Microbial Succession during a Field Evaluation of Phenol and Toluene as the Primary Substrates for Trichloroethene Cometabolism

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Microbial community composition and succession were studied in an aquifer that was amended with phenol, toluene, and chlorinated aliphatic hydrocarbons to evaluate the effectiveness of these aromatic substrates for stimulating trichloroethene (TCE) bioremediation. Samples were taken after the previous year's field studies, which used phenol as the primary substrate, and after three successive monthly treatments of phenol plus 1,1-dichloroethene (1,1-DCE) plus TCE, phenol plus TCE, and toluene plus TCE. Dominant eubacteria in the community were assessed after each of the four treatments by characterizing isolates from the most dilute most-probable-number tubes and by extracting DNA from aquifer samples. The succession of dominant phenol- and toluene-degrading strains was evaluated by genomic fingerprinting, cellular fatty acid methyl ester (FAME) analysis, and amplified ribosomal DNA restriction analysis (ARDRA). 1,1-DCE was found to drastically reduce microbial growth and species richness, which corresponded to the reduction in bioremediation effectiveness noted previously for this treatment (G. D. Hopkins and P. L. McCarty, *Environ. Sci. Technol.* 29:1628–1637, 1995). Only a few gram-positive isolates could be obtained after treatment with 1,1-DCE, and these were not seen after any other treatments. Microbial densities returned to their original levels following the subsequent phenol-TCE treatment, but the original species richness was not restored until after the subsequent toluene-TCE treatment. Genomic fingerprinting and FAME analysis indicated that six of the seven originally dominant microbial groups were still dominant after the last treatment, indicating that the community is quite resilient to toxic disturbance by 1,1-DCE. FAME analysis indicated that six microbial taxa were dominant: three members of the β subclass of the class *Proteobacteria* (*Comamonas-Variovorax*, *Azoarcus*, and *Burkholderia*) and three gram-positive groups (*Bacillus*, *Nocardia*, and an unidentified group). ARDRA revealed that the dominant community members were stable during the three nontoxic treatments and that virtually all of the bands could be accounted for by isolates from five of the dominant taxa, indicating that the isolation protocol used likely recovered most of the dominant members of this community.

Trichloroethene (TCE) is one of the most common pollutants of groundwater throughout the United States. Anaerobic degradation of TCE and tetrachloroethylene is well established (2, 16, 26, 27), but these transformations are often incomplete and can result in the accumulation of the less chlorinated metabolites 1,2-*cis*-dichloroethene (c-DCE), 1,2-*trans*-dichloroethene (t-DCE), 1,1-dichloroethene (1,1-DCE), and vinyl chloride (27). There has been much interest in the aerobic cometabolism of TCE and its dechlorination products since Wilson and Wilson (30) demonstrated cometabolism of TCE in soil enriched with natural gas. Later, several aromatic compounds, especially phenol and toluene, were also shown to stimulate TCE degradation (13, 28, 31). The oxygenases induced by these substrates are responsible for the cometabolic transformation of TCE and its dechlorination products.

Bioremediation remains potentially the most cost-effective cleanup technology for chlorinated aliphatic hydrocarbons (CAHs). Field experiments have been carried out at Moffett

Federal Airfield, Mountain View, Calif., to test the effectiveness of several primary carbon and energy sources for stimulating the bioremediation of CAHs. Methane was an effective substrate for the transformation of t-DCE and vinyl chloride but not for TCE and c-DCE (19, 22–24). Phenol was subsequently found to be effective for TCE and c-DCE removal (9, 10). The successful field demonstration of phenol as a stimulus of TCE removal prompted further investigations to optimize its use and to compare its effectiveness with that of other compounds for the removal of TCE and its less chlorinated products c-DCE, t-DCE, 1,1-DCE, and vinyl chloride (11).

Many studies have been carried out in the laboratory on selected culturable isolates capable of cometabolizing TCE, but no studies have focused on understanding the community selected in the field by such cometabolic treatments and the dynamics of that community in response to different treatments. In this work we studied microbial population succession as a result of injection of different aromatic substrates and CAHs into the aquifer. We evaluated community composition by classical growth-dependent microbiological procedures as well as by analysis of 16S ribosomal DNA restriction digestion patterns derived from DNA extracted from the entire community. The dominant populations selected were rather stable over four sequential treatments and quite resilient following

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FIG. 1. Cross section of the Moffett Field test site showing the glass bead (GB) and water (filter) sampling well, water sampling wells (SSE1 to SSE3), water injection well (SSE1), and water extraction well (P2) (19).

introduction of 1,1-DCE, the cometabolism of which produces highly toxic byproducts (3, 4).

MATERIALS AND METHODS

Field sample site description. The experimental site is located at the Moffett Federal Airfield (formerly the Moffett Naval Air Station). The aquifer characteristics have been described by Roberts et al. (19) and Hopkins et al. (10), with the relevant features for this study as follows. The aquifer is comprised of sand and gravel with 0.11% organic matter, lies 4.5 m below ground surface, and is 1.4 m thick. Groundwater velocity in the test zone is relatively high, at 1 m/day. The groundwater has <0.2 mg of O_2 /liter, 6 to 15 mg of NO_3^- /liter, 700 mg of SO_4^{2-} /liter, and <0.5 mg of phosphate/liter and is of low salinity (~1,500 mg of total dissolved solids/liter) and near-neutral pH. It is somewhat contaminated with chlorinated solvents from other sources, although the one of primary interest in this study, TCE, is not present. The only CAH of significance is 1,1,1-trichloroethane, which is found at a concentration of about 30 μ g/liter. These contaminants have posed no difficulties in the experiments conducted at the site, as they are either too low in concentration to be of significance or not reactive biologically under the experimental conditions.

Field treatments. The injection, sampling, and extraction wells and the protocol for substrate addition for this experiment have been described in detail by Hopkins and McCarty (11) (Fig. 1). The aquifer temperature was 18°C. Groundwater was extracted at one point from this aquifer at 10 liters/min to create a localized gradient. A portion (1.5 liters/min) of the extracted groundwater was filtered with a nominal 1- μ m-pore-size cotton filter, UV disinfected, saturated with pure oxygen to a dissolved oxygen concentration of 32 mg/liter, and then injected 7 m upgradient from the extraction well. The injected water was also augmented with various chlorinated ethenes: bromide, to serve as a conservative tracer, and either phenol or toluene during the four successive experimental treatments evaluated in this 1993 field study as summarized in Table 1. For the first 980 h of this study, phenol was added to the injected water for 5 min once every 8 h to produce a time-averaged concentration of 12.5 mg/liter for the 8-h period. Subsequently, toluene was added for 20 min once every 8 h to produce a time-averaged concentration of 9 mg/liter.

Microbiological sampling. Similar field experiments with a phenol amendment were conducted at the same location during the two previous (1991 and 1992) field seasons, and in the 1992 season TCE was the only CAH evaluated (9, 10). Sample I, the initial sample for biological analysis, was taken about 1 month before the start of the 1993 field season to represent the population remaining from the previous season's study, which had been terminated 8 months earlier. Three other microbiological samples (samples II, III, and IV) were collected over the course of the 1993 season during or following the different treatments listed in Table 1. All microbiological samples came from a monitoring well 1.5 m downgradient and about 6 h of travel time from the injection well, based upon bromide tracer studies. The microbiological sampling well consisted of a standard 5.1-cm-diameter polyvinyl chloride casing with a 1.52-m screen that fully penetrated the confined aquifer. For the first 2 years prior to this study, this well

was filled to the top of the screen with 2-mm-diameter glass beads that then became colonized with aquifer microorganisms. Sample I was obtained through hydraulic suction with a pump to withdraw the beads along with groundwater into a bottle. As this approach dislodged microorganisms from the glass beads, the water leaving the bottle (approximately 150 liters) was filtered through a cotton filter (Microwynd II; J. N. Fauver Company, Flint, Mich.) to catch dislodged microorganisms. Finally, after the glass beads were extracted from the well, 500 ml of additional water was removed from the well and filtered through a 0.22- μ m-pore-size filter for analysis.

For samples II, III, and IV, a different procedure was used. Ten bags were connected in a series, like a sausage, and lowered into the well to occupy the entire vertical space covered by the monitoring well screen. Each bag was made from nylon horsehair fabric and filled with 100 g of 2-mm sterilized glass beads. This procedure made it easier to remove the beads for analysis of the microbial communities that had colonized them during a single treatment period. Because new, uncolonized surfaces were provided before each treatment, the results reflected only colonization (growth) that resulted from the treatment rather than survival by indigenous flora, which would have been the case if aquifer solids had been sampled. The filters and bags of glass beads were shipped on ice by overnight mail to Michigan State University for microbiological analysis.

Analysis of the microbial community. To collect the biomass from the glass beads or filters, the samples were shaken with sterile saline solution, pH 7, in sterile 250-ml Nalgene plastic bottles. The supernatant was transferred to a

TABLE 1. Summary of substrate and CAH additions and microbiological sampling during 1993 field season

Operational period (days) ^a	Primary substrate added (concn [mg/liter]) ^b	Concn of CAH addition (μ g/liter)				Microbiological sample (day obtained)
		TCE	1,1-DCE	c-DCE ^c	t-DCE ^d	
Preoperation						I (-25)
0-8	Phenol (12.5)	230				
8-28	Phenol (12.5)	230	199 ^e			II (20)
28-41	Phenol (12.5)	230				III (40)
41-69	Toluene (9)	230		119	81	IV (67)

^a Dissolved oxygen (32 mg/liter) was added throughout the total operational period.

^b Time-averaged concentration shown.

^c c-DCE was added from day 55 to 59.

^d t-DCE was added from day 61 to 69.

^e 134 μ g of 1,1-DCE/liter was added from day 8 to 21, and 65 μ g/liter was added from day 21 to 28.

clean, sterile bottle and centrifuged. The pellet was retained and the supernatant was discarded. At least eight extractions were performed in this manner from each sample. The final pellet was resuspended in saline to yield a biomass concentration corresponding to 10 g of glass beads/ml of saline. The filter samples were resuspended to a visual turbidity similar to that of the glass bead samples. This resuspended turbid solution was used for most-probable-number (MPN) determinations, direct isolation of microbial populations, and total-microbial-community DNA extraction. DNA was extracted according to the direct-lysis protocol of Holben (8).

The protocol for the MPN determinations is described in the accompanying paper (6). The estimated population density and the 95% confidence intervals were calculated by standard methods (1). The dilution level from which each isolate was obtained was recorded to determine which strains recurred over time and their approximate population levels. Species richness (d) was calculated by the expression of Pielou (17), $d = (S - 1) / \log N$, in which S is the number of species and N is the number of individuals. We used identical genomic fingerprints, as determined by repetitive extragenic palindromic (REP)-PCR, as surrogates for bacterial species. While this does not reflect true species, it is a measure of genetic diversity in the community.

This study also analyzed the succession patterns of the phenol- and toluene-degrading isolates characterized in the accompanying paper (6). The fatty acid methyl ester (FAME) data reported in that paper is analyzed here by cluster analysis with NTSYS software (Applied Biostatistics, Inc., Setauket, N.Y.). A Euclidean similarity matrix was computed from nonstandardized data, and distances between strains were calculated by unpaired group mean averages. Goodness of fit between the Euclidean matrix and the resulting tree was computed with the cophonetic correlation coefficient (20). The taxonomic names reported for clusters are based on the criteria that most isolates in each cluster had indices of species similarity of >0.5 and appropriate Gram stains and morphologies. We also determined the fatty acid compositions of the following standard strains grown on the same medium as the isolates: *Hydrogenophaga pseudoflava* (MIDI database), *Arthrobacter* sp. strain 53 (7), *Pseudomonas putida* F1, *P. putida* PaW1, *Pseudomonas mendocina* KR, *Burkholderia cepacia* G-4, *Burkholderia pickettii* PKO1, and several *Azoarcus* strains.

Molecular methods. The method for determining REP-PCR patterns is described in the accompanying paper (6). Amplified ribosomal DNA restriction analysis (ARDRA) was performed on DNA extracted from glass beads, filters, and dominant isolates. Approximately 1,500 bp of the rRNA genetic loci were amplified by PCR with universal primers A and H of Ulrike et al. (25). In general, amplification was done in a 100- μ l total reaction mixture containing 100 ng of total-community-extracted DNA or DNA from pure cultures as the template, 1 μ M (each) primer, 250 μ M (each) deoxynucleoside triphosphate, 10 μ l of 10 $\times$ Taq buffer, and 2.5 U of Taq DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.). PCR was performed with an initial denaturation (95°C, 130 s) followed by 30 cycles of denaturation (92°C, 70 s), annealing (55°C, 30 s), and extension (72°C, 130 s) and a single final extension (72°C, 6 min). An aliquot of 10 μ l was run in a 0.7% agarose gel to evaluate the quality of the amplified fragment. In general, amplification yielded more than 5 μ g of PCR product. Amplified DNA from communities (25 μ l) or bacterial isolates (15 μ l) was digested according to the manufacturer's specifications for at least 3 h with restriction endonucleases (*Hae*III, *Hpa*II, and *Sau*3A; Boehringer Mannheim, Indianapolis, Ind.). The 16S ribosomal DNA restriction fragments were concentrated to a lesser volume by lyophilization for 10 min and then electrophoresed on a 4% (wt/vol) NuSieve 3:1 agarose gel (FMC, Indianapolis, Ind.) in 0.5 $\times$ Tris acetate-EDTA at 7 V/cm and stained in a 0.5- μ g/ml ethidium bromide solution. The resulting fragment patterns were then used as fingerprints for the comparison of populations.

RESULTS

Treatment effects on community density and diversity. No significant differences in the relative abundance of total heterotrophs, phenol degraders, toluene degraders, and phenol- or toluene-catalyzed TCE degraders were observed when TCE, c-DCE, or t-DCE was injected into the aquifer along with phenol or toluene (samples I, III, and IV) (Fig. 2A). In contrast, when 1,1-DCE was injected along with phenol and TCE (sample II), a drastic reduction in all populations was observed (Fig. 2A). After 1,1-DCE injection was stopped, population levels increased and reached densities similar to those at the beginning of the experiment. While 1,1-DCE had an effect on population densities, the primary carbon source did not, because the additions of phenol (sample III) and toluene (sample IV) produced the same population densities. It is noteworthy that the populations of toluene degraders rose markedly after feeding with phenol and that the density of phenol degraders was equal to that of toluene degraders after

FIG. 2. (A) Populations of total heterotrophs, phenol and toluene degraders, and TCE cometabolizers obtained from glass beads sampled following each of the four indicated treatments (I, II, III, and IV). For the initial sample, the populations of toluene and phenol degraders were obtained with 50 ppm of these chemicals used as substrates. Populations for samples II, III, and IV were obtained with 25 ppm of the indicated aromatic substrates. (B) Species richness index calculated as suggested by Pielou (17) by using isolates from glass beads with different REP-PCR sequences as the measure of diversity.

feeding with toluene. Also to be noted is that with the switch from phenol to toluene, toluene utilization was immediate and complete ($>99\%$) and TCE removal ($>90\%$) was not interrupted (11). Thus, the dominant population produced from phenol addition could also utilize toluene. When toluene was added, traces of *o*-cresol were formed, suggesting oxidation by the toluene *ortho*-monooxygenase pathway (11).

Species richness indices were used to express changes in relative diversity among successional stages. Due to technical difficulties in identifying a large number of bacterial isolates to species level, we used genomic fingerprints to reflect the effects of CAHs and primary substrates on the community. Diversity was greatly reduced by 1,1-DCE and remained low during the subsequent phenol feeding (sample III) but increased to original levels following toluene feeding (Fig. 2B). A taxonomic evaluation of the isolates showed that disturbance by 1,1-DCE led to the survival of relatively few populations compared with unperturbed communities and that these surviving populations were predominantly gram positive (four of the five different isolate genotypes) (6).

Succession determined by REP-PCR patterns of isolates. Isolates from successive treatments that appeared to have sim-

FIG. 3. Confirmation by REP-PCR patterns that isolates recovered from different successional treatments recurred over time. Strains were ordered by similarity in band patterns as determined by the Ambis clustering program and then rerun side by side for more accurate comparison of bands. Strain nomenclature: MF, Moffett Field; H (if present), isolate from heterotrophic medium. Numerals indicate aquifer samples as follows: sample I, two- and three-digit numbers ≤ 199 ; sample II, three-digit numbers from 200 to 299 and hyphenated numbers beginning with "2"; sample III, three-digit numbers from 300 to 399 and hyphenated numbers beginning with "3"; sample IV, three-digit numbers from 400 to 499 and hyphenated numbers beginning with "4." REP-PCR group numbers and FAME cluster letters are shown at the top. A, *Comamonas-Variovorax*; B, *Azoarcus*; C, *Bacillus*; D, *Nocardia*; E, unknown gram-positive isolates; F, *Burkholderia*.

ilar REP-PCR patterns were identified and run side by side on agarose gels to confirm their similarities (Fig. 3). Strains that were found at more than one sampling time from the most dilute MPN tubes and from several media were judged to be dominant in the field. The distribution of the isolated REP-PCR group members was influenced by the field treatments. Isolates represented by seven REP-PCR groups were the dominant populations in the initial sample (Fig. 4). However, after the phenol and 1,1-DCE treatment (sample II), very few different isolates were obtained, and none of these had REP-PCR patterns seen before. This suggests that one of these compounds was detrimental to the initial bacterial populations dominating the aquifer. Once 1,1-DCE was removed and only the phenol and TCE injections were continued, isolates with REP-PCR patterns 7 and 33 were again detected as the most commonly isolated populations (sample III). With the later switch from phenol to toluene, isolates representing six of the initial seven REP-PCR groups (sample IV) were again isolated at a high frequency (Fig. 4). Only the group represented by REP-PCR pattern 3 was not found after the addition and subsequent removal of 1,1-DCE.

Succession determined by FAME cluster analysis. FAME analysis was used to cluster isolates and as an independent method to evaluate which types were more frequently isolated over the four treatments. The analysis included isolates representing all of the different REP-PCR patterns found plus isolates with matching REP-PCR patterns which were isolated from different treatments. Initial populations present in the field were very diverse based on the number of different REP-PCR patterns represented (6) and on the number of FAME groups obtained. FAME analysis grouped the isolates from the four samples into 30 distinct groups at Euclidean distances of less than 20 (Fig. 5). MIDI literature suggests that the Euclidean distance scale permits a preliminary indication of the relatedness of entries at the genus, species, and subspecies levels, which are 25, 10, and 6 units of Euclidean distance, respec-

tively. We also included fatty acid data of five well-characterized strains that are known to harbor different pathways for toluene degradation. None of our isolates were clustered with these isolates (Euclidean distance, <20): *P. putida* F1, *P. putida* PaW1, *P. mendocina* KR, *B. cepacia* G-4, or a common soil isolate, *Arthrobacter* sp. strain 53. One isolate (MF-13) was

FIG. 4. REP-PCR genotypes that were dominant during the course of the experiment. The population levels for each isolate were estimated with the most dilute MPN tube from which the strain was isolated. The cluster letters for cross-referencing to FAME data and taxonomy are also shown. Members of REP-PCR group 3 were not found after later treatments, and this group was not further analyzed. G+, gram positive.

FIG. 5. Dendrogram based on the fatty acid profiles of the isolates found. FAME groups I through XXX were defined at a Euclidean distance of 20 ($r_{xy} = 0.86$). Isolate sample symbols: , sample I; , sample II; ★, sample III; , sample IV. Lettered brackets indicate groups of strains that were most common, and strains with the same REP-PCR patterns are boxed. A, *Comamonas-Variovorax*; B, *Azoarcus*; C, *Bacillus*; D, *Nocardia*; E, unknown gram-positive isolates; F, *Burkholderia*.

FIG. 6. ARDRA of aquifer community DNA and of pure cultures dominant in the aquifer. Results from DNA digested with *Hpa*II (A), *Hae*III (B), and *Sau*3A (C) are shown. The lanes show PCR products from aquifer glass beads (AQ. GB) of sample I, cells retained on the 0.22- μ m-pore-size filter (filter 1) and the cotton-fiber-filtered water (filter 2) from sample I, glass beads from sample III (phenol amendment), glass beads from sample IV (toluene amendment), and pure cultures from numbered REP-PCR groups. Numbers on the left are molecular size markers. FAME cluster letters: A, *Comamonas-Variovorax*; B, *Azoarcus*; E, unknown gram-positive isolates; F, *Burkholderia*.

clustered at a Euclidean distance of less than 10 with *B. picekettii* PKO1. However, many isolates and one previously described strain, *Azoarcus indigenus* VB32T, a nitrogen-fixing organism (18), also were clustered in this group at a Euclidean distance of less than 20. We also included in this analysis a recently described species of toluene-degrading denitrifiers, *Azoarcus tolulyticus* (32), since many of the Moffett Field isolates resembled this genus based on FAME analysis, toluene degradation, and denitrification properties (5). Gram-positive isolates (6) were clustered in 12 different groups (I, II, III, IV, V, VI, XVII, XXIV, XXVI, XXVIII, XXIX, and XXX). Most of the gram-positive isolates were retrieved in specific samples. However, group III gram-positive isolates were more common in the aquifer since isolates belonging to this group were obtained from samples I, III, and IV (Fig. 4).

The dominance and succession patterns obtained by REP-PCR analysis (Fig. 4) were supported by FAME analysis. The dominant strains with the same REP-PCR patterns are shown in Fig. 5 with related strains as determined by FAME cluster analysis. Lettered brackets are placed around the same or larger group of related strains, and the taxonomic identity of the strains as determined by FAME is shown. These cluster letters and taxonomic identities are also shown in Fig. 4 with the REP-PCR groups for clarity. All six isolates of cluster E, which stain gram positive, had no match in the MIDI database.

The upper branch of group XIV was identified as *P. putida* of the γ subclass of the class *Proteobacteria* and so is not included in cluster A, since *Comamonas* and *Variovorax* are members of the β subclass of *Proteobacteria*. Clusters C, D, and E stained gram positive, and the other three clusters were gram negative. Members of cluster C had spores. Members of clusters A and E were recovered after treatments I, III, and IV, while members of the other four clusters were found after treatments I and IV. Clusters A, B, C, E, and F had isolates recovered on heterotrophic medium from the highest dilution of inoculum (10^{-6} to 10^{-7}) as well as from the highest dilution of phenol or toluene MPN tubes (10^{-5}), providing further evidence of their dominance in the aquifer.

Community composition determined by ARDRA. We used ARDRA of community DNA to evaluate (i) how similar the glass bead biofilm community was to the community recovered from the filters, (ii) whether the community selected by phenol was different from the one selected by toluene, and (iii) whether the dominant strains determined from the isolate studies also appeared to be the dominant organisms by DNA analysis. The relatively low number of bands generated from aquifer community DNA suggests that the communities were low in diversity or that the diversity resides in a number of closely related organisms, i.e., those sharing the same restriction sites. The microbial-community ARDRA profile obtained from glass beads, representing the attached community, was very similar to the microbial-community profiles obtained from the filtered-water samples (Fig. 6).

The similarity in band patterns from the original community and communities obtained subsequently under different carbon sources strongly supports the hypothesis that the structure of the selected community did not change under the treatments compared (Fig. 6). The numbers of common bands per treatment, of the 15 restriction bands seen for all treatments (with three different restriction enzymes), were 13 for the initial sample, 14 after phenol treatment, and 14 after toluene

treatment. These ratios show considerable stability in the community over the course of treatments.

The ARDRA patterns observed for five aquifer strains initially thought to be dominant could also be discerned in the community DNA for the three samples under study (Fig. 6). This finding indicates that the dominant community profiles can be accounted for by the dominant isolates. Only one band (in the 460- to 510-bp range [Fig. 6B]) that was present in all community samples could not be accounted for by the bands from isolates from the five dominant groups.

DISCUSSION

We evaluated the succession of microbial populations in an aquifer field site in order to understand how different CAHs and two primary carbon sources affected the dynamics of that community. Similar population densities for total heterotrophs, toluene and phenol degraders, and TCE degraders were observed when the aquifer was amended with TCE, c-DCE, or t-DCE and with phenol or toluene as the primary carbon sources. However, there was a drastic reduction in all population sizes when 1,1-DCE was added. Products of TCE cometabolism, which are known to have a toxic effect on TCE metabolizers (28, 29, 31, 33), did not cause a measurable effect on the populations detected. In contrast, 1,1-DCE almost totally inhibited colonization of the glass beads. This result provides further evidence of the toxicity of products of 1,1-DCE cometabolism to TCE cometabolism that was previously reported (3, 4, 11).

The first signs of environmental stress usually occur at the population level, affecting especially sensitive species. If there is sufficient redundancy, other species may fill the functional niche occupied by the sensitive species (15, 21). Indeed, we observed a drastic reduction in the diversity of phenol and toluene degraders, as measured by the richness of REP-PCR patterns, during the injection of 1,1-DCE. The populations isolated after this field treatment were predominantly gram-positive bacteria that were able to degrade only phenol (6). These survivors may have a unique resistance to products of 1,1-DCE cometabolism, and most have poor CAH cometabolizing activity (6). None of the isolates obtained after injection of 1,1-DCE (sample II) carried the toluene *ortho*-monooxygenase gene, despite the fact that this was the most common gene observed in isolates from sample I (6). Perhaps all strains with the toluene *ortho*-monooxygenase gene attempted to oxidize 1,1-DCE but were killed by its toxic products.

Cluster analysis of cellular fatty acid data from the isolates shows that at least 30 different groups of toluene and phenol degraders were present; 12 of these were comprised of gram-positive organisms. This indicates that a taxonomically diverse community was selected even under a simple selection regimen. Members of certain groups, however, were more numerous, and most of these recurred over the successive treatments. These recurring groups may represent the most competitive taxonomic groups of indigenous aquifer phenol and toluene degraders expected under similar bioremediation treatments. These six groups included two that might be expected, the *Comamonas-Variovorax* group and the *Burkholderia* group, both from the β subclass of the class *Proteobacteria*, but four that were rather unexpected: *Azoarcus*, *Nocardia*, *Bacillus*, and one gram-positive group that has no match in the MIDI database. Considering the isolate and ARDRA data together, relative dominance among the six clusters was *Comamonas* > *Azoarcus* > *Burkholderia* > unknown gram-positive group > *Nocardia* = *Bacillus*.

One of the important topics in ecology is the resilience of

community composition, especially after severe disturbance (14). This study is one of few examples in microbial ecology in which this question has been evaluated in the field. This evaluation was facilitated by the toxicity of 1,1-DCE, which created a large disturbance in ecological terms, i.e., it was sufficiently lethal to release growth resources (12). The finding that six of the seven dominant microbial populations returned after severe disturbance suggests that these aquifer microbial communities are quite resilient. The recovery was not immediate, however, since the species richness remained low in the first treatment (phenol) after 1,1-DCE was removed. Perhaps the addition of another substrate (toluene) as well as additional time contributed to the return of the original species richness and dominant population clusters. It is noteworthy that the two most dominant strains that returned after the disturbance (MF-301 and MF-H-3-3) were very effective TCE degraders (reference 6 and unpublished data).

ARDRA provided an alternative method that does not rely on culturing for evaluating microbial community structure. ARDRA suggested that similar community structures were selected when phenol and toluene were the primary carbon sources, which is consistent with the interpretation of data from analysis of the dominant isolates. This is also consistent with the fact that many isolates grew on both substrates (6) and that toluene utilization was immediate and complete following the switch from phenol (11). Since the majority of bands observed in ARDRA patterns of community DNA were found in ARDRA patterns of the most common isolates, we conclude that unculturable populations were not important in this study. This result is more likely under conditions such as those used in this study, in which the growth substrate added in the field was also the substrate used for isolation.

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