Environ. Sci. Technol. **1998**, *32,* 3598−3611

Pilot-Scale Evaluation of Bioaugmentation for In-Situ Remediation of a Carbon Tetrachloride-Contaminated Aquifer

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An experiment was performed in a carbon tetrachloride (CT)- and nitrate-contaminated aquifer at Schoolcraft, MI, to evaluate bioaugmentation with *Pseudomonas stutzeri* KC, a denitrifying bacterium that degrades CT without producing chloroform (CF). A test section of the aquifer was treated to create pH conditions favorable for KC and then inoculated with culture grown aerobically on site. Activity was sustained with pulses of acetate-amended groundwater, followed by "chase" pulses of acetate-free water. In regions with effective substrate delivery, KC was detected, nitrate levels fell by 85%, pH levels increased, and CT levels decreased by ∼65%, with no significant increase in CF. After 3 weeks, denitrification and CT transformation activity decreased, and KC was no longer detected in groundwater from four wells. Loss of denitrification was attributed to the acetate-free chase. Upon eliminating the chase, CT transformation resumed, and KC was detected, but CF production was also observed, implicating indigenous organisms as agents of transformation. Final sediment analyses indicated 60−88% CT removal, little CF, and persistent KC. This work demonstrates subsurface pH adjustment, subsurface transport of KC, assimilation of KC into the aquifer community, CT removal without CF production after inoculation, and CF formation when KC activity declined.

Introduction

Pseudomonas stutzeri KC (ATCC Deposit No. 55595, DSM Deposit No. 7136) rapidly transforms carbon tetrachloride (CT) to CO_2, formate, and an unidentified nonvolatile product without either producing or transforming chloroform (CF) (*1*−*3*). The requirements for CT transformation by strain KC are (i) adequate concentrations of nitrate and electron donor, (ii) anoxic conditions, (iii) iron-limiting conditions, and (iv) trace levels of copper. Iron-limited conditions can be achieved by adjusting the pH to near 8.0 (*1*) or by adding iron chelators (*2*). Copper is required for CT transformation but is toxic at neutral pH (*4*). The transformation is cometabolic and linked to mechanisms for trace metal scavenging (*1, 2*). A novel aspect of the transformation is its mechanism. Under iron-limiting conditions, strain KC secretes a small (∼500 Da) biomolecule that transforms CT (*3*). The secreted biomolecule is regenerated or reactivated for transformation by actively growing cells. The regeneration step can be accomplished by diverse cell types, including aquifer microflora (*5*). Experiments with the secreted biomolecule have established that it is transported without retardation through aquifer sediments (*5*).

In the laboratory, CT transformation by strain KC was achieved in groundwater, soil, and aquifer sediment by raising the pH of the growth environment to 8.0−8.2 (*4*, *6*). Using this procedure, CT degradation was obtained in batch exchange columns (*7*) and in continuous flow columns packed with aquifer sediments (*8*).

Under denitrifying conditions, indigenous populations often do not transform CT or they do so slowly with CF production. Biostimulation of microflora native to aquifer sediments from Schoolcraft, MI, did not result in significant CT removal as compared to killed controls (*7*). In a field-scale experiment at the Moffett Naval Air Station, CF production was observed following the addition of acetate to stimulate indigenous denitrifying populations (*9*). In two columns packed with Schoolcraft aquifer sediments and bioaugmented with strain KC, CT was efficiently removed without CF production for a 2-month period of active operation (weekly acetate feedings), but CF production was observed in one of the two columns when weekly acetate addition was discontinued (*10*).

From laboratory findings, strain KC appeared to be a suitable candidate for field application, but important questions remained: (i) Would it be possible to maintain the pH of a subsurface environment at the slightly alkaline levels (pH > 7.6) needed for growth and CT transformation? (ii) Would strain KC survive in the face of competition from indigenous microflora under field conditions? (iii) Would strain KC remain active for CT transformation under field conditions? To address these questions, a field experiment was performed in a CT-contaminated aquifer at Schoolcraft, MI.

Project Description

Site Characterization. The pilot-scale experiment was performed in a test grid (Figure 1) constructed in the heart of a CT-contaminated aquifer at Schoolcraft, MI. The aquifer

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10.1021/es980200z CCC: $15.00
Published on Web 10/02/1998

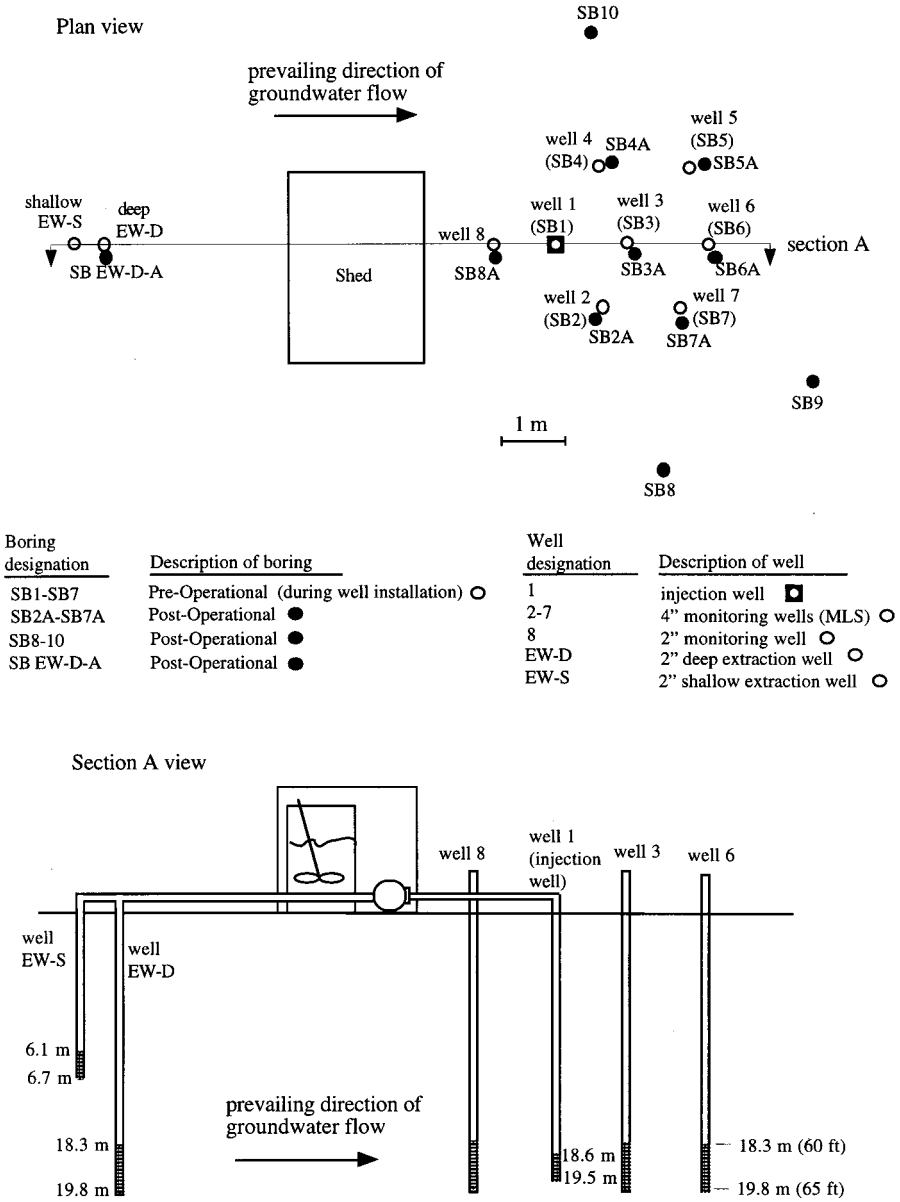

FIGURE 1. Layout of test grid and location of pre- and post-operational borings.

is comprised of relatively homogeneous glacial outwash sands. In slug tests performed during installation of test grid wells, hydraulic conductivity values ranged from 1.7×10^{-2} to 4.9×10^{-2} cm/s. These values agreed with computed values based on grain size distribution. Most of the grains (95−99%) fell within the size range of 0.30−10 mm. Acidities of groundwater and sediments within the test grid were 0.588 ± 0.13 and 0.84 ± 0.14 mequiv/kg, respectively. Groundwater alkalinity was 6.93 ± 0.11 mequiv/L (347 ± 5 mg/L as $CaCO_3$). Flow-through cell measurements during well development indicated a dissolved oxygen concentration of 3−5 mg/L and an oxidation reduction potential of +151 mV. Nitrate levels ranged from 57 to 68 mg/L.

Cores removed from the screened depth interval during well installation had a low percentage of organic carbon, with an average of 0.031 ± 0.013% ($n = 41$). The concentration of CT in the sediment was 23 ± 17 μg/kg ($n = 115$). Batch CT isotherms indicated a linear partition coefficient of 0.46 L/kg, over the range from 0 to 2500 μg/kg ($r^2 = 0.97$). The concentration of CF in the sediment was 3.0 ± 2.1 μg/kg ($n = 115$). Batch CF isotherms indicated a linear partition coefficient of 0.2 L/kg, over the range from 0 to 50 μg/kg.

Plate counts of aquifer flora on R2A agar (Difco Laboratories, Detroit, MI) gave 4.1×10^5 ($\pm 2.0 \times 10^5$) cfu/mL for water samples ($n = 18$) and 4.6×10^6 ($\pm 3.2 \times 10^6$) cfu/g for sediment samples ($n = 27$). Most probable number (MPN) assays on the denitrifying fraction of the indigenous community indicated 1.2×10^5 ($\pm 1.1 \times 10^6$) cfu/g ($n = 27$).

Layout of the Test Grid. A grid of PVC wells was constructed in a CT plume at Schoolcraft, MI. As shown in Figure 1, the grid consisted of one injection well (well 1); a tier of multi-level sampling wells (wells 2−4) located 1 m downgradient from the injection well; a second tier of monitoring wells (wells 5−7) located 2 m downgradient from the injection well; an upgradient monitoring well (well 8); a deep extraction well (EW-D); and a shallow extraction well (EW-S). EW-D had CT levels similar to those of wells 1−6 and served as a nonimpacted "control" and as a source of CT-contaminated water for injection into the test grid. EW-S was a source of CT-free groundwater. A groundwater flow of 15 cm/d transports materials injected at well 1 to the first tier of monitoring wells (wells 2−4) in 1 week and to the second tier of wells (wells 5−7) in 2 weeks. During well installation, cores of aquifer sediment were collected. The

TABLE 1. Major Field Activities Beginning with First Multi-Level Sampling Event[a]

phase	activity	day	sampling	injection pulse
pre-operational	baseline	0	X	
	monitoring	7	X	
		18	X	
		36		X
		39	X	
		39	X	
operational phase A	increase pH; monitor pH and bromide tracer	43	X	X
		43	X	
		46	XX	X
		50	XX	X
		52	X	
		53		X
		57	X	X
		60		X
		65		X
		70		X
		71	X	
		77		X
operational phase B	inoculate grid	80		XX
operational phase C (+ chase pulse)	monitor colonization and transformation	87	X	
		88		XX
		92	X	XX
		99	X	
		100		XX
		107	X	XX
operational phase D (− chase pulse)	monitor colonization and transformation	114	X	X
		121	X	X
		128	X	X
		135	X	X
		142	X	
post-operational phase	final drilling for cores; monitor recontamination	147	X	
		163	X	
		170	X	

[a] An X indicates that the operation was performed on the day indicated. XX for sampling indicates that samples were taken before and after injection; XX for injection pulses indicates that a pulse of nutrients was followed by a nutrient-free chase pulse.

location of the core samples and samples obtained at the end of the experiment are indicated in Figure 1.

Experimental Plan and Implementation. The pilot-scale experiment was conducted in six phases, as described below and detailed in Table 1:

(i) Pre-Operational Phase (Includes Days 0−42). Groundwater samples were analyzed to assess baseline conditions within the test grid.

(ii) Operational Phase A (Days 43−79). Pulse addition of base-amended groundwater was initiated on day 43 and repeated periodically thereafter to increase the pH of the grid. Between pulses, the groundwater flowed through the test grid at the velocity of background groundwater (15 cm/d). CT-contaminated groundwater was pumped from the deep extraction well to a Nalgene HDPE 2.1 m^3 tank where it was amended with bromide and stock solutions of NaOH (2.5 N) and phosphate (10.1%) to give final concentrations of 30 mg/L bromide, 0.75 mequiv/L NaOH (final pH of 8.3), and 10 mg/L phosphate. Base and phosphate were added with mechanical mixing. A floating layer of hollow polyethylene balls minimized losses of volatile organics. CT levels were monitored by headspace gas chromatography. Injections were initiated after stable readings for pH and CT were maintained for more than 30 min. A centrifugal pump was used to pump amended groundwater from the mixing tank to the injection well.

During operational phase A, base addition was performed nine times, with the last addition on day 77, 3 days before inoculation. In the first five pumping events (days 43, 46, 50, 53, and 57), 10.3 m^3 was pumped into the aquifer at a flow rate of 49 L/min. Multi-level sampling was performed on day 57, and vertical distribution profiles of pH and bromide were obtained. The results indicated that the initial pumping rate was insufficient for uniform delivery of bromide or base. To enhance delivery, the injection flow rate was increased to 150−189 L/min, and 3 m^3 pulses of base-amended groundwater was injected on days 60, 65, 70, and 77. Monitoring on day 71 indicated improved distribution of bromide and base, with pH levels within the desired range (>7.5).

(iii) Operational Phase B (Day 80). On day 80, the test grid was inoculated with an aerobically grown culture of *Pseudomonas stutzeri* KC. The inoculum (1.5 m^3) was grown on site in filter-sterilized groundwater (T = 20 °C, pH = 8.2, 10 ppm phosphate, 1.6 g/L acetate) from 12 L of starter culture grown aerobically on nutrient broth for 24 h. Growth was accompanied by a pH increase, and acetic acid (50%) was used to maintain the pH at 8−8.2. The inoculum was pumped into the injection well at a flow rate of 189 L/min. Inoculation was performed one time only.

(iv) Operational Phase C (Days 81−107). Distribution of cells for colonization of the test grid was achieved by a weekly pulse of groundwater (1.9 m^3) amended with acetate, phosphate, and base followed immediately by a "chase" pulse (1.1 m^3) of acetate-free groundwater (150−189 L/min). The groundwater for both pulses was extracted from the deep injection well. The purpose of the chase pulse was to achieve a more uniform distribution of organisms and to prevent plugging of the injection well.

(v) Operational Phase D (Days 108−142). Within 30 days of inoculation, CT removal and denitrification activity declined at the most communicative wells. The absence of detectable acetate and the presence of nitrate indicated insufficient acetate in the grid. The chase procedure was

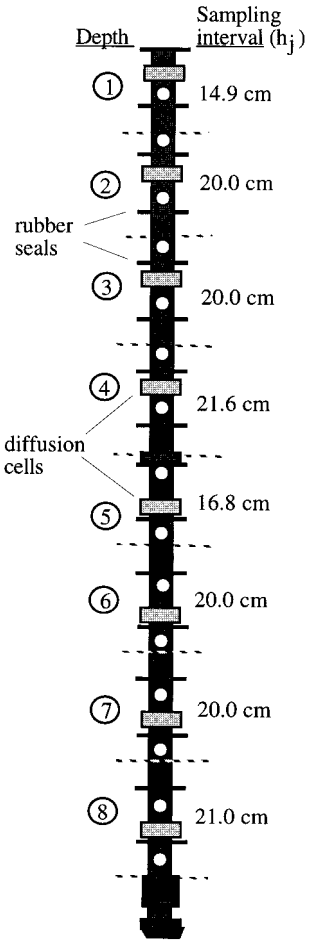

FIGURE 2. Multi-level sampling assembly with diffusion cells.

therefore discontinued on day 107. Beginning on day 114, 3 m^3 of nutrient- and base-amended groundwater was pulsed weekly into the aquifer.

(vi) Post-Operational Phase (Days 143−170). Groundwater sampling continued for 3 weeks following the final pulsing event. Drilling was performed on days 147−154. As indicated in Figure 1, borings for chemical and microbiological analysis were obtained from boreholes adjacent to the monitoring wells (SB 2A-7A), EW-D, and from borings 2 m outside the test grid (SB 8−10).

Analytical Methods

Groundwater Sampling. Multi-level sampling assemblies (Margan Ltd., Netanya, Israel) illustrated in Figure 2 were installed in wells 2−7. Each well contained eight cylindrical diffusion cells capable of sampling discrete vertical intervals. Each diffusion cell consisted of a short PVC cylinder threaded at each end and sealed with nylon membranes (10 μm) held in place by open-hole screw caps. Tests with PVC established that it did not sorb CT. Groundwater samples were collected with sanitized multi-level sampling devices using aseptic technique.

In general, the sampling assembly rested quiescently inside the well casing for 7 days, at which point it was withdrawn for analysis. The sole exception was a post-operational event in which one sampling assembly could not be removed from its well for two additional days. Laboratory and field experiments established that 3 days of static incubation were sufficient for equilibration of solutes inside and outside the diffusion cells. In the laboratory, bacteria concentrations inside and outside the diffusion cell also stabilized after 3−4 days, but recovery was incomplete with the concentration inside the cell only 20−40% of the concentration in bulk solution. After withdrawing the sampling assemblies from the wells, subsamples were removed for microbial enumeration and determinations of volatile organics, anions, and pH.

Sampling and Bulk Analyses of Aquifer Sediments. Aquifer sediments were obtained in the pre- and post operational periods using the Waterloo cohesionless continuous sand sampler. Cores (1.5 m sections) were collected from each borehole at three depth intervals between 16.8 and 21.3 m bgs. Post-operational borings were retrieved from positions located as close as possible to the corresponding pre-operational borings. Subsamples of cores were obtained by pushing plastic syringe-like samplers (D-Tech) through holes drilled in the core liner. Two to four cm^3 sediment plugs were placed into 20-mL headspace vials containing 0.25 mL of 40% w/v $NaHSO_4$ solution and 5 mL deionized water and were sealed with Teflon-lined septa. Samples were subsequently analyzed for organic carbon and volatile organics.

For grain-size determinations, aquifer sediments were dried in an oven at 105 °C to constant weight (<1% change) and then stored in a desiccator. Representative subsamples were characterized by sieve analysis.

Volatile Organics Analyses. For samples collected during the pre-operational phase, volatile organics were analyzed using a Hewlett-Packard (HP) 7694 headspace sampler interfaced to an HP 5890 II Plus gas chromatograph-HP 5972 mass selective detector (GC-MSD) system, outfitted with a 60-m fused silica capillary column HP-624. The gas chromatograph oven temperature was programmed for 40 °C for 5.0 min, a 5 °C/min ramp for 20 min, 1 min at 140 °C, and a 25 °C/min ramp for 3.2 min. The method detection limit for carbon tetrachloride and CF were 0.06 and 0.11 μg/L, respectively. For sediment samples, method detection limits were 0.88 and 1.8 μg/kg for CT and CF, respectively.

Groundwater samples collected during the operational phase and most of the aquifer solids collected during post-operational phase were analyzed by static headspace analysis with an Perkin-Elmer autosystem equipped with an electron capture detector by EPA Methods 3800 and 3810. Headspace analysis of liquid samples for CT and CF was performed with a Perkin-Elmer PE HS 40 headspace sampler equipped with an electron capture detector (350 °C) and a 50 m DB-624 column (oven temperature 80 °C). Nitrogen was used as both a carrier gas (10 mL/min through autosystem) and makeup gas (15 mL/min). All analyses were performed within 24 h of sample collection. Analytical runs included trip blanks, matrix blanks, and matrix spikes.

Inorganic Analyses. Acidity and alkalinity of the groundwater were measured as per Standard Methods 2310 and 2320 (*11*) with minor modifications. Acidity of aquifer sediments was determined by placing ~10 g (wet weight) of sediment in 50 mL of groundwater. The slurry was covered and mixed during titration to pH 8.3. Acidity of the solids was calculated as the difference between the acidity of the groundwater and the acidity of the slurry.

Anion analyses were performed using a Dionex model 2000i/SP ion chromatograph with suppressed conductivity detection equipped with a Dionex model AS4-A column and utilizing a 1.8 mM bicarbonate and 1.7 mM carbonate mobile phase at 3 mL/min (EPA Method 300). Five point external standard calibration curves were used for calibration. Analytical runs included trip blanks, matrix blanks, and matrix spikes.

Dissolved oxygen and oxidation reduction potential were measured during well development using a Purge-Saver Model FC2000 flow-through cell containing dissolved oxygen and ORP probes. Groundwater was pumped through the

cells until stable readings were obtained (<2% variation over 15 min).

Total Carbon and Organic Carbon. For total carbon determinations, duplicate subsamples of dried solids (150−250 mg each) were analyzed using a combustion- and infrared-based CO_2 analyzer, the CHN-1000 (LECO Corp.). To determine organic carbon, sediments were pretreated with HCl to remove inorganic carbon (*12*). Dried solids (1.5−2.0 g) were placed in tared 8 mL capped vials, acidified with 2 mL of acid, vigorously shaken, sonicated (5 min), dried, and then analyzed for total carbon with the CHN-1000. Inorganic carbon was calculated as the difference between total and organic carbon.

Microbiological Methods. Methods used to assess changes in the aquifer microflora included spread plate enumeration of culturable heterotrophs and strain KC, MPN analysis of the total denitrifying fraction, and characterization of community DNA. Spread plates using R2A agar were used to enumerate strain KC and aquifer flora. Strain KC exhibited a distinctive "fried egg" morphology on R2A agar.

To enumerate bacteria on sediments, bacterial cells were extracted (*13*), serial dilutions were prepared in sterile phosphate buffer (50 mM, pH 8.0), and aliquots (100 μL) of the dilutions were spread on R2A plates. Colony forming units were counted after 6 days of incubation at 20 °C. To enumerate denitrifiers, a most probable number method was employed (*14*).

Extraction of DNA from Groundwater and Aquifer Sediments. Total community DNA was extracted from groundwater and sediment samples for detection of strain KC using a strain-specific DNA probe and for analysis of microbial community structure by denaturing gradient gel electrophoresis (DGGE). Cell pellets were obtained from groundwater by centrifuging 10-mL groundwater samples for 20 min at 5000*g* at 4 °C. Cells were extracted from 10-g samples of sediment by shaking at 150 rpm for 15 min with 20 mL of 120 mM sodium phosphate buffer (pH 8.0) and then allowing the solids to settle for 5 min. The liquid phase was removed and saved, and the sediment was extracted again. A cell pellet was obtained by centrifuging the combined supernatants for 20 min at 5000*g* at 4 °C. Pellets were suspended in 300 μL of TE buffer (10 mM Tris, 5 mM EDTA). SDS was added to a final concentration of 0.5% followed by three cycles of freeze−thaw under liquid nitrogen (−65 °C). The proteinase K−CTAB procedure (*15*) was used to purify DNA, with the exception of the DNA precipitation step in which an equal volume of 2-propanol was added, and samples were incubated for 1 h at −20 °C.

DNA Probe Specific to Strain KC. DNA extracted from strain KC was digested with *Pst*I restriction enzyme and separated in 0.8% agarose gel. Fragments of 1−1.2 kb were recovered from the gel, purified, and cloned into a pUC19 vector. DNA from 20 different clones was labeled with ^{32}P using a nick translation kit (Promega) and screened by colony hybridization against 20 different *Pseudomonas* and *Escherichia coli* strains. Extraction of DNA from the gel, cloning, and hybridization were performed (*16*). Two fragments showing low levels of hybridization were selected for partial sequencing from both end points (250−300 bp) with *Taq* DNA polymerase using an DNA sequencer (Applied Biosystems 373A). Sequences were compared with sequences in GenBank using the GCG sequence analysis software package (Genetics Computer Group, Inc., Madison, WI). One of the two fragments (pKC40) was unlike any of the database sequences. This fragment was completely sequenced in both directions, and the complete sequence was compared with GenBank sequences to identify regions for primers with minimum similarity to known sequences. Three sets of primers (20−22 mers) were selected and tested for specificity by PCR amplification using these primers and DNA extracted from strain KC, the Schoolcraft groundwater, 17 different *Pseudomonas* strains, including closely related strains, and 3 *E. coli* strains. PCR reactions were carried out in 100-μL volumes containing 50 pmol each of two primers, 0.2 mM deoxynucleoside triphosphates, and 3 U of AmpliTag DNA polymerase (Perkin-Elmer) in a buffer containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), and 1.5 mM $MgCl_2$. Amplifications were performed with a DNA thermal cycler (Perkin-Elmer) with the following settings: 1 cycle at 94 °C for 2 min; 35 cycles at 94 °C for 1 min, at 64 °C for 2 min, and at 72 °C for 3 min, with a 7-min extension at 72 °C in the last cycle. Amplification products were separated in 1.5% agarose gel in TAE buffer with ethidium bromide. An intense band was obtained for strain KC, a less intense band for three closely related *Pseudomonas* strains, and little or no band for total community DNA and the *E. coli* strains. From these results and reevaluation of the sequence, a fourth primer pair was chosen, tested using the same protocol, and found to be specific for strain KC, producing no band from groundwater-extracted DNA. This pair, JMT 216 and JMT 219, had a primary structure 5′-TGGCATGGGTCTGGGCTCTAT-3′ and 5′-CCTGATGACCGATTACGACCA-3′. Using this primer pair, a 787 bp strain KC-specific DNA fragment was amplifed by PCR (GenBank Accession Number AF063250). Groundwater and sediment samples were analyzed for the presence of strain KC by extraction of total community DNA, addition of the primer pair JMT 216 and 219, PCR amplification, and gel separation. Samples were scored positive if the 787 bp fragment was observed.

Confirmation of Inoculum Identity and Transformation Activity. To confirm the capability of the inoculum to transform CT, samples from the field reactor were withdrawn, returned to the lab, and analyzed for growth by protein (*17*), CT degradation activity, and presence of strain KC using the DNA probe specific to strain KC.

Denaturing Gradient Gel Electrophoresis. Changes in community structure were monitored using denaturing gradient gel electrophoresis (DGGE) to resolve 16S rDNA genes amplified from total community DNA by PCR (*18*). The V6−V8 regions of the 16S rDNA genes were amplified using primers F984GC and R1378 (F968−984-GC: 5′-gc AAC GCG AAG AAC CTT AC-3′; R1378−1401: 5′-CGGTGTGTA-CAAGGCCCGGGAACG-3′; 5′-CGCCCGGGGCGCGCCCC-GGGCGGGGCGGGGGCACGGGGGG-3′) as per Heuer et al. (*19*). PCR products were analyzed using 6% acrylamide gels and a denaturing gradient of 40−60% (100% denaturant corresponds to 7 M urea plus 40% deionized formamide). DGGE was performed in 0.5 × TAE buffer at 60 °C at a constant voltage of 150 V for 6 h. A silver staining protocol was used for detection of DNA (*19*).

Averaging Procedures. To compute an average groundwater concentration over a specified vertical interval, the measured concentrations at each depth were multiplied by the corresponding sampling interval length, summed, and divided by the length of the total sampling interval:

$$\bar{C}_{ik} = \sum_j C_{ijk} h_j \Big/ \sum_j h_j$$

where $\bar{C}_{ik}$ is the average concentration of solute i (i = nitrate, acetate, CT, etc.) in well k (k = wells 2 and 3 combined, well 4 alone, wells 5 and 6 combined, or well 7 alone), C_{ijk} is the measured concentration of solute i in well k for sampling interval j (j = 1−8), h_j is the length of sampling interval j (Figure 2).

Results and Discussion

Delivery of Tracer and Base. A key question was whether it would be possible to create and maintain the slightly alkaline levels (pH > 7.6) needed for CT degradation and

TABLE 2. Average Bromide Concentrations (± One Standard Deviation for Four Samples) during the Operational and Post-Operational Phases[a]

		operational phase	post-operational phase[b]	
well	depths	Days 87−142	day 163	day 170
2/3	1−4	24.1 ± 2.5	8.6 ± 5.5	8.2 ± 4.2
	5−8	25.5 ± 1.8	6.7 ± 1.0	6.0 ± 5.4
4	1−4	4.6 ± 6.4	0.2	0.0
	5−8	5.0 ± 6.7	0.0	0.0
5/6	1−4	14.3 ± 1.3	19.8	12.4
	5−8	20.9 ± 3.0	13.3	5.2
7	1−4	22.7 ± 2.3	23.8	17.2
	5−8	24.1 ± 2.4	22.6	6.9

[a] Influent bromide concentration was 30 mg/L. [b] Duplicate samples only were available for wells 4−7.

competitiveness of strain KC. Alkalinity, acidity, and pH data indicated that this was achievable in certain grid locations but not others depending upon the extent of hydraulic communication with the injection well.

Hydraulic communication was evaluated in tracer studies using bromide. On the basis of a groundwater flow rate of 15 cm/d, we expected tracer to appear at the first tier wells 1 week after injection and at the second tier wells 2 weeks after injection. Arrival of bromide at the first tier wells 2 and 3 occurred 1 week prior to its arrival at second tier wells 5−7. In addition, arrival of bromide at the four lower depths (depths 5−8) occurred prior to its arrival at the four upper depths (1−4), suggesting a more conductive zone in the lower depths.

Throughout the pre-operational and operational phases, all water injected into the test grid was supplemented with bromide (30 mg/L). Bromide levels were therefore an indication of the extent of dilution of added water with background water. With the exception of well 4, steady concentrations of bromide were observed throughout the operational phase (Table 2). This indicates that bromide entering the grid between sampling events was approximately equal to bromide leaving the grid between sampling events. By dividing observed bromide concentrations by the injected concentration (30 mg/L), the percentage of groundwater receiving above-ground amendments can be calculated. For wells 2 and 3, the steady-state percentage of injected groundwater was 80% for upper depths and 85% for the lower depths; for well 7, 76% for upper depths and 80% for lower depths; for wells 5 and 6, 48% for upper depths and 70% for

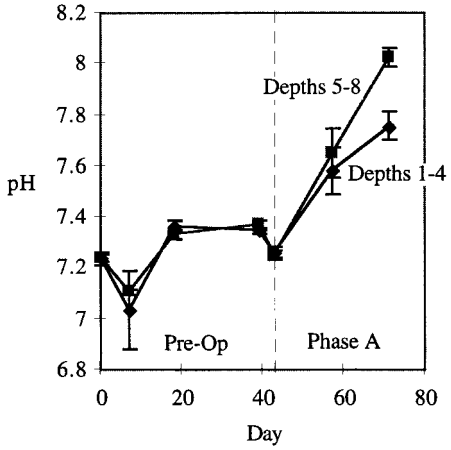

FIGURE 3. Depth-averaged changes in pH in wells 2 and 3 during the pre-operational phase and during operational phase A (first base addition on day 43). Error bars indicate the standard deviation for four samples.

TABLE 3. Detection of Planktonic Strain KC Cells in the First Tier of the Test Grid (Wells 2−4) As Indicated by DNA Probe Signal[a]

		day[b]								
depth	71	87	92	99	107	114	121	128	135	142
				Well 2						
1	not detected[c]		+	+	+	+	+	+	+	+
2			+	+	+	+	+	+	+	+
3			+	+	+	+	+	+	+	+
4			+	+	+	+	+	+	+	+
5		+	+	+	+	+	+	+	+	+
6		+	+	+	+	+	+	+	+	
7		+	+	+	+	+	+	+	+	+
8		+	+	+	+	+	+	+	+	+
				Well 3						
1	not detected[c]	+	+	+	+	+	+	NS	+	
2		+	NS	+	+	+	+	+	NS	+
3		+	+	+	+	+	+		NS	+
4			+	+	+	+	+		NS	+
5		+	+	+	+	+	+	+	NS	+
6		+	+	+	+	+	+	+	NS	+
7		+	+	+	+	+	+	+	NS	+
8		+	+	+	+	+	+	+	NS	+
				Well 4						
1	not detected[c]			NS				+		
2				+		+		+		
3				+						
4							NS			+
5				+				+		+
6										
7				NS				+		
8			+	+				+		

[a] A plus sign (+) cell indicates that strain KC was detected. An empty cell indicates that strain KC was not detected. NS, no sample. [b] Inoculation on day 80. [c] For all depths.

TABLE 4. Detection of Planktonic Strain KC Cells in the Second Tier of the Test Grid (Wells 5−7) As Indicated by DNA Probe Signal[a]

	Day[b]									
depth	71	87	92	99	107	114	121	128	135	142
				Well 5						
1	not detected[c]									
2										
3			+							
4				+						
5			+			+		+		
6										
7										+
8				+					NS	+
				Well 6						
1	not detected[c]									
2										
3				+						
4		NS								
5				+				NS		
6				+						
7			+	+						
8			+	+						
				Well 7						
1	not detected[c]			+			+		+	+
2			+	+		+	+		+	+
3			+	+			+		+	+
4			+	+			+	+	+	
5			+	+			+	+	NS	
6			+	+			+	+	+	
7			+	+			+		+	
8			+	+			+		+	+

[a] A plus sign (+) indicates that strain KC was detected in the groundwater. An empty cell indicates that strain KC was not detected. NS, no sample. [b] Inoculation on day 80. [c] For all depths.

lower depths; and for well 4, 15% for upper depths and 17% for lower depths. Thus, the effectiveness of chemical delivery (ranked from most effective to least effective) was wells 2, 3, and 7 (lower depths) > wells 2, 3, and 7 (upper depths) > wells 5 and 6 (lower depths) > wells 5 and 6 (upper depths) > well 4. During the post-operational phase, no additional bromide was introduced into the grid. Consequently, background groundwater flowed into the test grid and bromide levels fell.

Because bromide delivery was most effective at the lower four depths, delivery of other solutes was also expected to be greatest at these depths, and this was indeed the case. In well 2, pH increased (Figure 3) and alkalinity increased from 7.17 mequiv/L on day 43 to 7.44 mequiv/L on day 70, while acidity decreased from 0.576 to 0.366 mequiv/L. In well 3, alkalinity increased from 7.02 to 7.66 mequiv/L, and acidity simultaneously decreased from 0.504 to 0.35 mequiv/L. Because of differences in base delivery, in the period just prior to inoculation, the lower four depths of wells 2 and 3 were likely the most favorable locations within the test grid for colonization by strain KC.

On-Site Growth of *Pseudomonas stutzeri* KC and Aquifer Inoculation. One distinctive aspect of this study was on-site growth of inoculum using filtered groundwater. In 24 h, the inoculum culture grew to a final density of 2.3 (±1.3) × 10^7 cfu/mL of strain KC and 1.9 (±1.2) × 10^7 cfu/mL of contaminant organisms. The identity of strain KC was confirmed using the DNA probe specific to strain KC. The capacity of the inoculum to transform CT and denitrify was also confirmed.

Transport and Survival of *Pseudomonas stutzeri* KC. An important question motivating this study was whether strain KC would be transported and survive in the face of competition from indigenous microflora. Plate counts of samples from groundwater and results for the DNA probe indicated that strain KC was transported or migrated distances of at least 2 m following injection. Plate counts of sediments indicated long-term survival of strain KC at fairly high levels (10^6 cfu/g) on sediments close to the injection well. This was confirmed by analysis of total community DNA from the sediments using DGGE and the DNA probe. These results established that strain KC was able to colonize the sediments and assimilate into the aquifer community. Little growth of indigenous microflora was observed in the first weeks following inoculation, but shifts in community structure were observed with indigenous flora eventually achieving dominance in the aqueous phase as strain KC attached to the aquifer sediments. Evidence for these assertions is provided in the following paragraphs.

Detection of unattached or planktonic KC cells in the weeks following inoculation (Tables 3 and 4) generally correlated with the pattern of bromide delivery and the extent of pH adjustment. Strain KC appeared at the lower four depths of wells 2 and 3 within 1 week and in the upper four depths 5 days later. After a 1-week delay, strain KC cells were detected in wells 5−7. This lag was consistent with results for the bromide tracer and with the expected delay based on an estimated groundwater velocity of 15 cm/d. Plate count data confirmed DNA probe results, indicating rapid initial delivery of KC cells to the lower depth levels of wells 2 and 3 at concentrations of 10^5−10^6/mL, delayed delivery to the upper depths of wells 2 and 3, and delayed delivery to wells 5−7.

Groundwater from wells 2 and 3 tested positive for KC throughout the operational phase, while groundwater from wells 4−7 tested positive sporadically and inconsistently. By the end of operational phase C (day 107), strain KC cells were not detected in groundwater from wells 4−7. After eliminating the acetate-free chase (phase D), strain KC was again detected in groundwater from wells 4, 5, and 7.

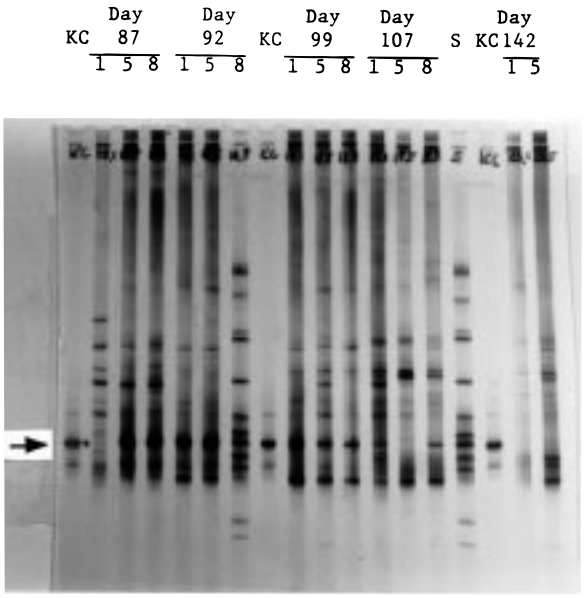

FIGURE 4. Use of denaturing gradient gel electrophoresis to resolve 16S rDNA genes of numerically dominant members of the microbial community in Schoolcraft groundwater. The samples analyzed were obtained from depths 1, 5, and 8 in well 3 on days 87, 92, 99, 107, and 142. Lanes designated KC contained PCR-amplified 16S rDNA from strain KC as a control. Lane designated S contained DNA from Schoolcraft groundwater amended with KC DNA. Arrow indicates band for strain KC.

TABLE 5. Concentrations of Indigenous Flora and Strain KC in Aquifer Sediments Obtained during the Post-Operational Phase (Days 147−154)

boring	depth (m)	indigenous (cfu/g)	KC (cfu/g)
2A	19.1	6.26E+06	5.64E+05
3A	19.1	3.79E+06	4.10E+05
	19.5	9.10E+06	6.78E+05
5A	19.1	8.95E+05	0.00E+00
7A	18.9	1.52E+06	1.16E+04
	19.1	6.49E+06	3.83E+05
	19.2	2.92E+06	1.15E+05
	19.5	2.66E+06	2.86E+04
8A	18.9	5.70E+06	3.33E+06
	19.2	1.41E+07	5.43E+06
	19.5	6.29E+06	1.26E+06
8	19.1	7.69E+05	5.10E+04
10	18.9	7.81E+05	0.00E+00
	19.2	5.33E+05	0.00E+00
	19.5	3.30E+07	4.18E+05

Plate count data for strain KC correlated well with results from the DNA probe. Plate count data for wells 2 and 3 indicated groundwater strain KC concentrations of 10^5-10^6/mL in the first two weeks following inoculation. These concentrations represented a large fraction (>∼50%) of the culturable planktonic biomass. After the first two weeks, however, groundwater plate counts for strain KC decreased to $\sim 10^2-10^3$ cfu/mL, representing only 0.1−1% of the culturable planktonic biomass. Indigenous microflora plate counts in wells 2 and 3 decreased slightly in the first two weeks following inoculation, but recovered to 10^5-10^6 cfu/mL for the balance of the study.

Figure 4 illustrates DGGE profiles for groundwater from well 3. Changes in aqueous phase community structure are visualized by comparing the relative intensity of the different bands on different days (87, 92, 99, 107, and 142). A more intense band suggests a higher number of organisms in the corresponding population. Of significance is the appearance of a band corresponding to strain KC in depths 5 and 8 one week after inoculation (day 87). This band is not observed in depth 1, and the pattern for this depth is therefore indicative of the microbial community structure prior to the arrival of strain KC. The same sequence of KC arrival was obtained with the DNA probe (Table 3) and with plate counts. Also of interest is the gradual loss of the strain KC band at the end of the operational phase (days 107 and 142) and the appearance of new bands, indicating a shift in community structure. These findings agreed with plate count data, although the DNA probe continued to detect aqueous phase strain KC, even at day 142 (Table 3), probably because of the high sensitivity of this method.

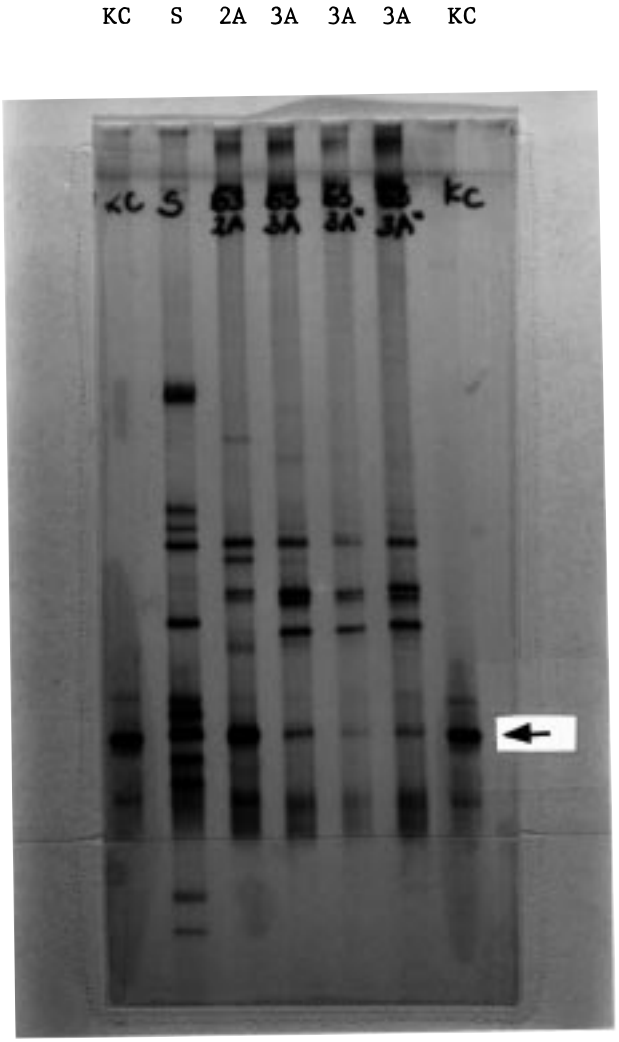

FIGURE 5. Use of denaturing gradient gel electrophoresis to resolve 16S rDNA genes of numerically dominant members of the microbial community in Schoolcraft aquifer sediments from post-operational borings 2A and 3A (depth of 19.2 m). Lanes designated KC contained PCR amplified 16S rDNA from strain KC as a control. Lanes designated S contained DNA from Schoolcraft groundwater amended with KC DNA. Arrow indicates band for strain KC.

The gradual loss of strain KC in groundwater did not mean that strain KC was no longer present in the test grid. Laboratory experiments with model aquifer columns established that strain KC preferentially colonized sediments within the treatment zone (*10*). This was also observed in the test grid. Figure 5 illustrates DGGE profiles in post-operational aquifer cores at a depth of 19.2 m (63 ft) in wells 2 and 3. DNA from sediment samples obtained near well 2 hydrobized strongly to the DNA probe specific for strain KC,

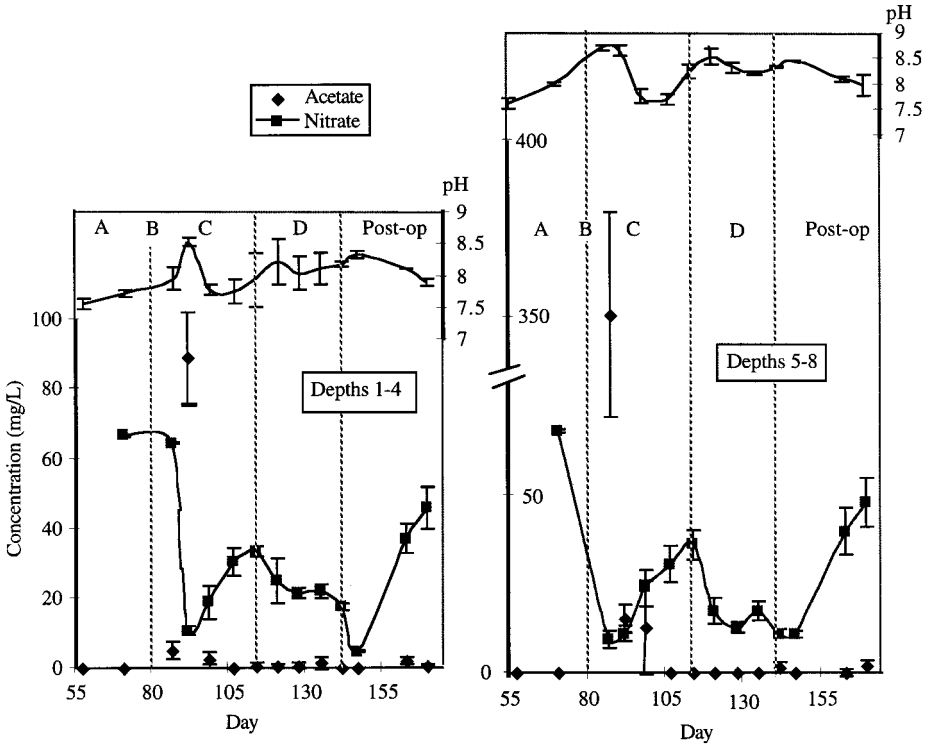

FIGURE 6. Wells 2 and 3: depth-averaged concentrations of acetate, nitrate, and pH for depths 1−4 and 5−8 during the operational and post-operational phases. Error bars indicate the standard deviation for four samples.

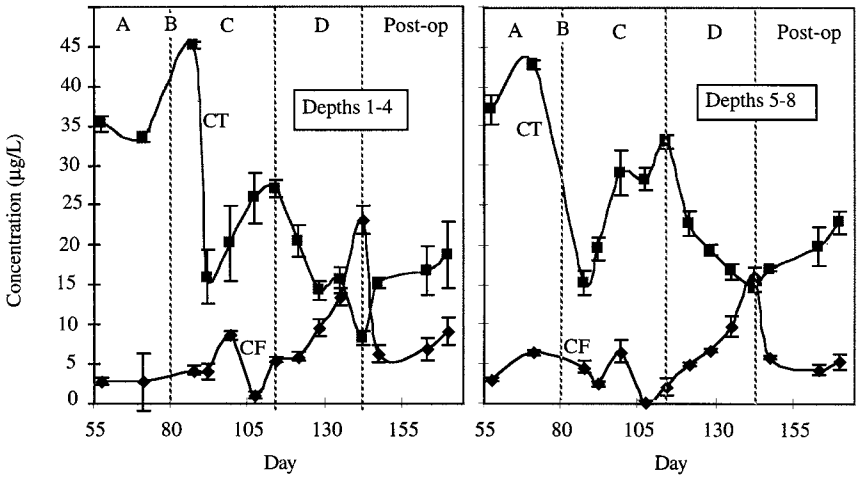

FIGURE 7. Wells 2 and 3: depth-averaged concentrations of CT and CF for depths 1−4 and 5−8 during the operational and post-operational phases. Error bars indicate the standard deviation for four samples.

and a similar, though less intense, band was observed in DNA extracted from sediments near well 3. Additional evidence for persistence and prevalence of strain KC on the sediment was obtained from plate counts for post-operational cores obtained on days 147−154 (Table 5). Strain KC was detected in sediments from several locations within the grid and was especially prevalent close to the injection well in boring 8A (Figure 1). Strain KC was also detected in boring 7A at intermediate levels, but it was only detected at the lowest depths in boring 10. The strain-specific DNA probe confirmed the presence of strain KC in borings 2A at 19.2 m below ground surface (bgs), 8A at 18.9 m bgs, and 8A at 19.2 m bgs. Pre-operational cores and other post-operational cores tested negative with the probe.

Carbon Tetrachloride Transformation, Chloroform Production, and Denitrification. A final question motivating this study was whether strain KC would remain active for CT transformation under field conditions. Evidence for a direct linkage between CT removal and strain KC addition was the correlation between detection of strain KC in groundwater at different monitoring well depths and the corresponding decrease in CT concentrations without CF production. The question of whether this activity can be sustained long-term in situ cannot be conclusively answered because of the loss of denitrification activity apparently caused by the chase pulse procedure. However, we have observed long-term continuous CT removal without CF production in model aquifer columns containing Schoolcraft sediments and its associated microflora (*8, 10*). We also observed CF production in one of two such columns when acetate, nutrient, and base addition was halted.

In general, three distinct patterns of biodegradation were observed: (i) wells 2, 3, and 7; (ii) wells 5 and 6; and (iii) well 4.The patterns for wells 2, 3, and 7 and for wells 5 and 6 correlated with bromide and base delivery (Figures 2 and 3, Table 2) and with delivery of strain KC (Table 3). Further

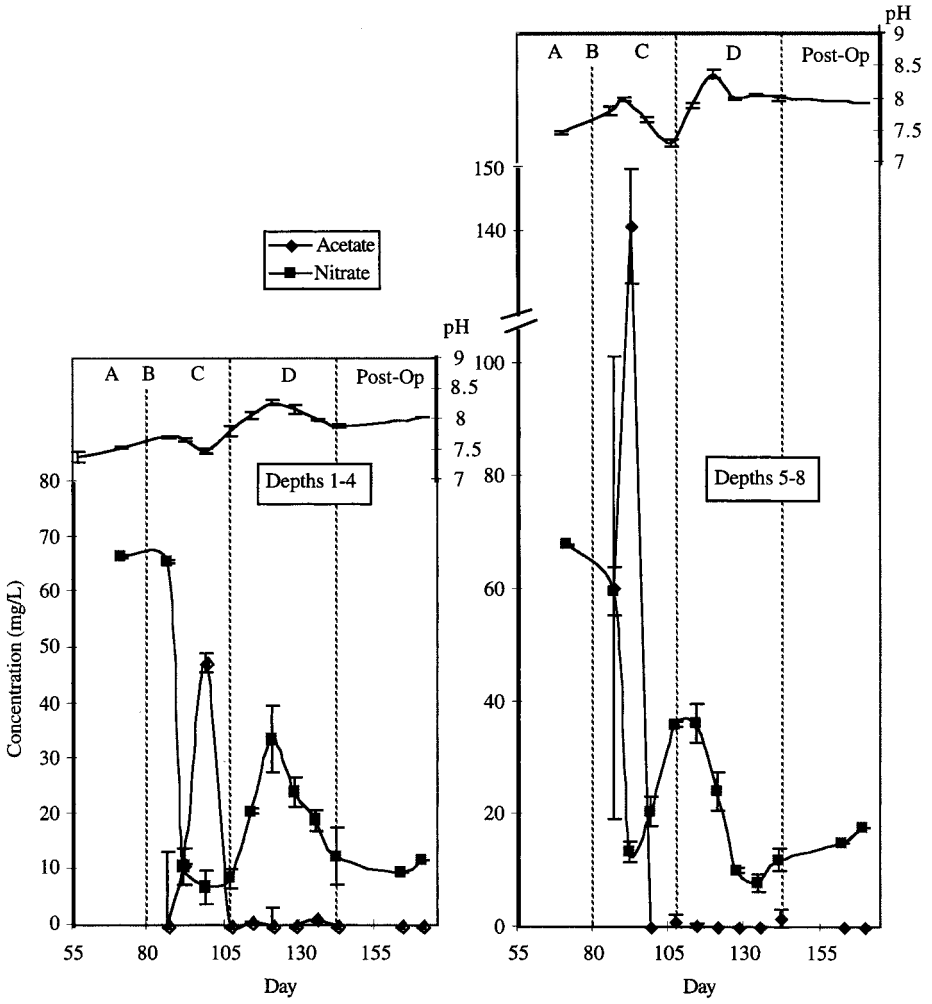

FIGURE 8. Well 7: depth-averaged concentrations of acetate, nitrate, and pH for depths 1−4 and 5−8 during the operational and post-operational phases. Error bars indicate the standard deviation for four samples.

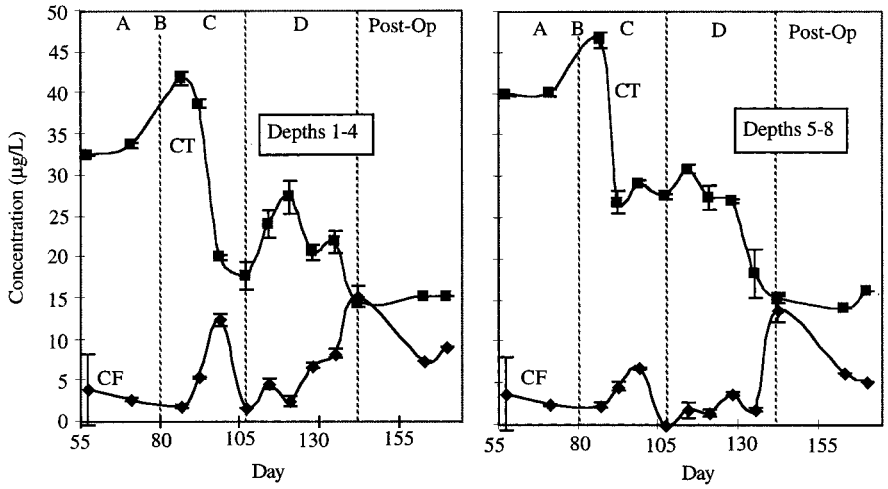

FIGURE 9. Well 7: depth-averaged concentrations of CT and CF for depths 1−4 and 5−8 during the operational and post-operational phases. Error bars indicate the standard deviation for four samples.

discussion of these patterns and the anomalous behavior of well 4 is provided in the following paragraphs.

Figures 6 and 7 illustrate changes in groundwater chemistry for wells 2 and 3 throughout the operational phase. Evidence for active denitrification in the first two weeks following inoculation includes the increase in pH to values in excess of the pH of the injected water (>8.1) together with the simultaneous decrease in nitrate and acetate concentrations (Figure 6). A similar response was observed in well 7, delayed by the 1-week transit time for drift of groundwater between the first and second tier of monitoring wells (Figures 8 and 9). CT degradation without CF production was observed in wells 2 and 3 and in the lower depths of well 7 (Figure 9). CT removal from groundwater during the operational phase may have been somewhat higher than indicated by the CT concentration data at the monitoring

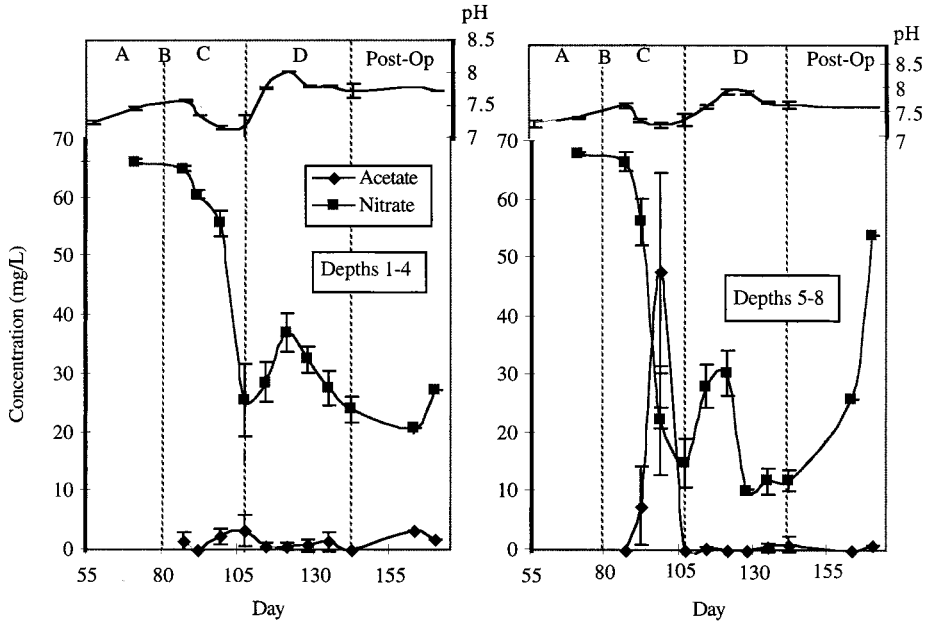

FIGURE 10. Wells 5 and 6: depth-averaged concentrations of acetate and nitrate and pH for depths 1−4 and 5−8 during the operational and post-operational phases. Error bars indicate the standard deviation for four samples.

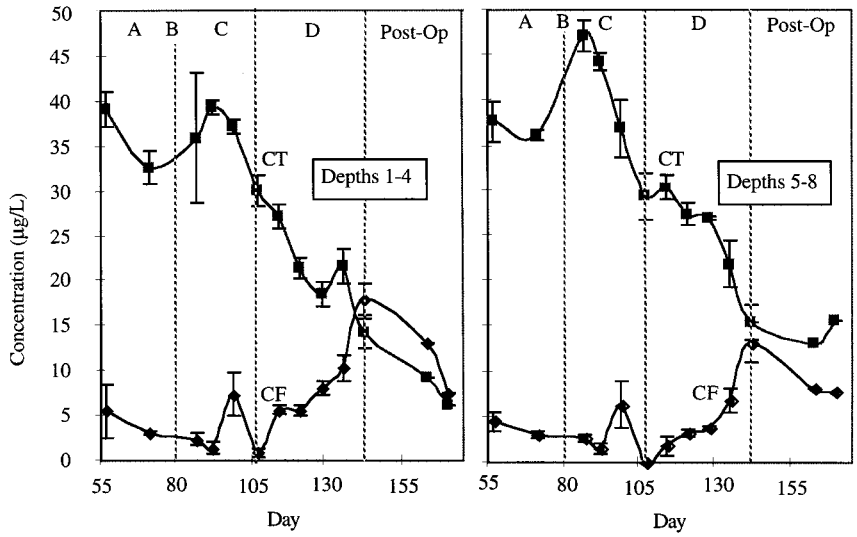

FIGURE 11. Wells 5 and 6: depth-averaged concentrations of CT and CF for depths 1−4 and 5−8 during the operational and post-operational phases. Error bars indicate the standard deviation for four samples.

wells. This is because groundwater introduced into the grid during pumping events was obtained from the upstream deep extraction well (Figure 1, EW-D) where CT levels were ~60% higher than the background groundwater in the grid (57 ± 8 μg/L vs ~35 μg/L for the background groundwater in the test grid). This practice may also account for the increase in CT levels at the end of the pre-operational phase, just prior to inoculation (Figures 7 and 9).

Nitrate removal and delivery of acetate were more extensive at the lower depths of the test grid, and this was especially apparent in wells 2, 3, and 7 (Figures 6 and 8). Acetate delivery patterns matched delivery patterns for bromide and base (Figure 3, Table 2) and for strain KC, as assayed by plate counts (data not shown), by DNA probe (Tables 3 and 4) and by DGGE (Figure 4). Appearance of strain KC corresponded to a significant drop in CT concentration. On day 87, only two of eight upper depth levels in wells 2 and 3 tested positive for strain KC whereas all eight lower depth levels tested positive (Table 3). There was correspondingly little change in CT and nitrate concentrations in the upper depths, but in the lower depths, CT and nitrate concentrations dropped by 65% and 85%, respectively. By day 92, KC cells were detected in the upper depths (Table 3) with a corresponding decline in nitrate (Figure 6) and CT (Figure 7).

At the end of phase C, nitrate and CT concentrations began to increase (Figures 6 and 7), pH declined, and DNA probe results indicated loss of strain KC (Table 3). Some increase in CF was detected. Loss of denitrification activity was presumed to have resulted from the flushing of acetate during the chase pulsing procedure. When the chase pulse was eliminated (phase D), denitrification resumed (pH increased, nitrate levels decreased), and strain KC was again detected (Tables 3 and 4). However, CT degradation was accompanied by CF production (Figure 7), implicating indigenous populations in CT degradation. During the post-operational period, no bromide, acetate, base, or other nutrients were introduced into the grid. As a result, bromide washed out (Table 2), pH levels fell (Figure 6), and nitrate levels increased (Figure 6). Nitrate levels increased quickly in depths 5−8 and gradually

in depths 1−4.

Changes in well 7 were similar to those of wells 2 and 3, except lagging in time as expected from the downgradient location of this well. Acetate was detected at the lower depth levels 7 days after inoculation (day 87) with a peak 12 days after inoculation (day 92). A delayed acetate response was observed in the upper depths, where peaks were not detected until 19 days after inoculation on day 99 (Figure 8). By day 92, strain KC cells were detected in all four lower depths, and three of the four upper depths (Table 3). In the lower depths, arrival of strain KC was accompanied by a 45% decrease in CT but without a corresponding increase in CF (a small lagging CF peak was observed). At the upper depths, the CT drop was delayed by 1 week and accompanied by a transient increase in CF. In operational phase D, CT levels increased, probably because of loss of transformation activity upstream followed by retarded CT transport. CT levels declined at the end of operational phase D with a concomitant increase in CF, likely resulting from biostimulation of indigenous microflora. DGGE profiles indicated a corresponding shift in microbial community structure (Figure 4).

Changes in the water quality of wells 5 and 6 are illustrated in Figures 10 and 11. A decline in CT and nitrate concentrations occurred first in the lower depths with a delayed response in the upper depths. CT concentrations declined througout operational phases C and D with CF production at the end of operational phase D. In the post-operational phase, bromide levels decreased (Table 2). CF levels also decreased (Figure 11), perhaps because of decreased CF production and subsequent washout from the test grid.

Sediments obtained during the post-operational phase were analyzed for CT and CF. A plot of solid-phase CT and CF within the test grid before and after the operational phase is provided in Figure 12. Overall, CT concentrations in the post-operational sediments ranged from the method detection limit (MDL), 0.88 μg/L, to 99 μg/kg. Highest concentrations were obtained for samples collected external to the test grid in SB-EWD-A and SB10.

In general, greater CT removal was observed at the lower depths This was expected given that bromide, acetate, and strain KC were most efficiently delivered to the lower depths (Table 2). Over the entire sampled interval of 16.8−21.3 m (55−70 ft) bgs, the CT concentration changed from a pre-operational level of 23 ± 17 μg/kg (n = 115) to a post-operational level of 10 ± 1.5 μg/kg (n = 81), a 56% decrease.

While the above comparison provides a general description of changes in CT distributions, the region of greatest interest extended over the screened interval from 18.3 to 19.8 m bgs. Over this interval, CT decreased significantly (36−87%) for all downgradient matched borings, with the exception of well 4. Among the post-operational borings, the highest average CT concentration for the 18.3−19.8 m (60−65 ft) bgs interval was 25 ± 5 μg/kg (n = 10) in SB-EWD-A. This is consistent with the upgradient location of this borehole. Sites external to the test grid but lying downgradient of the injection well included wells SB8, SB9, and SB10 with CT concentrations of 11 ± 2 (n = 20), 10 ± 2 (n = 6), and 16 ± 3 μg/kg (n = 5), respectively. These values are comparable to the post-operational concentrations within the test grid, suggesting that the influence of treatment extended to locations outside of the test grid itself.

CF was occasionally detected on post-operational aquifer sediments with a concentration across all borings and depths within the test grid (SB2A through SB7A) of 0.8 ± 0.8 μg/kg (n = 79), which is below the MDL of 2 μg/kg. Overall, post-operational CF concentrations were low with no consistent pattern.

Anamolous Behavior of Well 4. Several unusual phenomena were observed in well 4. Pre-operational borings at SB4 indicated a few relatively high CF levels with an average of 22 ± 13 μg/kg (n = 17). Pre-operational CT concentrations averaged only 10 ± 3 (n = 17), suggesting conversion of CT to CF by indigenous microflora.

Although well 4 was located only 1 m from the injection well, the pattern of bromide delivery was similar to that of wells located 2 m away (Figures 3 and 4). Bromide was delivered at low levels with more effective delivery to lower depths. Acetate (Figure 13) and strain KC (Table 3) were

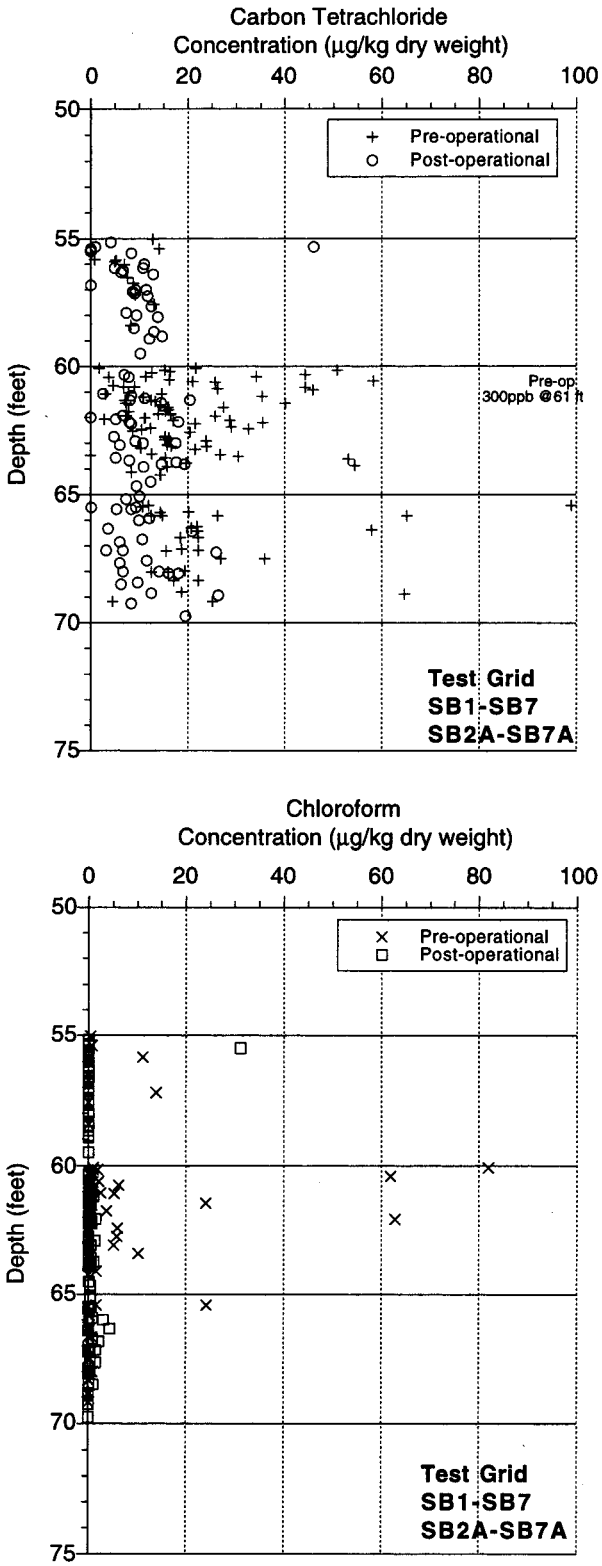

FIGURE 12. Pre- and post-operational concentrations of CT and CF on aquifer sediments from above, below, and within the test grid.

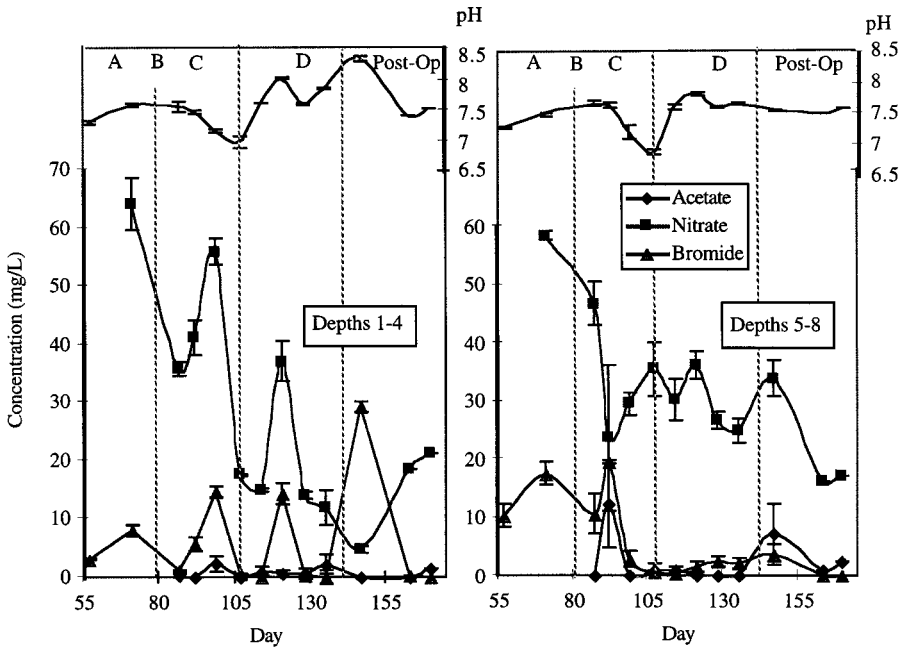

FIGURE 13. Well 4: depth-averaged concentrations of acetate, nitrate, and pH for depths 1−4 and 5−8 during the operational and post-operational phases. Error bars indicate the standard deviation for four samples.

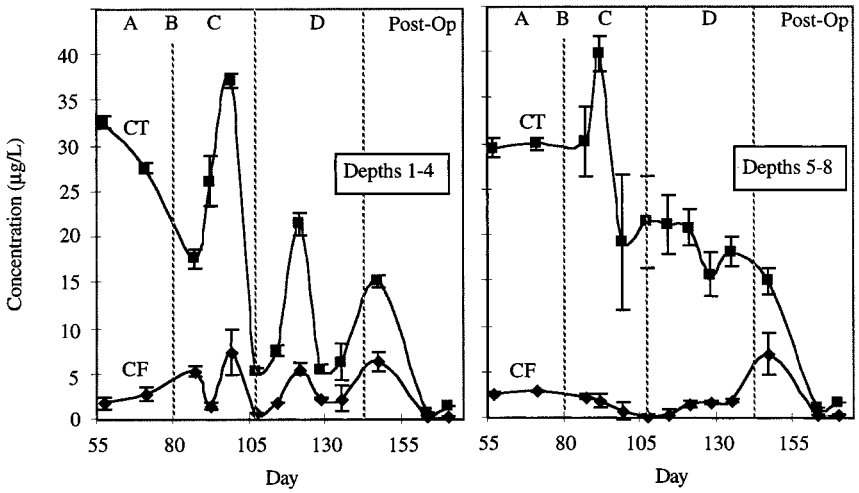

FIGURE 14. Well 4: depth-averaged concentrations of CT and CF for depths 1−4 and 5−8 during the operational and post-operational phases. Error bars indicate the standard deviation for four samples.

detected at these depths 12 days after inoculation but were not detected in the upper depths for an additional week. Following inoculation, bromide concentrations in the lower depths decreased dramatically, suggesting hydraulic alterations that prevented subsequent communication with the injection well (Table 2, Figure 13).

For nitrate and CT removal, the lower depths followed a pattern that was similar to that of wells 5 and 6. At the end of operational phase C, pH decreased and nitrate increased, indicating a loss of denitrification activity. CT transformation activity was subsequently restored, but CF production was observed.

The pattern of the upper depths is more difficult to explain. Throughout operational phases C and D, the concentrations of CT, CF, bromide, and nitrate rose and fell in tandem. The simplest explanation is that CT, CF, bromide, and nitrate introduced by pulsing were flushed out by background groundwater containing no bromide. However, this does not explain the gradual decrease in groundwater CT concentrations throughout the operational and post-operational phase (Figure 14). There is little evidence supporting biological activity as the removal mechanism. For indigenous Schoolcraft microflora, the normal CT degradation product is CF, but CF was not detected in well 4 groundwater or in post-operational sediments from SB4A. Strain KC was occasionally detected at low levels in the upper depths of well 4 (Table 3), but acetate delivery to this region was poor, making removal by strain KC unlikely unless it occurred in an upgradient location. Some of the removal can be attributed to increased sorption. Post-operational CT concentrations on sediments from SB4A ($17 \pm 2.8\ \mu g/kg$, $n = 15$) exceeded average sediment concentrations in the test grid ($10 \pm 1\ \mu g/kg$, $n = 81$, including well 4).

A final peculiarity of well 4 was its consistently low level of sulfate following inoculation (20−25 ppm vs 31−34 ppm for all other wells). Taken together, the results suggest an unusual indigenous microflora, capable of denitrification and possibly sulfate reduction; an unusual hydraulic regime, especially in the upper depths; and the possibility that increased CT sorption contributed to removal of CT from the groundwater.

Comparison with Bench-Scale Results and Scale-Up Considerations. Both laboratory studies and this pilot-scale study indicate that bioaugmentation with strain KC can be effective for CT remediation, but care must be taken to maintain an active population or CF levels can increase. In a bench-scale column study comparing biostimulation with bioaugmentation in Schoolcraft sediments, biostimulation alone did not result in CT removal rates that were significantly different from killed controls, and bioaugmentation with strain KC increased removal rates (*7*). In the present study, competition with indigenous flora became problematic following a period of poor chemical delivery, and CF levels increased. CF production was also observed in a model aquifer column containing Schoolcraft sediments after acetate feeding was discontinued (*10*).

In long-term studies using model aquifer columns packed with Schoolcraft aquifer sediment, CT removal efficiencies exceeded those of the present study (*8*, *10*). This is likely due to the engineering challenges associated with establishing hydraulic control and ensuring effective delivery of substrate, base, and strain KC in the pilot-scale system. One of the six monitoring wells exhibited notably different behavior with regard to delivery of tracer, organisms, nitrate, sulfate, and pH. Heterogeneous delivery of strain KC was also observed with highest concentrations persisting within 1 m of the injection well. Evidently, heterogeneity and the overall lack of hydraulic control within the test grid resulted in suboptimal CT removal as compared with the much higher efficiencies achieved in bench-scale column experiments where hydraulic control was assured. To improve CT removal and prevent CF formation, future work should focus on optimization of chemical and organism delivery.

Acknowledgments

Funding for was provided by the Michigan Department of Environmental Quality under Contract Y40386. Partial support for companion bench-scale studies was provided by the Great Lakes and Mid-Atlantic Hazardous Substance Research Center under Grant R-819605 from the Office of Research and Development, U.S. Environmental Protection Agency, and by the NSF Center for Microbial Ecology under Grant DEB-9120006. We thank Michael Szafranski, Greg Tatara, Randy Brown, Lance Warnick, and Xianda Zhao for laboratory work in support of the field project. Finally, we thank Paul Loconto, Yan Lyang Pan, Stanley Mathuram, Jiasong Fang, Michael Beebee, Keri Garver, Colleen Kelly, and Sinia Surya-Atmadja for their contributions.

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Received for review March 2, 1998. Revised manuscript received June 30, 1998. Accepted August 4, 1998.

ES980200Z