BIOREMEDIATION OF BENZENE, ETHYLBENZENE, AND XYLENES IN GROUNDWATER UNDER IRON-AMENDED, SULFATE-REDUCING CONDITIONS

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Abstract—Elevated concentrations of sulfide in groundwater (∼63 mg S²⁻/L in water and 500 mg dissolved H₂S/L dissipating from the wellhead) at a field site near South Lovedale (OK, USA) were inhibiting the activity of sulfate-reducing bacteria (SRB) that are known to degrade contaminants, including benzene, toluene, ethylbenzene, and xylenes. Elevated concentrations of these contaminants, except for toluene, also were present in this groundwater. Microcosms were established in the laboratory using groundwater and sediment collected from the field site and amended with various nutrient, substrate, and inhibitor treatments. All microcosms initially were amended with FeCl₂ to induce FeS precipitation and, thereby, to reduce aqueous sulfide concentrations. Complete removal of benzene, ethylbenzene, and m+p-xylene (BEX; o-xylene not detected) was observed within 39 d in treatments with various combinations of nutrient and substrate amendments, including treatments with no amendments (other than FeCl₂). This indicates that the elevated concentration of sulfide is the only limiting factor to BEX biodegradation at this site under anaerobic conditions and that treating the groundwater with FeCl₂ may be a simple remedy to both facilitate and enhance BEX degradation by the indigenous SRB population.

Keywords—Benzene Ethylbenzene Groundwater bioremediation Sulfate-reducing bacteria Xylene

INTRODUCTION

Groundwater contaminated by aromatic hydrocarbons, such as benzene, toluene, ethylbenzene, and xylenes (BTEX), is a major environmental problem because of the recalcitrance and potential toxic effects of BTEX, including liver, kidney, and nervous system damage and cancer ([1]; http://www.epa.gov/OGWDW/mcl.html). Biodegradation of BTEX under aerobic conditions is possible; however, oxygen depletion resulting from microbial respiration usually drives the geochemical environment to reduced, anaerobic conditions. Studies conducted within the past two decades have demonstrated that BTEX can be biodegraded under anaerobic conditions by organisms such as denitrifying bacteria [2–4] and sulfate-reducing bacteria (SRB) [5–16].

One potential drawback to BTEX degradation by SRB is that SRB use sulfate as the terminal electron acceptor and, thus, produce hydrogen sulfide (H₂S) as a metabolic by-product. Hydrogen sulfide production is a concern, because high sulfide concentrations (>56 mg/L, depending on pH) are potentially toxic and inhibitory to various anaerobic, hydrocarbon-degrading microorganisms, including SRB [17–23]. Sulfide produced through SRB activity, however, can form complexes with metals in solution and precipitate as metal–sulfide compounds, thereby decreasing aqueous sulfide and metal concentrations [24–26]. This implies that adding divalent transition metal salts (e.g., ferrous salts) might be a possible remedy for removing sulfide in low-ionic-strength waters with SRB activity.

The present study was conducted to determine the optimal nutrient amendments required for complete benzene, ethylbenzene, and m+p-xylene (BEX) biodegradation by SRB following a ferrous iron amendment to remove toxic sulfide from the system and to maintain low sulfide concentrations during the experiment. The present study serves as a verification of field remediation of BEX under sulfate-reducing conditions, both at sites where the environmental media samples were collected and at other sites exhibiting similar environmental characteristics. Microcosms in this work were established with groundwater and sediment collected from the field; therefore, the results were directly applicable to remediation of the field site.

MATERIALS AND METHODS

Site description

The field site is a former gas-compressor site near South Lovedale (OK, USA) challenged with elevated BEX and sulfide concentrations in the groundwater (Table 1). Site assessments have indicated that this site has been contaminated with BEX and other gasoline-range organics (GRO; i.e., compounds eluting with n-alkane markers ranging in size from C₄ to C₁₂) for more than a decade and that these elevated substrate concentrations have facilitated high aerobic microbial activity, which eventually lead to anaerobic conditions in the groundwater. Previous assessments indicated that hydrocarbon concentrations in this groundwater were declining, likely because of anaerobic hydrocarbon degradation, but concentrations eventually stabilized and have remained elevated. On-site gypsum beds, which theoretically could provide a source of sulfate as the electron acceptor, did not facilitate BEX biodegradation by SRB, initially suggesting that this system was either nitrogen and/or phosphorus limited. Toxic concentrations of sulfide, however, also were present in the groundwater (∼63 mg dis-
Table 1. Concentrations of various chemicals in groundwater collected from a representative monitoring well near South Lovedale (OK, USA) on December 17, 2004

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Groundwater</th>
<th>Method detection limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzene</td>
<td>700 µg/L</td>
<td>20 µg/L</td>
</tr>
<tr>
<td>Toluene</td>
<td>BDL*</td>
<td>5 µg/L</td>
</tr>
<tr>
<td>Ethylbenzene</td>
<td>220 µg/L</td>
<td>20 µg/L</td>
</tr>
<tr>
<td>m+p-Xylenes</td>
<td>310 µg/L</td>
<td>20 µg/L</td>
</tr>
<tr>
<td>GRO</td>
<td>3.07b mg/L</td>
<td>1.0 mg/L</td>
</tr>
<tr>
<td>Sulfate</td>
<td>1,730.0 mg/L</td>
<td>8.0 mg/L</td>
</tr>
<tr>
<td>Sulfide</td>
<td>63 mg/L</td>
<td>0.012 mg/L</td>
</tr>
<tr>
<td>Ammonia-N</td>
<td>2.0 mg/L</td>
<td>0.05 mg/L</td>
</tr>
<tr>
<td>Nitrate-N</td>
<td>4.3 mg/L</td>
<td>0.1 mg/L</td>
</tr>
</tbody>
</table>

* BDL = below the method detection limit.
* b Gasoline-range organic hydrocarbons (n = 5–10 carbons per chain).

solved S\(^2\)/L in the water and 500 mg dissolved H\(_2\)S/L dissipating from the wellhead) and likely inhibited SRB degradation of BEX regardless of the available nutrient concentrations.

Groundwater was collected with a peristaltic pump from a selected monitoring well at this site for chemical analyses and to fill microcosms in the laboratory. Groundwater samples were collected in glass/Teflon\(^\circ\) containers with zero headspace. A soil core was removed near the well, and sediment was collected from the uppermost portion of the phreatic zone of this core. This sediment and groundwater were used to establish microcosms in the laboratory.

**Groundwater analyses**

Groundwater samples from the field site were transported on ice to the Western Research Institute. Groundwater was analyzed for BEX, GRO, sulfate, sulfide, ammonia nitrogen, and nitrate nitrogen to establish baseline concentrations before addition to the microcosms. The pH of the groundwater also was measured at the field site during sample collection using an Orion\(^\circ\) model 720A+ pH meter (Thermo Electron Corporation, Waltham, MA, USA) equipped with an Orion Ag/AgCl combination electrode.

Nitrate nitrogen, nitrite, phosphate, and sulfate concentrations were measured with a DX-100 Ion Chromatograph ( Dionex Corporation, Sunnyvale, CA, USA) equipped with a 4 m x 250-mm IonPac AS14 anion-exchange column. Ammonium-nitrogen was measured by the indophenol blue method and ultraviolet–visible light spectroscopy [27]. Sulfide was analyzed using a modified version of the colorimetric/methylene blue method ([28]; http://www.epa.gov/region01/info/testmethods/pdfs/testmeth.pdf) using a ultraviolet–visible light spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD, USA). Aqueous BEX was analyzed by injecting aliquots into organics-free, distilled water mixed with 250 µL of methanol in 40-ml volatile organic analysis vials. The BEX concentrations were measured with a gas chromatograph–mass spectrometer (model 6890/5973; Agilent Technologies, Palo Alto, CA, USA) equipped with a model 3100 purge-and-trap concentrator (Tekmar Control Systems, Spokane, WA, USA). Aqueous GRO was analyzed with a gas chromatograph–flame ionization detector equipped with a Tekmar number-8 trap and a DB-5 column (30 m x 0.53 mm; Agilent Technologies) ([29]; http://www.epa.gov/region01/info/testmethods/pdfs/testmeth.pdf). The method detection limits (MDLs) for all analyses are listed in Table 1.

**Microcosms**

Anaerobic microcosms were established in 125-ml serum bottles (which actually hold >125 ml when filled to zero headspace) using groundwater and sediment collected from the field site. All microcosms were prepared under a N\(_2\) (O\(_2\)-free) atmosphere within a glove box. Each microcosm was filled with approximately 150 ml of groundwater to minimize headspace and contained 5% sediment (dry wt) based on the mass of water in the microcosms (~7.5 g of sediment). Ferrous iron (Fe\(^{2+}\)) was added at a molar ratio of 2:1 Fe\(^{2+}\):S\(^2\) to ensure the complexation of all sulfide that was present and could potentially be produced in the groundwater (based on the sulfate concentration; a molar ratio of 1:1 Fe\(^{2+}\):S\(^2\) is required for complete complexation). The Fe\(^{2+}\) was added to each microcosm as ferrous chloride tetrahydrate (FeCl\(_2\)·4H\(_2\)O; reagent grade; Sigma-Aldrich, St. Louis, MO, USA). The purpose of this FeCl\(_2\) addition was to remove sulfide in the groundwater through iron sulfide (FeS) precipitation as well as to remove sulfide that could potentially be produced through sulfate reduction during the experiment. The concentration of sulfate in the groundwater was approximately 100-fold the stoichiometric concentration required by SRB to fully degrade the BEX in the groundwater (a molar SO\(_4^−\) to BEX- and GRO-C ratio of 1:2 is required); therefore, no sulfate was added to the microcosms.

Various combinations of nutrients, substrate, and inhibitors were added to the microcosms to determine the optimal amendments for complete BEX degradation as well as to identify the most efficient biodegrading group in the microbial consortia present in the groundwater. The following amendment combinations were applied to microcosms set up in triplicate: No amendments; nitrogen and phosphorus; nitrogen, phosphorus, and methanol; nitrate and methanol; nitrate; nitrogen, phosphorus, methanol, and sodium molybdate; nitrogen, phosphorus, and nitrite; and nitrite and methanol. Potassium nitrate (KNO\(_3\); reagent grade; Sigma) was added to corresponding treatments at a molar BEX- and GRO-C ratio of 10:0 as an enhancement for denitrifying bacteria. Methanol (MeOH; reagent grade; Sigma) was added in a molar amount equal to the carbon substrate present in corresponding treatments to enhance BEX solubility and, possibly, BEX degradation. Ammonium chloride (NH\(_4\)Cl; reagent grade; Fisher Scientific International, Hampton, NH, USA) and dibasic potassium phosphate (K\(_2\)HPO\(_4\); reagent grade; Fisher Scientific) were used as nitrogen and phosphorus (nutrient) sources, respectively, and these were added to corresponding treatments in a molar C to N to P ratio of 100:10:2. Sodium molybdate (Na\(_2\)MoO\(_4\); reagent grade; Sigma) was added at a concentration of 20 mM molybdate (MoO\(_4^−\)) to corresponding treatments as a specific inhibitor to SRB [30]; and potassium nitrite (KNO\(_2\); reagent grade; Sigma) was added at a concentration of 3 mM nitrite (NO\(_3^−\)) to corresponding treatments as a competitive inhibitor of SRB [31–34].

Microcosms were sealed and incubated at room temperature (20°C) and then stored in the dark for 127 d. Samples were drawn from each microcosm on days 0 (after addition of sediment, amendments, and groundwater), 18, 39, 80, and 127 and analyzed for BEX; samples drawn on days 0, 18, 64, and 127 also were analyzed for sulfate and sulfide. On day 70, all microcosms were spiked with 700, 200, and 300 µg/L of ben-
We used p-xylene to spike the microcosms instead of m+p-xylene, because our analytical method did not distinguish between the two.

**Statistical analysis**

We conducted statistical comparisons with analysis of variance (α = 0.05), followed by the Tukey honestly significant difference post hoc pairwise comparisons using Minitab® (Ver 13.3.1; Minitab, State College, PA, USA) for data at each sampling period, but we did not compare data through time. Because of possible correlations in these time series data, we made a Bonferroni adjustment to our α-level from 0.05 to 0.01 for the BEX data (i.e., α = 0.05/5 sampling periods) and from 0.05 to 0.013 for the sulfate data (i.e., α = 0.05/4 sampling periods) for detecting significant differences between treatments that received different amendments.

**RESULTS**

Toluene concentrations were less than the MDL in the groundwater used to establish the microcosms in this experiment (Table 1); therefore, we only present the results concerning BEX degradation. The pH of groundwater samples and the background concentrations of BEX, GRO, sulfate, sulfide, and nitrogen are listed in Table 1. During the experiment, BEX and sulfate concentrations decreased in all treatments, but the most rapid and complete degradation occurred in the nonamended, nutrient-amended (nitrogen and phosphorus), and methanol–nutrient treatments. Therefore, for simplicity, we discuss only the results from these three treatments.

Mean benzene concentrations in the nonamended, nutrient-amended, and methanol–nutrient treatments decreased from an initial concentration of 700 μg/L (background concentration) (Table 1) to between 483 and 561 μg/L on day 0 after the addition of amendments and sediment to the microcosms. Concentrations continued to decrease to less than 38 μg/L during the first 39 d of the experiment. Ten days after the initial spiking of these microcosms with benzene, the benzene concentrations ranged from 202 to 353 μg/L, and complete benzene degradation (less than the MDL of 20 μg/L) occurred within 57 d of this additional benzene addition (Fig. 1a). Similarly, complete ethylbenzene and m+p-xylene degradation occurred in these microcosms during the first 39 d. Furthermore, after adding spikes of 200 and 300 μg/L of ethylbenzene and p-xylene on day 70, respectively, concentrations quickly decreased to less than 40 and 14 μg/L, respectively, within 10 d. Complete degradation of these two constituents occurred within 57 d of these additional spikes (Fig. 1b and 1c). No significant differences were found in the benzene, ethylbenzene, or m+p-xylene concentrations between any of these three treatments at any one sampling period.

Sulfate concentrations in the nonamended, nutrient-amended, and methanol–nutrient treatments increased, likely because of desorption from the sediment mixture in each microcosm, but then steadily decreased throughout this 127-d experiment to concentrations less than the MDL (8 mg/L) (Fig. 2). Sulfate concentrations in the methanol–nutrient treatments were much lower than those in the nonamended and nutrient-
amended treatments on day 64. Whether this difference was significant or not is debatable, because the p value was 0.013 and our α-level for this comparison also was 0.013. This, however, only illustrates that the sulfate concentration in the methanol–nutrient treatment decreased considerably faster than that in the other two treatments. As expected, sulfide concentrations in all microcosms remained very low and did not exceed 1.3 mg/L during the 127-d experiment.

**DISCUSSION**

The present study demonstrated BEX biodegradation under sulfate-reducing conditions using water and sediments from the actual contaminated field site without adding artificial enrichment media other than FeCl₂. Although some studies have demonstrated BTEX biodegradation by SRB using the water and sediments from a contaminated site [8, 12, 13], these studies have not addressed the production of sulfide and its toxic/inhibitory effects on SRB.

The groundwater and sediments at our field site contained high concentrations of BEX, which were being biodegraded through SRB activity that was facilitated by high sulfate concentrations at the site. Eventually, however, biodegradation of these contaminants was inhibited by high sulfide concentrations that accumulated because of the low ionic strength of the groundwater.

In these experiments, the initial decrease in BEX concentrations on day 0 in the microcosms likely resulted from adsorption to sediment and glass surfaces in the serum bottles. Following this initial decrease, BEX was completely degraded under anaerobic conditions within 39 d (Fig. 1), and the increased SRB activity, illustrated by the steadily decreasing sulfate concentrations (Fig. 2), suggests that SRB were using BEX as a substrate. Furthermore, BEX degradation occurred even faster following the BEX spike added to each microcosm on day 70 (Fig. 1). Interestingly, the treatment with no nutrient or substrate amendments (other than FeCl₂) performed as well on day 70 (Fig. 1). Interestingly, the treatment with no nutrient or substrate amendments (other than FeCl₂) performed as well on day 70 (Fig. 1), which suggests that SRB were using BEX as a substrate. Furthermore, BEX degradation occurred even faster following the BEX spike added to each microcosm on day 70 (Fig. 1). Therefore, it appears that the only limiting factor to BEX degradation in the groundwater at the field site was inhibition of the SRB by high sulfide concentrations.

The sulfate concentration decreased more rapidly in the methanol–nutrient treatment than in the other two treatments (Fig. 2), which suggests that SRB activity was highest in this treatment and, thus, that BEX degradation might have occurred more quickly in the presence of methanol. No significant differences, however, were found between the BEX concentrations in each treatment at the different sampling times; therefore, the addition of methanol did not enhance BEX degradation over the course of this 127-d experiment.

The SRB consortia in the groundwater from our field site were capable of rapidly (<39 d) biodegrading environmentally relevant BEX concentrations to less than the MDL under anaerobic conditions and without additional nutrient or substrate amendments (other than FeCl₂). Furthermore, because we used water and sediment samples from the field, the results of this experiment were successfully extrapolated to pilot-scale remediation of the field site (data not shown).

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**REFERENCES**