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Repeated inoculation as a strategy for the remediation of low concentrations of phenanthrene in soil

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Abstract

Phenanthrene, a polycyclic aromatic hydrocarbon, becomes increasingly unavailable to microorganisms for degradation as it ages in soil. Consequently, many bioaugmentation efforts to remediate polycyclic aromatic hydrocarbons in soil have failed. We studied the effect of repeatedly inoculating a soil with a phenanthrene-degrading *Arthrobacter* sp. on the mineralization kinetics of low concentrations of phenanthrene. After the first inoculation, the initial mineralization rate of 50 ng/g phenanthrene declined in a biphasic exponential pattern. By three hundred hours after inoculation, there was no difference in mineralization rates between the inoculated and uninoculated treatments even though a large fraction of the phenanthrene had not yet been mineralized. A second and third inoculation significantly increased the mineralization rate, suggesting that, though the mineralization rate declined, phenanthrene remained bioavailable. Restirring the soil, without inoculation, did not produce similar increases in mineralization rates, suggesting absence of contact between cells and phenanthrene on a larger spatial scale (>mm) is not the cause of the mineralization decline. Bacteria inoculated into soil 280 hours before the phenanthrene was added could not maintain phenanthrene degradation activity. We suggest sorption lowered bioavailability of phenanthrene below an induction threshold concentration for metabolic activity of phenanthrene-degrading bacteria.

Introduction

Polycyclic aromatic hydrocarbons (PAH's) are suspected toxins that accumulate in soils and sediments due to their insolubility in water and lack of volatility (Pignatello & Xing 1996; Scow & Johnson 1997). In a process termed aging, sorbed PAH's become increasingly resistant to extraction and degradation (Kelsey et al. 1997; Schwartz & Scow 1999). PAH's may migrate into rigidly structured forms of organic matter, soot, or in between the clay bi-layer from which diffusion into the soil solution is extremely slow (Luthy et al. 1997). Exposure from contaminated ground water requires that phenanthrene partitions from resistantly sorbed into bioavailable compartments. Presently, risk associated with PAH exposure is based upon the total concentration in soil and not just the bioavailable fraction in soil even though the majority of aged PAH's are resistantly sorbed (Kelsey & Alexander 1997).

Remediation of PAH's in soil is difficult because only low concentrations of pollutant are bioavailable despite the presence of extensive contamination in the sorbed phase (Hughes et al. 1997). It is unclear if aged PAH's are permanently sorbed in soil or if they desorb very slowly, thereby replenishing the fraction of available PAH's. If the latter is the case, activity of pollutant degrading-organisms on low available substrate concentrations must persist for extended periods of time.

In one remediation approach, termed bioaugmentation, soil is inoculated with pollutant-degrading organisms. This strategy often fails, not because the inoculum is unable to degrade phenanthrene in soil, but because the pollutants are not available. One approach in bioaugmentation relies on a single inocula-

tion to establish the inoculum in the soil community. However, successful persistence of bacterial strains through inoculation into non-sterile soils is rare (Acea et al. 1988). The consequences of a successful invasion on other ecosystem processes also remain poorly understood. A second approach to bioaugmentation is to require degradation activity by the inoculum only briefly. Temporary activity of an inoculum is much easier to achieve and abates concerns for unintended bioaugmentation consequences, but may remediate only a small fraction of pollution. Repeated inoculation may promote degradation of more contamination, including aged pollution that is slowly desorbing. In this paper we investigate the feasibility of repeatedly inoculating a non-sterile soil with a phenanthrene-degrading bacterium in order to promote the degradation of low concentrations of phenanthrene in soil.

Materials and methods

Mineralization experiments

Forbes soil (3.96% organic carbon, pH 5.6, cation exchange capacity 14.0 meq/100g, 47% sand, 38% silt, 15% clay, 0.333 bar moisture = 40.3%, further described in (Scow et al. 1994)) was passed through a 2 mm sieve and stored in a 4 °C cold room. One week prior to the experiments, the soil was brought up to a moisture content of 0.222 bar or 26.3% and stored at room temperature. A mixture of ^{14}C-labeled (Sigma Chemical Co., St Louis MO, >98% purity, specific activity of 59 mCi/mmol) and non-isotopically labeled phenanthrene was added to 20 grams dry weight soil in 100 μl of methylene chloride. Phenanthrene was mixed into the soil with a spatula by hand for 2 minutes. The soil was incubated at 28 °C in an air-tight pint mason jar with a trap containing 1 ml of 0.5 N NaOH. Three replicate samples were prepared for each experimental treatment. The base was periodically sampled, and its radioactivity was measured in a liquid scintillation counter (Beckman Instruments, Inc., Fullerton, CA). The mason jar was opened during sampling to ensure sufficient oxygen remained available over the course of the incubation.

Arthrobacter, strain RP17

Strain RP17 was isolated by Mark Fuller from an organic soil in the Sacramento River Delta through enrichment culturing on phenanthrene. Strain RP17 can use phenanthrene as its sole carbon and energy source and mineralize it to CO_2. Partial sequencing of the 16S ribosomal gene (GenBank accession # AY005127), as well as phospholipid fatty acid analysis, showed that strain RP17 is an *Arthrobacter* sp. Its closest known relative, based on 16S rDNA comparison, is *Arthrobacter polychromogenes*.

Preparation of inoculum

Cells of *Arthrobacter* strain RP17 were cultivated on 50 ml mineral media (3.47 g/L KH_2PO_4, 4.27 g/L K_2HPO_4, 1.23 g/L $(NH_4)_2SO_4$, 0.46 g/L MgSO4, 17.6 mg/L $CaCl_2$, 1 mg/L $FeSO_4$, 3mg/L H_3BO_3, 2 mg/L $CoCl_2$, 1 mg/L $ZnSO_4$, 0.3 mg/L $MnCl_2$, 0.3 mg/L Na_2MoO_4, 0.2 mg/L $NiCl_2$, 0.1 mg/l $CuCl_2$, 250ng/L starch, 250 ng/L peptone, 250 ng/L yeast extract) containing phenanthrene as the major carbon source. The cells were harvested through centrifugation at 8,000 rpm for 10 minutes and were subsequently washed three times as follows. The cells were resuspended in 40 ml of phospho-saline buffer (PSB) (50mM potassium phosphates, 0.85% NaCl, pH 6.9) and centrifuged at 1,000 rpm for 5 minutes. Solids, such as phenanthrene crystals, pelleted at that point, but cells remained in suspension. The supernatant was transferred to a new centrifuge tube and spun, again, at 8,000 rpm for 10 minutes. After the final wash, the cells were resuspended in 2 ml of PSB. Dilutions were made from this suspension using PSB. The cell densities of the inocula were measured through dilution plating on 1/10 strength (3 g/L) tryptic soy agar plates.

Population density experiment

The indigenous microbial community of Forbes soil does not degrade phenanthrene rapidly, but the mineralization rate can be increased substantially through inoculation with strain RP17. Cells were added to Forbes soil at densities of: 4.3×10^7, 2.83×10^6, and 2.67×10^5 cfu/g dry soil. Bacteria were inoculated into soil in the mason jars in 100 μl volumes of PSB dilutions and stirred with a spatula by hand for 2 minutes to promote even distribution within the soil. Initial inoculation occurred 24 hours after the phenanthrene had been added to soil.

Phenanthrene mineralization potential experiment

To test whether the inoculum retained the ability to mineralize phenanthrene in the absence of phenanthrene, 2.83×10^6 cfu/g dry soil cells were added to

soil 280 hours before phenanthrene was added. Bacteria and phenanthrene were added to soil as described above.

Repeated inoculation of soil microcosms

Selected microcosms were reinoculated twice more, 306 and 497 hours after the first inoculation, with 4.3 $\times$ 10^7 cfu/g dry soil and 4.1 $\times$ 10^7 cfu/g dry soil respectively. As in the other experiments, the cells were delivered in 100 μl of phospho saline buffer and mixed into the soil for 2 minutes by hand with a spatula. To test if mixing in inoculating soils repeatedly affected phenanthrene degradation kinetics, 100 μl of PSB without cells was stirred into soil with a spatula by hand for 2 minutes. Remixing occurred 306 and again 497 hours after the inoculum was added to soil. Immediately prior to restirring, the phenanthrene mineralization rate of inoculated treatments was similar to uninoculated control soils.

Effect of phenanthrene concentration on mineralization kinetics

The relationship between the concentration of phenanthrene added to soil and the fraction mineralized was investigated by adding three different concentrations, 50 ng/g, 5 μg/g, and 500 μg/g, of phenanthrene to soil and inoculating it with 5.55 $\times$ 10^5 cfu/g soil 24 hours later. The cells and phenanthrene were added to soil as described above.

Measurement of fraction of phenanthrene that evolved as CO_2 in mineral media

The fraction of 20 ng/ml phenanthrene that evolved as CO_2 was determined in 50 ml of mineral media inoculated with 5.83 $\times$ 10^8 cfu of RP17. The experiment was performed twice, and both times three replicate samples were prepared for each experimental treatment. Mineralization curves were fit with first order, single exponential equations using Kaleidagraph (Synergy Software, Reading, PA). Cells from a 10-ml aliquot were pelleted through centrifugation at 8,000 rpm for 10 minutes and subsequently washed three times with PSB. The pellet was resuspended in one ml of methanol and mixed with 5 ml of scintillation cocktail. The radioactivity was measured in a liquid scintillation counter. Radioactivity present in the supernatant as well as the wash solution was measured. These results were used to calculate the ratio between the radioactivity stored in cells and the sum of the radioactivity stored in cells and that evolved as CO_2.

Results

The indigenous population in Forbes soil mineralized 50 ng/g phenanthrene very slowly (Figure 1). After 280 hours, only 1.8% of the phenanthrene was mineralized. By 306 hours after Forbes soil was inoculated with 4.3 $\times$ 10^7, 2.83 $\times$ 10^6, or 2.67 $\times$ 10^5 cfu strain RP17/g dry soil, 19.6, 4.7, and 2.0% of the added phenanthrene had mineralized respectively. Phenanthrene mineralization in microcosms to which 4.3 $\times$ 10^7 or 2.83 $\times$ 10^6 cfu/g dry soil were added were fit well with a double exponential decay curve. Mineralization kinetics in the uninoculated soil and soil inoculated with 2.67 $\times$ 10^5 cfu/g dry soil were best fit with a zero order equation. Mineralization rates in all treatments diverged most approximately 26 hours after inoculation, at which time the mineralization rate in soil inoculated with the highest cell density was 41 times greater than in uninoculated soil. After 279 hours, degradation rates were similar in all treatments: 3.8 $\times$ 10^{-4} %/g hr, 3.8 $\times$ 10^{-4} %/g hr, 5.2 $\times$ 10^{-4} %/g hr, and 1.3 $\times$ 10^{-3} %/g hr for the uninoculated, 2.7 $\times$ 10^5, 2.8 $\times$ 10^6, and 4.3 $\times$ 10^7 cfu/g treatments, respectively.

The second inoculation resulted in an immediate increase in the mineralization rates in all treatments (Figure 1). Within 32 hours after reinoculation, mineralization rates increased 7.1, 13.7, and 13.8 times in soils initially inoculated with 4.3 $\times$ 10^7, 2.83 $\times$ 10^6, and 2.67 $\times$ 10^5 cfu/g dry soil, respectively. The third inoculation, at 497 hours, increased the mineralization rate, 2.7, 2.6, and 3.0 times in microcosms initially inoculated with 4.3 $\times$ 10^7, 2.83 $\times$ 10^6,and 2.67 $\times$ 10^5 cfu/g dry soil, respectively. The maximum mineralization rate occurred 27 hours after the third inoculation, similar to what was observed after the 1st and 2nd inoculations. Whereas only 7.3% of phenanthrene was mineralized in microcosms initially inoculated with 2.83 $\times$ 10^6 cfu/g soil, 25.6% more was mineralized when the soil was inoculated two additional times.

Restirring the soil, inoculated with 2.83 $\times$ 10^6 cells, 306 and 496 hours after inoculation resulted in a small, but significant increase in the phenanthrene mineralization rate ($p < 0.015$) (Figure 2). The mineralization rate increased 38% after the first restirring and an additional 25% after the second restirring. Approximately 5.1% and 6.5% of the initially added

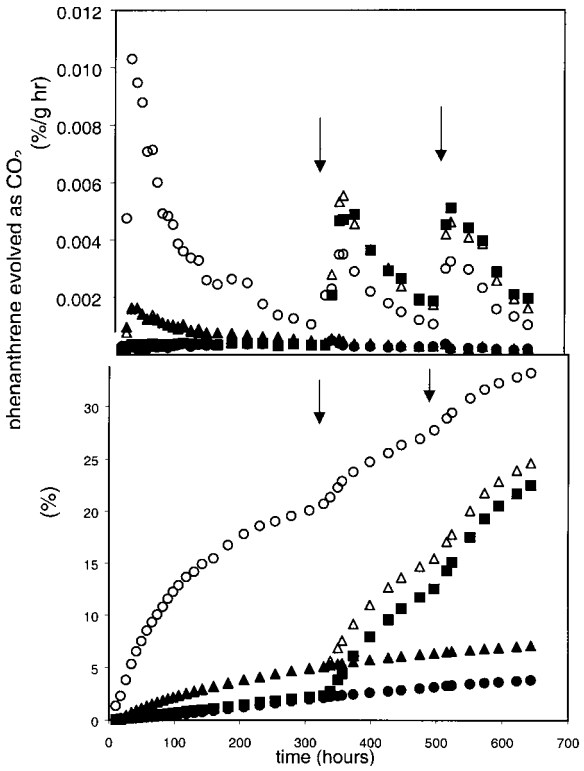

Figure 1. Phenanthrene mineralization rate (top) and cumulative mineralization (bottom) in soils inoculated with strain RP17 multiple times; 1st inoculation: 4.30×10^7 cfu/g, 2nd and 3rd inoculations: 4.10×10^7 cfu/g (○), 1st inoculation: 2.83×10^6 cfu/g, 2nd and 3rd inoculations: 4.10×10^7 cfu/g (△), 1st inoculation: 2.83×10^6 cfu/g, no further inoculations (▲), 1st inoculation: 2.67×10^5 cfu/g, 2nd and 3rd inoculations: 4.10×10^7 cfu/g (■), no inoculations (●). Arrows show times of 2nd and 3rd inoculations.

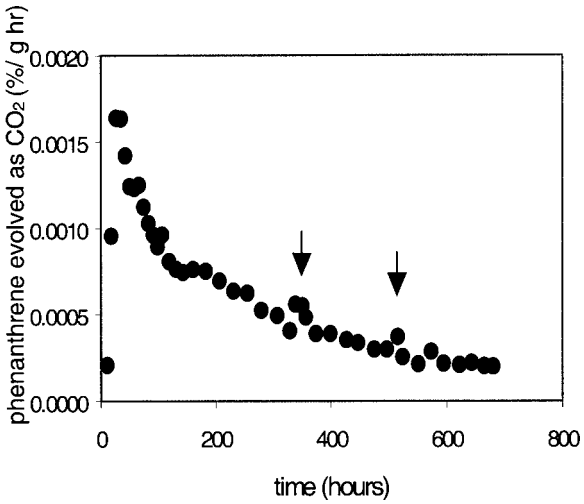

Figure 2. Phenanthrene mineralization rate of soil mixed 306 and 496 hours after 2.83×10^6 cells RP17/g soil were added. Arrows show times soils were mixed again.

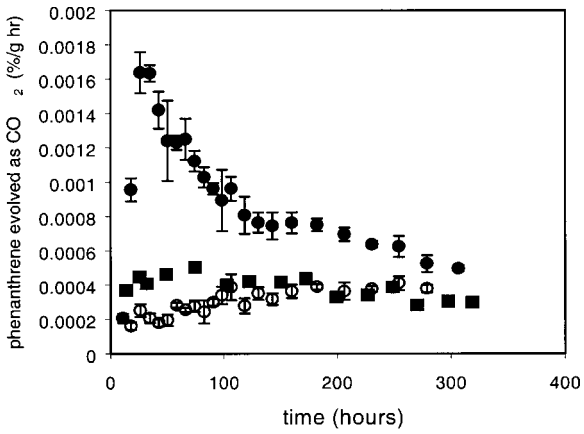

Figure 3. Phenanthrene mineralization rate of cells added to soil 280 hours before phenanthrene spike (■) in comparison to the mineralization rate of soil to which cells were added 24 hours *after* phenanthrene was added (●), uninoculated soil (○).

phenanthrene was mineralized by the first and second restirrings, respectively.

Arthrobacter strain RP17 cells did not maintain their full potential to mineralize phenanthrene 300 hours after being inoculated in Forbes soil (Figure 3). Twenty six hours after the phenanthrene was added, the mineralization rate in the soil to which 300 hours before 2.83×10^6 cfu/g cells were added was 0.00047 %/g hr, whereas in soil where 2.83×10^6 cfu/g cells were added 24 hours after the phenanthrene was added, the phenanthrene was mineralized at 0.001638 %/g hr.

Approximately 56.4% of the 500 μg/g phenanthrene added to Forbes soil and inoculated with 5.55×10^5 cfu/g RP17 was mineralized 425 hours after inoculation, whereas only 31.1% and 3.4% mineralized in microcosms containing 5 μg/g and 50 ng/g phenanthrene, respectively (Figure 4). The kinetics of mineralization of the three concentrations also differed. The mineralization rate in the 50 ng/g treatment remained relatively constant and was fit best by a zero order equation. The mineralization rate in soil spiked with 5 μg phenanthrene/g soil declined biphasically. The mineralization kinetics in the microcosms containing 500 μg/g phenanthrene showed a brief lag phase with a mineralization maximum not occurring until 200 hours after the cells were added to the soil and was subsequently followed by an exponential decline in the mineralization rate (Figure 5). Using a quantitative polymerase chain reaction method, Schwartz et al. (2000) showed that the lag phase was associated with growth of RP17.

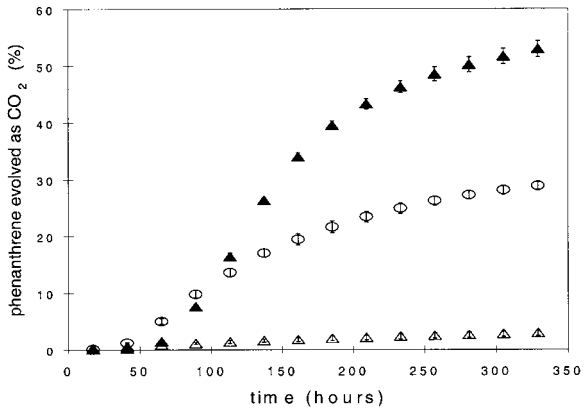

Figure 4. Cumulative phenanthrene mineralization in soil inoculated with 5.55×10^5 cfu/g *Arthrobacter*, strain RP17 and amended with phenanthrene: 500 μg/g phenanthrene (▲), 5 μg/g phenanthrene (○), 50 ng/g phenanthrene (△).

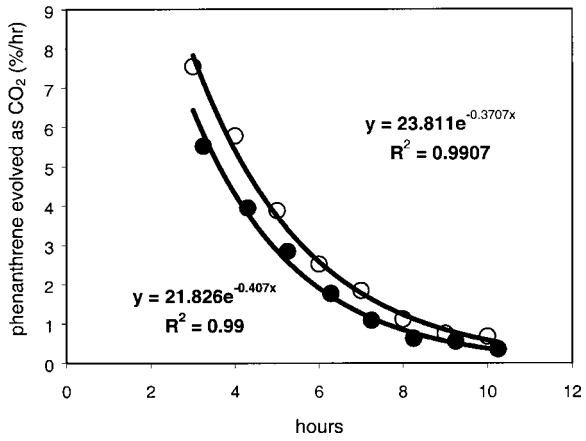

Figure 5. Mineralization rate of 20 ng/ml phenanthrene in mineral media inoculated with 5.83×10^8 cfu *Arthrobacter*, strain RP17. Experiment 1(○), experiment 2 (●).

Approximately 70% of 20 ng phenanthrene/ml mineral media added to a pure culture of RP17 cells evolved as CO_2,while the rest was stored in the cell's biomass. The degradation constant equaled 0.407/h in the first experiment and .371/h in the second experiment (Figure 5). The degradation kinetics of 20 ng/ml phenanthrene were fit well with a single exponential equation, as was expected with low concentrations of phenanthrene.

Discussion

In Forbes soil, the linear sorption isotherm for phenanthrene indicated that only 0.3% of the phenanthrene was present in the aqueous phase (Johnson 1997). Presumably, bacteria can only access pollutants dissolved in the aqueous phase for degradation (Ogram et al. 1985), though there are isolated reports of bacteria able to degrade sorbed chemicals (Guerin & Boyd 1992). In previous studies the availability of phenanthrene to RP17 declined in a biphasic curve, as phenanthrene aged in soil (Schwartz & Scow 1999). The first, rapid decline was completed within 150 hours after the phenanthrene was added to soil and was followed by a much slower decline in bioavailability. Thirty two hours after phenanthrene was added to soil, 2.85 times as much phenanthrene was available to RP17 than 611 hours after phenanthrene was added. Thus, RP17 can only access a small fraction of the phenanthrene at one time, and this fraction increasingly becomes smaller as phenanthrene ages in soil. Therefore, low bioavailability of phenanthrene could be the primary reason for recalcitrance of phenanthrene in soil and failure of bioaugmentation attempts.

One obvious explanation of the observation that degradation rates in all treatments became similar 300 hours after inoculation (Figure 1) was that the majority of phenanthrene had sorbed and that, therefore, the availability of phenanthrene, and not the population density of phenanthrene-degrading organisms, was determining the degradation rate. If this interpretation were correct, one would expect that adding cells a second or third time would not increase the mineralization rate, because any remaining phenanthrene would not be bioavailable. We observed, on the contrary, that re-inoculation improved the mineralization rate (Figure 1), indicating that bioavailability alone did not explain the decline in mineralization rate. Some of the phenanthrene must have remained bioavailable, but was not mineralized, because reinoculation increased the mineralization rate (Figure 1).

It is unlikely that the source of the $^{14}CO_2$ evolution after the 2nd and 3rd inoculation was the biomass that had previously grown up on ^{14}C-phenanthrene. The amount of $^{14}CO_2$ evolved from soil after the second inoculation is too great to be derived solely from biomass grown up on phenanthrene. For instance, at the time of the second inoculation (306 hours after the initial inoculation), five percent of phenanthrene had mineralized in soil inoculated with 2.83×10^6 cfu/g. In a pure culture study (Figure 5) in mineral media with phenanthrene as a sole carbon source, 71% of added phenanthrene evolved as CO_2, while the rest was stored in biomass. Using the yield measured in pure culture, 1.45% of the radioactivity was stored

in biomass before the 2nd inoculation in the soil experiment. After the second inoculation, 15.5% of the initially added phenanthrene evolved as CO_2 (Figure 1). Furthermore, it is unlikely the turnover of RP17 in soil was fast enough to account for any of the increases in mineralization rates observed in this study. Members of the genus *Arthrobacter*, which have a coccoid dormant cell morphology, can survive extensive times in a dormant state. Cells of *Arthrobacter crystallopoietes*, for instance, remained 100% viable after having been starved in phosphate buffer for 30 days (Boylen & Ensign 1970). Quantitative polymerase chain reaction measurements of the population dynamics of RP17 cells grown up in Forbes soil, amended with 500 ppm phenanthrene, showed the population remained stable, even though most of the phenanthrene had been mineralized (Schwartz et al. 2000). The turnover of microbial biomass in soil is often measured on the order of months and not hours. For instance, in a silty clay loam spiked with 500 μg/g ^{14}C-glucose, active soil microorganisms had a half life of 60 days (Wu et al. 1993).

It is unlikely mineralization increased upon repeated inoculations, because newly added bacteria were placed in the vicinity of phenanthrene that previously was spatially isolated from original inoculations. Soil is so spatially complex that it can be envisioned as containing a large number of isolated compartments, each of which must be filled with a phenanthrene-degrading bacterium to achieve remediation. This scenario suggests that the mineralization rate declined because the inoculum, though remaining active, had exhausted the phenanthrene in its immediate vicinity. Remixing the soil should allow sorbed bacteria of one compartment to come into contact with available phenanthrene in another compartment. However, restirring the soil led to only a slight increase in the phenanthrene mineralization rate (Figure 2). Also, a low density inoculum, of 5.55×10^5 cfu/g soil, mineralized a large fraction (56%) of high concentrations (500 ppm) of phenanthrene while it mineralized very little (<4%) of low concentrations (50 ppb) phenanthrene (Figure 4). Spatial heterogeneity should impede the mineralization of high concentrations of phenanthrene as much as low concentrations.

Population densities of RP17 cells measured with a quantitative competitive polymerase chain reaction method show the population was stable in soil even after phenanthrene mineralization had ceased (Schwartz et al. 2000). Therefore it is unlikely predation of RP17 by protozoa or viruses had an impact on the degradation of phenanthrene. Addition of new biomass to overcome a maintenance threshold that was high due to predation is not a likely explanation for the increase in mineralization rate upon reinoculation.

More likely, reinoculation cells were added with induced metabolic pathways that overcame an induction threshold. The concentration of bioavailable phenanthrene may not have been high enough to keep metabolic enzymes induced (Button 1985). Examples of substrate threshold concentrations include a *Corynebacterium* species, which could mineralize a large fraction of 100 ng/ml p-nitrophenol in sewage, but not 26 ng/ml or 17 ng/ml (Zaidi et al. 1988). A gram positive rod, bacteria strain T, was able to mineralize only 1.2% of 0.56 μg/l toluene in mineral media, but could mineralize 33.9% of 10 mg/L toluene (Roch & Alexander 1997). A greater fraction of high concentrations of phenanthrene was mineralized (Figure 4) because bioavailable phenanthrene concentrations were higher than the substrate threshold level in soils with 500 μg/g phenanthrene and not in soils spiked with 50 ng/g phenanthrene. Possibly, the inoculum had reserves, such as glycogen or poly-β-hydroxybutyrate, and once these were exhausted, subsequent inoculations were required to mineralize more phenanthrene.

Alternatively, Strain RP17 may have lost genes encoding for phenanthrene metabolism after it was inoculated into soil, and reinoculation provided fresh genetic potential to degrade phenanthrene. To date we have not been successful in isolating plasmids from the strain and therefore we do not know if genes for phenanthrene metabolism are encoded on a plasmid. The strain does retain the ability to degrade phenanthrene after it has been grown in 10% tryptic soy broth suggesting it does not lose its capacity to degrade phenanthrene rapidly.

Unlike most polluted sites, the phenanthrene in our studies was not aged extensively, and, therefore, it may be difficult to relate our results directly to environmental cleanup. However, it is possible that substrate threshold concentrations also play an important role in degradation kinetics of aged PAH's because in these cases desorption rates and hence the bioavailable concentrations are very low.

There are at least two major challenges to implementing repeated inoculations of soils in remediation efforts. The first is economic and concerns the cost of repeatedly inoculating a site. The second is the potential of stimulating predators, such as protozoa or nematodes, through repeatedly adding inoculum, or

prey, to soil. In one study, the decline of bacterial populations coincided with the increase of protozoan populations (Acea et al. 1988). Eventually, repeated inoculation may fail because predators consume the inoculum before it can degrade much of the pollutant.

In summary, we showed it was possible to mineralize a large fraction of low concentrations of phenanthrene in soil through repeated inoculation of the soil with phenanthrene-degrading bacteria. More phenanthrene was mineralized in soils that were inoculated repeatedly than in soils that were only inoculated once or that were not inoculated at all. We suggest that the bioavailability of small concentrations of phenanthrene in soil is so low it is below the minimum substrate threshold level and consequently that the activity of the phenanthrene-degrading population can not be maintained.

Acknowledgements

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