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Field-Scale Evaluation of in Situ Bioaugmentation for Remediation of Chlorinated Solvents in Groundwater

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Groundwater contaminated with 1000−2500 μg/L chlorinated ethenes (trichloroethene [TCE], dichloroethene [DCE], vinyl chloride [VC]) was treated by in situ bioaugmentation with a specialized microorganism, *Burkholderia cepacia* ENV435. The strain was selected for its limited adhesion to aquifer solids and its ability to degrade chlorinated ethenes in the absence of inducing cosubstrates. Approximately 550 L of the toluene *ortho*-monooxygenase-producing ENV435 culture (~100 g/L) was injected along with oxygen (20 mg/L) into a semi-confined silty-sand aquifer (test plot). An equal volume of basal salts medium was simultaneously injected into an adjacent control plot. The plots each measured 4.6 m wide by 12 m long, were spaced 9.2 m apart, and contained 18 multilevel monitoring wells. Groundwater ENV435 concentrations exceeded 1×10^8 cfu/mL 7 days after injection, and the strain traveled the 12 m from injection to recovery well with an average linear velocity of 0.37 to 0.54 m/day. By comparison, a bromide tracer passed through the same formation at a rate of 0.53 to 0.68 m/day. In one trial, the total mass of TCE, DCE, and VC in the treated area was reduced by as much as 78% within 2 days after injecting the organisms.

Introduction

Volatile organochloride compounds (VOCs) such as perchloroethylene (PCE), trichloroethylene (TCE), dichloroethylene (DCE), and vinyl chloride (VC) have been used extensively as industrial solvents, reagents, and cleaning agents, and they have become common groundwater contaminants throughout the United States (*1*). Significant resources have been expended to identify suitable remedial alternatives for these chemicals, and in situ bioremediation has emerged as a potentially important technology for destroying them.

A number of in situ biotreatment approaches have been developed and tested. The first approach, intrinsic bioremediation or natural attenuation, involves allowing indigenous microflora to destroy the contaminant of concern before it creates a significant risk to downgradient receptors (*2*, *3*). This has become a natural first choice of remediation alternatives where applicable because it requires no intervention, just monitoring of contaminant concentrations and modeling of the groundwater flow and natural degradation rates. Another approach is biostimulation whereby indigenous microbial populations are stimulated to degrade VOCs by adding specific nutrients, growth substrates, inducers, and/or oxygen to support their growth and degradative activity (*4*, *5*, *6*).

When intrinsic bioremediation or biostimulation are not appropriate for a given site, bioaugmentation might be utilized. Bioaugmentation involves injecting selected exogenous organisms with the desired catalytic capabilities directly into the contaminated zones along with any required nutrients to affect the biodegradation of target chemicals. Two distinct bioaugmentation approaches have been developed for remediating VOCs. In the first approach, degradative organisms are added to complement or replace the native microbial population (*7*, *8*, *9*). The added microorganisms can be selected for their ability to survive for extended periods or to occupy a specific niche within the contaminated environment. If needed, stimulants or selective cosubstrates can be added to improve survival or enhance activity of the added organism. Thus, the goal of this approach is to achieve prolonged survival and growth of the added organisms and degradation of the target pollutants.

In the second bioaugmentation approach, large numbers of degradative bacteria are added to a contaminated environment as biocatalysts that will degrade a significant amount of the target contaminant before becoming inactive or perishing (*10*). Additional microbes can be added as needed to complete the remediation process. Attempts can be made to increase the production of the degradative enzymes or to maximize catalytic efficiency or stability, but long-term survival, growth, and establishment of an active microbial population are not the primary goals of the treatment approach.

In this study, we evaluated the use of the latter bioaugmentation approach for remediating a chlorinated solvent-contaminated aquifer. A specialized bacterium, *Burkholderia cepacia* ENV435, was selected for the study because of several important characteristics including: (1) its limited adhesion to aquifer solids (adhesion deficiency), (2) its ability to degrade chlorinated ethenes in the absence of inducing cosubstrates (constitutive activity), (3) its ability to grow to high cell density (~100 g/L) in a fermentor, and (4) its accumulation of large amounts of internal energy reserves which can potentially be used to prolong VOC degradation and survival.

Materials and Methods

Strain Development. *B. cepacia* ENV435 (*11*) was developed by selecting adhesion deficient variants of the constitutive toluene *ortho*-monooxygenase-producing strain *B. cepacia* $PR1_{301}$ (*8*). Selection of the naturally occurring adhesion-deficient variants of the strain was essentially as previously described (*12*), and some of the adhesion characteristics of ENV435 have been reported (*11*). Strain ENV435 is resistant to naladixic acid (20 μg/mL), streptomycin (30 μg/mL), chloramphenicol (20 μg/mL), and cycloheximide (100 μg/mL). For routine monitoring of ENV435 in groundwater, samples were diluted in 0.1 M phosphate buffer (pH 7.0), spread plated (*13*) onto R2A agar plates containing 15 μg/mL naladixic acid, 30 μg/mL streptomycin, 20 μg/mL chloramphenicol, and 100 μg/mL cycloheximide, and incubated for 48 h at 30 °C. Background bacterial counts in test plot groundwater were $<1 \times 10^2$ cfu/ml on this media.

To produce large amounts of ENV435 for the field demonstration project, the cells were grown in a 750-L fermentor (ABEC, Inc. Allentown, PA) filled with 550 L of

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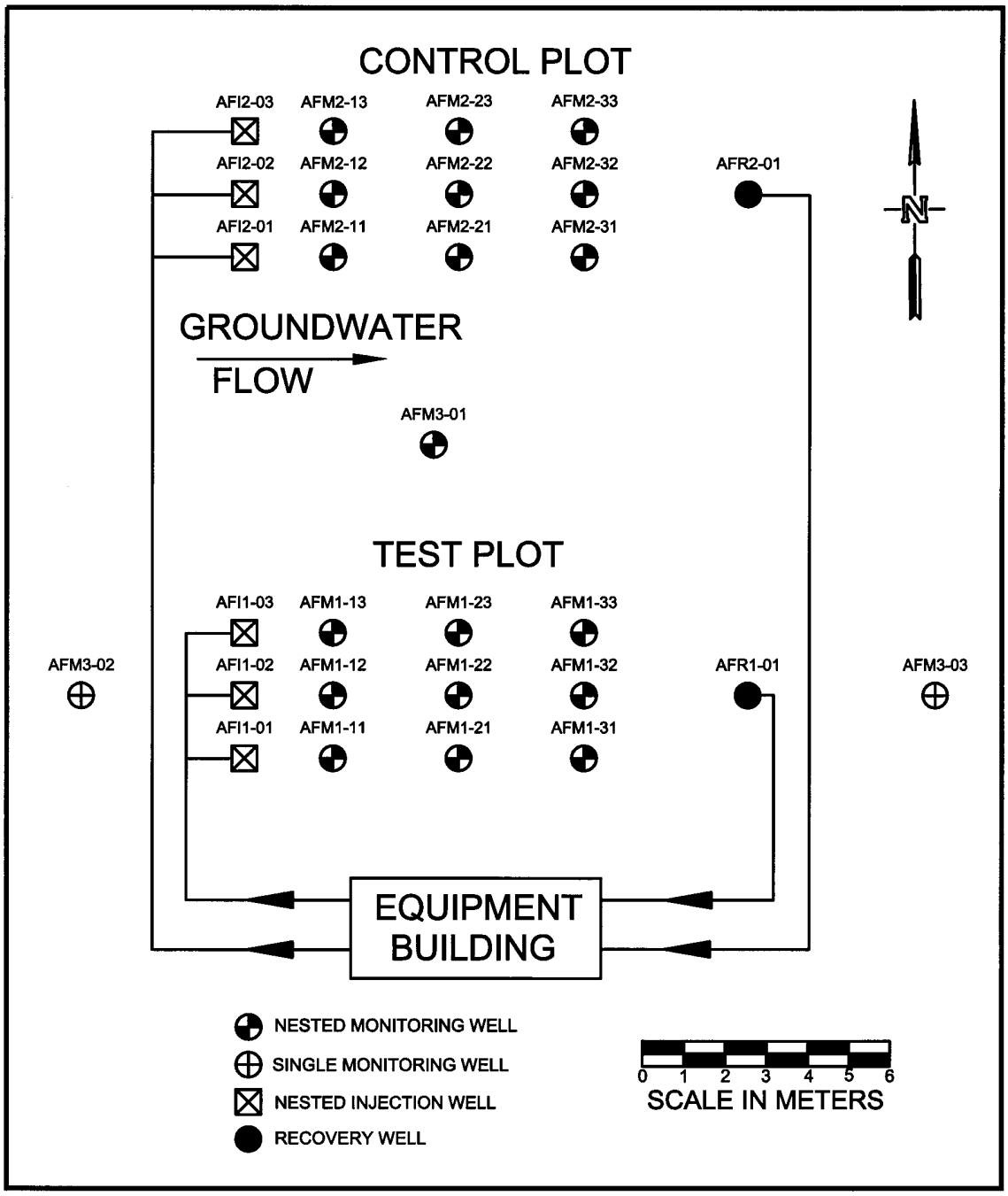

FIGURE 1. Plan view of the bioaugmentation demonstration site showing the location of injection, recovery, and monitoring wells in the control and test plot. Injection and monitoring wells were constructed as nested wells with one well screened in the shallow (upper one-third) of the formation and one well screened in the deep (lower one-third) region of the formation.

basal salts medium (BSM;*14*) containing 1.6% (w/w) sucrose. The pH of the culture was maintained at 7.0 by adding NaOH or H_2SO_4 as needed. As the sucrose within the reactor became depleted, as measured by an increase in dissolved oxygen (DO), the culture was fed alternating batches of sucrose and phenol as carbon sources. Bacterial storage polymers were produced naturally by the strain as the ammonium in the reactor became depleted. Cell density was determined by measuring the optical density of diluted samples at 550 nm (OD_{550}). The VOC-degrading activity of the culture was monitored by performing TCE bottle assays (*15*), and bacterial storage polymer concentrations were measured as previously described (*16*). The high cell density cultures of ENV435 were transferred to the field demonstration site as described below.

Site Characterization and Experimental Plots. The test system used in this study was located at an industrial facility in Pennsauken, New Jersey. The formation is heterogeneous and comprised of approximately 2.4 to 3 m of silty, fine to medium sands interspersed with thin (2 to 5 cm thick) lenses of moderately plastic gray clay. The test plots were installed in a portion of the formation that was confined above and below by a moderately plastic gray clay.

The field demonstration system consisted of a test plot and a control plot (Figure 1). The test plot served as a treatment cell and received ENV435, whereas the control plot did not receive ENV435. The plots measured 4.6 m wide by 12 m long, and were spaced 9.2 m apart as shown in Figure 1. The test and control plots each contained three sets

of dual nested injection wells (a total of six injection wells in each plot), and one recovery well installed approximately 12 m hydraulically downgradient of the injection wells (Figure 1). Nine sets of dual nested monitoring wells (one well in the lower third of the formation and one in the upper third) were installed between the injection wells and recovery well (3 each at approximately 2, 5, and 8 m downgradient of the injection wells) in each plot. One dual-nested well set and two individual monitoring wells were installed outside of the dimensions of the test plot to monitor for cross-contamination between the plots or migration of injected bacteria upgradient or downgradient of the test plot (Figure 1).

Injection and monitoring wells were constructed from 5.1 cm diameter Schedule 40 poly(vinyl chloride) (PVC) riser pipe. The screens on the injection and monitoring wells were 0.6 m long with 0.05 and 0.03 cm standard slot Schedule 40 PVC screens wells, respectively. Morie sand, grain sizes number 0 and number 00, was used to construct a sand pack around injection and monitoring wells, respectively. The sand pack typically extended 0.3 m above and below the top and bottom of the well screen. A minimum of 1.4 m of bentonite seal was installed above the well screen to hydraulically isolate the injection points. Recovery wells were constructed from 15-cm Schedule 40 PVC riser pipe and a 3-m stainless steel wire wrap, 0.03-cm slot, screen. Each recovery well was equipped with a 10-cm diameter Grundfos Redi-Flo4 5E12 submersible pump (Pumps of Oklahoma, Oklahoma City, OK).

Recovered groundwater was piped underground to a treatment building which housed a separate groundwater amendment and reinjection system for each plot. The recovered groundwater was passed through an oxygenation vessel (described below), a cartridge filtration system (three pleated cellulose 30-μm pore size filters piped in parallel) to remove sediment and precipitated metals (e.g., iron), and then into an injection manifold constructed with 12 Rate-Master flow meters (Dwyer, Inc., Michigan City, IN). The manifold distributed the oxygenated groundwater to the test and control plot injection wells via underground piping. Groundwater VOC concentrations were measured in both the recirculation system and monitoring wells by using EPA method 8260 (*17*).

Characterization of Site Hydraulic Parameters. Prior to injecting bacteria, the hydraulics of the test and control plots were evaluated by performing slug tests, pumping tests, and conservative tracer studies. Falling head and rising head slug tests were performed at each injection and monitoring well in accordance with ASTM standard test method D 4044-91 (*18*) to determine the variability in hydraulic conductivity throughout the pilot-test area. A series of five pumping tests were performed at recovery wells in the pilot-test area in accordance with ASTM standard test method D 4050-91 (*18*). Each pumping test was performed for approximately 24 h at a pumping rate ranging from 2.3 to 3.8 L/min. while water level elevations were recorded by using submersible pressure transducers linked to a data logger (In Situ, Inc., Laramie, WY). Pumping test data were analyzed by the Thiem method (*19*) for steady-state conditions, and by the Cooper-Jacob method (*20*) for transient conditions to calculate the transmissivity, hydraulic conductivity, and specific yield of the aquifer in the pilot-test area.

A convergent sodium bromide tracer test was performed to determine the predominant flow pathways and transport time of groundwater between the Test plot injection and recovery wells. A total of 57 L of NaBr solution (137 g/L) was injected into the six injection wells over a period of 20 min. To verify the lack of cross-contamination between the plots, the tracer test was performed while the recovery and injection wells in both the test and control plots were operating. The groundwater recovery and injection system operated at 3.8 L/min for 30 consecutive days after the tracer injection, with periodic sampling and bromide analysis. The same pumping conditions were maintained throughout the demonstration. Data from a similar, but earlier, bromide tracer test at the site was used to estimate effective porosity using a previously described method (*21*).

Oxygen Delivery Procedures. Two methods were employed for supplying oxygen to the subsurface during the pilot test. The first method, which was used throughout the demonstration, consisted of oxygenating recirculating groundwater prior to injection into the test and control plots. The recovered groundwater was passed through an oxygenating vessel that was constructed of Schedule 80 PVC equipped with aerosol spray nozzles and supplied with a constant flow of oxygen gas (99.5%). The oxygenation vessel was operated as a closed system to prevent stripping of VOCs, and VOC and DO concentrations were measured immediately prior to injecting the water into the formation.

The second method of supplying oxygen was by direct injection of oxygen (99.5%) into the subsurface through the existing monitoring wells. Monitoring wells screened in the lower formation of the test plot were fitted with locking airtight caps equipped with pneumatic quick connect fittings. A flexible oxygen supply line was used to periodically deliver oxygen to these monitoring wells. To prevent stripping of VOCs, only enough oxygen was added to each well to displace the water in the well, and thereby minimize sparging in the formation. Dissolved oxygen concentrations were monitored in the upper portions of the formation to detect the upward movement of oxygen (i.e., sparging) from the lower oxygen injection points.

Bacteria and Nutrient Injection Procedures. The bacteria and nutrient injection system consisted of two 750-L high-density polyethylene holding/mixing tanks (one for each plot), a microbe and nutrient feed pump, and a compressed oxygen supply system. The test plot holding/mixing tank was equipped with a compressor and an air diffuser to supply oxygen to microorganisms in the tank during injection. The feed pump was a skid-mounted, nonshearing positive displacement diaphragm pump (Neptune, Lansdale, PA), and was used to inject bacteria, nutrients, or inert tracer at flow rates of up to 3.8 L/min. Bacterial culture (550 L) was transferred to the field site in a 1100-L plastic tank equipped with an air stone and compressor and then pumped into the on-site holding tanks of the injection system.

Two bacteria injection trials were performed to evaluate the effect of different methods of bacteria deployment and oxygen delivery on the effectiveness of bioaugmentation for VOC remediation. In the first trial (trial number 1), bacteria were injected into the test plot through the upgradient injection wells. The culture was added to the recirculating groundwater to achieve an injection concentration of approximately 1×10^{11} cfu/ml. A total of 550 L of bacteria (1.2×10^{11} cfu/ml) were injected at a rate of 2.3 to 3.0 L/min. During the injection period, the groundwater pumping rate was maintained at 3.0 L/min. An equal volume (550 L) of $1 \times$ BSM solution was simultaneously injected into the control plot at the same rate. Oxygen was supplied to the test and control plots by oxygenating the recirculating groundwater.

The second trial was designed to rapidly distribute ENV435 throughout the test plot and to supply oxygen across the entire plot. An equal volume of ENV435 culture ($\sim 1 \times 10^{11}$ cfu/ml) was injected under pressures of 10 to 30 psi and a flow rate of 2.3 to 3.0 L/min into the monitoring wells screened in the lower formation of the test plot. Prior to and after inoculum injection, the monitoring wells were evacuated with compressed oxygen to force the culture into the formation. Oxygen was injected directly into the monitoring wells periodically after the bacteria injection to maintain a

TABLE 1. Results of Large-Scale Fermentation (550 L) of Strain ENV435

parameter	value
initial media	1 × BSM + 3 g/L NH_4Cl + 1.6% w/w sucrose
media volume	550 L
inoculum volume	5.5 L
initial optical density at 550 nm	0.06−0.08
initial cell counts	1.7−2.3 × 10^8 cfu/ml
growth rate	0.28−0.35 hr^{-1}
exponential growth rate	0.19−0.24 hr^{-1}
final cell counts	1.0−1.7 × 10^{11} cfu/ml
final optical density at 550 nm	55−61
cell yield	16−18 g/L (dwt)
storage polymer yield	50−64% (dwt)
specific activity of cells	
30 °C	0.074 μg TCE/mg cell protein/min
13 °C	0.027 μg TCE/mg cell protein/min
fermentation time	108−110 hr
growth yield (total)	0.25−0.28 g dwt/g sucrose
growth yield (during log phase)	0.51−0.53 g dwt/g sucrose

DO concentration above 8 mg/L. The recirculation system was operated during the first 14 days after bacteria injection, and the Control plot was not operated during the second trial.

Results

Production and Injection of ENV435. The results of large-scale fermentation of strain ENV435 are provided in Table 1. Approximately 2.5 h were required to transfer the bacteria from the fermentor to the transport tank and ultimately to the bacteria injection system at the test site. TCE degradation assays performed immediately after fermentation and periodically during the injection period showed no measurable decrease in specific activity of the culture during the approximately 3.7 h of injection. During trial number 1, a total of 394 L was delivered into the deep injection wells, and 157 L was injected into the shallow injection wells of the test plot. A total of 305 and 243 L of BSM were injected into the control plot deep and shallow injection wells, respectively, during the same time. The difference in the amount of culture injected into the two formations was due to the difference in hydraulic conductivity of the formations. During trial number 2, 61 L of ENV435 culture was injected into each of the nine deep monitoring wells.

Bacterial Transport. The hydrogeologic characteristics of the test aquifer are presented in Table 2. Preliminary groundwater flow modeling indicated that approximately 50% of the total flow of circulated groundwater would pass through the test plot. Bromide tracer in the test plot was transported fastest through the lower zone of the aquifer. The tracer initially traveled along the south side of the test plot and then migrated toward the north side of the plot. The bromide front traveled the length of the test plot (12 m) in approximately 13 days. The measured average linear velocity of the bromide tracer was approximately 0.53 and 0.68 m/day in the upper and lower regions of the aquifer, respectively (Figure 2).

ENV435, injected at an initial concentration of approximately 1 × 10^{11} cfu/ml, appeared to follow the same groundwater flow paths through the test plot as the bromide tracer (Figure 3). The strain was initially transported along the southern boundary of the test plot, with lesser concentrations of bacteria traveling through the north side and center of the plot. The highest measured groundwater concentration of ENV435 was 1.9 × 10^8 cfu/ml at monitoring well AFM1-11B located approximately 2 m hydraulically downgradient of the injection wells. A similar distribution, but lower concentration, of ENV435 was observed in the upper zone of the test plot, with the highest concentration of ENV435 (2.2 × 10^7 cfu/ml) at monitoring well AFM1-12A located 2 m downgradient of the injection wells. ENV435 were detected in the recovery well of the test plot 20 days after injection. No ENV435 were observed up- or downgradient of the test plot, nor in the control plot.

The ENV435 linear velocity was calculated on the basis of the movement of the ENV435 concentration peak through the test plot. The measured linear velocities of ENV435 ranged from approximately 0.23 to 0.80 m/day during trial number 1 and averaged 0.54 m/day and 0.37 m/day in the lower and upper regions of the test plot, respectively. The ratio of the tracer velocity (v_{Br}) to ENV435 velocity (v_{ENV}) in the upper and lower zone of the test plot was approximately 1.43 and 1.26, respectively. However, the several log decrease in ENV435 concentrations between the injection wells and the downgradient monitoring wells indicate that significant amounts of the strain were being filtered by the aquifer sediment or were perishing. A simple first- order decay analysis of the transport data suggested an ENV435 half-life of only 1 and 2 days in the shallow and deep zones of the aquifer, respectively (Table 3). By comparison, when ENV435 (1 × 10^9 cfu/ml) was added to aquifer microcosms constructed with site sediment and water samples, viable cell numbers decreased by about 2 orders of magnitude over a 35-day incubation (data not shown).

The trial number 2 direct injection of ENV435 (initially 1.1 × 10^{11} cfu/ml) into deep monitoring wells throughout

TABLE 2. Test Plot Hydrogeologic and ENV435 Transport Properties

parameter	notes	units	shallow zone	deep zone	test plot average
hydraulic conductivity, K_h	*a*	m/day	1.13	2.70	
hydraulic gradient, *i*	*b*	m/m			0.075
hydraulic gradient, *i*	*c*	m/m	0.002	0.0077	
effective porosity, η_e	*d*				0.16
total porosity, η	*e*				0.50
dispersivity, α	*d*	m			0.056
soil bulk density, ρ_b	*f*	kg/L			1.3
ENV435 partition coefficient, K_d	*g*	L/kg			0.051
groundwater linear velocity, v_{gw}	*b*	m/day			0.89
bromide linear velocity, v_{Br}	*d*	m/day	0.53	0.68	
ENV435 velocity v_{env}	*g*	m/day	0.37	0.54	
v_{Br}:v_{env}			1.43	1.26	

[a] Values calculated from pumping test results. [b] Values measured or calculated while pumping both the control and test plots at 3 L/min. [c] Values measured under static (nonpumping) conditions. [d] Value calculated from bromide tracer testing. [e] Value calculated from soil bulk density. [f] Value determined from laboratory analysis. [g] Values calculated from trial number 1 test results.

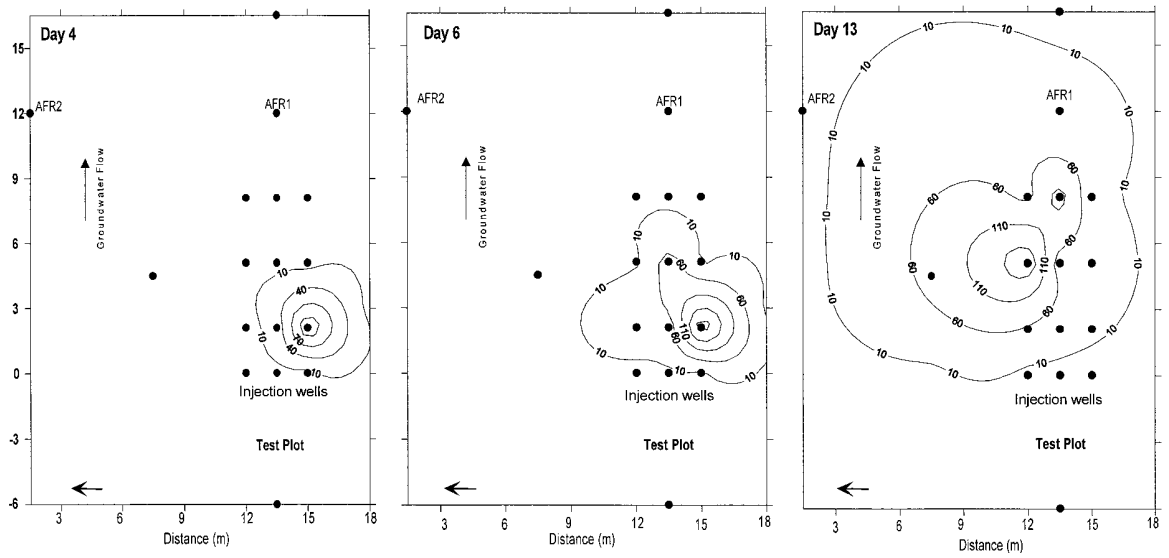

FIGURE 2. Isoconcentration contours of Br in mg/L in the test plot during trial number 1. The tracer test was performed with recovery wells in both the control and test plot pumping at 3.8 L/min.

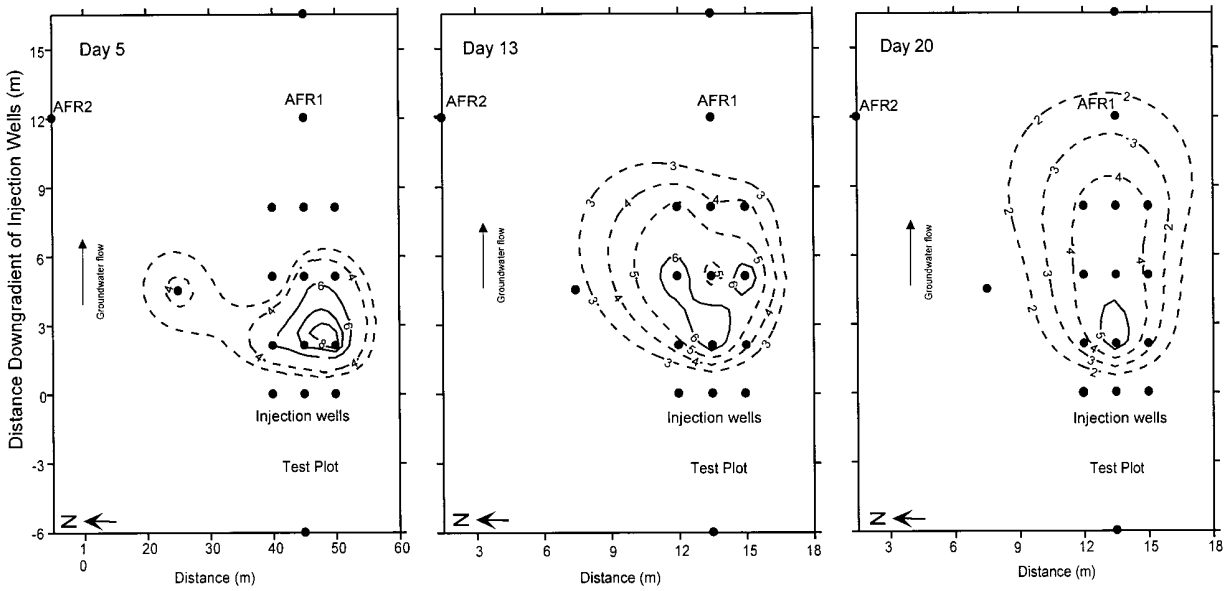

FIGURE 3. Isoconcentration contours of strain ENV435 in log cfu/ml in the test plot during trial number 1.

TABLE 3. Regression Analysis of Trial Number 1 Groundwater ENV435 Concentration Data and Estimates of ENV435 Half-Life

	shallow wells[a]			deep wells[b]		
parameter	**day 10**	**day 15**	**day 18**	**day 10**	**day 15**	**day 18**
slope, λ/v_{gw}	0.8227	0.4237	0.4215	0.4708	0.1237	0.1956
goodness of fit, R^2	0.99	0.93	0.88	0.93	0.93	0.90
linear GW velocity, v_{gw} (m/day)	0.53	0.53	0.53	0.68	0.68	0.68
first-order decay rate, λ (day^{-1})	1.440	0.741	0.738	1.045	0.275	0.434
first-order half-life, $t_{1/2}$ (day)	0.481	0.93	0.94	0.66	2.52	1.60

[a] AFM1-12A, AFM1-22A, AFM1-32A. [b] AFM1-12B, AFM1-22B, AFM1-32B.

the test plot (trial number 2) resulted in the highest observed groundwater ENV435 concentrations. ENV435 concentrations exceeded 1×10^9 cfu/ml in most test plot deep wells 2 days after injection, and were as high as 1.6×10^{10} cfu/mL in monitoring well AFM1-21B 5 days after injection. The exact distance traveled by the injected bacteria is not known because of the difficulty of collecting aquifer samples away from the monitoring wells, but trial number 1 transport data indicated that they should have moved approximately 0.8 m/day away from the injection points.

Oxygen Use and Distribution. Two methods were used for oxygenating groundwater during the bioaugmentation trials: (1) amending re-circulating groundwater with oxygen, and (2) directly injecting oxygen into the test area formation. In general, re-circulating groundwater was amended with oxygen to 20 mg/L, and direct injection of oxygen (trial

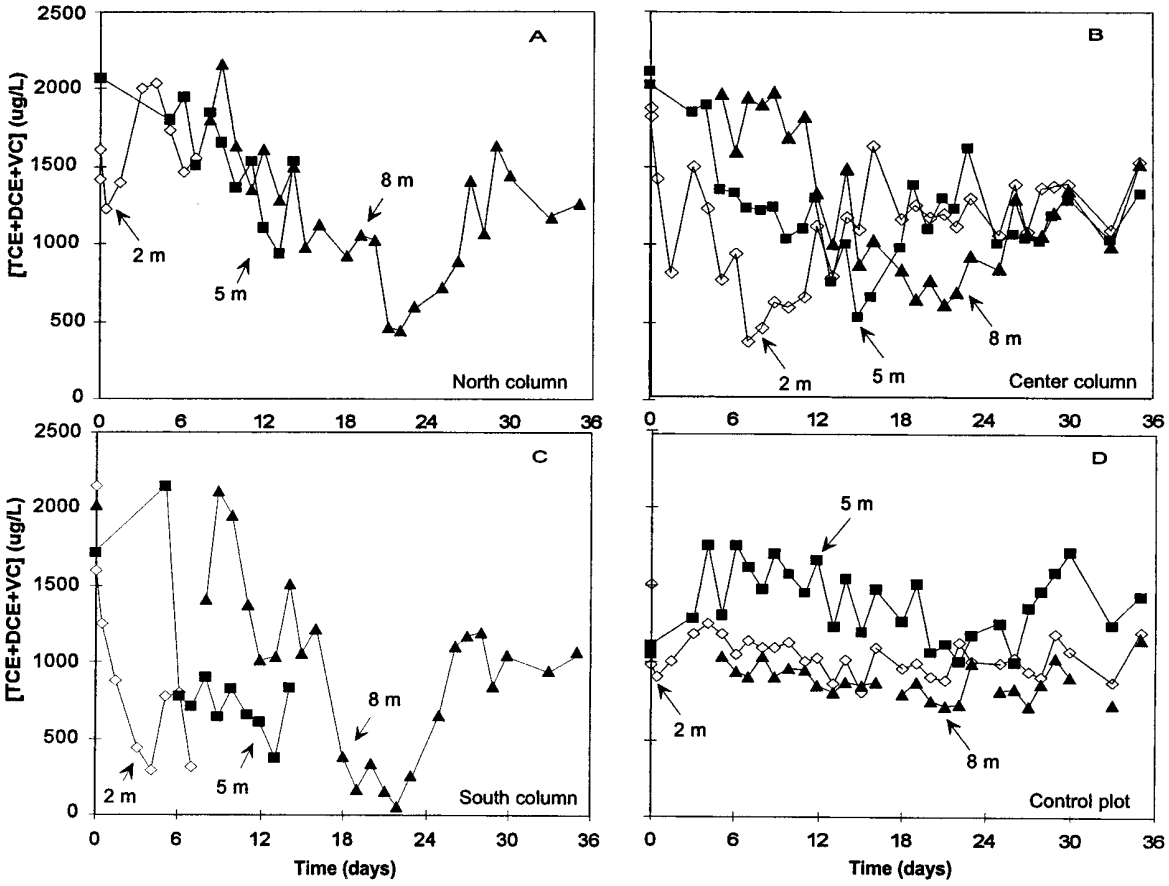

FIGURE 4. Groundwater VOC concentration in the deep zone of the test and control plots after injection of ENV435 during trial number 1. Panels A, B, and C represent the north, center, and south columns of monitoring wells in the test plot, respectively, and panel D represents the center column of the control plot. Diamonds, squares, and triangles represent the VOC concentration (μg/L) in monitoring wells located 2, 5, and 8 m downgradient of the injection wells, respectively. Groundwater pumped from the recovery wells was reinjected into the injection wells for the duration of the test.

number 2) was performed to maintain in situ groundwater DO concentrations above 8 mg/L. A total of 6.2 kg of oxygen was added during trial number 1, all via amendment of recirculating groundwater. During trial number 2, one kg of oxygen was added to the recirculating groundwater, and 11.4 kg of oxygen were directly injected into the formation. Because of the confined nature of the test formation, it was assumed that all of the added oxygen remained trapped within the formation below the overlying clay until dissolved.

During trial number 1, measurable levels of DO typically occurred only within about 2 m from the injection wells in both the upper and lower zones of the test plot, with concentrations >2 mg/L rarely observed in monitoring wells located 5 or 8 m downgradient. In the control plot, groundwater DO concentrations typically exceeded 20 mg/L in the first row of monitoring wells (2 m) and were consistently >2 mg/L at monitoring wells located 5 and 8 m downgradient of the injection wells.

Direct injection of oxygen into the deep monitoring wells of the test plot during trial number 2 resulted in significantly higher groundwater DO concentrations than observed during trial number 1. DO concentrations were consistently greater than 20 mg/L in all of the test plot deep monitoring wells immediately following oxygen injections, and they generally decreased to approximately 2 mg/L 3 to 5 days after injecting the bacteria.

VOC Degradation. To evaluate degradation of VOCs in the test aquifer, the total VOCs were separated into those that can be biodegraded by strain ENV435 ("T1": TCE, DCE, VC), and those that are not degraded ("T2": PCE, dichloroethane [DCA] and trichloroethane [TCA]). Thus, the nondegradable VOCs served as internal standards for evaluating biodegradation.

Groundwater concentrations of the T1 VOCs in deep monitoring wells of the test plot and the control plot during trial number 1 are presented in Figure 4. The T1 VOCs in the deep zone of the test plot decreased from approximately 2200 μg/L to less than 500 μg/L at most locations in the plot. The greatest degradation occurred along the southern edge of the test plot (Figure 4C). There, T1 VOCs decreased from approximately 2200 μg/L to 250 μg/L in the first 2 m, remained stable over the next 3 m, and then decreased to less than 50 ppb over the following 3 m (measured at AFM1-31). The concentrations of the individual T1 compounds at AFM1-11 are shown in Figure 5. The progressive decrease in T1 VOC concentrations with distance suggested that the organisms continued to degrade VOCs as the organisms and treated water moved downgradient.

In the center row of the test plot (Figure 4B) degradation was greatest in the first 2 m, but degradative activity appeared to decrease or cease as the organisms moved downgradient. Degradation appeared minimal in the first 2 m of the north edge of the test plot (Figure 4A) which received lower numbers of ENV435, but it increased in the downgradient areas of the north edge of the plot. A similar trend in total degradable VOC concentrations was observed in test plot shallow monitoring wells (data not shown), but overall the magnitude of VOC degradation was less, presumably because of the lower volume of inoculum and oxygenated water injected into the shallow formation.

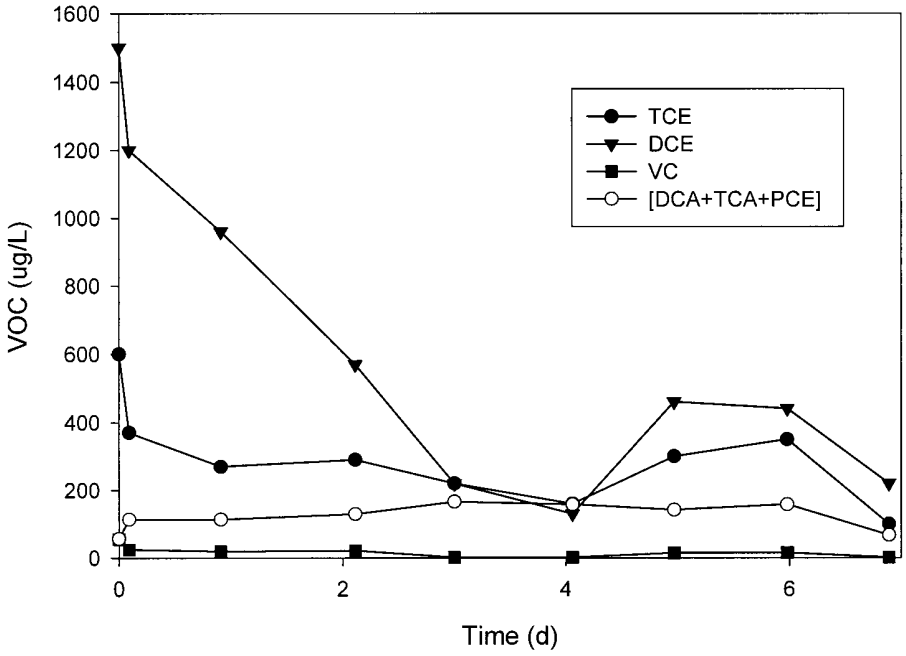

FIGURE 5. Concentration of individual degradable VOCs and total nondegradable VOCs (PCE, DCA, TCA) at deep monitoring well AFM1-11B (south column, 2 m from injection well). Symbols: TCE, ●; *c*DCE, ▼; VC, ■; total nondegradable VOCs, ○.

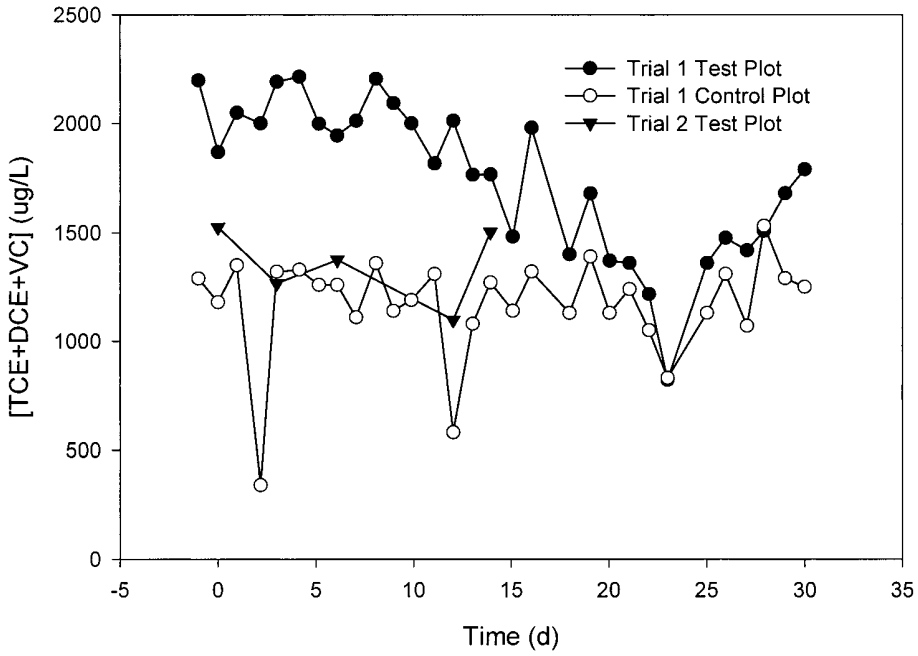

FIGURE 6. Concentration of degradable VOCs (μg/L) in the reinjected groundwater entering the test plot during trial number 1 (●) and trial number 2 (▼), and the control plot during trial number 1 (○).

In each case the total VOC concentration in the test plot rebounded several days after injecting the organisms. This rebound was likely due to the continuous reinjection of contaminated groundwater from the recovery well which collected water both from the test plot and the more heavily contaminated plume (up to 4000 μg/L VOCs) immediately downgradient of the test plot (Figure 6). Some amount of rebound was also possible as a result of desorption of VOCs from the aquifer sediments. The total concentration of T1 VOCs measured in the control plot remained relatively stable throughout the 60-day monitoring period of trial number 1 (Figure 4D), and they were not measurably affected by the injection of BSM.

A comparison of the ratio of degradable to nondegradable VOCs (T1:T2) and the concentration of total T2 compounds in the test plot monitoring wells is provided in Figure 7. The T1:T2 ratio decreased from approximately 14 to less than 2 in most locations of the test plot that received inoculum, and did not change in the control plot. The observed decrease in T1:T2 ratios indicated that the measured decrease in groundwater total VOC concentration is attributable to biologically mediated degradation and not physical processes such as dilution or displacement, even though some dilution of the T2 compounds was apparent.

The greatest decrease in groundwater VOC concentrations during the entire study was observed in trial number 2 when

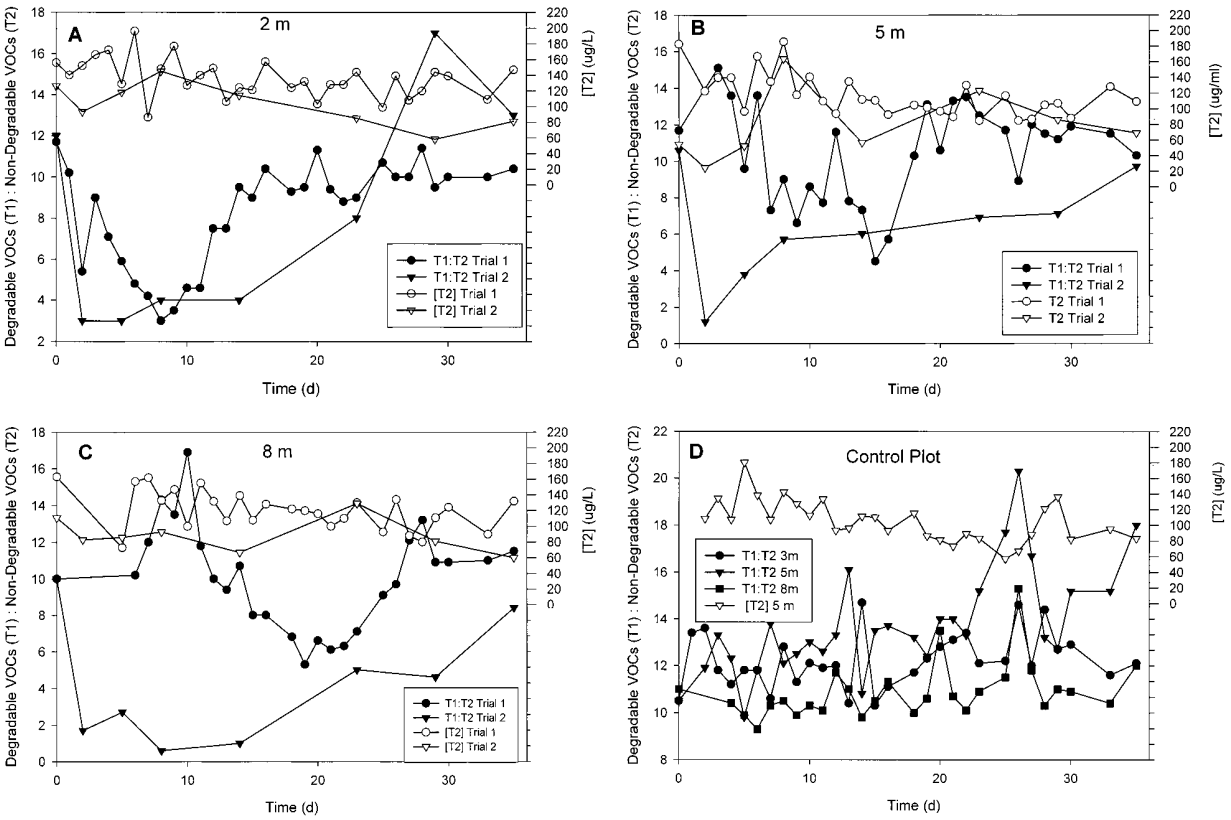

FIGURE 7. Ratio of degradable VOCs (T1: TCE, DCE, VC) to nondegradable VOCs (T2: PCE, DCA, TCA) in the test and control plots. Panels A, B, and C represent deep center column monitoring wells in the test plot located 2, 5, and 8 m downgradient of the injection wells, respectively. Symbols are: trial number 1 T1:T2, (●); trial number 2 T1:T2, (▼); trial number 1 total T2 compounds (μg/L), (○); trial number 2 concentration of total T2 compounds (μg/L), (▽). Panel D represents the control plot trial number 1 T1:T2 in the deep center column monitoring wells located 2 m (●), 5 m (▼), and 8 m (■) downgradient of the injection wells, and total T2 compounds (μg/L), (▽).

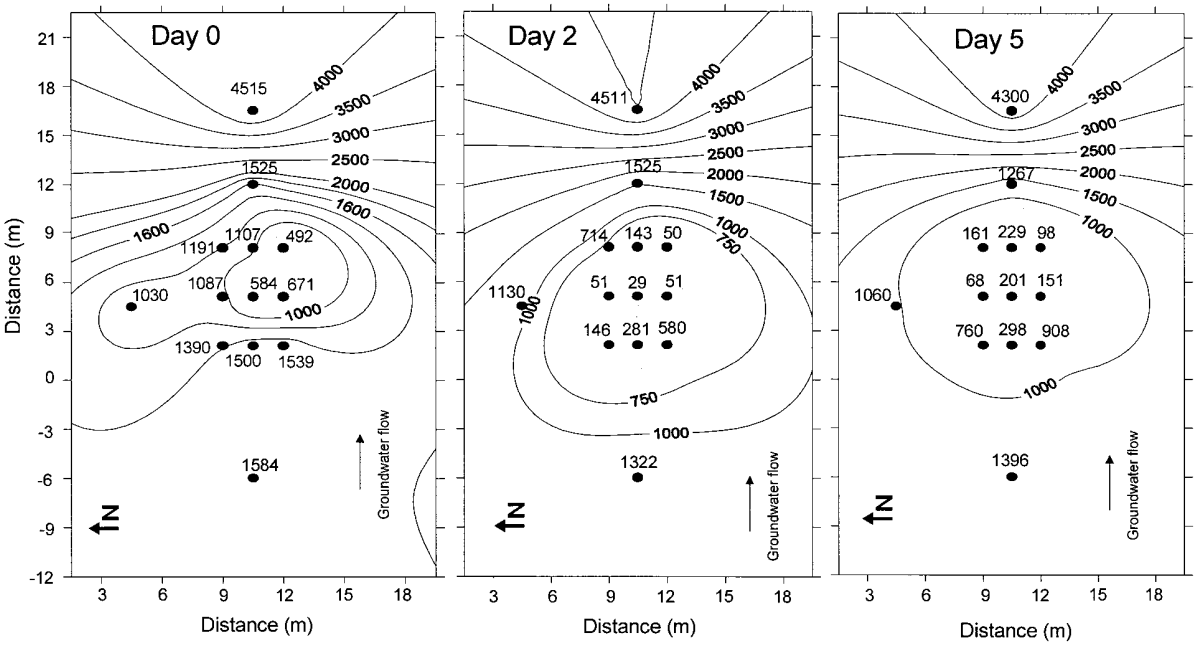

FIGURE 8. Isoconcentration contours of degradable VOCs (TCE, DCE, VC) in the test plot during trial number 2. Values are in μg/L. Groundwater pumped from the recovery well was reinjected into the injection wells during the first 14 days after ENV435 injection.

bacteria and oxygen were directly injected through deep monitoring wells located throughout the test plot (Figure 8). The most dramatic decrease occurred near the center of the plot where the concentration of T1 VOCs was reduced to approximately 50 μg/L. The concentration of T1 VOCs rebounded slightly by day 5, presumably due to recirculating, contaminated groundwater into the test plot (Figure 6).

As observed in trial number 1, the ratio of degradable to nondegradable VOCs decreased rapidly in each well after injecting ENV435 (Figure 7). In trial number 2, however, the

magnitude of the decrease was similar throughout entire plot; it was not less in the downgradient wells than in the upgradient wells. Like the total VOC concentration, the T1: T2 ratio within the test plot rebounded with time, presumably because of reinjecting contaminated water into the plot (Figure 6).

Estimate of VOC Mass Removal. Two methods were used to estimate the mass of VOCs degraded in the test plot. In the first method, the amount of VOCs circulated into the test plot at the injection wells was compared to the amount of VOCs passing the first row of monitoring wells, 2 m away from the injection wells. Assuming that 50% of the recirculating groundwater passed through the test plot, approximately 107 g of VOCs were injected into the test plot during the first 21 days of trial number 1 after injecting ENV435. On the basis of the mean concentration of VOCs at the first row of monitoring wells, approximately 31 g of VOCs were removed in the lower zone of the aquifer, and 15 g were removed in the upper zone (total of 46 g VOCs removed). This represented a removal of about 44% of the injected degradable VOCs. After the first 21 days of trial number 1, additional mass removal in the first 2 m of the test plot was negligible.

In the second analysis, the total mass of VOCs in the test plot was estimated by multiplying the average VOC concentration at all of the monitoring wells by the estimated volume of the treated zone (assuming a total porosity of 0.5, calculated from the measured soil bulk density). Mass removed was calculated by comparing total VOC mass prior to injection of ENV435 to the lowest total mass in the plot after injecting the organisms. During trial number 1, the initial estimated dissolved mass of VOCs in the plot was 244 g (125 g in the shallow zone, and 119 g in the deep zone). On day 13 after injecting the organisms, there was an estimated 54 g of VOCs remaining in the shallow zone and 49 g remaining in the deep zone. Thus, at day 13 estimated VOC masses of 71 and 70 g were removed in the shallow and deep zones, respectively (total of 141 g VOCs removed). This represented a total mass removal of approximately 58% of the VOCs in the test plot.

The same analysis performed with data from trial number 2 indicated an estimated initial VOC mass in the lower zone of the test plot of 60 g. An estimated 13 and 23 g total VOCs were remaining in the plot on days 2 and 8, respectively. This represented 78% removal of VOCs in the test plot during the first 2 days.

Stripping of VOCs was assumed to be minimal during this study. During trial number 1, oxygen was added only to recirculating groundwater prior to reinjection, and the high oxygen demand of the injected organisms and natural sinks such as high ferrous iron likely limited off gassing of the added oxygen. Likewise, during trial number 2, oxygen was added to the formation by pressurizing deep wells with pure oxygen while attempting to minimize sparging. Because the aquifer was confined above and below by clay layers, any oxygen or VOCs stripped from the lower area of the formation would likely dissolve into the upper formation groundwater causing an increase in VOC and DO concentrations. No such increases in VOC or DO concentrations were observed in the upper formation.

Discussion

This study was designed to test the utility of bioaugmentation for remediating aquifers contaminated with VOCs. The ability of an exogenous organism to survive and compete for resources against indigenous organisms has long been an area of interest in both macro and microbial ecology, and in most cases added organisms do not fare well (7, 22, 23). The lack of competitiveness of exogenous strains can be exacerbated during co-metabolic degradation of VOCs because oxidation of these compounds can produce toxic intermediates that damage the degradative organisms (24, 25) and further reduce their competitiveness (23). The difficulty in establishing functional microbial populations of exogenous organisms prompted us to evaluate a second approach for applying bioaugmentation. To apply the technology, large amounts of degradative organisms were grown ex situ in a fermentor and then injected directly into a contaminant plume without the expectation of long-term survival. For this technique to be an effective remedial alternative, enough contaminant must be removed to allow closure of the site, or degradative organisms must be repeatedly cultured and added until contaminant levels are sufficiently reduced.

Ex situ fermentation and bioaugmentation may have an advantage over in situ biostimulation technologies because optimum growth conditions for specific biocatalysts can be developed and maintained. For example, during this study, highly active ENV435 cultures were produced by growing the strain in a fermentor on sucrose with the periodic addition of phenol to maintain full induction of the catalytic genes. In a full-scale field fermentor the periodic addition of phenol also may help maintain the integrity of the culture during prolonged fermentation by inhibiting the growth of contaminating organisms. During this study, growth rates as great as 0.19 hr^{-1} were achieved, the cells could be grown to high cell density ($>1 \times 10^{11}$ cfu/ml), and the resulting cells had a high specific activity for degrading VOCs (Table 2). It would be difficult to obtain such growth rates and high specific activity during in situ biostimulation (4, 5) because of colder temperatures, insufficient mixing, and the inability to supply sufficient amounts of oxygen and growth substrates.

During fermentation, ENV435 naturally produced large amounts of high-energy polyalkanoate storage polymers. Although the exact effect of these polymers on the catalytic activity or survival of ENV435 in situ was not evaluated during this study, such polymers have been shown to supply reducing energy (i.e., NADH) for degrading VOCs (26, 27, 28), and possibly increase the inherent degradative capacity of the organism (26) in laboratory studies.

Prior field studies of bioaugmentation for VOC degradation (9, 10) have met with mixed results, largely due to the poor transport properties of the microorganisms that prevented them from coming into contact with the target contaminant. Likewise, the potential for well-head or formation plugging also exists when injecting large numbers of naturally adhesive microorganisms. In this study, two microorganism delivery protocols were used to test the transport properties of the adhesion-deficient cultures and to ensure contact with the contaminants: (1) upgradient injection perpendicular to groundwater flow (trial number 1) and (2) direct injection of the organisms throughout the contaminant plume (trial number 2). In trial number 1, ENV435 was transported through the aquifer at a rate relative to bromide (v_{Br}: v_{ENV}) of only 1.26 in the deep sediments, and the organisms were detected throughout the test plot. Although the injected organisms were transported throughout the test plot, much of the degradative activity appeared to occur near the injection wells. This was likely a result of the sieving or entrapment of much of the added bacteria by aquifer sediments and the depletion of DO.

The direct injection of ENV435 throughout the test plot (trial number 2) was more effective for distributing active biomass and removing VOCs throughout the test plot than upgradient injection (trial number 1) because it resulted in a more rapid distribution of the organisms across the entire plot. Thus, it was not necessary to maintain high levels of degradative activity for an extended period, and the higher local cell concentrations resulted in rapid degradation of VOCs. Furthermore, oxygen limitations were minimized because oxygen could be added in the immediate vicinity of

the injected bacteria, thereby eliminating the need for long distance oxygen transport across the plot.

During full scale application of bioaugmentation, transport of the catalyst may still be a limitation at some sites because of the existing hydrogeology (e.g., low effective porosity) or the inability to induce groundwater flow or promote mixing as done in this study. However, other mechanisms for distributing microorganisms throughout aquifers are available (29, 30), and they may reduce the measured decay rate by minimizing sieving and rapidly dispersing the bacteria over greater distances. In another field-scale test of bioaugmentation, strain ENV435 was injected with high-pressure air directly into a bedrock aquifer during pneumatic fracturing (30), and the bacteria were distributed through an 8-m radius from the injection well within minutes. The high-pressure airflow also promoted mixing of the injected culture with contaminated groundwater. Likewise, applying the organisms with microbubble foams appeared to improve their transport and distribution in model aquifers (29), and nutrients and growth substrates can be added to the foams to help prolong survival. The potential for using these alternative delivery techniques in aquifer materials similar to those encountered during this project has not yet been tested.

One of the critical issues faced when injecting large quantities of aerobic microorganisms into an aquifer is satisfying the oxygen demand of the organisms and reactive chemical species in the groundwater. Oxygen limitation also has been encountered when applying other aerobic in situ biotreatment technologies (4, 5, 6). In this work, DO concentrations in the test plot decreased significantly after injecting ENV435, and they did not recover even after three weeks of injecting aerated water into the plot. This depletion of oxygen likely reduced the efficiency of VOC degradation by the added strain. As seen in Figures 4 and 7, most of the VOC degradative activity during trial number 1 occurred near the injection wells, and it decreased as the organisms moved downgradient. Mass removal calculations suggested that about 33% of the VOC removal occurred between the injection wells and the monitoring wells located 2 m downgradient. If degradation continued at high levels, VOC concentrations in the groundwater should have continued to decline as the organisms and water moved downgradient, as was observed along the southern edge of the test plot (Figure 4C).

Dissolved oxygen limitations were reduced in this study by injecting the biocatalyst and pure oxygen throughout the test plot (trial number 2). In this case, high oxygen concentrations (>2 mg/L) were maintained without sparging, and TCE degradation occurred throughout the plot (Figure 8). Likewise, the T1:T2 ratio decreased significantly at all of the monitoring wells of the test plot after injection of the organisms (Figure 7), indicating that the T1 compounds were degraded throughout the plot. Other approaches for adding oxygen, such as injecting hydrogen peroxide (5), may also supply sufficient oxygen in some situations, but the cost and potential toxicity of the added compound must be considered.

During trial number 1, most of the degradation in the test plot occurred within the first 3 to 5 m downgradient of the injection wells where the organisms were concentrated and oxygen was most available. We estimate that at least 141 g of VOCs were degraded during trial number 1 by 38.5 kg of ENV435. This represents an apparent transformation ratio (g VOC/g biomass) of approximately 0.004 which is only 10% of the transformation capacity reported for phenol degraders (0.031) (31), and 45% of those reported for other toluene degraders (0.0073) (31) in laboratory studies. The actual in situ transformation capacity of the added strains, however, may be greater than that measured here. For example, the low concentrations of VOCs present and limited oxygen availability may have prevented an accurate assessment of the amount of VOCs that could be degraded by the organisms under different conditions.

Two of the most attractive characteristics of the bioaugmentation approach demonstrated here are (1) that it can result in rapid degradation of VOCs and (2) it can be applied over wide areas and/or targeted directly toward the source of contamination. By comparison, biostimulation approaches can require several months of application before sufficient degradation is achieved (4, 5), and depending on how they are applied, they can require in situ transport of the contaminants to a treatment zone (5) not unlike pump and treat approaches. Thus, the results of this work demonstrate a technology that can be applied at sites where the hydrogeological conditions and access are favorable and rapid treatment TCE, DCE, and/or VC is desired.

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