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Bioremediation of atrazine-contaminated soil by repeated applications of atrazine-degrading bacteria

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Abstract Bioaugmentation has previously been unreliable for the in situ clean-up of contaminated soils because of problems with poor survival and the rapid decline in activity of the bacterial inoculum. In an attempt to solve these problems, a 500-l batch fermenter was investigated for its ability to deliver inoculum repeatedly to contaminated soils via irrigation lines. In a field experiment, mesocosms were filled with 350 kg soil containing 100 mg kg^{-1} atrazine, and inoculated one, four or eight times with an atrazine-degrading bacterial consortium that was produced in the fermenter. After 12 weeks, no significant degradation of atrazine had occurred in soil that was inoculated only once; whereas, mesocosms inoculated four and eight times mineralized 38% and 72% of the atrazine respectively. Similar results were obtained in a laboratory experiment using soil contaminated with 100 mg kg^{-1} [^{14}C]atrazine. After 35 days, soil that was inoculated once with 10^8 cfu ml^{-1} of the consortium or with the atrazine-degrading bacterium, *Pseudomonas* sp. strain ADP, mineralized 17% and 35% of the atrazine respectively. In comparison, microcosms inoculated every 3 days with the consortium or with *Pseudomonas* sp. (ADP) mineralized 64% or 90% of the atrazine over this same period. Results of these experiments suggest that repeated inoculation from an automated fermenter may provide a strategy for bioaugmentation of contaminated soil with xenobiotic-degrading bacteria.

Introduction

Bioremediation techniques were first pioneered by the petroleum industry and have become a popular alternative to chemical or physical remediation because of their relatively low cost and minimal impact on the environment. Techniques using microorganisms to degrade contaminants in polluted sites have been commercially available since the 1970s, but gained particular attention following the successful decontamination of the Alaskan beaches after the Exxon Valdez oil spill (Forsythe et al. 1995). Soils contaminated with petroleum products are most often treated by stimulating indigenous microflora that degrade pollutants. In contrast, bioaugmentation, which is the addition of bacteria to a contaminated environment to enhance degradation of pollutants (Vogel 1996), is not considered reliable because inoculated organisms often fail to survive at an effective population size or do not maintain high levels of degradation activity (Alexander 1994).

Despite the general lack of success of bioaugmentation, there is still considerable interest in this technology. A large fraction of bioaugmentation research has focused on identifying degrader organisms that can be cultured in the laboratory, or that might be genetically engineered to facilitate bioremediation where natural attenuation of the pollutant is too slow. In situations where indigenous degraders cannot rapidly degrade recalcitrant chemicals, bioaugmentation may be the only means for successful bioremediation (Baud-Grasset and Vogel 1995). This is particularly true for soil contaminants that are dispersed over large land areas that cannot be treated cost-effectively by intensive approaches such as excavation and landfilling, or thermal desorption and vapor extraction.

Atrazine is a model xenobiotic pollutant for bioaugmentation studies, because, in the past 30 years, it has been one of the most widely used herbicides in the world. Concern has grown in the U.S. because atrazine and its metabolites have been detected in groundwater above allowable Environmental Protection Agency (EPA) levels (Belluck et al. 1991; U.S. EPA 1990a, b). Many studies have examined the ability of indigenous microbial communities to degrade atrazine in soil (Dousset et al. 1997; Kishore and Green 1971). However, com-

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plete mineralization of atrazine appears to be rare, as shown by the persistence of atrazine and current problems with groundwater contamination. In the past few years, several soil bacteria that can mineralize atrazine in liquid media have been identified (Alvey and Crowley 1995; Radosevich et al. 1995; Yanze-Kontchou and Gschwind 1994; Mandelbaum et al. 1993a). Only a few studies with these bacteria have examined their usefulness for bioaugmentation. Alvey and Crowley (1996) and Mandelbaum et al. (1993a) observed greater mineralization rates in highly contaminated soils inoculated with a degrader organism than in non-bioaugmented soils. Radosevich et al. (1997) and Alvey and Crowley (1996) also examined degradation of atrazine at low concentrations typical of residue levels remaining after a normal herbicide application. However, in most instances when the soils is inoculated with a single application of degrader organisms, degradation activity slows or ceases before the contaminant has been completely removed from the soil.

A technology has now been developed by EcoSoil Systems Inc. (San Diego, Calif.) that allows repeated inoculation of soils with microorganisms, using a field-based fermenter system that automatically cultures and injects bacteria into irrigation water. This concept has not yet been examined for bioaugmentation of soils with xenobiotic-degrader microorganisms, but could solve many of the problems associated with bioaugmentation by allowing repeated application of inocula, as well as providing a cost-effective method for treating large land areas. The objective of this research was to examine the continuous-application concept in a laboratory-bench study using soil microcosms contaminated with atrazine. A second objective was to develop an application method for repeated delivery of large quantities of atrazine-degrading bacteria to contaminated soil in mesocosms, using a batch fermenter operated in the field.

Materials and methods

Chemicals

[*ring*-UL-^{14}C]Atrazine (100 μCi g^{-1}) was purchased from Sigma Chemical company (St Louis, Mo.) and had a purity greater than 98%. Non-radioactive atrazine metabolites, hydroxyatrazine (97.8%), deethylatrazine (99.1%), deisopropylatrazine (94%), and diaminoatrazine (97%) were purchased from Chemservice (Westchester, Pennsylvania).

Bacterial strains and growth conditions

An atrazine-degrading consortium (Alvey and Crowley 1996) and an atrazine-degrading bacterial isolate *Pseudomonas* sp. strain ADP (Mandelbaum et al. 1993a) were maintained at room temperature in chloride-free mineral salts (MS) medium containing glucose (1000 mg kg^{-1}) as a carbon source and atrazine (90–100 mg kg^{-1}) as the sole nitrogen source. The medium contained 10 mM $KHPO_4$, 3 mM NaH_2PO_4, 1 mM $MgSO_4$, and 10 ml chloride-free trace element solution, which consisted of the following (mg l^{-1}): $CaSO_4$, 200; $FeSO_4 \cdot 7H_2O$, 200; $MnSO_4 \cdot H_2O$, 20; $NaMoO_4 \cdot 2\ H_2O$, 10; $CuSO_4$, 20; $CoSO_4 \cdot 7\ H_2O$, 10; H_3BO_3, 5 (Focht 1994). The consortium consisted of a *Clavibacter michiganese* ATZ1, *Pseudomonas* sp. strain CN1 (Alvey and Crowley 1996), and strain of *Cytophaga* sp. that was later shown to be nonessential (de Souza et al. 1998). In brief *C. michiganese* is responsible for the dechlorination of atrazine to hydroxyatrazine. Furthermore, *C. michiganese* can *N*-dealkylate hydroxyatrazine, producing *N*-ethylammelide. The *Pseudomonas* sp. strain CN1 can *N*-dealkylate the *N*-ethylammelide to form cyanuric acid. Cyanuric acid is further mineralized to CO_2 by *Pseudomonas* sp. strain CN1. *Pseudomonas* sp. strain ADP alone is able to mineralize atrazine completely to CO_2 (Mandelbaum et al. 1993a). The atrazine degradation pathway utilized by *Pseudomonas* sp. strain ADP is subtly different. *Pseudomonas* sp. strain ADP *N*-dealkylates hydroxyatrazine to form *N*-isopropylammelide and then cyanuric acid, which is completely mineralized to CO_2 (de Souza et al. 1997; Mandelbaum et al. 1993a).

Atrazine mineralization in soil microcosms

Soil microcosms were established in 175-ml glass jars at room temperature. The top 15 cm of a Handford loamy sand that had not previously been exposed to atrazine was obtained from an orange grove at the Agricultural Operations field on the campus of U.C. Riverside. The soil was shaken through a 2-mm sieve and 100-g air-dried samples were weighed into each jar. Each jar was sealed with a rubber stopper equipped with two holes for a gas inlet and outlet. CO_2-free air was introduced into the microcosms, using a vacuum to pull ambient air through a column filled with a desiccant and then through a 1-L 2 M NaOH trap. Air from the outlet port of each microcosm was pulled through tandem 10-ml, 2 M NaOH traps to capture [^{14}C]CO_2. The experimental design consisted of 20 microcosms in a randomized block configuration with five treatment and four replications per treatment.

Atrazine and [^{14}C]atrazine were dissolved in methanol and added to each microcosm in 1-ml aliquots. The final concentration of atrazine in the soil was 100 mg kg^{-1} and it had an activity of 0.5 μCi/microcosm. The soils were stirred, brought up to 15% moisture w/w (approximately −0.30 Mpa) and aged 2 weeks before the addition of microorganisms.

The experimental treatments included an uninoculated control, two treatments in which the soil was inoculated once with either the consortium or with *Pseudomonas* sp. (ADP), and two treatments that were inoculated with the consortium or with *Pseudomonas* sp. (ADP) every 3 days over a 35-day period for a total of 11 inoculations. Inocula were produced in MS/glucose/atrazine medium. Cultures of 200 ml were shaken at room temperature on a rotary shaker at 90 rpm for 3 days, and then harvested by centrifugation at 15 000 rpm for 10 min. The supernatant was discarded and the cells resuspended in 1 ml double-deionized water. Aliquots of 200 μl were stirred into each inoculated microcosm with a sterile spatula, providing a final density of 1.1×10^8 cfu g^{-1} soil, as determined by plating on tryptone-soy-agar. Soil moisture in all of the microcosms was checked daily and maintained at approximately 15% (w/w) or approximately −0.3 MPa.

Atrazine mineralization in the field

Soil mesocosms were established in plastic concrete-mixing tubs at the Agricultural Operations field on the campus of U.C. Riverside under ambient conditions. The tubs, 116 cm long × 71 cm wide × 25 cm high, were filled with approximately 350 kg Dello loamy sand (typic Psammaquent, sandy alluvium; pH 8). Atrazine in the form of the commercial product, Aatrex 90, was incorporated into the soil with a rake at a concentration of 100 mg kg^{-1} soil. The Aatrex 90 was in granular form so it was reduced to sand-sized particles with a mortar and pestle and added to 1 kg soil before incorporation into the mesocosms. Each mesocosm was mixed thoroughly, saturated with water and aged for 3 weeks prior to inoculation. The tubs did not allow the soil to drain freely. However, evaporation was sufficient to require the addition of water or inoculum to maintain a moisture content of 15% (w/w).

The mesocosms received 1, 4 or 8 applications of the atrazine-degrading consortium as inoculum. Four repetitions of each treatment were arranged in a randomized block design. The treatment inoculum was prepared on site in a modified 500-l batch fermenter (Bioject; EcoSoil Systems Inc., San Diego, Calif.). To produce the glucose MS medium, irrigation-quality water was passed through tandem 100-µm and 10-µm filters and an ultraviolet sterilization unit before entering the fermenter and reaching a final volume of 124 l. Stock solutions of glucose and the MS components as 100-fold concentrates were added in 1-l aliquots to 122 l water to complete the final medium. Float valves located inside the fermentation vessel maintained the high- and low-volume settings by turning on and off the water automatically during inoculation cycles. Two air compressor pumps maintained aeration of the inoculum and pressurized 19-l tanks containing concentrated glucose and MS medium. Activation of solenoid valves controlled the amount of concentrated medium components entering the tank during the refilling phase. Initially 12.4 l cells grown under laboratory conditions were transported to the field and added to 124 l of the glucose MS medium in the reactor. Thereafter 12.4 l culture was left in the tank, after delivery to the mesocosms, to act as the carryover inoculum for the next batch. Technical-grade atrazine was added by hand to the fermenter at the beginning of each cycle, yielding a final concentration of 50 mg l^{-1}. Approximately 32 l fresh inoculum was used to purge the irrigation lines before each cycle to remove any residual inoculum from the previous application. Each mesocosm received approximately 5 l inoculum/application. Applications were made, on average, once every 1.5 weeks, with the entire experiment lasting 12 weeks. Since atrazine was a component of the growth medium, samples were taken and analyzed by HPLC before each application of the inoculum to the mesocosms. In all cases the treatment inoculum contained less than 1 ppm atrazine.

An Oakfield sampler (Ben Meadows Inc.) was used to collect soil samples and was sanitized with 70% ethanol before each sample was taken. Samples were placed in plastic bags and kept on ice for preservation until they were analyzed. Inoculum density and number of atrazine-degrading units present in the soil were monitored by the most-probable-number method adapted from Alvey and Crowley (1995). Vials were scored positive if the concentration of atrazine in the supernatant was less than 4 mg kg^{-1}. Control samples showed no significant mineralization of atrazine.

Analytical methods

Extraction of atrazine and metabolites from soil samples involved three successive extractions with a soil to extractant ratio of 4:1. The extractant consisted of a 90% acetonitrile 10% 0.1 M HCl solution (Alvey and Crowley 1996). Concentrations of atrazine and metabolites were measured by HPLC analysis. The HPLC method was adapted from Rustum et al. (1990), and employed UV detection at 215 nm and 230 nm after separation by reverse-phase chromatography on a C-18 column. A Hewlett-Packard 1050 series HPLC (Hewlett-Packard Company, Fullerton, Calif.) was used for all separations. Solvents were acetonitrile and a 0.01 M KH_2PO_4 buffer adjusted to pH 2.0, with a mobile-phase flow rate of 1 ml min^{-1}. The time course was as follows [time (min), acetonitrile (%): % buffer(%)]: 0–10 min, 5:95; 10–21 min, 15:85; 21–31 min, 70:30; 31–32, 40:60; 32–35 min, 5:95. Retention times were determined for purified standards of atrazine, hydroxyatrazine, deethylatrazine, deisopropylatrazine, and diaminoatrazine. The most-probable-number assays utilized a more rapid method that separated atrazine from its metabolites, but did not distinguish between the metabolites. This method used an isocratic gradient with 50% acetonitrile and 50% double-deionized water.

Statistical analyses

Data comparing degradation of atrazine in the different treatments for the microcosm and mesocosm experiments were analyzed by analysis of variance using Sigma Stat (Jandel). Statistical differences between treatment means ($P > 0.05$) were determined by Duncan's mean separation test.

Results

Atrazine mineralization in soil microcosms

Mineralization rates of atrazine were significantly enhanced in all of the inoculated soils as compared to uninoculated control soils. After 35 days, uninoculated soil microcosms mineralized less than 1% of the added atrazine (Fig. 1). In contrast, soil that was inoculated every 3 days with *Pseudomonas* sp. (ADP) had mineralized 72% of the atrazine by day 18, and approximately 90% of the atrazine was mineralized by day 35 (Fig. 1). Repeated application of the consortium resulted in slower degradation rates with 20% degradation of the atrazine by day 6, which continued at a steady rate such that 64% of the atrazine was mineralized by day 35. In comparison, soil that that had received only a single inoculation of either the atrazine-degrading isolate or the consortium mineralized much less atrazine. In soil inoculated once with *Pseudomonas* sp. (ADP), only 33% of the atrazine was mineralized by day 12, after which degradation slowed and only 3% more of the atrazine was mineralized during the remainder of the experiment. Similarly, soil inoculated once with the atrazine-degrading consortium mineralized 11% of the added atrazine by day 6, after which mineralization slowed such that, by the end of the

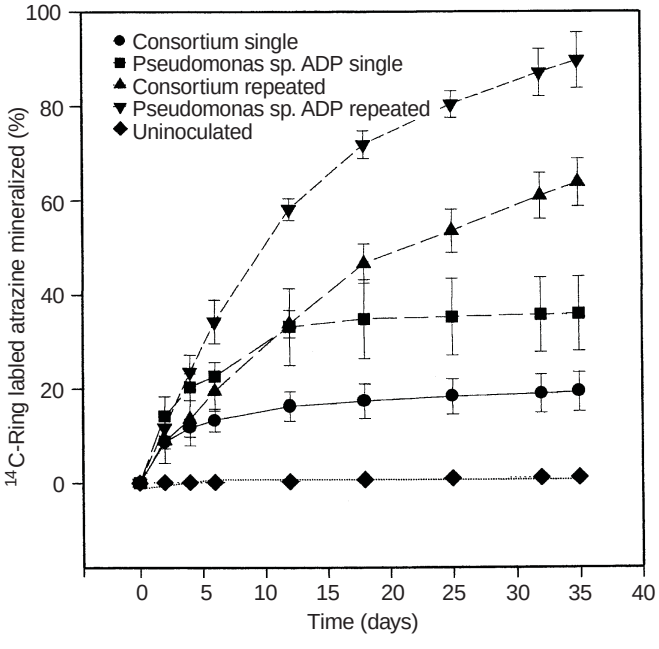

Fig. 1 Mineralization of ring-labeled [^{14}C]atrazine in soil microcosms. Each point represents the mean of four replicates. Error bars represent one standard deviation from the mean

experiment, only 17% of the total atrazine present in the soil had been degraded.

Mineralization of atrazine and survival of degraders under field conditions

Atrazine degradation was compared for soils treated with one, four or eight applications of the atrazine-degrading consortium over an 11-week period. A single application of the consortium did not significantly decrease the concentration of atrazine over the time course of the experiment (Fig. 2). Mesocosms receiving four applications of the consortium alternating with four applications of water showed a 38% decrease in atrazine concentration to 62 mg kg^{-1}; however, owing to high variability between replicates, the quantities degraded did not differ significantly from those following a single application. Only repeated applications of the consortium caused significant mineralization of atrazine, resulting in the disappearance of 72% of the atrazine after 11 weeks (Fig. 2). No atrazine metabolites were detected in any of the soils during the experiment.

The consortium was added to the mesocosms at an average density of 10^5 cfu g^{-1} soil at each inoculation. Most-probable-number analyses performed at the start of the experiment showed that culturable indigenous atrazine degraders were not present. After 6 weeks, mesocosms receiving a single application of the consortium contained $10^{3.4}$ atrazine-degrading-units (ADU) g^{-1} soil (Fig. 3). At the same time, mesocosms that had received two inoculations alternating with two applications of water yielded $10^{2.7}$ ADU g^{-1} soil. The treatment that had been inoculated with four applications of the consortium yielded $10^{5.3}$ ADU g^{-1} soil, almost 100 times more than the other treatments. After 12 weeks, the population densities of atrazine degraders in all of the mesocosms had increased as compared to those at week 6 (Fig. 3). The soils receiving a single application showed a slight increase from $10^{3.4}$ to $10^{3.8}$ ADU g^{-1} soil, whereas atrazine degradation in soils that received alternating water and inoculum treatments increased from $10^{2.7}$ to $10^{3.4}$ ADU during the last 5 weeks. Atrazine degraders in the mesocosms that were repeatedly inoculated eight times continued to have the highest cell densities, increasing slightly from $10^{5.3}$ at week 6 to $10^{5.8}$ ADU g^{-1} soil at week 11.

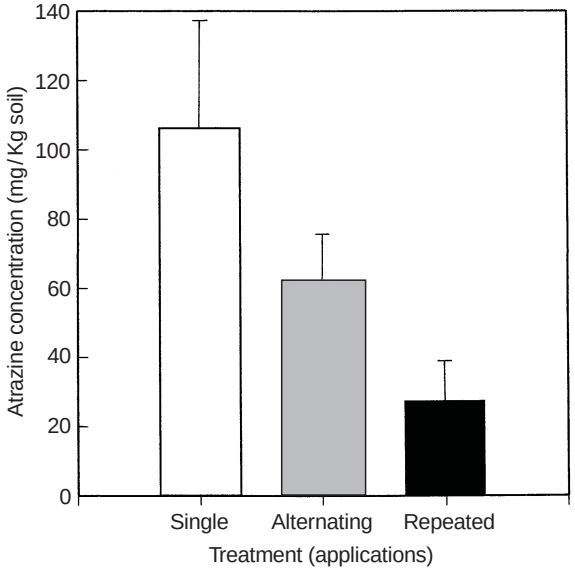

Fig. 2 Mineralization of atrazine in soil mesocosms after 11 weeks of treatments where one, four and eight applications of an atrazine-degrading bacterial consortium were administered. Each column represents the mean of four replicates. Error bars represent the standard error of the mean

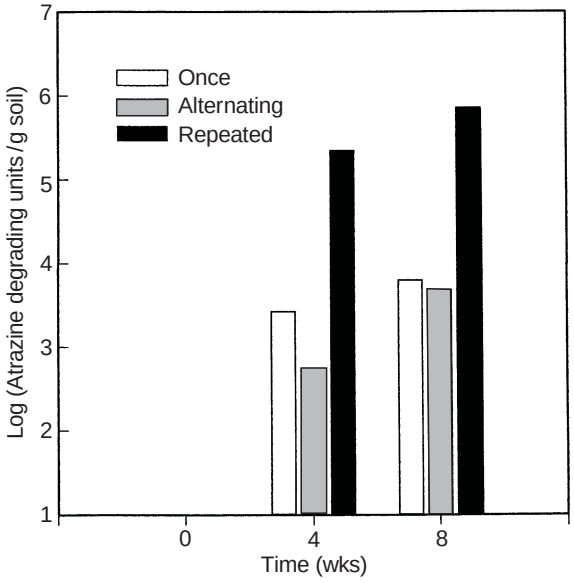

Fig. 3 Population density of atrazine-degrading bacterial consortium units after 11 weeks of treatments delivering one, four and eight applications of inoculum

Discussion

Successful bioremediation of xenobiotic-contaminated sites that do not contain indigenous degraders, or where the activity of indigenous degraders is low, may require bioaugmentation to enhance the rate of degradation to a level that will prevent groundwater contamination (Belluck et al. 1991). In the case of atrazine, the absence of indigenous atrazine degraders is a common phenomenon that has caused concern over non-point-source pollution from herbicide residues. In many soils, atrazine is reported to have a half-life ranging from months to years. The recent identification of bacterial isolates and consortia with the ability to mineralize atrazine provides a unique opportunity for increasing the mineralization of this contaminant through bioaugmentation (Alvey and Crowley 1996; Radosevich et al. 1995; Mandelbaum et al. 1993a). To date, however, single inoculations with atrazine-degrading bacteria have given inconsistent results (Dousset et al. 1997;

Kishore and Green 1971; Radosevich et al. 1997), and most experiments, including the research reported here, suggest that degradation activity rapidly declines when soils are treated with only a single inoculation of atrazine degraders.

In an attempt to overcome this problem, this research examined the use of a batch fermenter to apply atrazine-degrading bacteria repeatedly to soil that did not have indigenous degraders. With the batch fermenter, repeated inoculation potentially could be used to treat localized areas of contamination with very high concentrations of bacteria, or used to produce and deliver bacteria at lower population densities over large land areas to reduce herbicide residues. For example, assuming bacterial population densities of 10^9 cfu ml^{-1}, a 500-l fermenter can treat 10^4 kg soil with 5×10^7 cfu g^{-1} soil at each application. When diluted over a 1-km^2 area, bacterial densities equivalent to 10^5 cfu g^{-1} soil in the upper 10 cm soil could be delivered. Problems associated with long-term survival of the inoculum are essentially eliminated since the bacteria must only survive for the period between each inoculation cycle.

The benefits of repeated inoculation were especially highlighted by the contrasting results obtained in the laboratory and field. In the laboratory, single inoculation treatments with *Pseudomonas* sp. (ADP) or the consortium resulted initially in rapid degradation, which slowed after the first week such that most of the atrazine still remained at the end of the experiment. In contrast, under field conditions, both the single-inoculation treatment and the treatment with four inoculations were ineffective, and only treatment with eight repeated applications showed significant reduction of atrazine.

Initial rates of degradation, observed here after a single inoculation, were much lower than those reported by others (Alvey and Crowley 1995; Mandelbaum et al. 1993a), even though the cells were produced similarly and were in active log-phase growth at the time of inoculation. To optimize the benefit of repeated inoculation, future experiments will need to examine the relationship between population density and degradation rate. Data from the field experiment suggested that a minimum of 10^5 cfu g^{-1} soil was necessary to obtain significant degradation over a 12-week period. From a practical standpoint, these data suggest that repeated applications of atrazine degraders may be necessary for removing residual atrazine present at threshold concentrations not capable of supporting the growth of the organisms. For example (Radosevich et al. 1997) showed a decrease in mineralization rates of atrazine after a single inoculation of strain M91-3 as a function of aging in soil. When biodegradation is limited by simple sorption/desorption kinetics, repeated applications would allow microorganisms to be present at a high density and in a physiological condition that would allow them to degrade the substrate actively as it desorbs from soil.

This research also compared the performance of an atrazine-degrading isolate to the atrazine-degrading bacterial consortium. In the microcosm experiment, *Pseudomonas* sp. (ADP) mineralized a greater amount of atrazine than the consortium in both single and repeated inoculation treatments. Use of atrazine as a carbon and nitrogen source may be more energetically feasible for an isolate than a consortium in which the metabolites must diffuse between cells. The consortium used here required atrazine in the growth medium or loss of degradative capabilities occurred. Laboratory studies were conducted to test the feasibility of using commercially available liquid atrazine concentrate. The consortium did not grow as well on the liquid concentrate as on pure atrazine. However, *Pseudomonas* sp. (ADP) grew very efficiently on Aatrex 4L as a nitrogen source (data not shown). Since Aatrex 4L is in a liquid form, an automated delivery is possible, using the new generation of BioJect batch reactors.

In summary, repeated inoculation of xenobiotic-degrading bacteria may be useful for increasing degrader bacterial population densities under conditions in which it is difficult or impractical to enrich for the growth of indigenous degraders selectively. It may also be useful for maintaining high population densities of introduced degraders when toxic co-contaminants cause rapid declines in population size and activity. Lastly, repeated applications of bacteria may be useful for maintaining high population densities of degraders that cometabolize organic contaminants. In this situation, active cells can be grown in pure culture on the co-substrate and added to the soil, thus by-passing problems with competition for the co-substrate by indigenous microorganisms, or environmental problems associated with the addition of hazardous chemicals that are sometimes used as co-substrates. While there are many factors that may need to be optimized for different situations, the ability to deliver degrader organisms with repeated applications from an automated fermenter offers a novel strategy with many advantages over the conventional single-inoculation method for bioaugmentation.

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