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Succession and Convergence of Biofilm Communities in Fixed-Film Reactors Treating Aromatic Hydrocarbons in Groundwater

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Community composition, succession, and performance were compared in three fluidized bed reactors (FBR) operated to test preemptive colonization and the influence of toluene compared with a mixture of benzene, toluene, and *p*-xylene (BTX) as feeds. One reactor was inoculated with toluene-degrading strains *Pseudomonas putida* PaW1, *Burkholderia cepacia* G4, and *B. pickettii* PKO1. PaW1 outcompeted the other two strains. When groundwater strains were allowed to challenge the steady-state biofilm developed by inoculated strains, they readily displaced the inoculated strains and further reduced the toluene effluent concentration from 0.140 to 0.063 mg/liter for 98% removal. Amplified ribosomal DNA restriction analysis (ARDRA) of reactor community DNA showed a succession of populations to a pattern that was stable for at least 4 months of operation. Parallel reactors fed toluene and BTX but inoculated directly from groundwater had the same treatment performance and the same ARDRA profiles as each other and as the seeded reactor once the groundwater community took over. Convergence and stability of populations were confirmed by genotype analysis of 120 isolates taken from all reactors and at several times. Ninety percent of the isolates were of 4 of the 12 genotypes found, and their ARDRA patterns accounted for most of the community ARDRA patterns. Estimates of the maximum specific growth rates (μ_{max}), half-saturation constants (K_m), and maximum substrate utilization rates (V_{max}) of the 12 genotypes isolated revealed a rather high diversity of toluene use kinetics even though the toluene in the feed was constant. The climax populations, however, generally showed kinetic parameters indicative of greater competitiveness than the inocula. rRNA sequence analysis of three codominant strains showed them to be members of the alpha, beta, and gamma subdivisions of the *Proteobacteria*. Two were similar to *Comamonas* and *Pseudomonas putida*, but the member of the alpha group was somewhat distant from any organism in the rRNA database. The convergence of communities to the same composition from three different starting conditions and their constancy over several months suggests that a rather stable community was selected.

Bioremediation of groundwater contaminated with benzene, toluene, and xylene (BTX) can be accomplished either in situ or ex situ depending on site conditions. In the latter case, fluidized bed reactors (FBR) can be used instead of carbon adsorption to remove BTX compounds in pump-and-treat operations. We used an FBR operated in a manner used for commercial operations to evaluate whether microbial inoculants could compete effectively with indigenous groundwater organisms, to determine whether the final communities that developed in initially inoculated reactors were different from those communities inoculated with groundwater, and to determine whether communities selected by feeding only toluene were different from those fed BTX. We also evaluated the performance of each of these communities in removing toluene or BTX.

For inocula, we chose well-studied, toluene-degrading strains that carried three of the five different toluene pathways that have been characterized (28). These were *Burkholderia cepacia* G4, which carries the toluene *ortho*-monooxygenase pathway (8); *B. pickettii* PKO1, which carries the toluene *meta*-monooxygenase pathway (13); and *Pseudomonas putida* PaW1, which oxidizes the methyl group of toluene, forming benzoate as an intermediate (9, 12). These strains are also of interest because two of them, G4 and PKO1, are good trichloroethylene cooxidizers, while PaW1 has no trichloroethylene-metabolizing ability (6). Hence, the competitiveness of these strains in different reactor systems is of additional interest. Previous work on competition of different toluene-degrading populations in mixed chemostats suggested that strains which initially hydroxylate the aromatic ring were competitively superior to those which oxidized the methyl group (5).

The FBRs contained granular activated carbon (GAC) as the biomass carrier. The surface adhesion of bacterial populations and the subsequent cell binary fission and exopolymer production lead to the formation of biofilm communities (2, 24), and FBRs that were fed BTX developed biofilms more than 100 μm thick (16). We used growth-dependent counting and isolation methods, community DNA fingerprints of rRNA genes, and fatty acid analyses to study succession in biofilm communities from inoculated and uninoculated reactors and from reactors fed toluene or BTX. We found convergence of populations in the seeded and the natural groundwater-colonized communities on GAC regardless of the source of inoculum and the carbon source. Although all inoculated strains were outcompeted by the indigenous groundwater populations, the methyl group-oxidizing strain (PaW1) was the only one to survive, but it survived only at very low levels in mature biofilms.

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MATERIALS AND METHODS

GAC-FBR and support medium. The reactors were 2.54- by 195-cm glass columns packed with approximately 100 g of sterile GAC and operated as a single pass with an aqueous upflow rate of 0.2 liter/min. Oxygen, nutrients, and 3 mg of toluene per liter or 1 mg each of benzene, toluene, and *p*-xylene per liter in a mixture were constantly supplied in the feed. A detailed description of the bioreactor and the operating parameters was given previously (16). The following FBR systems were operated in parallel for this study: (i) a reactor colonized by indigenous aquifer microbial populations with toluene as the sole carbon source (referred to as the TOL.NS reactor [toluene.nonsterile]); (ii) a reactor colonized by indigenous aquifer microbial populations with BTX added as the sole carbon source (referred to as the BTX.NS reactor); (iii) a reactor seeded with three toluene-degrading species (initially referred to as TOL.S [seeded]). This bioreactor was operated in sequential stages: (i) on days 0 to 53, filter-sterilized groundwater amended with toluene was fed as the sole carbon source; (ii) on days 53 to 124, the filter was removed and groundwater with toluene continued to feed the reactor; and (iii) on days 124 to 175, the groundwater feed was amended with BTX rather than toluene. This latter configuration is referred to as the BTX.S reactor. The seeded reactor was sterilized prior to inoculation by running a 6 N HCl solution through the reactor for 48 h prior to adding autoclaved GAC. The filter-sterilization system consisted of (in series) a 0.30-μm-pore-size fiber filter, an activated carbon filter, and two 0.45-μm-pore-size membrane filters [Polycap 75 ASW/Bell]. The water was university tap (drinking) water which is taken from 60-m-deep wells under university farms and forest preserves.

Physicochemical analyses of all FBR, including measurements of BTX, oxygen consumption, temperature, and pH, were generally conducted at 2- to 3-day intervals by methods described previously (16).

Bacterial strains and growth conditions. The filter-sterilized FBR (TOL.S reactor) was inoculated with *B. cepacia* G4, *B. pickettii* PKO1, and *P. putida* PaW1. All the strains were provided by Ron Olsen, University of Michigan. The strains were grown to mid-exponential phase in basal salt medium (BSM) (18) with toluene as the sole carbon source. Cells of the three strains were mixed in equal numbers (approximately 10^9 cells per strain) and used to inoculate the sterile GAC reactor. After recirculation of the inoculum for 2 h, cell adsorption to the carbon was apparent since the liquid phase changed from turbid to clear. At this time, continuous feeding of toluene-amended, filter-sterilized groundwater was started.

Selective media were designed from antibiotic resistance patterns and BIOLOG substrate use data and used to determine the population sizes of inoculant strains. All media were made with distilled water and solidified with 2.2% (wt/vol) Noble agar. For strain G4, 1% (wt/vol) D-sorbitol was used as the sole carbon source in BSM; for strain PKO1, 10 μg of kanamycin per ml and γ-hydroxybutyric acid (1%, wt/vol) was used in BSM; and for strain PaW1, the medium was composed of the following (grams per liter): sucrose, 10; $NaHCO_3$, 1; $MgSO_4 \cdot 7H_2O$, 5; K_2HPO_4, 2.3; and *N*-lauroylsarcosine sodium salt, 1.2; as well as 20 mg of trimethoprim (after sterilization) and 10 ml of glycerol.

Bacterial counting, isolation, and characterization. Biofilm samples were collected aseptically from the bottom column sampling port every 2 to 7 days for the duration of reactor operation. Cells were removed and homogenized as previously described (24). Viable bacterial numbers from GAC samples were determined by using R2A medium (Difco, Detroit, Mich.), which was designed for improved recovery of environmental heterotrophs. R2A plates were incubated at 25°C for 3 to 4 days, and total counts and relative densities of different colony types were recorded. Populations of the inoculated strains were determined with selective media after incubation at 25°C. Total bacterial cell counts were determined by acridine orange epifluorescence microscopy, using the method of Zimmerman et al. (27) with modifications (16). The extracted GAC sample was desiccated at 195°C for a week or until constant weight for reporting cell density per gram of GAC.

To isolate numerically dominant bacteria from GAC biofilm communities, dispersed biofilm bacteria from steady-state reactors were diluted and then plated on R2A solid medium. Isolates picked from the terminal dilutions were subcultured three times to ensure purity and screened by the repetitive extragenic palindromic (REP) PCR technique (3). One isolate from all groups with different band patterns was selected for further study. To determine the substrate range, these isolates were grown on R2A plates, washed and resuspended in BSM, and transferred to sterile vials containing BSM with 10 mg of toluene, benzene, or *p*-xylene per liter in triplicate sets. The inoculated vials were sealed with Teflon-coated septa and incubated stationary for 2 weeks at 25°C. Substrate removal was analyzed by using a gas chromatograph equipped with a flame ionization detector. Positive activity was indicated when more than 80% of substrate was removed relative to the uninoculated control vial.

Isolates representing three dominant populations were further characterized by partial 16S rRNA gene sequence analyses. The 16S rRNA gene was amplified from genomic DNA by PCR and cloned into a plasmid vector (26). The 16S rRNA gene was sequenced in both directions by using an ABI automated fluorescence sequencer with the forward and reverse primers, which span *Escherichia coli* 16S rRNA gene positions 785 to 805 and 1115 to 1100, respectively. The DNA sequences were analyzed using the programs in the Genetics Computer Group software package (4) and in the PHYLIP phylogeny inference package (7).

Kinetic parameter estimation of isolates. The dominant biofilm populations were characterized for their kinetic parameters of toluene use. Duplicate sets of exponentially growing cultures in BSM containing 50 mg of toluene per liter were used to determine maximum growth rates by optical density measurements at 550 nm. K_m and V_{max} values were estimated by fitting progress curve data to the integrated Michaelis-Menten equation, using a nonlinear analysis. Both kinetic parameters were calculated from a single substrate depletion curve as a function of time. Cells freshly grown on toluene (50 mg/liter) were harvested, transferred to fresh preoxygenated medium with 3 mg of toluene per liter, and immediately drawn into a 50-ml gastight syringe containing a small magnetic stir bar; this system was developed to avoid creating any headspace. The biomass concentration was determined empirically for each culture (high enough not to increase with the amount of substrate added and low enough to allow collection of samples at reasonable time intervals). All assays were performed at room temperature (25°C). Samples of 1 ml were collected in 10-ml sterile vials sealed with Teflon-coated septa every 5 to 15 min, fixed with approximately 0.05 ml of 7 N HCl, and stored at 4°C until headspace analysis was performed (generally within a week). Cell numbers were determined before and after the assay by direct plating on R2A medium. No significant increase in the cell number was observed. Three of the cultures were independently assayed twice; their V_{max} and K_m values fell within the 95% confidence intervals of the first estimate.

Amplified ribosomal DNA restriction analysis (ARDRA). Nucleic acids were extracted from cell pellets of bacterial isolates or GAC communities by a lysozyme-sodium dodecyl sulfate method (15). Universal primers were used to amplify by PCR an approximately 1,500-bp 16S rDNA fragment with a forward primer which corresponds to nucleotide positions 49 to 68 (5′-AGAGTTTGA TCCTGGCTCAG-3′; primer A) of *Escherichia coli* 16S rRNA and a reverse primer which corresponds to the complement of positions 1541 to 1518 (5-AA GGAGGTGATCC AGCCGCA-3′; primer H) (22). All primers were synthesized with an Applied Biosystems DNA synthesizer at the Macromolecular Structure and Sequencing Facility, Michigan State University. In general, amplification was done in a 100-μl total reaction volume containing 100 ng of DNA from communities or isolates as the template, 1 μM each primer, 250 μM each deoxynucleoside triphosphate, 10 μl of 10× *Taq* buffer, and 2.5 U of *Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.) (17). PCR amplification was performed in an automated thermal cycler (no. 9600; Perkin-Elmer) with an initial denaturation (92°C for 130 s) followed by 30 cycles of denaturation (92°C for 70 s), annealing (48°C for 30 s), and extension (72°C for 130 s) and a single final extension (72°C for 6 min). An aliquot of 5 μl of PCR product was initially run in a 0.7% agarose gel to evaluate the quality of the amplified fragment. In general, amplification yielded greater than 10 μg of PCR product.

Community (5 μg) or bacterial (1 μg) amplified DNA was digested at 37°C for at least 3 h in a 15-μl final volume with *Hae*III, *Hpa*II, *Mse*I, or *Hin*f (Boehringer Mannheim, Indianapolis, Ind.). The 16S rDNA restriction fragments were then separated by electrophoresis on 4% (wt/vol) NuSieve 3:1 agarose gel (FMC, Indianapolis, Ind.) in 0.5× TAE (Tris acetate-EDTA) at 7 V per cm and stained in ethidium bromide solution (0.5 μg per ml). The resulting restriction fragment patterns were used as a fingerprint to recognize whether communities were similar.

Specific primers complementary to the V3 region of the 16S rRNA gene of strains G4 and PKO1 were constructed for a more sensitive and specific detection of these strains by PCR amplification. PCR was performed as described above but at an annealing temperature of 55°C in 50-μl reaction volumes. After amplification, 10 μl of the PCR product was separated by electrophoresis on a 0.7% (wt/vol) agarose gel in 1× TAE buffer.

Community fatty acid methyl ester (FAME) analysis. Total fatty acids of microbial communities were analyzed by using the Microbial ID system and protocol (MIDI, Newark, Del.). Duplicate samples of cells collected from fresh GAC samples (approximately 0.1 g [wet weight]) were analyzed.

RESULTS

Groundwater. The influent cell concentration in the groundwater was approximately 10^7 microscopic cell counts per liter. These microbial populations were a natural and continuous inoculum source for colonization of the nonseeded toluene- and BTX-fed reactors, as well as a challenge to the seeded toluene reactor after removal of the filter sterilization system. The groundwater at the bioreactor influent was 13 ± 1°C and had a pH of 7.1 ± 0.3 throughout the course of the experiments.

Nonseeded reactors. The toluene (TOL.NS reactor)- and BTX (BTX.NS reactor)-fed reactors were operated in parallel at the same organic loading rate. A description of the startup conditions and overall performance is given in Table 1. Steady state, defined as the phase when the dissolved-oxygen (DO)

TABLE 1. GAC-FBR startup phase and performance under steady-state conditions

Reactor designation	Inoculum source	Length of operation (days)	Carbon source	Lag (days)	Mean DO consumption (mg/liter) (SE)[a]	Toluene or BTX[b]	
						Mean concn in effluent (mg/liter) (SE)[a]	Removal efficiency (%)
NS.TOL	Aquifer	22	Toluene	6–7	4.1 (0.8)	0.066 (0.051)	98.0
NS.BTX	Aquifer	98	BTX	5–6	6.1 (2.6)	0.040 (0.035)	99.5
TOL.S	G4, PKO1, PaW1	0–53	Toluene	13–14	5.7 (0.7)	0.140 (0.031)	95.8
	+Aquifer[c]	53–129	Toluene	0	4.9 (0.7)	0.063 (0.041)	98.2
	+Aquifer[d]	129–175	BTX	0	4.8 (1.2)	0.094 (0.053)	97.5

[a] Mean (and standard error of the mean [SE]) based on $n = 7$ to 20.
[b] The reactor feed was 3 mg of toluene per liter or a mixture of 1 mg each of benzene, toluene, and *p*-xylene per liter.
[c] The filter sterilization system was removed 53 days after inoculation.
[d] The carbon source was shifted to BTX after 124 days.

consumption and substrate consumption were constant, was generally achieved after 15 days. At this time, the substrate removal efficiency was 98 to 99%. DO and toluene concentrations declined with depth in the reactor column (Fig. 1). More than 80% of the toluene was consumed in the first 30% of GAC bed height. Similar results were obtained with the column-fed BTX as substrate (data not shown).

Seeded reactor. To determine the competitiveness among seeded strains, equal numbers (2×10^7 cells/g of GAC) of strains G4, PKO1, and PaW1 grown on toluene were inoculated into the sterilized, toluene-fed FBR. Significant oxygen consumption and biomass accumulation began after a lag phase of 14 days. A phase of transient acclimation to toluene occurred from days 14 to 18. This was followed by a 10-day biofilm growth phase until maximum cell density was reached.

Analysis of specific population types by selective plating during the lag phase (Fig. 2), showed a decrease in the densities of strains PKO1 and G4 but growth of strain PaW1. During the growth phase, PaW1 was the sole recoverable strain. The increase in the cell density of PaW1 corresponded to an increase in DO consumption, which confirmed that toluene oxidation (not adsorption by carbon) was occurring. At steady state, the average amount of DO consumed was 5.7 mg/liter and the toluene effluent concentration was 0.140 mg/liter, for a 96% removal efficiency (Table 1). After 35 days, however, new bacterial types appeared on R2A plates, which suggested that groundwater strains may have breached the filter. On day 53, the filtration system was removed to allow aquifer populations to freely enter the already colonized GAC. The steady-state effluent toluene concentration declined from 0.140 to 0.063 mg/liter (Table 1) after the filter was removed.

To evaluate the effects of carbon sources on community structure, the toluene-selected community (TOL.S reactor) was switched at 129 days (Table 1) to groundwater amended with an equal mixture of BTX compounds at the same organic loading rate. The carbon removal rate and DO consumption did not change, even immediately after the shift.

Characterization of biofilm isolates. The density of aerobic heterotrophs in the steady-state reactors was 10^9 to 10^{10} CFU/g of GAC, which corresponds to 20 to 45% of the direct counts. Isolates from all reactors were obtained at several sampling times after the steady state was achieved. Only 5 of 120 isolates from the different reactors failed to transform any of the BTX substrates as sole carbon sources. At least 12 different toluene-degrading populations were isolated as determined by unique REP-PCR and ARDRA patterns (Fig. 3). Inoculum strain PaW1 was recovered from a 113-day-old GAC sample but only after prior enrichment on *p*-xylene. The recovered clone showed identical substrate range and REP-PCR fingerprint to PaW1. Isolates GF161, GF261, GF262, and GF281 were the numerically dominant isolates. They were found in all the reactor treatments and constituted more than 90% of all isolates. Their ratios changed somewhat with time and substrate feed.

The dominant GAC isolates with different REP-PCR pat-

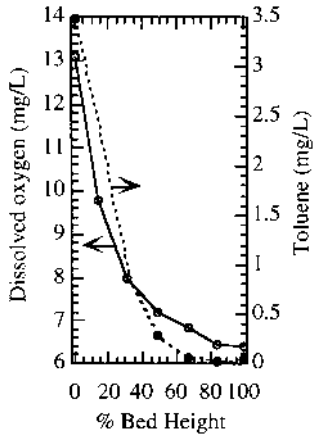

FIG. 1. Profile of DO (—) and toluene (····) concentrations with respect to depth in the bioreactor column.

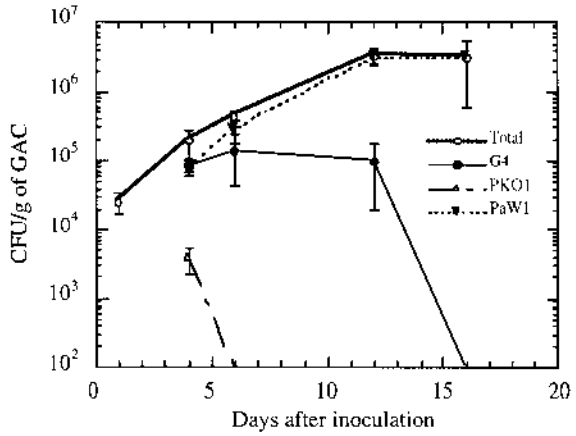

FIG. 2. Densities of inoculated strains as determined by selective plate counts over time in the TOL.S reactor. Error bars indicate 95% confidence intervals.

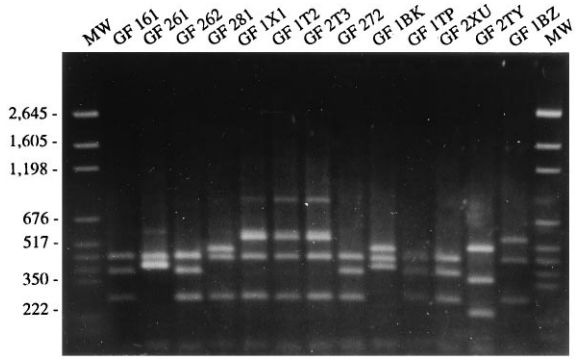

FIG. 3. ARDRA patterns of the dominant biofilm populations obtained with *Mse*I. The sizes of marker DNA fragments (lanes MW, in base pairs) are indicated on the left.

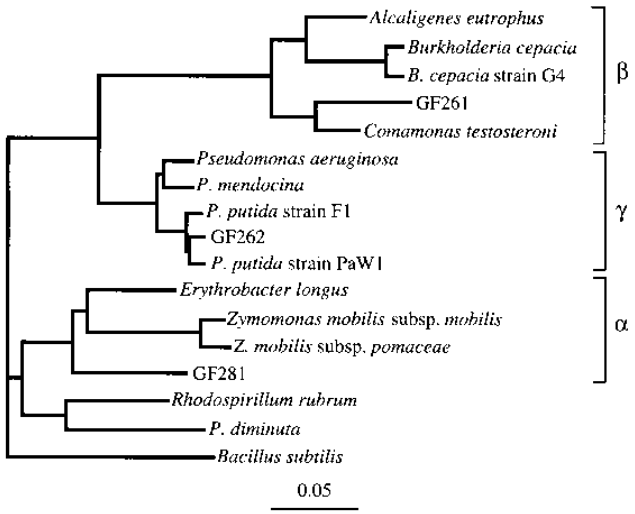

FIG. 4. Phylogenetic distance tree produced from partial 16S rRNA sequences. Representative members of the various subgroups of the *Proteobacteria* and three dominant toluene degraders from GAC biofilm communities are shown. The 16S rRNA sequence of *Bacillus subtilis* was used as the outgroup.

terns are described in Table 2. The apparent values for V_{max} ranged from 0.06 to 2.0 mg of toluene/10^{10} cells/h for strains G4 and GF272, respectively. K_m values ranged from a low of 0.074 mg/liter for strain GF161 to a high of 1.7 mg of toluene per liter for strain GF272. In general, GAC isolated strains showed lower K_m and/or higher V_{max} values than the seeded populations. Although strain GF272 has a relatively high K_m compared to the other isolates, it also has the highest substrate utilization rate constant. The majority of the isolates showed the ability to utilize more than one of the monoaromatic compounds tested. Estimates of μ_{max} on toluene ranged from 0.10 h^{-1} (doubling time [T_d] = 6.9 h) to 0.52 h^{-1} (T_d = 0.93 h).

Phylogenetic analyses of partial sequences established by the neighbor-joining method showed that three of the dominant strains were affiliated to the *Proteobacteria* (Fig. 4). Strain GF261 was related to *Comamonas testosteroni* in the beta subdivision, with a similarity of 93%; strain GF262 was closely related to *Pseudomonas putida* in the gamma subdivision, with a similarity of 99%; and strain GF281 was associated with *Erythrobacter longus* and *Zymomonas mobilis* with a similarity of 90%. Very similar tree topologies were also obtained by the maximum-parsimony and maximum-likelihood methods (data not shown).

Bacterial and community ARDRA fingerprints. More than 65% of the biofilm cells were lysed as determined by direct fluorescent cell counts. Approximately 60 μg of DNA/g of GAC was recovered at steady state. The DNA was of high molecular mass (>23.1 kb) and digestable by restriction endonucleases. Each of the inoculated strains showed a distinct restriction fragment pattern with the amplified rDNA when digested by *Hpa*II (Fig. 5, lanes G4, PKO1, and PaW1). These three patterns could also be discerned in mixed samples taken after inoculation (Fig. 5, lane Day 0). By day 22, however, DNA isolated from the GAC biofilm showed only a PaW1-like pattern. This finding is consistent with the results obtained from selective plating. Further changes in band pattern profiles can be seen over time, reflecting further changes in community composition, but the community profiles remained stable after day 53. Interestingly, band patterns similar to those generated from G4 and PKO1 can be seen at these later time points. Because of the pure-culture results (Fig. 3), we suspected that these bands were from different populations that shared restriction sites with G4 and PKO1. To examine whether these strains were present, we used the species-specific oligonucleotide primers for these strains for PCR amplification. Samples from time zero showed amplification products for these species-specific primers, but bands for G4 and PKO1 were not

TABLE 2. Description and comparison of toluene growth kinetic coefficients of inoculum strains and dominant GAC-FBR isolates

Strain	Reactor[a]	Description	Substrate range	Mean μ_{max} (h^{-1}) (SD)	V_{max} (mg of toluene/10^{10} cells/h)	K_m (mg of toluene/liter)	V_{max}/K_m
G4		*Burkholderia cepacia*	B, T	0.22 (0.04)	0.061	0.44	0.14
PKO1		*Pseudomonas pickettii*	B, T	0.16 (0.03)	0.010	0.20	0.49
PaW1		*P. putida*	T, X	0.40 (0.08)	0.52	0.13	4.0
GF161	1, 2, 3, 4	*Pseudomonas* spp.[c]	B, T, X	0.33 (0.07)	0.72	0.07	9.7
GF261	1, 2, 3, 4	β subdivision[b]	B, T, X	0.10 (0.02)	0.40	0.10	4.1
GF262	1, 2, 3, 4	γ subdivision[b]	B, T, X	0.25 (0.05)	1.6	0.09	17.0
GF281	1, 2, 3, 4	α subdivision[b]	B, T, X		0.26	0.10	2.6
GF272	1	*Pseudomonas* spp.[c]	B, T	0.52 (0.10)	2.1	1.7	1.2
GF1TP	2	*Pseudomonas* spp.[c]	T, X	0.38 (0.08)	1.2	0.19	6.3
GF2TY	1	*Aeromonas* spp.[c]	B, T	0.44 (0.09)			

[a] Reactor from which the isolates, or isolates with the same REP profile, were obtained: 1, TOl.S; 2, BTX.S; 3, TOL.NS; 4, BTX.NS. Isolates for reactor 1 were taken only after filter removal.

[b] Based on 16S rRNA sequence analysis.

[c] Based on the FAME microbial identification system.

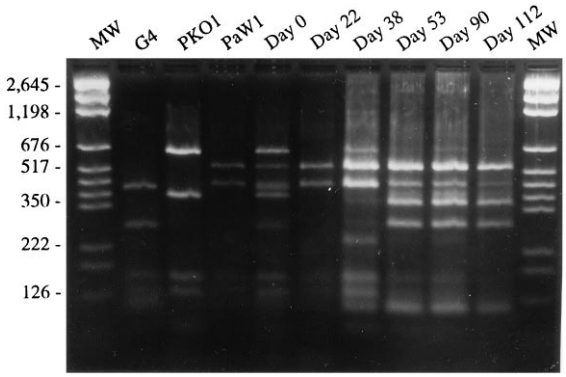

FIG. 5. ARDRA patterns of a bioreactor seeded with bacterial strains G4, PKO1, and PaW1. The filtration system was removed on day 53. Shown is an *Hpa*II digestion of the amplified 16S rDNA from reference cultures (lanes G4, PKO1, and PaW1) and the GAC community on days 0, 22, 38, 53, 90, and 112. The sizes of marker DNA fragments (lanes MW, in base pairs) are indicated on the left.

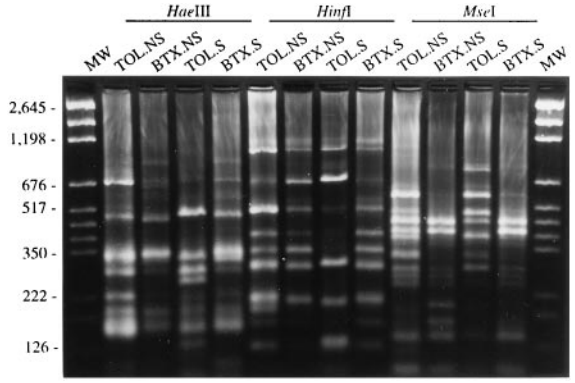

FIG. 7. ARDRA patterns from mature GAC communities digested with *Hae*III, *Hinf*I, and *Mse*I. The lane designations correspond to the bioreactor treatments. The sizes of marker DNA fragments (lanes MW, in base pairs) are indicated on the left.

detected on day 22, 38, 53, 90, or 112, confirming that the inoculated strains were not successful colonizers at these stages.

To determine whether position within the bioreactor treatment column led to differences in bacterial composition, samples collected from the bottom, middle, and top of the GAC bed were subjected to ARDRA (Fig. 6). Identical patterns were observed at all of these heights with all three endonucleases tested. Hence, we assumed that routine sampling at the bottom reflects the populations throughout the column.

The composition of mature GAC communities in all FBRs was also compared by ARDRA (Fig. 7). Similar DNA patterns were observed between the seeded (TOL.S) and naturally colonized (TOL.NS) toluene-fed reactors and between the seeded (BTX.S) and naturally colonized (BTX.NS) BTX-fed reactors with each of the three endonucleases. The toluene- and BTX-fed communities seem to show some differences from each other, as was particularly noted with the *Mse*I restriction endonuclease digestion, in which two signature fragments of approximately 460 and 410 bp were more apparent in the two BTX-fed reactors. A thorough evaluation of faint bands as well as the comparison by other enzymes showed no band pattern differences between the systems fed with the two substrates. ARDRA band sizes from the reactor DNA corresponded well to ARDRA band sizes of isolates (Fig. 3). Summing the data from three restriction enzymes, 37 total bands from community DNA matched 34 bands from the 12 isolates and 25 bands from the dominant 4 isolates. Hence, the isolates appeared to represent the community well.

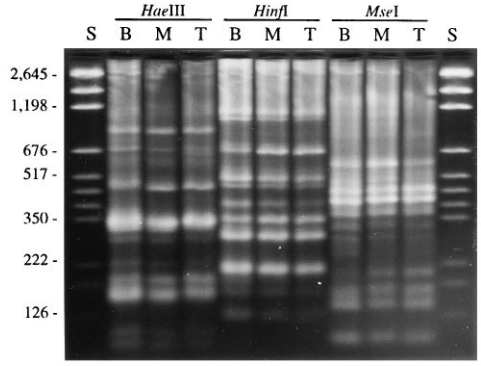

FIG. 6. ARDRA patterns from the BTX.S reactor on day 159. Samples correspond to the 20, 60, and 90% reactor column bed height (lanes B, M, and T respectively) digested with *Hae*III, *Hinf*I, and *Mse*I. The sizes of marker DNA fragments (lanes S, in base pairs) are indicated on the left.

FAME community analysis. The reactor community composition was also compared by FAME profile analysis (Table 3). At least 22 fatty acids were identified among the different bioreactor treatments. Fatty acids 16:1 ω7c and 18:1 ω7c/ω9t/ω12t have been reported to be characteristic of the genus *Pseudomonas* (11). Significant amounts of both of these fatty acids were observed in all FBR systems. Some fatty acids were

TABLE 3. Relative abundance of fatty acids of the GAC-FBR communities

Fatty acid[a]	Relative abundance of fatty acid (% of total)[b] in:				BTX.NS (day 22)	BTX.S (day 151)
	Day 48	Day 98	Day 53	Day 129		
10:0	0	2.31	0	1.69	0	2.49
10:0 3OH	3.28	5.94	2.84	1.64	6.37	7.75
12:0	3.91	21.62	1.59	21.11	4.35	25.07
12:0 2OH	0.75	1.61	1.91	0.85	0	5.12
12:0 3OH	0.91	1.00	1.42	0.65	1.99	3.99
14:0	2.20	8.24	1.02	9.43	0	7.68
i15:0	0	1.39	1.24	0	2.55	0
15:0	0	0.53	0	0.69	0	0
14:0 2OH	2.45	0.82	5.31	2.93	0	0
16:1 ω7c	44.21	28.24	30.37	23.68	47.52	21.34
i15:0 2OH, 16:1 ω7t[c]	0	0	0	0	14.04	8.76
16:0	15.60	11.74	7.12	9.64	17.43	9.85
17:1 ω6c	0	0	2.64	0.50	0	0
17:0 CYCLO	0	1.51	0	0	0	2.37
i16:0 3OH	0	0	1.13	0.85	0	0
18:2 ω6, 9c, a18:0[c]	0	1.94	0	0.55	0	0
18:1 ω9c	0	1.11	0	0	0	0
18:1 ω7c, ω9t, ω12t[c]	25.66	8.58	41.22	22.63	5.74	5.57
18:0	0	1.49	0	1.85	0	0
20:4 ω6, ω9, ω12, ω15c[c]	0	1.24	0	0	0	0
20:0	1.03	0	2.18	0.69	0	0

[a] Fatty acids are designated by the number of carbon atoms, number of double bonds, and position relative to the aliphatic (ω) end of the molecule; prefixes i and a refer to iso and anteiso branching, respectively; CYCLO and OH indicate cyclopropane and hydroxyl substitutions, respectively.

[b] Data are means of replicate samples.

[c] A group that MIDI-FAME does not reliably separate.

detected in only a particular treatment, for example, 14:0 2OH in toluene-fed systems and i15:0 2OH/16:1 ω7t in BTX-amended reactors. There is also a trend of a higher percentage of fatty acid 12:0 in older samples regardless of the carbon source. Although this fatty acid can be found at low levels in some *Pseudomonas* species (11), it has been generally associated with microeukaryotic organisms (20). Yeasts and protozoans were observed by microscopy in these biofilms at older stages (16).

DISCUSSION

A prominent concept in microbiology has been to inoculate a habitat with a strain that has desired properties to initiate or enhance a desired process. An equally prominent fact is that it is usually difficult to establish the inoculated population. One approach to enhance the success of inoculation is to preemptively colonize a new habitat so that the invading strains have less chance to gain a foothold. We evaluated this approach by using FBR reactors, initially evaluating the competitive outcome among three inoculated strains and subsequently evaluating their competitiveness against invaders from the groundwater. The competitive rank of the three strains was PaW1 > G4 > PKO1, which correlated with their specific growth rates and competitive coefficients for toluene uptake, V_{max}/K_m ratio, indicating that these traits were a consistent (but perhaps incomplete) explanation. These results are particularly interesting for evaluating whether the superior TCE-cooxidizing strain, G4, would be competitive in an FBR fed with toluene. The fact that strain PaW1, which is totally inactive on TCE, won the competition suggests that it would be difficult to maintain G4 in such a reactor.

Challenges to the established PaW1 biofilm occurred in two stages; the first resulted from strains breaching the filter system after day 35, and the second occurred after the filter was removed. In both cases, the invading strains were more competitive than the former community, resulting in a succession of dominant community types. The new inocula appeared to rapidly dominate, suggesting that the established community was not very resistant to the invaders. The invaders did generally have higher competitive coefficients for toluene than the original inocula. Particularly important was the decrease in toluene effluent concentration from 0.14 to 0.063 mg/liter after the filter was removed. This suggests that the indigenous groundwater populations are much better suited to scavenging low toluene concentrations than are the inocula or early invaders. The groundwater population came from deep wells under university farm property, which has no record of prior petroleum exposure or measurable petroleum content. Hence, bacteria with diverse toluene use kinetics probably reside in aquifer communities, even in pristine sites. The poor competitiveness of the inocula, which are extensively studied laboratory strains, may originate from the fact that these strains, as well as most toluene-degrading laboratory strains, were isolated under saturating toluene concentrations (~600 mg/liter) and hence may have particularly poor competitive coefficients for toluene concentrations found in typical pump-and-treat operations. The constant high flux of inocula from groundwater (2×10^6 cells/min), of which a significant number are probably toluene degraders (1), may also explain why the invaders so rapidly overgrew the established community. The density of invaders is known from ecology studies to be an important factor in determining the success of a founding population (23). Hence, the high inoculum flux from groundwater suggests that it would be very difficult to maintain an inoculum in any flowthrough reactor if the influent contained a reasonable diversity of organisms that could grow on the primary substrate(s).

Populations in all reactors converged to the same climax community irrespective of seeding, time of start-up, or particular BTX substrate as indicated by ARDRA, isolate studies, and FAME analyses. The reactors fed the same substrates, i.e. TOL.S and TOL.NS versus BTX.S and BTX.NS, were particularly similar. The reactors fed the different substrates had the same members, but their relative ratios were probably shifted since both ARDRA band intensities and particular fatty acids varied in amounts. The four dominant isolates degraded all three BTX substrates and were found in the reactors fed toluene only, showing that these reactors had the genetic capacity to immediately metabolize benzene and xylene and hence did not need to shift community composition. The finding of a common climax community among these treatments and over time suggests that a rather stable community was selected and was unlikely to be displaced by operational problems such as shock-loading and short-term periods of anoxia. Indeed, three failures in the O_2 feed caused 24- to 48-h periods of anoxia, but no shifts in ARDRA patterns or substrate removal was noticed once the O_2 supply was restored.

Current ecological theories of competition are based heavily on the concept of competitive exclusion and the assumption that communities exist at competitive equilibrium (10). Therefore, success or survival is based mainly on the physiological fitness of each competitor in a stable, uniform environment. Since competitive equilibrium requires that the rates of change of all competitors be zero, and since the physical environment, predation, and other factors are changing constantly, equilibrium rarely occurs in nature (14, 25). This reactor, however, was operated under conditions seemingly much more constant than nature, yet some diversity was apparent in the climax community. The particles slowly migrate up and down the column due to microbial growth decreasing the density and to shearing of excess cells and sloughing of dead cells increasing the density. The net effect is a gradual exposure, on a temporal basis, of each GAC-biofilm complex to changes in both substrate and O_2 concentration (Fig. 1). A change in location will represent a mild disturbance or regeneration of a niche, which may be advantageous for a given group; hence, a state of nonequilibrium and coexistence of trophic equivalent species is maintained.

It is also important to note that a biofilm can serve as a refugium for a competitively inferior population. Strain PaW1 was isolated from a 113-day-old film more than 60 days after it had been displaced as a dominant member of the community. The complex structure of this biofilm, as shown by confocal laser-scanning microscopy (16), helps explain how biofilms can be refugees as well as show niche heterogeniety.

Finding a member of the alpha, beta, and gamma (true *Pseudomonas*) *Proteobacteria* groups among the four most dominant organisms suggests that all *Proteobacteria* subdivisions have effective competitors for toluene. The isolates for the beta and gamma groups appear to be similar to the well-known biodegraders *Comamonas* and *Pseudomonas putida*, respectively. The alpha *Proteobacteria* isolate is associated with the *Sphingomonas* group, and its nearest relative was *Erythrobacter*, which is found on the surface of high-tide seaweeds and sand and in surface seawater (21).

The ARDRA approach greatly simplifies the characterization of communities over previous rRNA approaches based on analyzing individual clones of PCR-amplified 16S rRNA libraries (19), because the information is obtained in one or a few reactions. The limitations of the community ARDRA method, compared with analysis of clone libraries, is the lack of sensi-

tivity for detection of nondominant members, the limited resolving power of band sizes on agarose gels, and the lack of quantitative information. From a practical point of view, however, the complexity of the community-level pattern can be controlled by the cutting frequency of the restriction endonuclease used. This feature allowed us to quickly screen and characterize cultures at different time points as well as to recognize if strains were missing from the isolate collection. The correlation between fingerprints determined from GAC samples and those determined for isolated strains was very good. The ARDRA analysis with three enzymes represents a moderate level of genetic resolution, and it must be recognized that considerable phenotypic and strain diversity could still be present in different organisms that have the same bands.

In this study, we used community-level DNA fingerprint, isolate, and FAME studies to characterize competition, the resulting succession, and eventually community stability in GAC-FBRs. Particularly important were the poor competitiveness of the laboratory selected inocula; the convergence to a stable community irrespective of inocula, time, or BTX substrate; and the success of strains with a variety of kinetic parameters in what might have been thought to be a highly selectively constant environment.

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REFERENCES

1. **Aelion, C. M., and P. M. Bradley.** 1991. Aerobic biodegradation potential of subsurface microorganisms from a jet fuel-contaminated aquifer. Appl. Environ. Microbiol. **57:**57–63.
2. **Costerton, J. W., K.-J. Cheng, G. G. Geesey, T. I. Ladd, J. C. Nickel, M. Dasgupta, and T. J. Marrie.** 1987. Bacterial biofilms in nature and disease. Annu. Rev. Microbiol. **41:**435–464.
3. **De Bruijn, F. J.** 1992. Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intrageneric consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. Appl. Environ. Microbiol. **58:**2180–2187.
4. **Devereaux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
5. **Duetz, W. A., C. de Jong, P. A. Williams, and J. G. van Andel.** 1994. Competition in chemostat culture between *Pseudomonas* strains that use different pathways for the degradation of toluene. Appl. Environ. Microbiol. **60:**2858–2863.
6. **Ensley, B. D.** 1991. Biochemical diversity of trichloroethylene metabolism. Annu. Rev. Microbiol. **45:**283–299.
7. **Felsenstein, J.** 1989. PHYLIP—phylogeny inference package (version 3.2). Cladistics **5:**164–166.
8. **Folsom, B. R., P. J. Chapman, and P. H. Pritchard.** 1990. Phenol and trichloroethylene degradation by *Pseudomonas cepacia* G4: kinetics and interactions between substrates. Appl. Environ. Microbiol. **56:**1279–1285.
9. **Franklin, F. C. H., M. Bagdasarian, M. M. Bagdasarian, and K. N. Timmis.** 1981. Molecular and function analysis of the TOL plasmid pWWO from *Pseudomonas putida* and cloning of genes for the entire regulated aromatic ring meta cleavage pathway. Proc. Natl. Acad. Sci. USA **78:**7458–7462.
10. **Fredrickson, A. G., and G. Stephanopoulos.** 1981. Microbial competition. Science **213:**972–979.
11. **Haack, S. K., H. Garchow, D. A. Odelson, L. J. Forney, and M. J. Klug.** 1994. Accuracy, reproducibility and interpretation of fatty acid methyl ester profiles of model bacterial communities. Appl. Environ. Microbiol. **60:**2483–2493.
12. **Harayama, S., M. Rekik, M. Wubbolts, K. Rose, R. A. Leppik, and K. M. Timmis.** 1989. Characterization of five genes in the upper-pathway operon of *tol* plasmid pWWO from *Pseudomonas putida* and identification of the gene products. J. Bacteriol. **171:**5048–5055.
13. **Kaphammer, B. J., J. J. Kukor, and R. H. Olsen.** 1991. Characterization of a novel toluene degradative pathway from *Pseudomonas pickettii* PKO1, p. 571–572. *In* H. W. Rossmore (ed.), Biodeterioration and biodegradation. Elsevier Applied Science, London, United Kingdom.
14. **Levin, B. R.** 1972. Coexistence of two asexual strains on a single resource. Science **175:**1272–1274.
15. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
16. **Massol-Deyá, A., J. Whallon, R. F. Hickey, and J. M. Tiedje.** 1995. Channel structures in aerobic biofilms of fixed-film reactors treating contaminated groundwater. Appl. Environ. Microbiol. **61:**769–777.
17. **Massol-Deyá, A., D. A. Odelson, R. F. Hickey, and J. M. Tiedje.** 1995. Bacterial community fingerprinting of amplified 16S and 16-23S ribosomal DNA gene sequences and restriction endonuclease analysis (ARDRA), p. 3.3.2:1–3.3.2:8. *In* A. D. L. Akkermans, J. D. van Elas, and F. J. de Bruijn (ed.), Molecular microbial ecology manual. Kluwer Academic Publishers, Dordrecht, The Netherlands.
18. **Owens, J. D., and R. M. Keddie.** 1969. The nitrogen nutrition of soil and herbage coryneform bacteria. J. Appl. Bacteriol. **32:**338–347.
19. **Schmidt, T. M., E. F. DeLong, and N. R. Pace.** 1991. Analysis of a marine picoplankton community by 16S rRNA gene cloning and sequencing. J. Bacteriol. **173:**4371–4378.
20. **Shaw, R.** 1966. The polyunsaturated fatty acids of microorganisms. Adv. Lipid Res. **4:**107–174.
21. **Shiba, T., U. Simidu, and N. Tagan.** 1979. Distribution of aerobic bacteria which contain bacteriochlorophyll α. Appl. Environ. Microbiol. **38:**43–45.
22. **Ulrike, E., T. Rogall, H. Blocker, M. Emde, and E. C. Bottger.** 1989. Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. Nucleic Acids Res. **17:**7843–7853.
23. **Wagner, M., R. Erhart, W. Manz, R. Amman, H. Lemmer, D. Wedi, and K.-H. Scheifer.** 1994. Development of an rRNA-targeted oligonucleotide probe specific for the genus *Acinetobacter* and its application for in situ monitoring in activated sludge. Appl. Environ. Microbiol. **60:**792–800.
24. **Warren, T. M., V. Williams, and M. Fletcher.** 1992. Influence of solid surface, adhesive ability, and inoculum size on bacterial colonization in microcosm studies. Appl. Environ. Microbiol. **58:**2954–2959.
25. **Wiens, J. A.** 1983. Competition or peaceful coexistence? Nat. History **3:**30–34.
26. **Zhou, J.-Z., M. R. Fries, J. C. Chee-Sanford, and J. M. Tiedje.** 1995. Phylogenetic analysis of a new group of denitrifiers capable of anaerobic growth on toluene and description of *Azoarcus tolulyticus* sp. nov. Int. J. Syst. Bacteriol. **45:**500–506.
27. **Zimmerman, R., R. Iturriaga, and J. Becker.** 1978. Simultaneous determination of the total number of aquatic bacteria and the number thereof involved in respiration. Appl. Environ. Microbiol. **36:**926–935.
28. **Zylstra, G. J., and D. T. Gibson.** 1991. Aromatic hydrocarbon degradation: a molecular approach. Gen. Eng. **13:**183–203.