Ground Water Monitoring&Remediation

Monitoring Subsurface Microbial Ecology in a Sulfate-Amended, Gasoline-Contaminated Aquifer

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Abstract

An unconfined, gasoline-contaminated aquifer (2.3 to 5 m water table) in fractured sedimentary bedrock has been under remediation since 1999 in Bellingham, Washington. The central portion of the ground water plume has been difficult to remediate because of the fractured sedimentary bedrock and the presence of significant utilities that have prevented aerobic in situ cleanup methods. A 15-month field trial was conducted to determine the efficacy of enhancing the attenuation of benzene, toluene, ethylbenzene, and xylenes (BTEX) hydrocarbons at the site by continuously amending the aquifer with sulfate that was initially introduced into the aquifer using an infiltration trench in 3.8-m^3 batches at a concentration of 500 mg/L. The goal was to stimulate anaerobic bacteria and enhance dissolved-phase gasoline and BTEX biodegradation rates. The subsurface microbial ecology in the aquifer was monitored during sulfate injection using Bio-Sep® biotraps (Microbial Insights Inc., Rockford, Tennessee). Bio-Sep beads have been shown to be very effective in collecting biofilms, which are more indicative of in situ microbial ecology than planktonic organisms from ground water samples. Sulfate injection was shown to increase the rates of biodegradation of BTEX components by more than twofold for toluene, for example, and about fivefold for gasoline-range hydrocarbons. The subsurface microbial community became more anaerobic in character as sulfate utilization increased as evidenced by its depletion in the aquifer. Bead biofilms were also compared to planktonic samples from ground water monitoring wells. During sulfate injection, bead biofilm biomarkers were shown to correlate with changes in ground water geochemistry, while planktonic samples from ground water remained relatively unchanged over time.

Introduction

Monitored natural attenuation (intrinsic bioremediation) can be a successful management strategy for ground water contaminated with hydrocarbons if the requisite microorganisms are present and the geochemistry is conducive to maintaining appropriate rates of biodegradation to protect environmental receptors. When active intervention is necessary to increase biodegradation rates, the most common remediation amendments are electron acceptors (dissolved oxygen [D.O.], sulfate, nitrate, etc.). Injection of electron acceptors such as nitrate (Pombo et al. 2002) or sulfate (Schroth et al. 2001) have been shown in push-pull tests to stimulate biodegradation of petroleum hydrocarbons. Injection of sulfate has also been shown to stimulate benzene biodegradation remote from the injection well (Anderson and Lovely 2000).

In addition to monitoring site geochemistry, the prudent site manager will also monitor the subsurface microbial ecology of the aquifer to ensure that remediation amendments have the desired effect throughout the zone of interest. The subsurface microbial ecology of a contaminated aquifer is often investigated by sampling ground water from monitoring wells in the plume. However, the pre- or postamendment microbial ecology of a contaminated aquifer is better represented by in situ biofilms grown during active remediation than planktonic organisms in sampled ground water (White et al. 2003). By conventional means, subsurface biofilm sampling requires coring of aquifer sediments and extraction of viable microorganisms or biomarkers (lipids, deoxyribonucleic acid (DNA), etc.). However, the efficiency of these extractions varies with the geochemistry of the sediments (White et al. 1998). We propose that biofilms characteristic of aquifer conditions can be rapidly and efficiently collected using a biofilm-sampling system based on Bio-Sep® technology. Bio-Sep consists of 3- to 4-mm-diameter spherical beads engineered from a composite of 25% aramid polymer (Nomex, DuPont, Wilmington, DE) and 75% powdered activated carbon (PAC) with a porosity of 75%. The

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median pore diameter is 1.9 μm; however, large macropores (>20 μm) also exist inside the beads. Beads are surrounded by an ultrafiltration-like membrane with pores of 1 to 10 μm, and the internal surface area is >600 m^2/g. Bio-Sep beads may be heated to 300°C for sterilization and to render the beads free of fossil biomarkers.

Biomarkers are more efficiently extracted from Bio-Sep beads than aquifer sediments and provide measures of viable biomass, redox environment, microbial community composition, and nutritional status (White and Ringelberg 1998). Bio-Sep beads are also more efficient collectors of biofilms than materials like glass wool. When like bulk volumes of Bio-Sep beads and glass wool were incubated in a perchloroethylene (PCE)-contaminated aquifer for 30 d, the beads collected over seven times as much viable biomass (as represented by extracted phospholipids) as glass wool (Sublette et al. 2002). Bio-Sep beads have also been shown to collect detectable biofilms in a drinking water distribution system in 1 d (White et al. 2003). The efficiency of biofilm formation in Bio-Sep has been attributed to the high internal surface area, low-shear conditions in the bead, the concentration of limiting nutrients by the PAC, and the rapid formation of preconditioning films. The feasibility of using Bio-Sep biotraps suspended down-well for microbial biofilm formation and collection under in situ conditions has been successfully tested at PCE-contaminated sites (Sublette et al. 2002, 2003), at a site where uranium reduction and precipitation was affected by acetate injection (Istok et al. 2004), and in several benzene, toluene, ethylbenzene, and xylenes (BTEX)–contaminated aquifers (K. Sublette, unpublished results). Most recently, we have introduced Bio-Sep beads "baited" with ^{13}C-benzene and ^{13}C-toluene into aquifers contaminated with BTEX and demonstrated in situ biodegradation potential by demonstrating ^{13}C incorporation into biomass phospholipids (Geyer et al. 2005).

In this paper, we describe the use of Bio-Sep bead biotraps to investigate the subsurface microbial ecology of a gasoline-contaminated aquifer postinjection of a sulfate amendment during a 15-month pilot study. Sulfate injection was shown to increase the rates of biodegradation of BTEX components and gasoline-range hydrocarbons. The subsurface microbial community became more anaerobic in character as sulfate utilization increased as evidenced by its depletion in the aquifer. Bead biofilms were also compared to planktonic samples from ground water monitoring wells. During sulfate injection, bead biofilms were also shown to correlate with changes in ground water geochemistry while planktonic samples from ground water remained relatively unchanged over time.

Site Description

The site used in these experiments is a shallow, unconfined, fractured-rock aquifer contaminated by rupture of a gasoline pipeline in Bellingham, Washington. The 1999 release of 870 m^3 occurred between Whatcom Creek and its tributary, Hannah Creek. The area between the creeks contains a complex network of subsurface water supply distribution and related piping. The majority of the gasoline flowed overland to Hannah Creek. However, a significant amount infiltrated into soil near the source area and migrated into underlying sedimentary rock fractures extending toward Whatcom Creek.

The near-surface geology of the site consists of sandstone, siltstone, shale, and coal of the Eocene Chuckanut Formation. The bedrock is overlain by up to 4.5 m of fill and/or silty fine sand to silty clay glacial sediment. In some locations within the release area, a thin veneer of forest duff or topsoil directly overlies bedrock. In the immediate vicinity of the release area, the sedimentary bedding planes dip steeply downward to the east (from 45° to near vertical). The bedding planes in this area have a north-south orientation, nearly perpendicular to the main creek (Whatcom Creek). Gasoline migrated through two primary pathways in the subsurface: (1) utility backfill surrounding the large-diameter water supply piping and (2) the near-vertical bedding planes and fractures in sedimentary bedrock.

The site was initially treated by excavation of >7500 m^3 of impacted soil, installation of a horizontal/vertical well couplet, traditional recovery wells, air sparging, and soil vapor extraction (Carlisle et al. 2002). No significant amounts of free-product gasoline were present or recovered after December 1999, 5 months after excavation of >7500 m^3 of gasoline-contaminated soil at the source. Vapor extraction was initiated in late 1999 and was terminated in all vapor extraction wells, including RW1, in late 2002. RW1 is actually a vertical recovery well component connected to a 131-m long, 10-m-deep, horizontally drilled boring back-reamed with perforated polyvinyl chloride pipe positioned to protect Whatcom Creek. Although originally designed to remove free-phase gasoline, RW1 continues to operate as a last line of defense to prevent dissolved-phase gasoline from migrating through rock fractures into the creek. Air sparging (primarily in the source zone) started in late 2001 and continues at present in a reduced manner. Collectively (free product + vapor) ~3.8 m^3 of gasoline had been removed from the aquifer by mid-2003.

BTEX concentrations were monitored monthly downgradient of the source zone by a network of monitoring wells from 1999 to the present. Even after much of the free product was removed, dissolved-phase concentrations of BTEX in the central untreated area of the plume attenuated only very slowly. Sulfate was seen to be depleted in the plume (<1 mg/L) relative to upgradient (5 to 12 mg/L) suggesting the presence of sulfate-reducing bacteria (SRB) and active sulfate reduction. These observations along with operational limitations, the presence of subsurface utilities, and difficulties associated with remediating a fractured-bedrock aquifer using aerobic methods prompted a decision to conduct a sulfate injection pilot test. Given the greater solubility of sulfate, injection of this electron acceptor has the potential to be more economical than energy-intensive aeration. In April 2003, a 61-m-long, 1.2-m-deep infiltration gallery was installed (Figure 1) to facilitate batch additions of Na_2SO_4 solution. As Figure 1 shows, the ground water flow pattern in the vicinity of the infiltration gallery is complex, but generally at this site, ground water travels in bedding plane fractures running north-south and structural features that crosscut bedding running northwest-southeast.

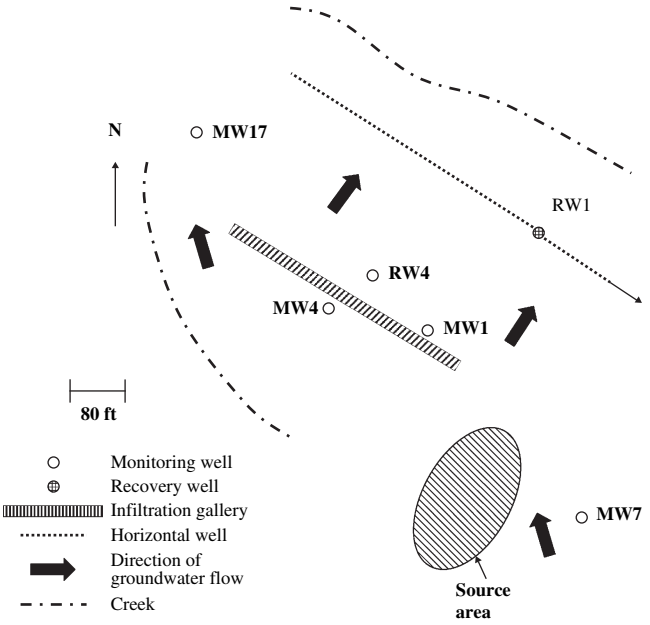

Figure 1. Site map. Scale approximate.

The infiltration gallery was designed to crosscut these fractures in the underlying bedrock. This concept was shown to be successful with the horizontal/vertical well concept. Monitoring wells MW4, MW1, RW4, and MW17 are under the direct influence of the infiltration gallery, and MW1, RW4, and MW17 are within the plume. MW4 was considered on the plume fringe. MW7 was upgradient of the source zone. RW4 was operated as a product recovery well from early 2000 until late 2001 when it was converted to a monitoring well. BTEX concentrations in MW1 during the 12 months prior to sulfate injection are shown in Figure 2.

Depth to ground water (mean ± standard deviation) during the 2-year period of 2003 to 2004 was 2.8 ± 0.4 m (MW1), 2.3 ± 0.5 m (MW4), 3.6 ± 0.2 m (MW17), 5.0 ± 0.4 m (MW7), and 3.3 ± 0.3 (RW4). Mean (± standard deviation) temperatures at three sampling times in 2004 were 10.8 ± 1.0°C (February), 10.1 ± 0.4°C (June), and 11.9 ± 0.9°C (December).

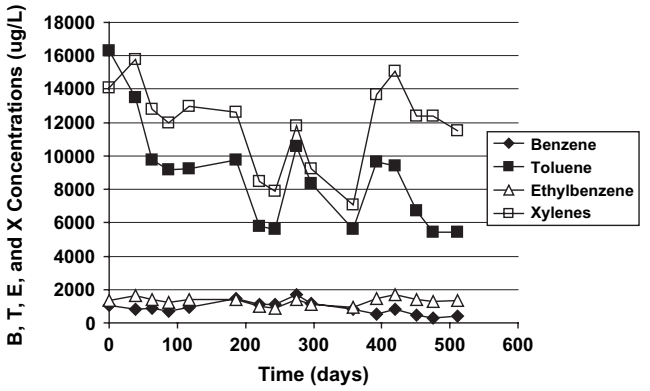

Figure 2. BTEX concentrations in MW1 during the 12 months prior to the initiation of sulfate injection. $t = 0$ corresponds to January 3, 2002.

Materials and Methods

Bio-Sep Biotraps

The Bio-Sep biotraps used in this work were composed of ~120 Bio-Sep beads in 11.4-cm polyfluorylalkoxy (PFA) tubing (12.7-mm outside diameter, 9.5-mm inner diameter [ID]). The tubing was perforated with six rows of 2.3-mm holes spaced 0.6 cm apart to provide contact between the beads and the ground water. Beads were held in place in the tubing with glass wool and nylon plugs. Prior to use, all biotrap materials that could not be heat treated were washed with methanol. Assembly of the biotraps took place on a surface of aluminum foil that had been fired at 300°C. All parts were handled with sterile gloves, and assembled biotraps were stored in sterile Whirl-pak® bags (Nasco, Ft. Alkinson, WI) at 4°C until deployed. Biotraps were transported to the field site in a cooler at 4°C as a further precaution against contamination.

Sulfate Injection Pilot Test

Sulfate injection began on June 2, 2003, with an injection of 3.8 m^3 of 500 mg/L sulfate as Na_2SO_4. Injections were repeated approximately every other day for a total of 15 per month through September 2003. In October, the injection frequency was reduced to eight injections per month and the sulfate concentration reduced to 245 mg/L. The injection frequency averaged four to seven injections per month through September 2004. The volume of each injection remained at 3.8 m^3, but the sulfate concentration was increased to 368 mg/L in mid-April and then to 500 mg/L in mid-August of 2004. The water used to initially prepare the sulfate solution for injection was saturated with air. Therefore, 3.8 m^3 of the sulfate solution initially delivered 1.9 kg SO_4^{-2} and 0.03 kg O_2. After 7 months of operation, the feed water was changed to treated ground water from the site, which was sparged with nitrogen to remove oxygen. The cumulative mass of sulfate delivered through September 2004 was 200 kg.

Sampling and Analysis

Geochemistry

Pre- and post–sulfate injection ground water was collected in completely filled volatile organic analysis (VOA) vials from purged wells once a month and analyzed for gasoline-range hydrocarbons (Washington State Department of Ecology Method NWTPH-G) and BTEX (EPA Method 8021B) and sulfate (EPA method 300) using 20% duplicate analysis. Ground water samples were obtained using low-flow sampling techniques with dedicated downhole electric Whale® pumps (CSP Outdoors, Shreveport, Louisiana) and polyethylene tubing. A Horiba U-22 flow cell field meter was used to evaluate various parameters including D.O.

Ground Water and Biotrap Microbial Ecology

One-liter samples of ground water were collected from several wells pre- and post–sulfate injection in completely filled sterile high density polyethylene (HDPE) bottles. Samples were chilled on ice in the field and shipped cold

by overnight delivery to Microbial Insights Inc. (Rockford, Tennessee) for phospholipid fatty acid (PLFA) analysis.

Biotraps were first installed (one per well) in MW1, MW4, MW17, MW7, and RW4 on May 9, 2003, in order to establish preamendment conditions. Biotraps were attached to a nylon line and positioned so that the biotrap was ~30 cm below the water level in the well. Biotraps were retrieved on June 2 in sterile Whirl-pak bags, chilled in the field, and shipped by overnight delivery to Microbial Insights Inc. for PLFA analysis. Biotraps were retrieved and replaced with fresh biotraps over the next 15 months during the sulfate injection pilot test, each with an incubation period of ~30 d.

PLFA Analysis

PLFA analysis is based on the extraction and separation of lipid classes, followed by quantitative analysis using gas chromatography/mass spectrometry. Lipids are essential components of the cell membranes of all microbial cells. This method is superior to plate counts since both culturable and nonculturable microorganisms are enumerated and characterized. Signature lipid biomarker analysis also provides quantitative insight into three important attributes of microbial communities: viable biomass, community structure, and nutritional/physiological status (White et al. 1998).

PLFA analysis was performed using previously reported important precautions to achieve quantitative results (Pinkart et al. 2002). Ground water was filtered using 0.45-μm membrane filters. Biomass-laden filters or Bio-Sep beads were extracted with the single-phase, chloroform-methanol buffer system of Bligh and Dyer (1959). The total lipid extract was fractionated into neutral lipids, glycolipids, and polar lipids by silicic acid column chromatography. The polar lipids were transesterified to the fatty acid methyl esters (FAMEs) by a mild alkaline methanolysis. The FAMEs from the PLFA were analyzed by gas chromatography/mass spectroscopy using an Agilent 6890 series gas chromatograph interfaced to an Agilent 5973 mass selective detector with a 50-m nonpolar column (0.2-mm ID, 0.11-μm film thickness) using the conditions described by Pinkart et al. (2002).

Fatty acid nomenclature is of the form "A:BωC" where "A" designates the total number of carbon atoms, "B" the number of double bonds, and "C" the distance of the closest unsaturation from the aliphatic end of the molecule. The suffixes "c" for cis, and "t" for trans refer to double-bound isomers. The prefixes "i" and "a" refer to iso and anteiso methyl-branching, respectively, whereas "br" assigns an undetermined position of the methyl-branching; "cy" assigns a cyclopropyl fatty acid (Radledge and Wilkinson 1988).

Real-Time Polymerase Chain Reaction for bssA

The bssA targets a catabolic gene (benzylsuccinate synthase) associated with the first step of anaerobic toluene and xylene degradation (Beller et al. 2002). This gene is an excellent screening tool for sites, which are contaminated with aromatic hydrocarbons by quantifying the number of bacteria that are genetically capable of anaerobic toluene or xylene degradation in a given sample.

Bead samples were extracted using Mobio Laboratories (Solana Beach, California) Power Soil DNA kits following the manufacturer's recommendations. Real-time polymerase chain reaction (PCR) was then performed on each sample using oligonucleotides that are designed to target the region of interest. One of the oligonucleotides was a probe containing 6-carboxy-fluorescein (FAM) as a reporter fluorochrome on the 5′ end, and *N*,*N*,*N*′,*N*′-tetramethyl-6-carboxy-rhodamine (TAMRA) as a quencher on the 3′ end. Each 30-μL reaction contained 1X TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, California); forward primer (625 nM), reverse primer (625 nM), and TaqMan probe (200 nM); and DNA template from the extracted samples. The PCR conditions were as follows: 2 min at 50°C and 10 min at 95°C, followed by 50 cycles of 15 s at 95°C and 1 min at 58°C. The PCR reaction was carried out in an ABI Prism 7000 Sequence Detection System (Applied Biosystems). A calibration curve was obtained by using a serial dilution of a known concentration of positive control DNA. The cycle threshold values obtained from each sample were then compared with the standard curve to determine the original sample DNA concentration.

Statistical Analysis

All statistical analyses were done using Statistica Version 7.0 (StatSoft, Tulsa, Oklahoma). Independent *t*-tests were used for comparison of means. Slope comparisons used a univariate test of significance of slopes (analysis of covariance/ANCOVA). Repeated measures were analyzed using a repeated measures analysis of variance with Fisher LSD post hoc test for homogeneous groups (Winer et al. 1991).

Results and Discussion

Preinjection Geochemistry

Figure 2 gives the concentrations of BTEX components in MW1 in the 12 months preceding the injection of sulfate. Some decrease in concentrations of toluene and xylenes are evident, but benzene and ethylbenzene concentrations were relatively unchanged over this period. Figure 3 shows the median D.O. concentrations in the plume wells in the 12 months prior to the initiation of sulfate injection. Except for MW4, D.O. concentrations in the plume were typically 0 to 1 mg/L. During this same period, sulfate concentrations in the plume were generally below 1 mg/L.

Postinjection Geochemistry

Figure 4 gives the concentrations of BTEX components in MW1 during sulfate injection. Both the pre- and post-injection depletions of BTEX components were modeled as first-order reactions, and apparent first-order rate constants were obtained for the plume wells with the highest BTEX concentrations and tabulated in Table 1. First-order rate constants were consistent with those observed from

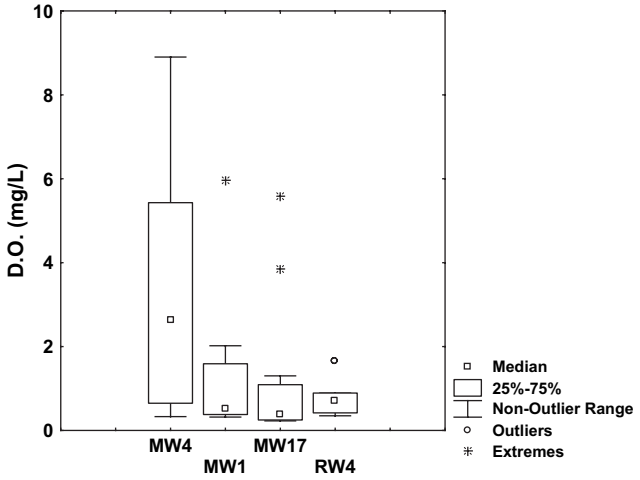

Figure 3. Median D.O. concentrations in plume wells in the 12 months prior to initiation of sulfate injection.

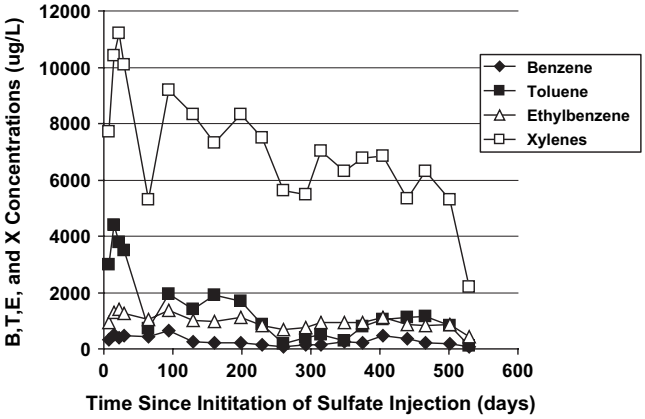

Figure 4. BTEX concentrations in MW1 during sulfate injection.

previously reported field studies (Rifai and Newell 2002). Using a multiple-regression analysis, pre- and postinjection rate constants were compared to determine if any significant differences existed.

As seen in Table 1, significant increases in the apparent first-order rate constants were seen in all three plume wells for toluene and xylenes, in two wells for benzene and gasoline-range total petroleum hydrocarbons (TPH-G), and in one well for ethylbenzene in the 15 months following initiation of sulfate injection. Since toluene and o-xylene are the most rapidly degraded of the BTEX components under anaerobic conditions, these results are consistent with a stimulation of biodegradation as the major mechanism of attenuation following sulfate injection (Hutchins et al. 1991; Lovely and Lonergan 1990; Edwards et al. 1992; Grbic-Galic and Vogel 1987). Further, as shown in Figure 5, the ratio of the toluene concentration to the total BTEX concentration declined during sulfate injection, reflecting relatively higher rates of removal for toluene and again suggesting biodegradation as the operative mechanism. Even if ethylbenzene is assumed to be a conservative marker and used to "correct" toluene concentrations for dilution and dispersion, toluene attenuation rates were still significantly greater after sulfate injection in MW1 ($p < 0.013$) and MW17 ($p < 0.002$) compared to preinjection depletion rates.

Figure 6 shows concentrations of sulfate in the plume wells during sulfate injection. As seen here, there was initially an accumulation of sulfate in the aquifer following initiation of sulfate injection; however, sulfate concentrations subsequently decreased presumably due to use of sulfate by SRB using BTEX hydrocarbons as electron donors. Sulfate concentrations were seen to rise again after 350 to 400 d as BTEX concentrations decreased (Figure 4).

D.O. concentrations postinjection are shown in Figure 7. Early on, there was an increase in D.O. concentrations after initiation of sulfate injection. This was due to the use of water saturated with air to prepare the sulfate solutions for injection. After the increase in D.O. was noted, the source water was changed to treated ground water from the site, which was nitrogen sparged to reduce or eliminate D.O. Subsequently, D.O. concentrations declined due to the change in dilution water and increased levels of aerobes, which then effectively scrubbed the oxygen leaving sulfate as the most abundant and dominant electron acceptor. In summary, comparisons of the geochemistry of the ground water pre- and postinjection were consistent with a stimulation of SRB by sulfate injection and biodegradation of BTEX hydrocarbons.

Table 1
Comparison of Pre- and Post–Sulfate Injection Apparent First-Order Rate Constants for Depletion of BTEX Components from Plume Wells (Rate Constants × 10^3 d^{-1})

Monitoring Well	Injection	Benzene	Toluene	Ethylbenzene	Xylenes	TPH-G
MW1	Preinjection	1.3	1.4**	0**	0.3***	0.2*
	Postinjection	2.0	3.9	1.0	1.4	1.1
MW17	Preinjection	2.0*	3.2***	0	0**	0**
	Postinjection	3.3	7.1	1.3	2.4	2.4
RW4	Preinjection	0.2*	5.7**	0	0.4**	0.2
	Postinjection	2.3	6.5	0.9	3.0	2.0

*$p < 0.1$, **$p < 0.05$, ***$p < 0.01$ indicated level of significance in differences in pre- and post–sulfate injection first-order rate constants.

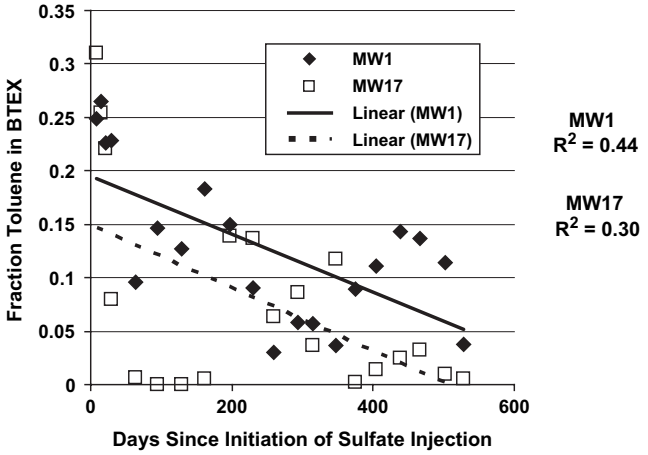

Figure 5. Ratio of toluene concentrations to total BTEX concentration in MW1 and MW17 during sulfate injection (C_o for toluene = 2980 and 5220 parts per billion in MW1 and MW17, respectively).

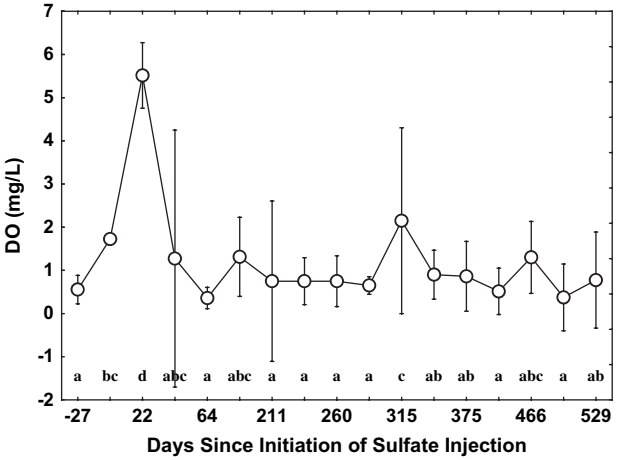

Figure 7. D.O. concentrations in plume wells during sulfate injection. Bars represent 95% confidence limits. $N = 4$ in each case. Different letters under data points indicate samples that are significantly different from each other at $p < 0.05$.

Subsurface Microbial Ecology during Sulfate Injection

As noted previously, biotraps were retrieved approximately every month from MW1, MW4, MW17, RW4, and MW7 each after ~30 d of incubation. PLFA analysis was used to determine the relative proportions of the fatty acid structural groups. As an example, Figure 8 shows the relative proportions of branched monounsaturated (BrMonos) PLFA from plume biotraps following initiation of sulfate injection. As discussed previously, the concentrations of sulfate and D.O. in the plume wells were seen to rise as sulfate (and oxygen) were injected into the aquifer; however, after ~200 d both sulfate and oxygen concentrations were similar to preinjection levels. The declines in sulfate and oxygen concentrations were caused by their use as electron acceptors. An independent *t*-test showed that the mean sulfate concentrations in the four plume wells pre- and post-200 d were significantly different (pre-200 d, 38.4 ± 42.4 mg/L [mean ± standard deviation], $N = 36$; post-200 d, 10.5 ± 12.3 mg/L, $N = 44$, $p < 0.0001$). A similar analysis of D.O. concentrations also showed a significant difference in pre- and post–200 d levels (pre-200 d, 2.6 ± 2.3 mg/L, $N = 24$; post-200 d, 0.9 ± 0.8 mg/L, $N = 44$, $p < 0.0001$). These electron acceptor patterns were used to block PLFA data to evaluate the effects of the sulfate injection on subsurface microbial ecology. Table 2 gives the results of this analysis.

The most significant difference identified in Table 2 was in the relative proportions of cyclopropyl fatty acids (cy17 + cy19) in biofilms pre- and post-200 d. Cyclopropyl fatty acids are commonly found in anaerobic bacteria and are major components of *Desulfobacter* species (Guckert et al. 1986; Baird and White 1985; White 1988; Guckert et al. 1985; Dowling et al. 1986). Fang and Barcelona (1998) found that there was a linear correlation in the concentrations of cyclopropyl fatty acids and redox potential and concentrations of D.O. and sulfate in ground water

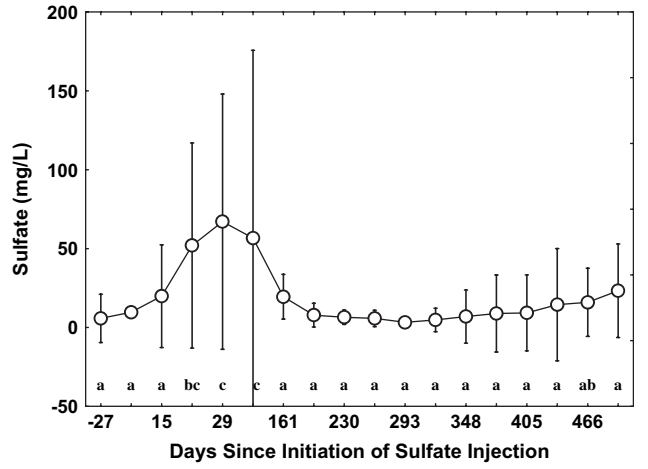

Figure 6. Sulfate concentrations in plume wells over the course of sulfate injection. Bars represent 95% confidence limits. $N = 4$ in each case. Different letters under data points indicate samples that are significantly different from each other at $p < 0.05$.

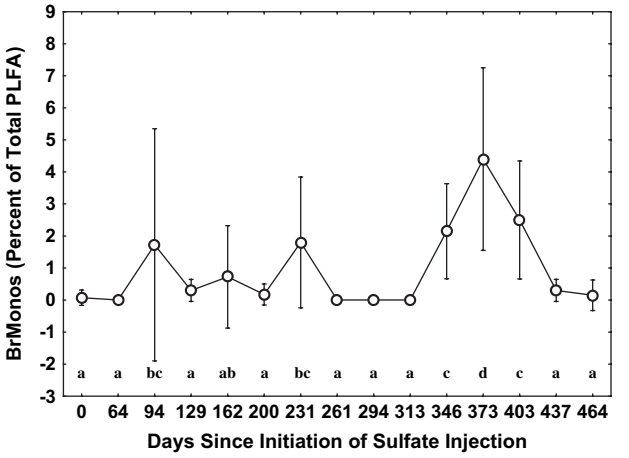

Figure 8. Relative proportions of branched monoenoic fatty acids from plume biotraps during sulfate injection. Bars represent 95% confidence limits. $N = 4$ in each case. Different letters under data points indicate samples that are significantly different from each other at $p < 0.05$.

Table 2
Analysis of the Effects of Sulfate Injection on Various PLFA Parameters

Parameter	Pre-200 d[1] (N = 20)	Post-200 d[1] (N = 40)	p Value[2]
TerBrSats	2.9 ± 2.6	5.1 ± 8.9	0.28
BrMonos	0.56 ± 1.19	1.2 ± 1.6	0.16
MidBrSats	0.95 ± 1.13	1.7 ± 1.8	0.10
C16 Monos	26.0 ± 12.0	30.9 ± 15.6	0.26
C18 Monos	31.2 ± 15.8	35.3 ± 15.9	0.38
n-Sats	32.5 ± 21.4	23.1 ± 7.0	0.01
Polyenoics	1.9 ± 1.6	3.1 ± 3.5	0.16
cy17 + cy19	0.76 ± 1.18	3.8 ± 3.4	0.0003
18:1ω7c	7.1 ± 3.9	11.2 ± 10.8	0.11
pmol PLFA/bead	57.8 ± 43.7	25.6 ± 26.9	0.0008

[1]Mean ± standard deviation.
[2]Independent t-test for pre-200 d vs. post-200 d.

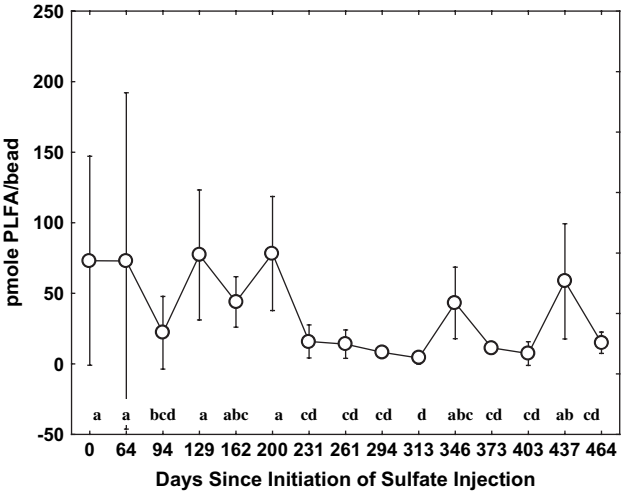

Figure 9. PLFA concentrations (pmol/bead) in biotraps during sulfate injection. Bars represent 95% confidence limits. $N = 4$ in each case. Different letters under data points indicate samples that are significantly different from each other at $p < 0.05$.

impacted by JP-4. The increased percentage of cyclopropyl fatty acids post-200 d indicates increasing proportions of anaerobic bacteria following injection and adaptation to increased availability of sulfate in the aquifer. This conclusion is supported by increases in relative proportions of 18:1ω7c, which is formed by the anaerobic fatty acid desaturase pathway (White et al. 1983).

Increased proportions of BrMonos fatty acids (primarily i17:1ω7c and br19:1a) and mid-branched saturated (MidBrSats) fatty acids (primarily 10me16:0, 10me17:0, and 10me18:0) were also seen post-200 d. These increases are also indicative of increased anaerobic activity post-200 d as well as increased activity of SRB (Pinkart et al. 2002). Relative proportions of C16 and C18 monoenoics and terminally branched saturates (TerBrSats) were not significantly different pre- or post-200 d.

Table 2 also indicates that there was a highly significant decrease in the pmol PLFA collected per bead after 200 d (see Figure 9). Our interpretation of an increase or decrease in the amount of biomass collected per bead is not an increase or decrease in total biomass in the aquifer. To be detected in the beads, microbes must enter the bead and grow there to produce sufficient biomass. Therefore, the beads sample the active fraction of the subsurface community in an integrative manner during the time of incubation. The most active (fastest growing) organisms of the subsurface microbial community are the most likely to be collected in detectable amounts. If biotraps of identical geometry are deployed over time in a like manner, then an increase or decrease in the amount of biomass collected represents a change in microbial activity in the aquifer. A decrease, for example, would indicate a decrease in numbers and/or growth rates of some members of the community. These variables interact to determine the concentration of biomass collected. An analysis of collected biofilms over time can determine what groups or specific organisms have become more or less active over the observation period.

The limited amount of real-time PCR data for the bssA gene supports conclusions from lipid analysis. As shown in Table 3, during the initial phases of sulfate injection, there was an increase in the concentration of bssA genes in biotrap beads. In MW4 and RW4, those increases were many orders of magnitude.

These results taken together indicate that sulfate injection resulted in a shift in the subsurface microbial community structure to increased anaerobic character and increased proportions of SRB. This shift to a predominance of anaerobic growth was accompanied by a subsequent reduction in growth rates as readily degradable electron acceptors were depleted.

Biotrap vs. Ground Water Sampling of Subsurface Microbial Community Structure

In order to determine whether ground water sampling of plume wells could have led to the same conclusions regarding shifts in microbial community structure as the biotraps, a comparison was made between PLFA results obtained from biotraps as summarized previously and the results of PLFA analysis of ground water samples also taken from plume wells pre- and post-200 d. Table 4 compares the results of PLFA analysis of ground water and biotraps pre- and post-200 d. As seen in Table 4, ground water and biotraps gave significantly different results with almost

Table 3
Concentrations of the bssA Gene in Bio-Sep Beads from Biotraps

Days[1]	MW1[2]	MW4[2]	MW17[2]	RW4[2]
94		0	0	130
162	104	0	45	30
200	970	5.1×10^5	910	1.4×10^5

[1]Days since initiation of sulfate injection.
[2]Gene copies per bead ($N = 2$).

Table 4
PLFA Parameters in Ground Water Samples and *p* Values for Comparison to Biotrap Samples in the Same Time Period

Parameter[1]	Ground Water Pre-200 d; $N = 12$	Biotraps Pre-200 d; $N = 20$	*p* value[2]	Ground Water Post-200 d; $N = 8$	Biotraps Pre-200 d; $N = 40$	*p* Value[2]
TerBrSats	0.84 ± 0.4	2.9 ± 2.6	0.24	3.0 ± 2.2	5.1 ± 8.9	0.35
BrMonos	1.5 ± 0.4	0.56 ± 1.19	0.04	1.2 ± 0.9	1.2 ± 1.6	0.88
MidBrSats	1.9 ± 1.1	0.95 ± 1.13	0.04	1.4 ± 1.0	1.7 ± 1.8	0.52
C16 Monos	41.9 ± 9.3	26.0 ± 12.0	0.0008	42.8 ± 6.2	30.9 ± 15.6	0.04
C18 Monos	17.7 ± 5.1	31.2 ± 15.8	0.003	19.5 ± 4.1	35.3 ± 15.9	0.008
cy17 + cy19	5.7 ± 2.1	0.76 ± 1.18	0	5.5 ± 2.3	3.8 ± 3.4	0.18
18:1ω7c	10.8 ± 1.9	7.1 ± 3.9	0.005	13.1 ± 2.2	11.2 ± 10.8	0.62

[1]Percent of total PLFA.
[2]Independent *t*-test for ground water vs. biotraps.

every parameter pre-200 d. Proportions of TerBrSats were the exception. Few differences were seen when ground water and biotraps were compared post-200 d. Finally, there were no significant differences between pre- and post-200 d samples of ground water ($p = 0.19$ for TerBrSats and $p > 0.5$ for all other parameters). Clearly, ground water sampling did not indicate any significant changes in subsurface microbial ecology during sulfate injection, whereas biotrap sampling did and was consistent with geochemistry. The difference is due to the requirement for growth when sampling with biotraps. Ground water organisms are detected and quantified whether actively growing or not. Biotrap samples are more indicative of the microbial activity actually being expressed in the aquifer. Further, a ground water sample is a grab sample indicative of only a moment in time, whereas a biotrap sample is integrative of subsurface conditions during the entire period of incubation in the aquifer.

In conclusion, biodegradation of BTEX components and gasoline-range hydrocarbons were shown to be enhanced by sulfate injection at this site. Bio-Sep biotraps deployed on a monthly basis in a gasoline-contaminated aquifer undergoing sulfate injection indicated changes in subsurface microbial ecology, which were consistent with observed changes in geochemistry such as sulfate concentrations, D.O. concentrations, and rates of BTEX biodegradation. Further, these changes in subsurface microbial ecology were not detected by biomarker analysis of ground water samples. Biotraps provided an integrative sample of the active fraction of the subsurface community and better reflected the dominant in situ microbial processes occurring in the aquifer.

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References

Anderson, R.T., and D.R. Lovely. 2000. Anaerobic bioremediation of benzene under sulfate-reducing conditions in a petroleum-contaminated aquifer. *Environmental Science and Technology* 34, no. 11: 2261–2266.

Baird, B.H., and D.C. White. 1985. Biomass and community structure of the abyssal microbiota determined from the ester-linked phospholipids recovered from Venezuela Basin and Puerto Rico Trench sediments. *Marine Geology* 68, no. 1–4: 217–231.

Beller, H.R., S.R. Kane, T.C. Legler, and P.J. Alvarez. 2002. A real time polymerase chain reaction method for monitoring anaerobic, hydrocarbon-degrading bacteria based on a catabolic gene. *Environmental Science and Technology* 36, no. 18: 3977–3984.

Bligh, E.G., and W.J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology* 37, no. 8: 911–917.

Carlisle, D., D.A. Cook, and J.A. Miller. 2002. Successful use of an innovative horizontal/vertical well couplet in fractured bedrock to intercept a mobile gasoline plume. *Ground Water Monitoring & Remediation* 22, no. 2: 82–87.

Dowling, N., J.E. Widdel, and D.C. White. 1986. Analysis of phospholipids ester-linked fatty acid biomarkers of acetate-oxidizing sulfate reducers and other sulfide forming bacteria. *Journal of General Microbiology* 132, no. 3: 1815–1825.

Edwards, E.A., L.E. Wills, M. Reinhard, and D. Grbic-Galic. 1992. Anaerobic degradation of toluene and xylene by aquifer microorganisms under sulfate-reducing conditions. *Applied and Environmental Microbiology* 58, no. 3: 794–800.

Fang, J., and M.J. Barcelona. 1998. Biogeochemical evidence for microbial community change in a jet fuel hydrocarbons-contaminated aquifer. *Organic Geochemistry* 29, no. 4: 899–907.

Geyer, R., A.D. Peacock, A. Miltner, H.-H. Richnow, D.C. White, K.L. Sublette, and M. Kästner. 2005. *In situ* assessment of microbial activity using microcosms loaded with ^{13}C-labeled benzene or toluene. *Environmental Science and Technology* 39, no. 13: 4983–4989.

Grbic-Galic, D., and T.M. Vogel. 1987. Transformation of toluene and benzene by mixed methanogenic cultures. *Applied and Environmental Microbiology* 53, no. 2: 254–260.

Guckert, J.B., C.P. Antworth, P.D. Nichols, and D.C White. 1985. Phospholipid ester-linked fatty acid profile as reproducible assays for changes in prokaryote community structure of estuarine marine sediments. *FEMS Microbiology Ecology* 31, no. 3: 147–158.

Guckert, J.B., M.A. Hood, and D.C. White. 1986. Phospholipid ester-linked fatty acid profile changes during nutrient deprivation of *Vibrio cholerae*: Increases in the trans/cis ratio and proportions of cyclopropyl fatty acids. *Applied and Environmental Microbiology* 52, no. 4: 794–801.

Hutchins, S.R. 1991. Biodegradation of monoaromatic hydrocarbons by aquifer microorganisms using oxygen, nitrate, and nitrous oxide as the terminal electron acceptor. *Applied and Environmental Microbiology* 57, no. 8: 2403–2407.

Istok, J.D., J.M. Senko, L.R. Krumholz, D. Watson, M. Bogle, A. Peacock, Y.J. Chang, and D.C. White. 2004. *In situ* bioreduction of technetium and uranium in a nitrate-contaminated aquifer. *Environmental Science and Technology* 38, no. 2: 468–475.

Lovely, D.R., and D.J. Lonergan. 1990. Anaerobic oxidation of toluene, phenol, and p-cresol by the dissimilatory iron reducing organisms, GS-15. *Applied and Environmental Microbiology* 56, no. 6: 1858–1864.

Pinkart, H.C., D.B. Ringelberg, Y.M. Piceno, S.J. MacNaughton, and D.C. White. 2002. Biochemical approaches to biomass measurements and community structure analysis. In *Manual of Environmental Microbiology*, 2nd ed., ed. C.J. Hurst, R.L. Crawford, M.J. McInerney, and L.D. Stetzenbach, 101–113. Washington, DC: ASM Press.

Pombo, S.A., O. Pelz, M.H. Schroth, and J. Zeyer. 2002. Field-scale ^{13}C-labeling of phospholipid fatty acid (PLFA) and dissolved inorganic carbon: Tracing acetate assimilation and mineralization in a petroleum hydrocarbon-contaminated aquifer. *FEMS Microbiology Ecology* 41, no. 3: 259–267.

Radledge, C., and S.G. Wilkinson. 1988. An overview of microbial lipids. In *Microbial Lipids*, vol. 1, ed. C. Radledge and S.G. Wilkinson, 3–21. London, UK: Academic Press.

Rifai, D.W., and C.J. Newell. 2002. *Estimating First-Order Decay Constants for Petroleum Hydrocarbon Biodegradation in Groundwater.* Washington, D.C.: American Petroleum Institute, Soil/Groundwater Technical Task Force.

Schroth, M.H., J. Kleikemper, C. Bollinger, S.M. Bernasconi, and J. Zeyer. 2001. *In situ* assessment of microbial sulfate reduction in a petroleum-contaminated aquifer using push-pull tests and stable sulfur isotope analysis. *Journal of Contaminant Hydrology* 51, no. 3–4: 179–195.

Sublette, K.L., A.D. Peacock, G.A. Davis, M.C. Harrison, R. Geyer, and D.C. White. 2003. *In situ* monitoring of the remediation of chlorinated hydrocarbons using "bug traps". In *Paper presented at the 7th International Symposium on In Situ and On-Site Bioremediation*, Orlando, Florida. Eds. V.S. Mager and M.E. Kelley. Paper I-11. Columbus, Ohio: Battelle Press.

Sublette, K.L., A.D. Peacock, G.A. Davis, M.C. Harrison, R. Geyer, and D.C. White. 2002. Convenient, down-well, *in situ* monitoring of chlorinated hydrocarbon remediation with sterilizable "bug traps" containing Bio Sep® beads. In *Paper presented at the International Symposium on Subsurface Microbiology*, Copenhagen, Denmark. Eds. H-J. Albrechtsen and J. Aamand. International Society for Subsurface Microbiology.

White, D.C. 1988. Validation of quantitative analysis for microbial biomass, community structure, and metabolic activity. *Archiv fur Hydrobiologie Beih Ergebnisse der Limnologie* 31: 1–18.

White, D.C., C.A. Fleming, K.T. Leung, and S.J. MacNaughton. 1998. *In situ* microbial ecology for quantitative appraisal, monitoring, and risk assessment of pollution remediation in soils, the subsurface, the rhizosphere and in biofilms. *J. Microbiological Methods* 32, no. 2: 93–105.

White, D.C., J.S. Gouffon, A.D. Peacock, R. Geyer, A. Biernacki, G.A. Davis, M. Pryor, M.B. Tabacco, and K.L. Sublette. 2003. Forensic analysis by comprehensive rapid detection of pathogens and contamination concentrated in biofilms in drinking water systems for water resource protection and management. *Environmental Forensics* 4, no. 1: 63–74.

White, D.C., and D.B. Ringelberg. 1998. Signature lipid biomarker analysis. In *Techniques in Microbial Ecology*, ed. R.S. Burlage, R. Atlas, D. Stahl, G. Geesey, and G. Sayler, 255–272. New York: Oxford University Press.

White, D.C., G.A. Smith, M.J. Gehron, J.H. Parker, R.H. Findley, R.F. Martz, and H.L. Fredrickson. 1983. The groundwater aquifer microbiota: Biomass, community structure, and nutritional status. *Developments in Industrial Microbiology* 24, Chapter 15: 201–211.

Winer, B.J., D.R. Brown, and K.M. Michels. 1991. *Statistical Principals in Experimental Design*, 3rd ed. New York: McGraw Hill.

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