

Geochemical and Microbial Evidence of Fuel Biodegradation in a Contaminated Karst Aquifer in Southern Kentucky, June 1999

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Abstract

Complex hydrogeologic conditions coupled with poorly understood biodegradation processes in karst aquifers have led many to believe that the potential for natural attenuation of petroleum fuel hydrocarbons is limited. This research addressed the capacity for biodegradation processes in karst. Ground-water samples were collected for bacteria and geochemical analysis from several contaminated monitoring wells in an unconsolidated regolith and karst aquifer that had varying concentrations of dissolved fuel. Bacteria concentrations were greatest in ground-water samples containing the greatest fuel contamination. Additionally, bacteria isolated from fuel-contaminated ground-water samples readily grew in Petri dishes with dissolved gasoline fuel as the only source of food. The wells with screens intersecting less contaminated sections of the aquifer had greater dissolved oxygen concentrations (6.3 milligrams per liter) than those intersecting more contaminated sections (dissolved oxygen less than 0.1 milligrams per liter). Also, where the oxygen concentrations were diminished, geochemical evidence indicated that anaerobic processes were active. This evidence includes elevated levels of ammonia and ferrous iron in the fuel-contaminated ground-water samples. Based on these results, biodegradation of fuel constituents in the karst aquifer is indicated, and therefore, natural attenuation should not be disregarded because of preconceptions about low microbial activity in karst aquifers.

INTRODUCTION

Approximately 40 percent of the United States east of the Mississippi River is underlain by various types of karst aquifers (Quinlan, 1989), and more than two-thirds of the State of Tennessee is underlain by carbonate rocks that are classified as karst (Wolfe and others, 1997). Potential sources of ground-water contamination are numerous in karst regions; however, the fate of ground-water contaminants such as petroleum hydrocarbons in karst aquifers is poorly understood because of the complex hydrology (Field, 1993) and the lack of biodegradation studies. Furthermore, ground-water models that simulate flow or predict the fate and transport of contaminants in unconsolidated, porous-media aquifers have limited application to karst aquifers. Most natural attenuation and bioremediation guidelines specify that these models are not applicable in fractured rock or karst aquifers (U.S. Environmental Protection Agency, 1997).

The U.S. Geological Survey (USGS) conducted a preliminary study to evaluate whether microbes in a karst aquifer have a capacity to biodegrade the fuel-related compounds benzene, toluene, ethyl-benzene, and xylenes (BTEX). This report presents field and laboratory data collected to determine the potential for biodegradation of jet-fuel contamination in a karst aquifer in Southern Kentucky. The study site was selected because of the known presence of BTEX in the ground water and the availability of hydrologic and

historic water-quality data. The objective of the study was to characterize the microbial population and geochemical conditions present in ground water at the study site to determine if BTEX biodegradation in the karst aquifer was significant enough to warrant further investigation.

METHODS AND MATERIALS

Ground-water samples were collected from seven monitoring wells located within a 5-hectare study area with a known jet-fuel spill, north of Clarksville, Tennessee, in southern Kentucky (fig.1). The wells were selected based on depths of screens and recent reports of fuel contaminant concentrations. The goal was to examine the geochemistry and bacteria present under a broad range of hydrogeologic (fig. 1) and contaminant conditions. The monitoring wells selected were screened in regolith, epikarst, and bedrock.

Water-quality samples were collected by the USGS during June 1999. Ground-water samples were collected from the wells using a submersible pump. Wells were micropurged as according to Puls and Paul (1995). The pump was lowered to the middle of the screen before starting. Water was pumped at a rate of approximately 1 liter per minute. An electronic tape measure was used to concurrently monitor the water level in the well casing. Water recharging the well was considered to be equal to the water being pumped when the water level became steady. At the point when water recharge equaled pump discharge and the

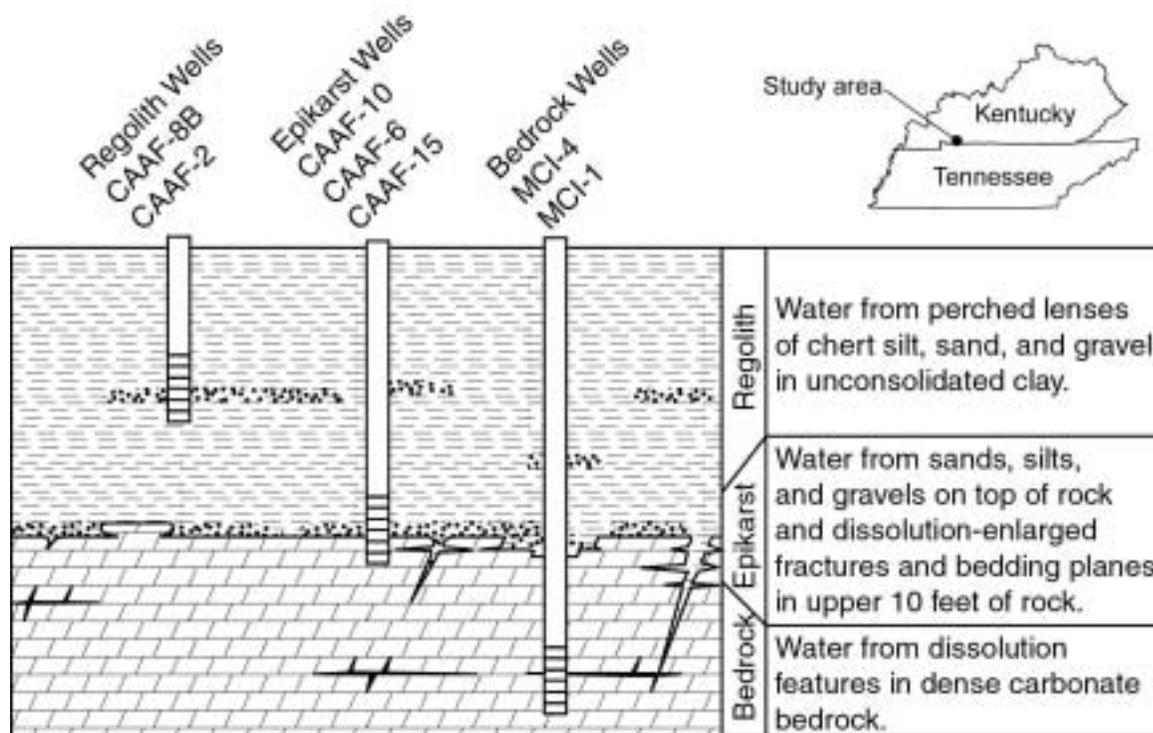


Figure 1. Generalized hydrogeologic setting of wells sampled for the biodegradation assessment of a karst aquifer in southern Kentucky.

specific conductance, turbidity, and fluorescence values in the pumped water no longer changed over a 1-minute period, water samples were collected. The first ground-water samples collected were for volatile organic compounds, followed by bacteria, and geochemical analyses respectively. Bacteria growth tests were begun within 6 hours of sample collection. Dissolved oxygen, alkalinity, carbon dioxide, pH, specific conductance, and temperature were measured immediately upon sample collection (Wood, 1981). The other ground-water samples were preserved according to Wood (1981) and the Hach Company (1992), and were analyzed within the holding period. The water sample for BTEX analysis was collected in three 40-milliliter (mL) vials and analyzed by gas chromatography at the USGS, National Water Quality Laboratory in Arvada, Colo. Analyses for nutrients and geochemical constituents were conducted using spectrophotometric methods described by Hach Company (1992), and, by Byl and Williams (2000).

Bacteria were enumerated and identified in ground-water samples from the monitoring wells to supplement the interpretation of the geochemical information. Bacteria were enumerated from four of the seven monitoring wells using two methods (data from the remaining three wells were not used because

the samples were not processed within 24 hours of collection). The facultative and aerobic heterotrophic bacteria were enumerated using dilution buffer and tryptic soy agar plate counts as described by Eaton and others (1995). Aerobic and facultative bacteria capable of using gasoline as their sole food source also were enumerated using sterile media pads soaked with dilution buffer containing dissolved gasoline. Sterile filters with 0.45-micrometer pore sizes were used to inoculate the growth media. A 0.01- or 1-mL aliquot of sample water was transferred to 20 mL of sterile dilution buffer and drawn onto the filter as described in the membrane-filtration method (Britton and Greeson, 1989). The filters were placed on the growth media using sterile forceps, and the plates were placed in an incubator at 35 °C for 5 days. Bacteria colony-forming units were counted after 24, 48, and 120 hours. Results are reported as colony-forming units per milliliter of sample water using data from the 48-hour count (table 1).

Bacteria types present in the samples were identified by the use of the ribonucleic (RNA)-oligonucleotide hybridization method (Amann and others, 1995; Byl and others, 1997). The RNA-oligonucleotide hybridization method is a technique that exploits unique nucleotide sequences in the

ribosomal RNA (rRNA) to identify bacteria (Amann and others, 1995). The method can be used to identify groups of bacteria such as sulfate-reducing bacteria or specific genera such as *Nitrosomonas* sp. The level of identification used in this study was general bacteria groups such as iron oxidizers, methanotrophs, ammonia oxidizers, and sulfate reducers (Byl and others, 1997; Farmer and others, 1998).

RESULTS AND DISCUSSION

Results of the analyses demonstrated geochemical conditions that are indicative of fuel biodegradation in contaminated wells. A strong

inverse pattern was found between dissolved oxygen (DO) levels and BTEX concentrations in the water samples, implying a biological oxygen demand resulting from BTEX consumption (tables 1 and 2). In addition, a great quantity of bacteria was present in each of the samples, with the largest populations associated with the most contaminated wells. Subsurface bacteria known to influence BTEX degradation processes were identified using RNA-hybridization probes (Farmer and others, 1998, Chapelle, 1993).

Table 1. Summary of benzene, toluene, ethylbenzene, and xylenes (BTEX) and bacteria analysis for wells in study area, June 9-11, 1999. Descending down the chart, wells are arranged in relative order of shallow to deepest and least to most fuel contaminated. The blank was collected from equipment (pump) rinse water between sampling at wells CAAF-2 and CAAF-6. Bacteria were enumerated using media pads soaked with dilution buffer containing gasoline as a food source. No bacteria grew in the control treatments when the gasoline was absent. Bacteria samples also were tested with different RNA-hybridization probes to identify bacteria. Bacteria types identified were: *Pseudomonas aureginosa* (known to efficiently biodegrade BTEX in soils), *Pseudomonas* species (a broader, more encompassing group of *Pseudomonas* bacteria), ammonia-oxidizing bacteria, and iron-oxidizing bacteria. The intensity of the bacteria identified by RNA-hybridization is indicated by "+" symbols. Three +++ symbols means greater than 100 bacteria present per milliliter, two ++ indicate a moderate number of bacteria (10 - 100 bacteria / mL), one + indicates there were only a few bacteria present (1 - 10 bacteria/mL).

[Bnz, benzene; Tol, toluene; Eth, ethyl-benzene; Xyl, xylenes; CFU, colony-forming units per 1 milliliter; µg/L, microgram per liter; mL, milliliter; <, less than; RNA, ribonucleic acid; BD = below detection; ND = No data; Pseudo., *Pseudomonas*; sp., species; aure, *aureginosa*; Ammonia ox., ammonia oxidizers]

Well and date samples were collected	Hydro-geologic setting	Bnz µg/L	Tol µg/L	Eth µg/L	Xyl µg/L	CFU per mL	RNA hybridization results
CAAF-8B 6/9/99	Regolith	<0.5	1.00	<0.5	1.6	500	Pseudo. aure.+, Pseudo. sp. +
CAAF-10 6/10/99	Epikarst	BD	0.63	<0.5	0.6	325	Pseudo. aure.+, Pseudo. sp. +
MCI-4 6/9/99	Bedrock	<0.5	0.75	<0.5	0.6	93	Pseudo. sp. ++
CAAF-2 6/11/99	Regolith	2.4	BD	11.0	5.5	ND	Pseudo. sp. ++, Ammonia ox. +
CAAF-6 6/11/99	Epikarst	16.0	3.50	8.8	25.0	ND	Pseudo. aure.+, Pseudo. sp. ++, Ammonia ox. ++
CAAF-15 6/11/99	Epikarst	220.	BD	3.1	360.0	ND	Pseudo. aure.+++ , Pseudo. sp. +++, Iron oxidizer +++, Ammonia ox. ++
MCI-1 6/10/99	Bedrock	75.0	14.0	26.0	38.0	2550	Pseudo. aure.+++ , Pseudo. sp. +++, Ammonia ox. +
BLANK		0.71	BD	4.3	2.9	ND	(None)

Table 2. Summary of selected geochemical data for wells in study area, June 9-11, 1999.

[$\mu\text{S/cm}$, microsiemen per centimeter; mg/L, milligram per liter; CaCO_3 , calcium carbonate; DO, dissolved oxygen; NO_3 , nitrate; NH_3 , ammonia; Aq Fe^{2+} , aqueous ferrous iron; <, less than)

Well and date samples were collected	Hydro-geologic setting	pH	Specific conductance [$\mu\text{S/cm}$]	Alkalinity [mg/L CaCO_3]	DO [mg/L]	NO_3 [mg/L]	NH_3 [mg/L]	Aq Fe^{2+} [mg/L]
CAAF-8B 6/9/99	Regolith	5.0	31	9	6.3	3.5	<0.01	0.01
CAAF-10 6/10/99	Epikarst	7.3	266	143	5.5	2.6	<0.01	0.06
MCI-4 6/9/99	Bedrock	6.9	373	145	3.6	14.5	<0.01	0.01
CAAF-2 6/11/99	Regolith	6.0	548	305	<0.1	4.4	2.59	16.20
CAAF-6 6/11/99	Epikarst	7.4	233	121	0.3	0.9	<0.01	0.06
CAAF-15 6/11/99	Epikarst	7.3	257	120	0.5	1.3	0.19	0.14
MCI-1 6/10/99	Bedrock	6.3	549	293	<0.1	4.4	0.80	5.80

The bacteria identified were *Pseudomonas* sp., *Pseudomonas aureginosa*, and two groups of bacteria known as ammonia-oxidizing and iron-oxidizing bacteria. *Pseudomonas* bacteria, one of the most common soil bacteria, contain a variety of enzymes that degrade BTEX, such as toluene di-oxygenase and catechol di-oxygenase (Chapelle, 1993). *Pseudomonas aureginosa* is a specific species in the *Pseudomonas* family that has been shown to be important in BTEX biodegradation (Houghton and Shanley, 1994). The number of *Pseudomonas aureginosa* generally increased with increasing BTEX concentrations in the samples, indicating they were flourishing in the contaminated waters.

Ammonia-oxidizing bacteria also were identified in samples from four of the wells. These bacteria types have an enzyme called ammonia mono-oxygenase that can degrade BTEX in the presence of DO (Bedard and Knowles, 1989). Bacteria associated with iron geochemistry also have been shown to degrade BTEX (Chapelle, 1993). Only one highly contaminated well (CAAF-15) tested positive for iron-oxidizing bacteria. Elevated concentrations of dissolved iron in wells CAAF-2 and MCI-1 indicated that iron-reducing bacteria were active in other parts of the aquifer. In general, a consortium of aerobic and anaerobic bacteria appeared to be present in water samples from the

contaminated wells implying that environmental conditions change in the aquifer or that heterogeneous niches exist within the karst aquifer with regard to redox conditions.

Perhaps the most direct evidence that bacteria in the karst aquifer at this site are capable of degrading fuel came when bacteria were grown directly on gasoline as the sole food source. Filters containing indigenous bacteria from the aquifers were placed on sterile media pads soaked with dilution buffer (Eaton and others, 1995) with gasoline dissolved in it. The control tests contained sterile media pads soaked with dilution buffer only, and no gasoline. Results of these tests are shown by the number of colony-forming units (CFU) per milliliter that grew on the pads (table 1). Bacteria in water samples from well MCI-1, a karst bedrock aquifer well, grew rapidly on the gasoline-amended media, while nothing grew on the filters placed on the control pads containing dilution buffer only, indicating the bacteria used the gasoline as a food source to grow and replicate. Bacteria from three other well samples, CAAF-8B, CAAF-10, and MCI-4, also grew on gasoline-amended media, but not on the control pads soaked with only dilution buffer. Samples from wells CAAF-2, CAAF-6, and CAAF-15 were not tested for growth on gasoline because of time constraints.

Results from these tests indicate several preliminary lines of evidence exist that bacteria are actively degrading fuels in the karst aquifer at the site. The geochemical evidence includes such indicators as oxygen consumption, ammonia production, iron dissolution, and sulfur reduction as bacteria use these constituents in their metabolic processes (table 2). The bacteria evidence includes identification of bacteria known to consume BTEX, high bacteria counts, and growth of indigenous bacteria using fuel as their only food source. These data demonstrate that bacteria thrive in water from both the deep and shallow wells at the site. Based on these results, biological degradation of BTEX is believed to occur in both unconsolidated regolith and bedrock parts of the fuel-contaminated aquifer.

Implications of the findings

The concentrations of bacteria present in the bedrock aquifer samples were high enough to be comparable with concentrations reported for contaminated sand aquifers (Ghiorse and Wilson, 1988). Thus, some of the fuel biodegradation utilization capacity derived for sand aquifers may apply to ground water in regolith, epikarst, and karst aquifers. A stoichiometric balance of terminal electron acceptors utilized in the consumption of BTEX by bacteria is

used to derive a utilization capacity (Newell and others, 1996). Comparing geochemical constituents in relatively clean wells to concentrations in more contaminated wells derives the estimated concentration of terminal electron acceptors in an aquifer. Using the methods and stoichiometric equations found in the U.S. Environmental Protection Agency model BIOSCREEN: Natural Attenuation Decision Support System (Newell and others, 1996), the biodegradation capacity would be nearly 4 and 3 milligrams BTEX per liter of water based on the concentrations of DO, nitrate (NO₃), ferrous iron (Fe²⁺) and sulfate (SO₄²⁻) found in samples from the regolith and bedrock aquifers, respectively (table 3). These results imply that BTEX dissolved in the regolith and karst aquifers are likely to be consumed by bacteria given sufficient time (the highest BTEX concentrations identified in the karst aquifer have been less than 1 mg/L). Note that the biodegradation capacity does not provide a rate of biodegradation, so factors such as ground-water flow velocity and contaminant retention time in the aquifer are important unknown variables. A study that integrates a more detailed assessment of the hydrogeology, along with further geochemistry and biology would be required to determine if BTEX biodegradation occurs at a rate sufficient to protect target receptors.

Table 3. Concentration of terminal electron acceptors used to determine BTEX biodegradation capacity as described in Newell and others (1996)

[BTEX, benzene, toluene, ethyle-benzene, xylenes; mg, milligram; mg/L, milligrams per liter]

Terminal electron acceptor molecule (TEA) or byproduct	BTEX Utilization Factor (mg TEA consumed / mg of BTEX degraded)	Estimated concentrations of TEA in regolith water	Biodegradation capacity in regolith (mg of BTEX degraded per liter of water)	Estimated concentrations of TEA in bedrock water	Biodegradation capacity in bedrock aquifer (mg of BTEX degraded per liter of water)
Dissolved oxygen (DO)	3.14 mg DO / mg BTEX	6.3 mg / L	2.00	3.6 mg /L	1.15
Nitrate (NO ₃)	4.9 mg NO ₃ / mg BTEX	3.5 mg / L	0.61	5.0 mg/L	1.00
Ferrous (Fe ²⁺) iron from ferric (Fe ³⁺)	21.8 mg Fe ³⁺ transformed / mg BTEX	0.05 mg / L	0.73	16 mg/L	0.27
Sulfate (SO ₄ ²⁻)	4.7 mg SO ₄ ²⁻ / mg BTEX	4.0 mg / L	0.43	2.0 mg/L	0.43

Estimated total biodegradation capacity = 3.77 mg

2.85 mg

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