, signifying that it was degraded within the three feet between injection wells and the first monitoring transect AA (Fig. 1). A similarly high rate of degradation was observed in the previous experiment (Mackay et al. 2006), where while ethanol was not detected in any of the release wells, its degradation products (acetate and propionate) were detected at significant levels downstream of the injection wells. TBA was already present and detectable at high concentrations at the start of the injection phase due to the effects of previously injected ethanol into this area, which stimulated the transformation of TBA from residual MTBE (Fig. 3b). At the end of the injection period, bromide concentrations and plume size were similar to what was measured at the start of the experiment within a short period after starting the injections.

In Lane A (ethanol and MTBE injected), a plume of TBA was formed. Despite its small size, the plume was distinct from the residual and injected TBA in Lane B (Fig. 3d). In Lane B, the center of the TBA plume (region with highest concentrations) did not shift over the course of 7 months while TBA was being injected, while a thin connecting plume formed between the injection source and the plume center. TBA was thus being removed from the center of the plume, either through biological reactions or physical sequestration in the less permeable layers confining the aquifer.

Upon completion of the injection phase of the experiment, TBA persisted for a much longer period of time than what was expected based on its solubility and properties of the aquifer (e.g. sorption was not expected to be significant based on organic matter content in aquifer sediment). Bromide, which has solubility comparable to TBA, was eluted from the test region of the field site within 7 months of cessation of injection. In addition to slow movement with groundwater flow, the TBA plume resulted in high levels of tailing, supporting the action of a sequestration process. Another possibility is that ethanol stimulated the conversion of previously sequestered MTBE to TBA. A direct mass balance approach to account for injected MTBE and TBA is confounded by the presence of significant residual chemicals. This question will be investigated using a modeling effort, currently under way.

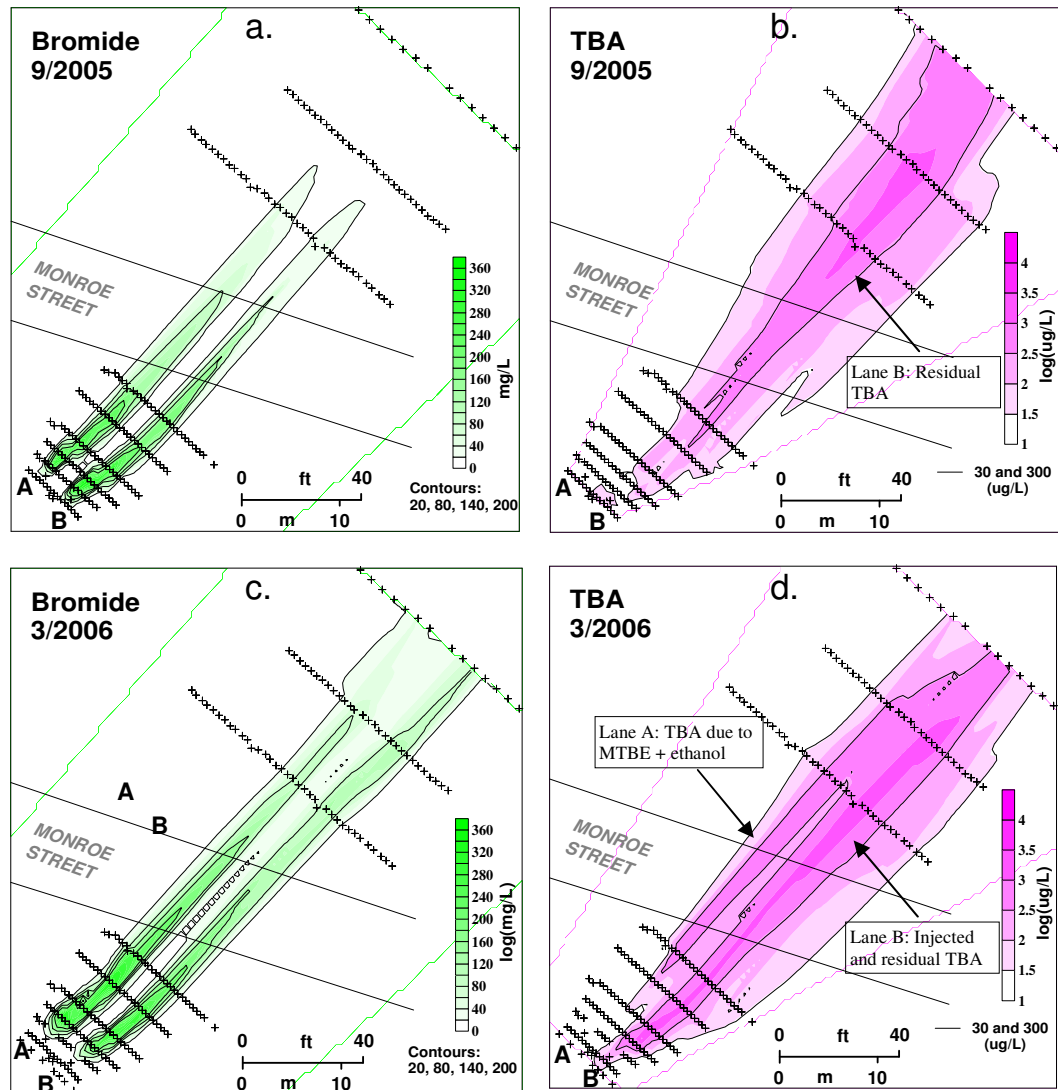
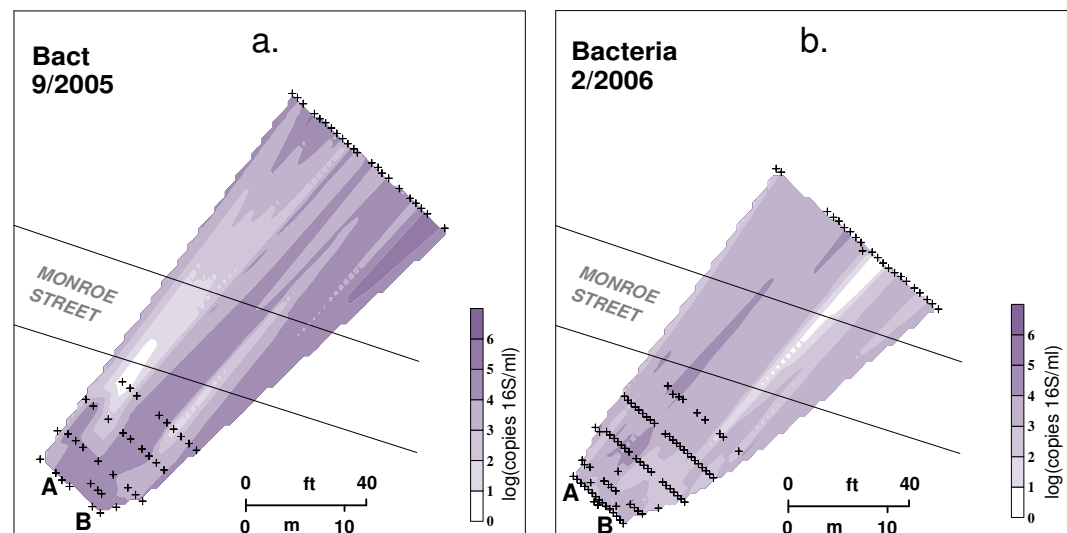


Figure 3. Bromide (a,c) and TBA (b, d) concentrations in September 2005 and March 2006. Bromide concentrations indicate the formation of two distinct lanes of solute, with very little lateral (inter-lane) mixing. TBA injected in Lane B resulted in the maintained levels of TBA similar to pre-injection levels, and ethanol and MTBE injected into Lane A resulted in the formation of a distinct TBA plume in that lane (d). Wells that were sampled for TBA and bromide are indicated as X on the contour maps.

Bacterial and archaeal distributions based on qPCR

The distribution of bacterial and archaeal populations was correlated with TBA concentration. At the end of 7 months, high concentrations of TBA (~10 ppm) in the centre of Lane B appear to have inhibited populations of archaea and bacteria, the latter demonstrated by the low densities of bacteria in transect H (Fig. 3b, d vs Fig. 4b, d). These concentrations of TBA are only slightly higher than what was measured at this point at the start of the experiment, at which time bacteria were present at relatively higher numbers (Fig. 4a). Although on a larger scale bacterial and archaeal densities appear to increase over time, there are localized decreases in bacterial populations in zones with high TBA concentrations. Interestingly, despite a recovery period of several months between the current and previous field studies (Fig. 2), densities of bacteria and archaea are reduced in Lane A near the injection zone, potentially indicating long term negative effects of the contaminant release. The effect, however, is also highly localized in the areas of highest TBA concentration.

The above discussion focuses only on the relationship between microbial concentrations and concentrations of TBA. However, TBA concentration is only one of a number of variables that could affect microbial communities during the injection experiments. Other factors may be directly responsible for changes in microbial communities and the relationship with TBA concentration may be an indirect one, where TBA is only an indicator of the change actually influencing microbial communities. As observed following ethanol release in the previous experiment (2004-2005), ethanol was transformed into degradation products that moved in a plume, downstream, changing microbial communities during that time (Feris et al., 2008).



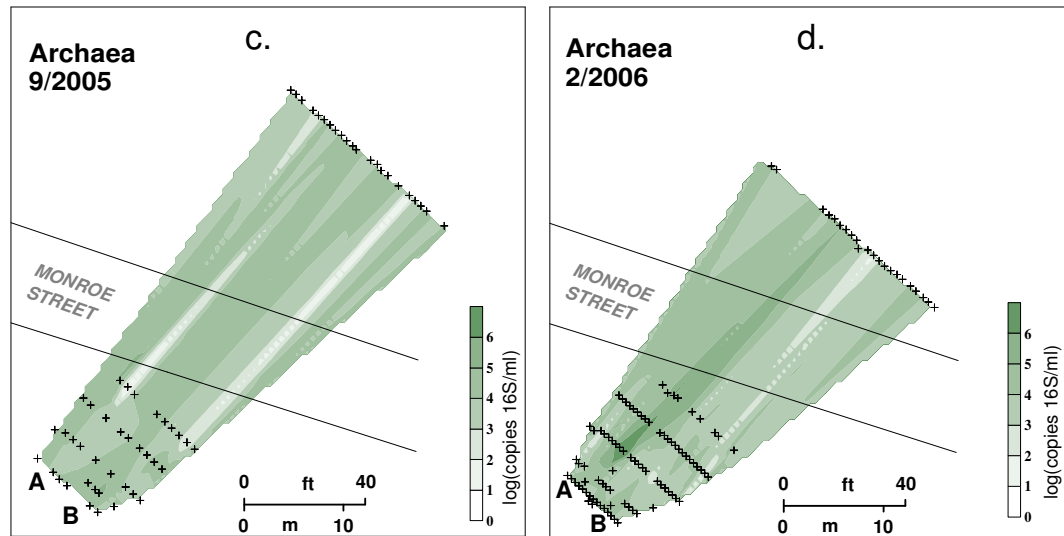


Figure 4. Numbers of bacteria and archaea as determined by qPCR before and at the end of the injection experiments.

Canonical correspondence analysis and correlation of microbial and VOC results

With CCA, we attempt to explain the changes in bacterial communities by determining whether variance in the microbial communities can be explained by changes in measured environmental factors (Fig. 5). CCA is advantageous in its ability to analyze multiple microbial community and environmental relationships simultaneously. CCA analysis displays distributions of samples in a 2D space, with samples plotted as points at distances that best represent similarities based on TRFLP fingerprints. The two ordination axes (Axis 1 and Axis 2 in Fig. 5), explain some of the variation in microbial communities. Specifically in CCA, these axes are also chosen because of their correlation with environmental variables for the same set of samples. The position of the samples **along** the environmental variable axis approximately indicates the relative value of that variable in that sample, irrespective of the **distance from** an axis for that sample. Samples near the origin of the environmental axes are those that have average values of those variables. As an example, although samples D08_2-6 and C08_2-6 have different community compositions (relative distance between samples), they have a similar concentration of methane (relative distance to axis) (Fig. 5).

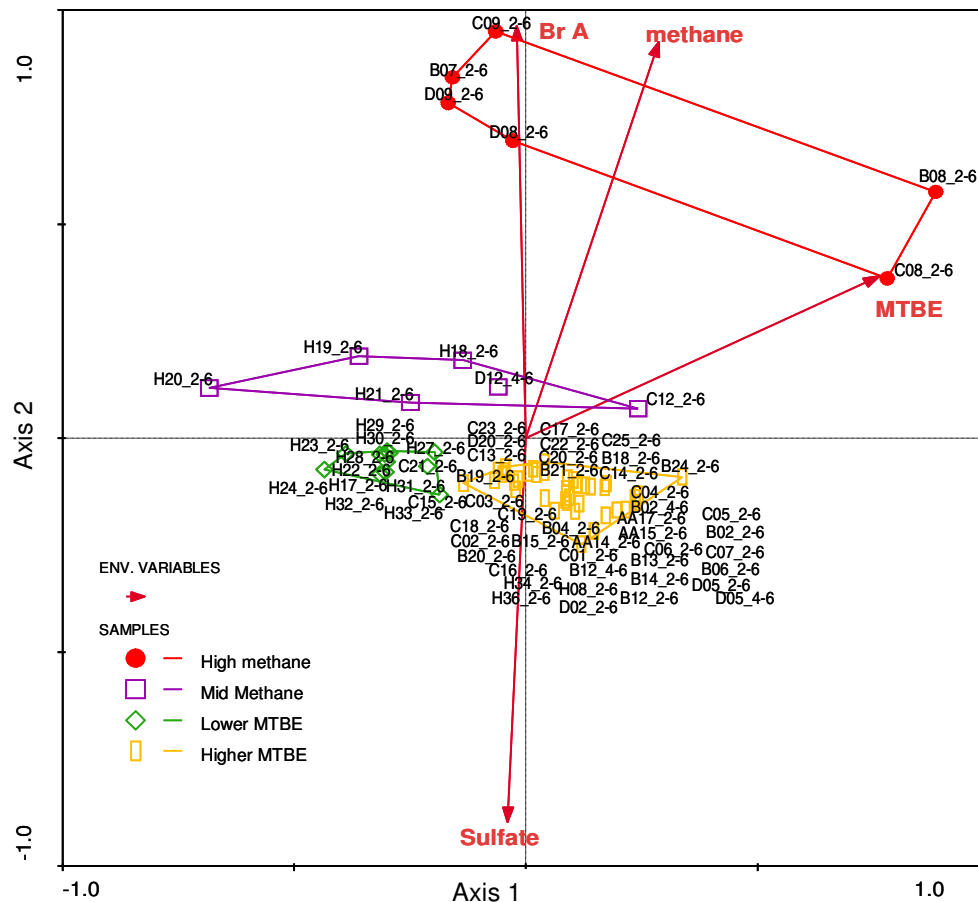


Figure 5. CCA of bacterial TRFLP profiles and statistically significant environmental variables. “2-6” in sample name refers to the February 2006 sampling point, and 4-6 refers to the April 2006 sampling point.

Eight environmental variables were hypothesized to be explanatory axes of microbial community composition: measured concentrations of 1) MTBE, 2) TBA, 3) methane and 4) sulfate; 5) total bacteria and 6) total archaea based on qPCR; and two variables derived from the bromide data to reflect the position of the sample in the each lane, 7) Br A and 8) Br B. The two bromide variables were derived by using available bromide data to designate in a quantitative manner whether a sample was in Lane A or Lane B. Thus, each sample had a Br A and a Br B value associated with it. For example, sample B07_2-6, located in the center of Lane A, with a concentration of bromide of 306.8 mg/L, was assigned a value of 306.8 for Br A, and a value of 2 for Br B (background concentration of bromide at the site was 2 mg/L). Similarly, a sample with a high level of bromide in Lane B would be assigned 2 mg/L as its Br A value, and the actual measured concentration for its Br B value.

The two ordination axes in Fig. 5 explain 49.5 % (Axis 1: 29.4 % and Axis 2: 20.1 %) of the variation in bacterial composition of the samples. The most significant determinants of community structure were Br A ($p=0.013$), methane ($p=0.077$), MTBE ($p=0.036$) and sulfate ($p=0.11$). The other parameters tested (Br B, total Br, TBA, bacteria, archaea) did not provide *additional* explanatory power

for the sample distribution in ordination space, partially due to their high degrees of correlation with variables included in the CCA (Table 1).

Multiple correlations between environmental variables

Environmental variables fell into two groups based on their correlation coefficients. In Group A, a strong negative correlation of sulfate with methane, Br A, bacterial and archaeal numbers was observed, where methane, Br A, and bacterial and archaeal numbers were strongly positively correlated. Group B showed a strong negative correlation of MTBE with TBA and Br B (Table 1). On the other hand, low correlations were obtained for variables across these two groups. Among the cross-group correlations, the most significant relationships were between Br B and bacteria, and between bacteria and methane. Total bromide (Br) is also shown as a variable. As expected, in all cases where there were significant correlations with total bromide, there were also correlations with either Br B or Br A.

	log (MTBE)	log (TBA)	log (CH4)	log (arch)	log (bact)	log (SO4)	BrA	BrB	Br	
log (MTBE)		0.000	0.026	0.207	0.910	0.138	0.053	0.219	0.758	p
log (TBA)	-0.619		0.566	0.556	0.102	0.739	0.929	0.000	0.000	
log (CH4)	0.271	-0.071		0.000	0.008	0.000	0.000	0.369	0.000	
log (arch)	0.156	-0.073	0.448		0.000	0.000	0.000	0.264	0.004	
log (bact)	-0.014	-0.202	0.320	0.559		0.001	0.001	0.022	0.223	
log (SO4)	-0.183	0.041	-0.638	-0.485	-0.394		0.000	0.108	0.001	
BrA	0.238	0.011	0.727	0.468	0.402	-0.643		0.159	0.000	
BrB	-0.152	0.477	-0.112	-0.139	-0.279	0.198	-0.174		0.000	
Br	0.038	0.440	0.464	0.350	0.151	-0.403	0.622	0.546		

Table 1. Multiple correlation between environmental variables and microbial levels used in CCA. Spearman’s R values between all pairs of columns are shown in lower left segment, while probabilities that the columns are uncorrelated are given in the upper. Variables and significant correlations and their probabilities are red for Group A and blue for Group B.

Spatial patterns in microbial communities at the field site.

To visualize the significance and spatial relationships of the CCA patterns shown in Figure 5, the samples in each group were plotted, in association with the wells from which they were sampled, on a contour map of bromide (Fig. 6). The CCA groupings are designated according to the same scheme as in Figure 5. The most distinct CCA group (red, “high methane”) is confined to the area near the injection wells (transects B, C and D). Two of six of the samples in this group are associated with very high MTBE levels, and have distinctly different community composition from adjacent sampling points. Thus, the effect of conditions present in the center of Lane A, near the release zone, appears to be quite pronounced: microbial communities within a few feet which are not under the impact of such conditions are distinctly different.

Further upstream in Lane A across the H transect, microbial communities also form a distinct group (purple, “mid methane”). The environmental

explanatory variable most likely to differentiate this group of organisms correlates with the ordination Axis 1, but such a variable was not measured in our study and remains unidentified. Based on CCA environmental variable axes projections this variable is independent of Br A/sulfate (Group A in Table 1). However, it is partially related to MTBE (and thus potentially other Group B variables). Therefore, it is likely that this variable is related to the breakdown of MTBE and TBA. Considering the downstream location of transect H with respect to the release zone, degradation products may be generated here, or are transported to this area from the source zone. “Mid methane” type communities are also present between the lanes, where degradation is likely to be enhanced at plume fringes, supporting the hypothesis that unidentified degradation products are a key determinant in microbial community structure and composition.

The last two groups (green, “lower MTBE” and orange, “higher MTBE”), can also be distinguished by this hypothetical variable, including most of the microbial communities near the source zone in the center of Lane B, and surrounding both Lanes, as well as background upstream communities. As expected lower MTBE concentration is a determinant of microbial communities in these communities as a result of its degradation over the length of the plume.

No effect on bacterial community composition due to TBA exposure over the experimental period (Lane B) could be resolved using CCA. Communities exposed to the highest TBA levels did not appear to be different from communities in areas of very low TBA exposure. However, the abundance of total bacteria (based on qPCR data), may have been reduced by TBA levels ($R = -0.20$, $p = 0.10$, Table 1).

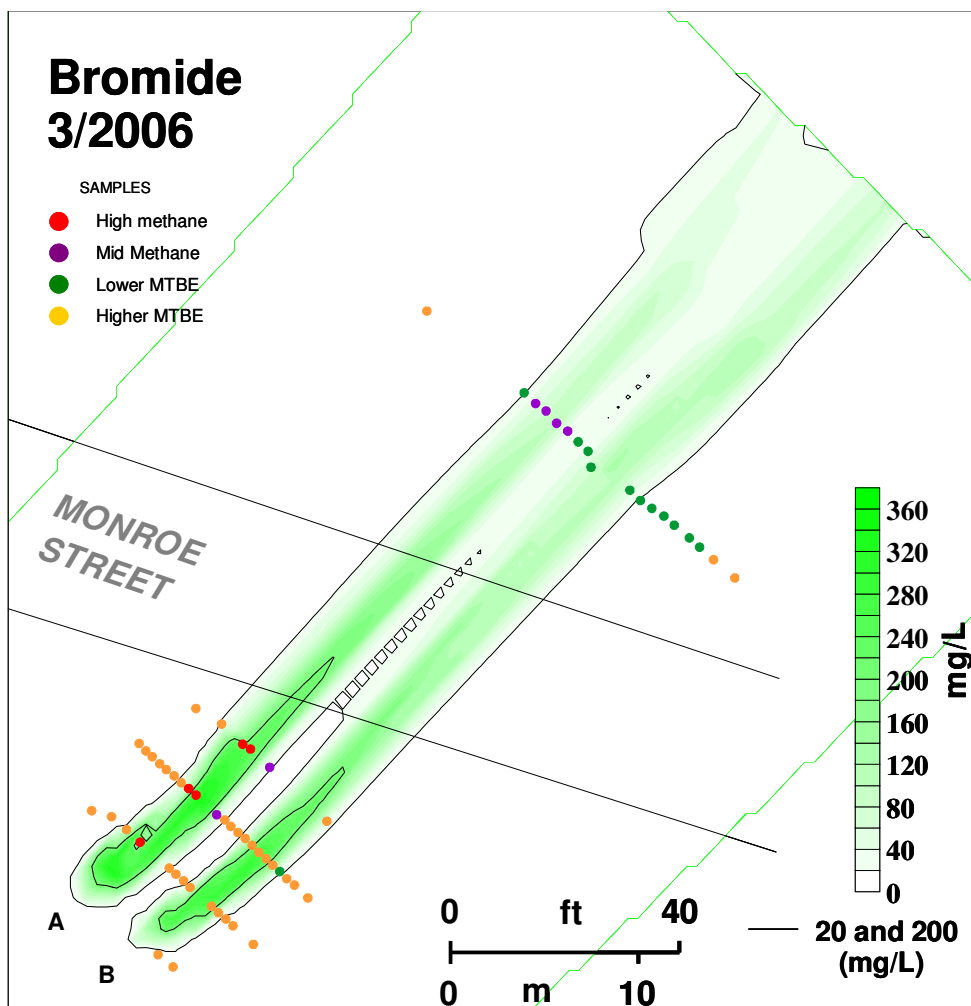


Figure 6. Concentration contours of bromide overlaid with sample well location color-coded by CCA group type. The legend for colors of CCA groups is the same as in Fig 5.

Conclusions

Our work shows that ethanol and MTBE are strong determinants of community structure at the VAFB site. Concentrations of bacteria and archaea in groundwater increased in the region of ethanol and MTBE injection. Microbial communities also remained distinct in the portion of the aquifer downstream of this zone, although they became more similar to those in the TBA release lane and those outside the acute impact area of Lane A. Another strong determinant of microbial community structure was related to degradation products of MTBE and/or TBA. Further analysis of this data set will show whether such changes in communities persist over time.

List of publications

Feris K, DM Mackay, N de Sieyes, I Chakraborty, M Einarson, K Hristova, and K Scow. Effect of ethanol on microbial community structure and function during natural attenuation of benzene, toluene and o-xylene in a sulfate-reducing aquifer, *Environ Science & Tech* (accepted Jan 2008)

Mackay DM, N de Sieyes, M Einarson, K Feris, A Pappas, I Wood, L Jacobsen, L Justice, M Noske, J Wilson, C Adair and KM Scow. Impact of ethanol on the natural attenuation of MTBE in a normally sulfate-reducing aquifer. 2007. *Environ Science & Tech.*41 (6):2015 -2021

MacKay D, N Sieyes, M Einarons, K Feris, A Pappas, I Wood, L Jacobson, L Justice, M Noske, K Scow and J Wilson. Impact of ethanol on the natural attenuation of benzene, toluene, and o-xylene in a normally sulfate-reducing aquifer. 2006. *Environ Science & Tech*, 40 (19), 6123 -6130

Nakatsu CH, K Hristova, S Hanada, X-Y Meng, JR Hanson, KM Scow and Y Kamagata. *Methylbium petroleiphilum* gen. nov., sp. nov., a novel methyl tert-butyl ether-degrading methylotroph of the Betaproteobacteria. 2006 *Int J Syst Evol Microbiol* 56: 983-989