BIODEGRADATION DURING CONTAMINANT TRANSPORT IN POROUS MEDIA: 7. IMPACT OF MULTIPLE-DEGRADER COMMUNITY DYNAMICS

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Abstract—The biodegradation and transport of phenanthrene in porous media containing multiple populations of phenanthrene degraders is examined with a series of miscible-displacement experiments. A long-term experiment was conducted with a soil containing an indigenous microbial community comprised of 25 identified phenanthrene-degrading isolates. The rate and magnitude of phenanthrene biodegradation oscillated throughout the six-month experiment. This behavior, at least in part, is attributed to multiple-population dynamics associated with the indigenous community of phenanthrene degraders, the composition of which changed during the experiment. This hypothesis is supported by the results of experiments conducted using sterilized porous media that were inoculated with selected isolates obtained from the indigenous soil community. The results of experiments conducted with sterilized soil inoculated with isolate A exhibited an initial extended period of steady phenanthrene effluent concentrations, followed by a uniform decline. The results of experiments conducted using sterilized sand for single-isolate systems with one of three selected isolates and for systems of two-isolate combinations, indicate the existence of apparent synergistic and antagonistic interactions among the isolates. For example, phenanthrene biodegradation was relatively extensive and occurred without a lag phase for isolate A alone. However, biodegradation was constrained when isolate A and B were combined, indicating an antagonistic interaction. Conversely, whereas extensive lag phases were exhibited by both isolates B and C for the single-isolate experiments, there was minimal lag when isolates B and C were combined, indicating a synergistic interaction.

Keywords—Biodegradation Transport Microbial community

INTRODUCTION

Subsurface environments typically contain complex microbial communities comprised of multiple populations of bacteria and other microorganisms. Thus, it is reasonable to expect that more than one member of the community may be capable of degrading a given solute. It also is reasonable to expect that each population will have its own affinity and utilization rate for that solute. Furthermore, the presence of multiple populations affords the opportunity for interpopulation interactions, such as competition, cooperation, gene transfer, and predation.

For environmental systems, the use of multiple microbial populations (i.e., mixed cultures) is prevalent for wastewater treatment applications. The influence of interactions among populations on biodegradation activity has been examined in these systems (e.g., [1–5]). Recently, investigators have begun to examine temporal variability of microbial community composition for petroleum hydrocarbon–contaminated systems [6–11]. The results of this research show that the diversity and composition of degrader communities changed over time in the systems studied. The potential impact of microbial community dynamics on coupled biodegradation-transport of contaminants in subsurface environments has received limited attention to date. The purpose of this study was to investigate the influence of multiple populations of degraders on biodegradation and transport of organic compounds in porous media.

MATERIALS AND METHODS

Materials

Phenanthrene (purity > 98%) and pentafluorobenzoic acid ([PFBA]; >99%) were purchased from Aldrich Chemical (Mil-

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Microcosm experiments.

For phenanthrene, which was confirmed by the results of batch limited number of effluent fractions. These three were selected from the soil at the end of the experiment. Isolate B was recovered from effluent fractions throughout the experiment, as well as from the indigenous community. The three isolates representing 19 genera, were described.

Several experiments were conducted using isolates collected from the indigenous community. The three isolates selected, labeled A, B, and C, are noted in Table 1. The selection of these isolates was based on their differing recoveries in the miscible-displacement experiment conducted with the indigenous community. Isolate A was recovered from column effluent fractions throughout the experiment, as well as from the soil at the end of the experiment. Isolate B was recovered from a limited number of effluent fractions and from the soil at the end of the experiment. Isolate C was recovered only from a limited number of effluent fractions. These three were selected under the assumption that they would have different affinities for phenanthrene, which was confirmed by the results of batch microcosm experiments.

**Batch microcosm experiments**

Batch experiments were conducted to characterize phenanthrene biodegradation activity for the three isolates. These experiments used three separate methods, measuring phenanthrene loss, mineralization, and cell density. Direct analysis of phenanthrene utilization was measured using aqueous solutions of phenanthrene (1 mg/L) and mineral salts inoculated with 10^7 colony forming units (CFU)/ml of the selected isolate. The solutions were contained in Erlenmeyer flasks and placed on a rotary shaker (200 rpm) for 72 h. One-milliliter samples were collected periodically and analyzed for phenanthrene. The experiment was conducted in triplicate and was repeated.

Mineralization of phenanthrene was characterized by measuring CO₂ evolution. Specially modified 250-ml Erlenmeyer flasks designed to connect to a gas-flushing manifold were used [13]. The inside bottom of each flask was coated with phenanthrene spiked with radiolabeled phenanthrene-9-14C to provide excess substrate. Twenty-five milliliters of mineral salts solution was added. The flasks then were inoculated with the selected isolate (~10^6 CFU/ml) and flushed periodically to collect CO₂, which was analyzed using liquid scintillation counting. The experiment was conducted for 80 h and was conducted in triplicate.

Population growth was measured by monitoring changes in cell density. Erlenmeyer flasks (250-ml) were used, and the inside bottom of each flask was coated with phenanthrene to provide excess substrate. Mineral salts solution was added to the flasks, which then were inoculated with the selected isolate (~10^5 CFU/ml). After 14 d, samples were diluted serially and plated to determine final cell density. The experiment was conducted in duplicate.

**Miscible-displacement experiments**

The apparatus and methods employed for the miscible-displacement studies were similar to those used previously in our laboratory (e.g., [14,15]). Precision-bore stainless steel chromatography columns (Alltech Associates, Deerfield, IL, USA), with inner diameters of 2.1 cm and length of 15.0 cm, were sterilized (autoclaved) before use. Stainless steel distribution plates and stainless steel porous plates (0.5-μm nominal pore diameter) were used at both ends of the column to help ensure uniform flow and to serve as porous-medium supports. In addition, all tubing and fittings were stainless steel to minimize sorption of phenanthrene. The system (fittings, tubing, pump head) was sterilized with 2% bleach solution before each experiment. The disinfectant was neutralized by flushing with 0.01% sodium thiosulphate that was followed by flushing with sterilized, distilled water. All solutions were filter-sterilized before use and placed in closed containers to prevent contamination during the experiments.

Each column was packed with porous media in a manner to obtain a uniform bulk density (soil ~ 1.4 g/cm³, sand ~ 1.65 g/cm³) and porosity (soil ~ 0.4, sand ~ 0.37). The packed columns were wetted slowly from the bottom with electrolyte solution to establish saturation. The column was connected to a single-piston liquid chromatography pump (Scientific Systems, SSI Model 300, State College, PA, USA) to generate steady state flow. The hydrodynamic characteristics of the packed columns were determined by conducting tracer tests with sterile PFBA solutions (250 mg/L). Phenanthrene solution (1.2 ± 0.1 mg/L) then was injected to initiate the biodegradation-transport experiment.

Four sets of column experiments were conducted. The first was conducted using sterile soil to examine the influence of sorption and potential abiotic transformation processes on phenanthrene transport. A 390 pore-volume pulse of sterile phenanthrene solution was applied, followed by a 1530 pore-volume pulse of sterile 0.01 N CaCl₂ solution. The flow rate was 1.86 ml/min, which is equivalent to a pore water velocity of 67 cm/h and a hydraulic residence time of 0.2 h.

The second experiment employed nonsterilized mixed soil that contained an indigenous phenanthrene-degrading community. The initial cell density of culturable phenanthrene degraders was 7.4 × 10⁶ CFU/g of dry soil. Phenanthrene was injected into this system continuously for six months (equivalent to 11,648 pore volumes) to investigate the influence of the multiple indigenous populations of phenanthrene degraders on phenanthrene biodegradation and transport. Dissolved oxygen in the influent and effluent was monitored by use of an oxygen meter (Microelectrodes, Model OM-4, Bedford, NH, USA) equipped with a MI-730 micro-oxygen electrode. The flow rate and residence time were similar to those of the sterile experiment.

The third set of experiments was conducted using sterilized mixed soil that was inoculated with a culture of isolate A (Table 1). Two experiments were conducted, one with more than 2,000 pore volumes of phenanthrene solution input and one with approximately 6,000 pore volumes of input. The soil was inoculated targeting an initial cell density of approximately 10⁵ CFU/g dry soil, similar to the initial cell density of isolate A.

<table>
<thead>
<tr>
<th>Study ID</th>
<th>Strain/accession no.</th>
<th>Nearest relative (accession no.; % homology)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>11/AY177359</td>
<td><em>Acinetobacter junii</em> DSM 6964 (AJ16SRRND; 99%)</td>
</tr>
<tr>
<td>B</td>
<td>11/AY177351</td>
<td><em>Methyllobacterium</em> sp. A4 (AF361189; 99%)</td>
</tr>
<tr>
<td>C</td>
<td>M13/AY177356</td>
<td><em>Pseudomonas oleovorans</em> (PSE1AM17; 99%)</td>
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in experiment 2 (indigenous system). Soil subsamples collected during column packing, as well as column influent and effluent, were checked for bacterial contamination. The flow rate and residence time were similar to those of experiments 1 and 2.

The fourth set of experiments was conducted using sterile sand inoculated with one or two of the three selected phenanthrene-degrading isolates obtained from the indigenous community associated with the mixed soil (Table 1). Cultures of the three isolates were used to inoculate sterilized sand, which then was packed into the sterilized column. The inoculations produced initial cell densities of approximately 10^7 CFU/g of dry soil, similar to the initial cell density for culturable phenanthrene degraders for experiment 2. Sand subsamples collected during column packing, as well as column influent and effluent, were assessed for bacterial contamination. These shorter-term (~400 pore volume) experiments were conducted at a flow rate equivalent to a pore-water velocity of 9 cm/h (hydraulic residence time of 1.67 h). The single-isolate experiments were conducted in triplicate, and the two-isolate experiments were conducted in duplicate.

Chemical and microbial analysis

Effluent samples were collected during all four sets of experiments to monitor phenanthrene concentration and microbial cell densities. At the completion of each experiment, subsamples of the porous medium were collected and subjected to solvent extraction to determine residual phenanthrene concentrations. In addition, subsamples of porous media were collected during packing of the columns and after the completion of each experiment to measure solid-phase cell densities.

Effluent samples collected during the miscible-displacement experiments were analyzed for phenanthrene using fluorescence spectroscopy (Hitachi, Model F-2000, Tokyo, Japan), with an excitation wavelength of 250 nm and an emission wavelength of 365 nm (quantifiable detection limit = 1 µg/L). Preliminary analyses indicated no measurable interferences from possible metabolites. A flow-through, variable-wavelength ultraviolet-visible spectrophotometer (Gilson, Model 115, Middleton, WI, USA) was used to continuously monitor the PFBA effluent concentration at a wavelength of 243 nm. The effluent concentrations were normalized by the injection concentration to obtain relative concentrations, which was plotted against pore volume (discharge normalized by column capacity) to produce breakthrough curves.

Following each experiment, six 1-g subsamples of porous media were removed aseptically from uniform intervals along the column axis for microbial enumeration. These samples were dilution plated on phenanthrene–mineral salts plates. The samples were plated in triplicate and enumerated after 14 d of incubation at room temperature.

RESULTS AND DISCUSSION

PFBA and phenanthrene transport under sterile conditions

Breakthrough curves for PFBA transport in the packed columns are symmetrical and relatively sharp for all cases, indicating that the columns were packed homogeneously and that flow was ideal (data not shown). In contrast to PFBA, the breakthrough curve obtained for phenanthrene transport in the sterile soil is noticeably asymmetrical, suggesting that its transport is influenced by rate-limited sorption/desorption (data not shown). A large retardation factor, R = 362 (obtained from moment analysis), indicates that the soil possesses significant sorption capacity for phenanthrene, which presumably is due to the high organic carbon content of the soil. This large retardation resulted in a residence time of approximately 72 h for phenanthrene, compared to the hydraulic residence time of 0.2 h. Phenanthrene retardation was minimal (R ≈ 2.5) for the silica sand. Mass recovery for phenanthrene in the sterile systems essentially was 100%, indicating no measurable mass loss due to abiotic transformation reactions or other processes.

Phenanthrene biodegradation and transport in the indigenous multiple-population system

Breakthrough for phenanthrene transport in the column packed with the nonsterilized soil (Fig. 1) was much later (~2,000 pore volumes) than for the sterilized soil experiment (~200 pore volumes). It is possible that some of the difference in transport behavior observed between the two experiments is related to the impact of soil sterilization on sorption. For example, Dao et al. [16] found that autoclaving reduced the sorption of aniline and diuron by up to 94% for three soils with organic carbon contents similar to the soil used herein. However, the difference in sorption between the sterilized and nonsterilized soil would need to be greater than 500% if the phenanthrene-arrival differential solely was due to the impact of autoclaving. This great a difference does not seem likely. It also is possible that sorption of phenanthrene by the bacterial biomass may have contributed to the phenanthrene-arrival differential. However, the equivalent soil organic carbon fraction associated with the initial total biomass, based on a cell mass of 9.5 × 10^{-13} g [17], is approximately one-tenth of the mea-
sured soil organic carbon content. Thus, it is unlikely that biosorption of phenanthrene is the primary cause of the observed phenanthrene-arrival differential.

Considering sorption effects to be of minor importance, the significant delay in phenanthrene arrival observed for the nonsterilized experiment indicates that an extensive degree of biodegradation took place within the column, with minimal observable lag. This is supported by the results of the phenanthrene mass-balance calculations for the experiment. A total of 40.7 mg of phenanthrene was eluted in the column effluent, which is equivalent to 11.4% of the total mass applied to the column (355.2 mg). The results of the postexperiment soil extractions show that only a very small quantity of phenanthrene (1.6 mg, 0.5%) remained sorbed to the soil at the end of the experiment. The resulting mass loss (312.9 mg) is attributed to biodegradation. The overall mass balance indicates that approximately 88% of the input phenanthrene mass was biodegraded by the microbial community.

The phenanthrene effluent concentrations oscillated throughout the course of the experiment (Fig. 1). Phenanthrene first was detected in the effluent at pore volume 2,028. The concentration then oscillated through three cycles. The first, largest wave had the highest peak concentration and occurred from 2,028 to 4,680 pore volumes. The second wave had a slightly lower peak and spanned from 4,680 to 7,007 pore volumes. The third, smallest wave had the lowest peak and started at pore volume 7,007 and extended to approximately pore volume 8,600, after which the phenanthrene concentration remained undetectable throughout the completion of the experiment (11,648 pore volumes). This oscillation in effluent concentrations indicates that the rate and magnitude of phenanthrene biodegradation changed during the course of the experiment.

Dissolved oxygen concentrations in the column effluent were lower than the influent concentration, fluctuating between 4 and 8 mg/L, reflecting biodegradation activity. The oxygen concentration in the column effluent exhibited oscillatory behavior similar to that of phenanthrene, with a small temporal lag. This suggests that the variation in dissolved oxygen concentration was influenced by changes in activity of the phenanthrene degraders.

Elution of bacterial cells from the column was observed, averaging approximately $8 \times 10^5$ CFU/pore volume. The spatial distribution of culturable phenanthrene-degrader cell density in the column was nonuniform at the completion of the experiment, ranging from $2 \times 10^7$ at the influent end to $6 \times 10^5$ CFU/g dry soil at the effluent end. Such a distribution is typical of that found for miscible-displacement experiments (e.g., [18]). No significant increase in total biomass of phenanthrene degraders was observed.

Bodour et al. [12] examined the composition of phenanthrene degraders in the effluent and soil for this experiment. Of special note, analysis of aqueous- and soil-associated bacteria indicates that the composition of the phenanthrene-degrader community changed during the experiment. For example, certain isolates were detected only in soil samples analyzed before the experiment, some were detected only in the column effluent, and yet others were detected only in soil samples collected at the end of the experiment. These results suggest that different degrader populations were dominant at different points during the experiment. It is plausible that interpopulation interactions occurred under such conditions, which may have influenced the biodegradation activity of the system.

The temporal oscillating-concentration profile observed in Figure 1 indicates that the rate and magnitude of biodegradation changed during the course of the experiment, as noted above. Such behavior may be caused by a number of factors or processes: Global changes in substrate, electron acceptor, or nutrient supply; global changes in environmental conditions (pH, temperature); predation by other microorganisms (e.g., protozoans); inhibition/toxicity effects on metabolic activity; and interpopulation interactions (competition, cooperation, succession). For the experiments reported herein, the first two factors may be eliminated. Although the third factor cannot be ruled out completely for the nonsterile soil experiment (see this section), it can be for the sterilized/inoculated experiments to be presented below (see Phenanthrene biodegradation and transport in the inoculated systems section). These latter experiments were conducted to evaluate the potential existence of interpopulation interactions in this system.

Phenanthrene biodegradation and transport in the inoculated systems

Batch experiments were conducted to evaluate phenanthrene biodegradation activity of the three isolates. This was done using three separate measures: Direct analysis of phenanthrene loss, generation of CO$_2$ (mineralization), and production of biomass. The results of all three sets of batch experiments were consistent. Isolate A was shown to biodegrade phenanthrene appreciably with minimal lag. Conversely, isolates B and C exhibited extensive lag phases and degraded phenanthrene to a lesser extent. For example, isolate A mineralized 13% of the initial mass of phenanthrene during an 80-h period, whereas isolates B and C mineralized only 2 and 0.3%, respectively.

The results of the two miscible-displacement experiments conducted with sterilized soil inoculated with isolate A are shown in Figure 2. Breakthrough of phenanthrene occurs similarly to that observed for the experiment conducted with sterilized soil, and much earlier than for the experiment conducted with the nonsterilized soil containing the indigenous degrader community. In addition, the phenanthrene effluent concentrations are much higher for the single-isolate experiments, compared to those for the experiment conducted with the nonsterilized soil containing the indigenous degrader community.
These differences likely reflect the differences in the initial cell densities, as well as the influence of community composition.

The phenanthrene effluent concentration remains relatively constant for approximately 3,000 pore volumes for the single-isolate experiments. After this period of steady state behavior, the phenanthrene concentration begins to decline at a relatively uniform rate, indicating a significant increase in the rate and magnitude of biodegradation. This behavior is a result of long-term growth of the isolate A population, wherein a two order-of-magnitude increase in total cells occurred as determined from the cell mass balance (tabulation of total cells in the effluent and the initial and final soil-phase cell densities). The behavior observed for this system, which occurred in the absence of multiple-population dynamics, is in distinct contrast to that observed for the indigenous soil system.

The results of the experiments conducted with the sterilized silica sand inoculated with one of three selected isolates are illustrated in Figure 3. The results of the replicate experiments were very similar for each of the three isolates. The reproducibility of these inoculated experiments is illustrated by the data presented in Figure 4, which show the three replicate breakthrough curves for the experiments conducted with isolate A. Phenanthrene retardation was relatively minimal for these experiments conducted with the sand. Potential effects of autoclaving on sorption are unlikely to be significant for the sand. Ball and Roberts [19] report no measurable effect of autoclaving on sorption for a low organic carbon sandy porous medium. Biosorption also is unlikely of significance for the inoculated sand system, as the estimated equivalent biomass organic carbon content is 100 times lower than the measured organic carbon content of the sand.

Phenanthrene biodegradation was relatively extensive (~30% mass loss) and occurred without a lag phase for the experiments conducted with isolate A (Figure 3). Conversely, extensive lag phases were exhibited by both isolate B and C, wherein biodegradation did not commence until approximately 120 pore volumes. These results are very consistent with the results obtained from the batch experiments noted above (see this section). Once initiated, the rate of phenanthrene biodegradation essentially was constant for approximately 100 pore volumes (as indicated by constant effluent concentrations), after which the phenanthrene concentrations increased and remained constant until the end of the experiments. Given the controlled conditions of these experiments (constant phenanthrene, oxygen, and nutrient supply; use of silica sand), the rebound in phenanthrene effluent concentrations suggests that toxicity/inhibitory effects associated with production of intermediates may have influenced microbial activity.

The results of the experiments conducted with the sterilized silica sand inoculated with combinations of two of the three selected isolates are shown in Figure 5. The results of the duplicate experiments were very similar for each of the three sets. Recall that isolate A biodegraded phenanthrene appreciably and without lag for the single-isolate experiment. However, the activity of isolate A appears to be constrained by the presence of isolate B, with biodegradation exhibiting a lag phase and reduced magnitude (~7% mass loss) when isolate A and B were combined (Fig. 5A). These results suggest that an antagonistic interaction occurred between isolates A and B. Conversely, the coexistence of isolates B and C appeared to produce a synergistic effect, because there was minimal lag when isolate B and C were combined (compared to extensive lag phases for each one separately). Interestingly, biodegradation slowed considerably for the isolate B–C system after approximately 150 pore volumes, suggesting the advent of antagonistic or inhibitory conditions in the later stages of the experiment. The results of these experiments indicate the apparent existence of synergistic and antagonistic interactions among the isolates.

CONCLUSION

A study was conducted to examine the influence of multiple populations of phenanthrene degraders on biodegradation and transport of phenanthrene in soil. The breakthrough curve obtained for phenanthrene transport in a column packed with a soil containing an indigenous community of phenanthrene degraders exhibited oscillatory behavior. This behavior, at least in part, is attributed to the dynamics of the diverse multiple-population community of phenanthrene degraders associated with the soil, wherein biodegradation likely is mediated by competition, cooperation, and other multipopulation interactions. This hypothesis is supported by the observation that the composition of the phenanthrene-degrader community changed during the course of the indigenous-soil experiment. The hypothesis also is supported by the results obtained from the experiments conducted using sterilized porous media inoculated with selected isolates, which indicate the occurrence of both synergistic and antagonistic interactions among the three isolates examined. The results presented herein illustrate...
that the dynamics of multiple-degrader microbial communities can influence biodegradation and transport and, thus, should be considered when evaluating contaminant fate in subsurface systems.

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REFERENCES