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«Οι σύγχρονες τεχνικές βιο-ανάλυσης στην υγεία, τη γεωργία, το περιβάλλον και τη διατροφή»

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Field-scale remediation of atrazine-contaminated soil using recombinant *Escherichia coli* expressing atrazine chlorohydrolase

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Summary

We performed the first field-scale atrazine remediation study in the United States using chemically killed, recombinant organisms. This field study compared biostimulation methods for enhancing atrazine degradation with a novel bioaugmentation protocol using a killed and stabilized whole-cell suspension of recombinant *Escherichia coli* engineered to overproduce atrazine chlorohyrolase, AtzA. AtzA dechlorinates atrazine, producing non-toxic and non-phytotoxic hydroxyatrazine. Soil contaminated by an accidental spill of atrazine (up to 29 000 p.p.m.) supported significant populations of indigenous microorganisms capable of atrazine catabolism. Laboratory experiments indicated that supplementing soil with carbon inhibited atrazine biodegradation, but inorganic phosphate stimulated atrazine biodegradation. A subsequent field-scale study consisting of nine (0.75 m^3) treatment plots was designed to test four treatment protocols in triplicate. Control plots contained moistened soil; biostimulation plots received 300 p.p.m. phosphate; bioaugmentation plots received 0.5% (w/w) killed, recombinant *E. coli* cells encapsulating AtzA; and combination plots received phosphate plus the enzyme-containing cells. After 8 weeks, atrazine levels declined 52% in plots containing killed recombinant *E. coli* cells, and 77% in combination plots. In contrast, atrazine levels in control and biostimulation plots did not decline significantly. These data indicate that genetically engineered bacteria overexpressing catabolic genes significantly increased degradation in this soil heavily contaminated with atrazine.

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Introduction

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-*s*-triazine) is a herbicide widely used in the United States for the control of broad-leaved weeds in corn, sorghum and sugar cane. Atrazine is relatively recalcitrant in soil (Best and Weber, 1974; Cohen *et al*., 1984; Koskinen and Clay, 1997), especially below the soil surface, where it is commonly detected after 1 year. Atrazine has been reported to have a half-life greater than 170 days in soils known to contain atrazine-degrading microorganisms (Radosevich *et al*., 1996), and is significantly more persistent in soil when present at concentrations higher than the suggested application rate of 1 p.p.m. (Davidson *et al*., 1980). Such a situation may result from normal pesticide handling techniques that predate current environmental regulations (Fadullon *et al*., 1998), or from accidental spills.

Atrazine removal from the soil environment can occur by several different mechanisms. At typical soil pH, atrazine is only very slowly chemically hydrolysed (half-life of 200 days) to produce hydroxyatrazine (Armstrong *et al*., 1967; Plust *et al*., 1981). A more significant degradation mechanism for atrazine in soils is microbial metabolism (Mandelbaum *et al*., 1993a; Radosevich *et al*., 1995). Microbial degradation of atrazine has been demonstrated to occur via dealkylation (Giardina *et al*., 1982; Giardi *et al*., 1985; Behki and Khan, 1986; Behki *et al*., 1993; Masaphy *et al*., 1993; Behki and Khan, 1994; Hickey *et al*., 1994; Mougin *et al*., 1994; Nagy *et al*., 1995), deamination (Giardina *et al*., 1980) or dechlorination (Mandelbaum *et al*., 1993b; Bouquard *et al*., 1997) reactions. Complete biodegradation pathways are shown in the Biocatalysis and Biodegradation Database web page (Ellis and Wackett, 1999).

For decontamination purposes, the most efficient method of transforming atrazine into a less harmful endproduct is by biostimulation or bioaugmentation (Liu and Suflita, 1993). Biostimulation involves supplementing the contaminated soils to change the physical state of the contaminant, thereby converting it to a bioavailable form (Atlas and Bartha, 1992), or supplying a nutritional

supplement or co-substrate to increase the population of indigenous bacteria capable of catabolizing the contaminant (Adriaens and Focht, 1990). Bioaugmentation involves inoculating soils with a non-indigenous microorganism capable of catabolizing the contaminant (Brodkorb and Legge, 1992).

The ability of introduced live cultures of atrazine-degrading bacteria to increase biodegradation has been investigated in laboratory studies. In studies performed using non-sterile soil, the success of bioaugmentation was inversely related to population levels of indigenous atrazine-degrading microorganisms (Kontchou and Gschwind, 1993; Radosevich *et al*., 1996; Struthers *et al*., 1998). In sterile soils devoid of indigenous atrazine-degrading bacteria, it has been reported that atrazine concentration was reduced by 70% (from 20 p.p.m. to 6 p.p.m.) in 30 days (Fadullon *et al*., 1998), or eliminated from 15 p.p.m. in 5 days (Wenk *et al*., 1998).

In view of these studies, we decided to test the performance of a novel form of bioaugmentation: direct addition of killed and stabilized suspensions of whole recombinant *Escherichia coli* cells engineered to overexpress the enzyme atrazine chlorohyrolase (AtzA). AtzA dechlorinates atrazine in a single step to produce hydroxyatrazine, which is non-toxic to plants. The *atzA* gene was cloned from *Pseudomonas* strain ADP, a bacterium capable of mineralizing atrazine rapidly (deSouza *et al*., 1995; Mandelbaum *et al*., 1995).

In this paper, laboratory studies are described that were performed to determine the extent to which bacteria indigenous to spill-contaminated soil were capable of degrading atrazine. We tested a range of biostimulation supplements to optimize the nutritional additives and took this information to the field to test systematically the ability of different bioremediation methods to remediate soil heavily contaminated with up to 29 000 p.p.m. atrazine by an accidental spill. In this study, we show that, in field-scale experiments, bioaugmentation of the contaminated soil with killed and stabilized recombinant *E. coli* expressing AtzA, in conjunction with phosphate supplementation, was effective in removing 77% of atrazine from the contaminated soil.

Results

Enzyme activity

Viability tests of the killed cell suspension showed no cell growth on solid LB medium or LB plus atrazine and chloramphenicol. Cross-linking to kill cells resulted in some enzyme inactivation; immediately after cross-linking, killed cells retained 65% of enzyme activity compared with the live cell activity. The A_{270} in the suspension containing 30 p.p.m. atrazine and killed cells dropped 0.25 absorption units in 55 min, corresponding to an AtzA enzyme velocity of 167 mg day^{-1} g^{-1} cross-linked cells (data not shown).

Cross-linked cell suspensions retain significant enzyme activity over a long storage time, if stored properly. Suspensions stored at room temperature for 7 months retained only 24% of their original enzyme activity when stored in complex media consisting of the growth media plus cross-linking chemicals and neutralizers, as described in *Experimental procedures*, but they retained 55% of the original enzyme activity if stored in pH 7 phosphate buffer. After 8 and 9 months of storage in pH 7 phosphate buffer, cross-linked cells retained 51% and 41% of the original enzyme activity respectively. Cross-linked cells that were frozen showed no enzyme activity after 7 months.

Microbial mechanism for atrazine degradation

The results of a bench test for microbial degradation of atrazine in the spill-contaminated soil are shown in Fig. 1. In unsterilized soil, the atrazine concentration declined by 84% from 17 100 p.p.m. to 2700 p.p.m. over the 5 week sampling period. The first-order reaction rate constant was 0.08/day ($R^2 = 0.95$). This is well within the 0.01–0.2 day^{-1} range of rate constants for atrazine mineralization in soil under laboratory conditions measured by Radosevich *et al*. (1996). In sterilized soil, no atrazine degradation occurred, and atrazine concentration remained constant at 15 100 p.p.m. The sterilized soil remained sterile for the length of the experiment, as determined by plating assays. This indicates strongly that microorganisms are responsible for atrazine degradation in soil, and is consistent with other reports in the literature (Jones *et al*., 1982; Smith and Walker, 1989; Winkelmann and Klaine, 1991).

Biostimulation bench-top experiments

Soil from the atrazine spill site that was directly plated onto

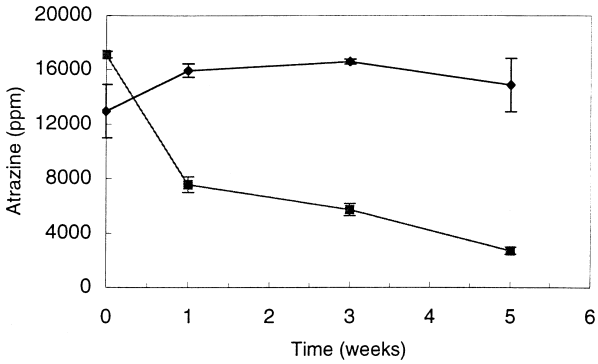

Fig. 1. Microbial degradation of atrazine in bench test studies. (♦) sterilized soil; (■) non-sterilized soil. Error bars represent 1 standard deviation.

Table 1. Influence of soil amendments on degradation of atrazine in spill-site soil.

	Amendment	Degradation (%)
1	0.5 ml of soybean oil	42
2	1 ml of soybean oil	12
3	1 ml of 0.1% sodium citrate + 0.1% glucose	21
4	2 ml of 0.1% sodium citrate + 0.1% glucose	48
5	1 ml of glycerol	36
6	2 ml of glycerol	16
7	1 ml of non-fat whey	37
8	2 ml of non-fat whey	27
9	1 g of ground corn	33
10	2 g of ground corn	39
11	Buffer only	43
12	Water only, no buffer	26
13	No amendment	11

Amendments were added to 45 g of soil, and degradation was measured19 days after addition. Initial atrazine concentration in each sample was individually determined and ranged from 3400 p.p.m. to 6700 p.p.m.

Table 2. Experimental design to analyse simultaneously the effects of soil modifications on atrazine degradation.

	pH	Carbon ($g\,45\,g^{-1}$ soil)	Phosphorus (p.p.m.)	Final [atrazine] (p.p.m.)
1	7	0.08	150	1370
2	8	0.08	150	1970
3	7	0.8	150	1705
4	8	0.8	150	1350
5	7	0.08	450	2280
6	8	0.08	450	1090
7	7	0.8	450	3515
8	8	0.8	450	1870
9	7.5	0.44	300	1840
10	7.5	0.44	300	1320
11	6.5	0.44	300	1420
12	8.5	0.44	300	1475
13	7.5	0	300	900
14	7.5	1.16	300	1910
15	7.5	0.44	0	2540
16	7.5	0.44	600	1730

Initial atrazine concentration was 4500 p.p.m. Amendments were added to 45 g of soil, and degradation was measured 13 days after addition. Multiple regression of the results shows that carbon addition reduces atrazine degradation (P-value = 0.002), and phosphorus addition increases atrazine degradation (P-value = 0.03).

minimal media with no nitrogen source supported no bacterial growth. However, when this soil was plated directly onto minimal media augmented with 500 p.p.m. atrazine as the sole nitrogen source, abundant bacterial growth resulted. This confirmed the existence of microbes in the soil, whose capability for catabolizing atrazine might be enhanced by biostimulation.

Preliminary screening of potential biostimulation agents indicated that atrazine degradation in this soil appeared to be most influenced by phosphate buffer and perhaps simple sugars (Table 1). Further testing of the effects of pH, carbon addition and phosphate addition on atrazine degradation was implemented using a statistically designed central composite experiment. These results are shown in Table 2. Carbon addition decreased atrazine degradation (P-value = 0.002), and phosphorus addition increased atrazine degradation (P-value = 0.03). The effect of pH manipulation was statistically insignificant. Optimal atrazine degradation occurred at 300 p.p.m. phosphorus addition. These results were used to construct the four field site treatment protocols described in *Experimental procedures*.

Field studies

Atrazine concentration as a function of elapsed time in field test plots is presented in Fig. 2. After 12 weeks, atrazine levels in plots containing atrazine-degrading enzyme in the form of 0.5% (w/w) killed recombinant *E. coli* cells declined by 53% from 3800 p.p.m. to 1800 p.p.m. (this is statistically significant at P-value = 0.16). In plots augmented with the combination of phosphate and killed recombinant cells, atrazine degradation was 77%, with levels declining from 6700 p.p.m. to 1450 p.p.m. (significant at P-value = 0.03). In contrast, plots not treated with enzyme (the control plots and plots augmented with 300 p.p.m. phosphate) exhibited no significant degradation (P-values of 0.43 and 0.73 respectively), and atrazine concentration remained at its initial average of 2500 p.p.m. Soil temperature at 10 cm depth dropped from 19.2°C to 3.6°C over the experimental time period (data not shown). Significant degradation in the test plots continued until the soil temperature dropped below 7°C. This analysis shows that no significant degradation occurred during this time period when the soil temperature averaged 3.6°C.

Discussion

In the 18 months that this soil was stored on site following contamination by an accidental atrazine spill, a significant population of indigenous microorganisms capable of catabolizing atrazine developed. In laboratory experiments, atrazine levels in non-amended moistened soil declined by 84% from 17 100 p.p.m. to 2700 p.p.m. over a 5 week period. Results from our studies show that, when microbial populations were killed by autoclaving, no atrazine degradation occurred, and atrazine levels remained constant at 15 100 p.p.m.

Despite the presence of significant populations of indigenous atrazine-degrading microorganisms, the ability of these bacteria to reduce the atrazine concentration significantly under field conditions appears to be limited. If the original atrazine was uniformly distributed in the 26 m^3 of containment soil, the concentration of atrazine would average ≈ 11 500 p.p.m. Atrazine was not uniformly distributed, however, and ranged from 400 to 29 000 p.p.m.

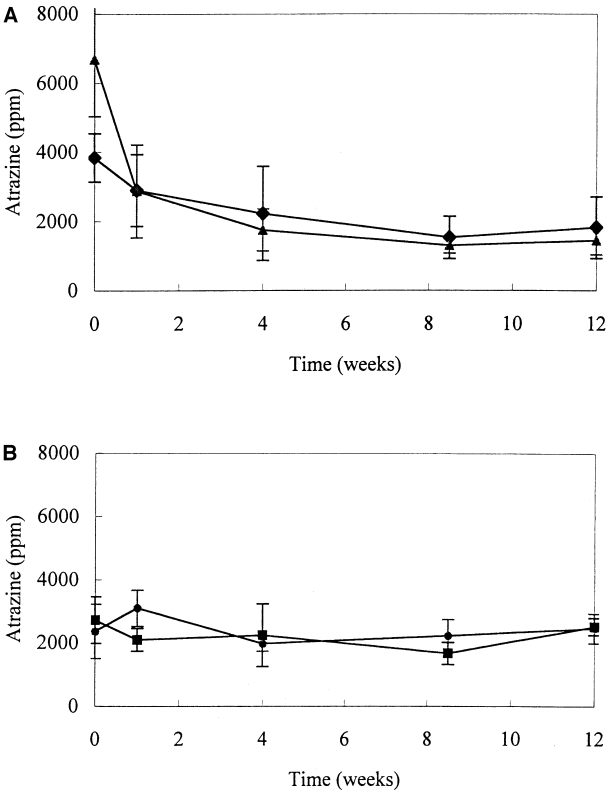

Fig. 2. Atrazine biodegradation in field test plots.
A. (♦) Bioaugmentation plots (0.5% killed recombinant *E. coli* cells) and (▲) biostimulation plus bioaugmentation plots (0.5% killed recombinant *E. coli* cells plus 300 p.p.m. phosphate).
B. (●) Control and (■) biostimulation (300 p.p.m. phosphate) plots. Error bars represent 1 standard deviation. Soil temperature over the experimental time period at 10 cm depth dropped from 19.2°C to 3.6°C.

(Fig. 3). For our test procedures, the portions of the soil with the highest atrazine concentrations were selected and homogenized. The weighted average of the initial atrazine concentration in the field test plots was 3500 p.p.m. This means that, over the 18 months of storage, the indigenous population reduced the atrazine concentration by ≈ 70%, corresponding to a half-life of 300 days. This is longer than the reported 170 day half-life of atrazine in soil environments (Radosevich *et al.*, 1995), but is not surprising because of the very large initial concentration. While our results show that significant degradation is occurring naturally in this soil, the ability to store large amounts of soil for long periods of time while waiting for natural degradation to occur is not feasible; therefore, developing a more rapid remediation procedure is desirable.

To address this issue, we have attempted the first use of killed, recombinant organisms in field remediation studies in the United States. We have demonstrated the ability to effect a 77% reduction in atrazine concentration (from 6700 p.p.m.) in only 8 weeks by adding a suspension of stabilized, killed recombinant bacterial cells containing active atrazine chlorohydrolase. This result is likely to be at the lower limit of treatment efficacy, because the experiment was performed in the late autumn when temperatures were falling rapidly, and our data indicate that remediation using this method is likely to be more effective at higher temperatures. Because atrazine is typically applied to fields during the spring, accidents are most likely to occur at this time. Remediation can be implemented immediately during the season in which temperatures are rising and stay elevated for many weeks.

There was a reduction in atrazine concentration from 6700 p.p.m. to 1450 p.p.m. in the enzyme plus phosphate-treated plots. This occurred while soil temperatures were dropping from 19°C to 7°C (67°F to 38°F). While the atrazine-degrading ability of the recombinant organism is clearly temperature related, it is also related to other factors. To show this, enzyme activity as a function of temperature is presented as an Arrhenius plot in Fig. 4. In this form, the line slopes represent activation energy for the first-order reaction. If this atrazine dechlorination reaction (catalysed by whole stabilized cell suspension) was a simple first-order reaction, the slopes of all the lines would be equal, but the slopes increase with increasing media complexity.

Cells stored on the bench top in clear buffer retain the highest reaction activity. Reaction activity decreases when cells are stored in complex media, compared with that measured in an identical batch of cells stored as a moistened pellet. Finally, reaction activity decreases to a minimum for the cells used at the field remediation site. This indicates

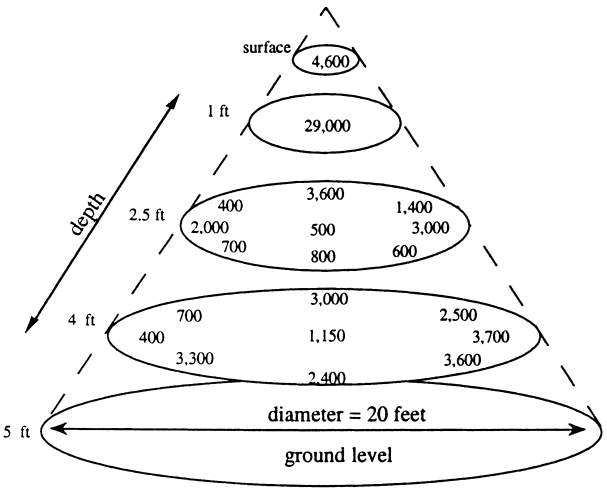

Fig. 3. Distribution of atrazine (p.p.m.) in the 26 m^3 soil excavated after an accidental spill. Values are from independent determinations of atrazine concentration at each test point. If atrazine were uniformly distributed, which it clearly is not, average concentration would be 11 500 p.p.m., based on the known amount of atrazine spilled.

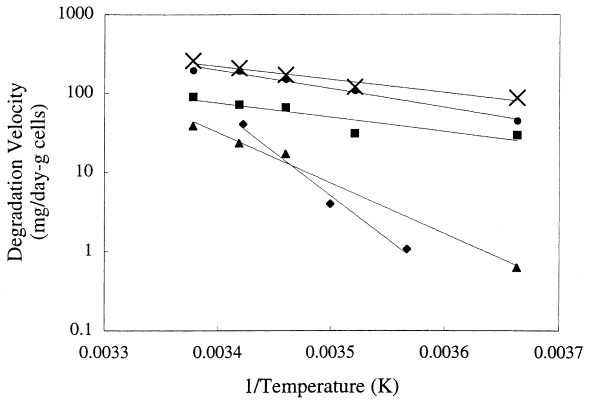

Fig. 4. Arrhenius plot of enzyme activity as a function of temperature. Enzyme was introduced in the form of killed, cross-linked, recombinant cells. Four of the data sets are laboratory data: the same cells used in the field study, stored for 7 months on the bench top as a moist cell pellet (■) or stored in neutralized cross-linking media (▲), and cells stored on the bench top for 8 months (●) or 9 months (×) in 30 mM phosphate buffer, pH 7.2. The fifth data set is from data from the field remediation study (◆).

that perhaps the drop in measurable activity may be associated with chemical interactions occurring between the enzyme embedded in the cross-linked cells and other molecules present in the complex soil environment.

While it is true that the enzymatic activity of killed cells decreases in the chemically complex soil environment, and that the chemical process used to kill the cells results in a 35% initial loss of enzyme activity, this field experiment has demonstrated that the overall enzyme activity remains acceptably high for soil remediation work. There is a beneficial trade-off involved in the use of enzyme encapsulated in whole cells to be considered as well: cells that have been chemically cross-linked show stabilization of enzyme activity during storage. After 8 months of storage at room temperature, cross-linked cells retained more than 50% of their original enzyme activity. This compares extremely favourably with storage of purified enzymes, which must be maintained under special conditions to retain activity over time. In addition, it should be noted that major costs of cell preparation and on-site engineering are clearly cheaper than incineration, or transport of the soil to a land-fill rated to accept hazardous waste. These factors combine to make the use of atrazine-degrading enzymes encapsulated in genetically engineered bacteria an economically viable and environmentally effective remediation method, even when implemented under suboptimal environmental conditions.

Experimental procedures

Chemicals

Authentic atrazine, provided by Novartis Crop Protection, was used for gas chromatography standards, and enzyme activity and plate clearing assays. High-performance liquid chromatography (HPLC)-grade dichloromethane used for atrazine extraction, 50% aqueous glutaraldehyde (photographic quality), Tris base and sodium tetraborate used in cell cross-linking were obtained from Fisher.

Bacterial strains, plasmids, cross-linking and growth conditions

Atrazine chlorohydrolase, AtzA, was produced by growing a large quantity of *E. coli* DH5α containing plasmid pMD4 (deSouza *et al.*, 1995). Cells (300 l) were grown at 37°C in a batch fermentor at 207 mmHg (4 psi), pH 7.0, with a stir rate of 400 r.p.m. Air flow was maintained at 125 l min^{-1} with oxygen supplementation if dissolved oxygen fell below 50%. Medium (66 l) containing 12 g l^{-1} tryptone, 24 g l^{-1} yeast extract, 1.1 g l^{-1} KH_2PO_4, 4.7 g l^{-1} K_2HPO_4, 4 g l^{-1} glucose and 25 mg l^{-1} chloramphenicol was fed continuously into the reactor. Feed was controlled with a peristaltic pump, and feed rate was increased from 2 to 5 l h^{-1} over 14 h. Cells were killed and cross-linked by the addition of 0.3% glutaraldehyde. Cross-linked cells were allowed to incubate for 1 h at 22°C, with stirring at 300 r.p.m. After incubation, 7.5 kg of sodium tetraborate was added to the 370 l culture, and the pH was adjusted to 8.8 with the addition of H_3PO_4. After another hour of incubation, 1 kg of Tris base was added and the pH adjusted to 8.6 with H_3PO_4. The suspension was stirred overnight at 22°C; then, the cells were harvested by centrifugation at 15 000 × *g* in a Sharples AS-16 centrifuge. Killed and cross-linked whole cells were used as enzyme carriers. To determine whether the cross-linked *E. coli* cell suspension contained only dead cells, aliquots of the killed cell suspension were plated onto LB agar medium (Sambrook *et al.*, 1989) and LB medium containing 500 p.p.m. atrazine and 30 μg ml^{-1} chloramphenicol.

Enzyme activity

The kinetics of the purified AtzA enzyme have been described previously (deSouza *et al.*, 1996). Enzyme velocity in killed whole cells was determined by adding 10 μl of 20% (w/v) killed cell suspension to 1 ml of 30 p.p.m. atrazine in 30 mM Tris-HCl buffer at pH 7.5. The reaction was allowed to proceed at room temperature for ≈ 1 h and stopped by adding 12 μl of 6 M HCl to 0.8 ml of the mixture. Tubes were centrifuged for 10 min at 10 000 *g* to remove cells from the suspension. Atrazine concentration was determined using a modification of the spectrophotometric method for determining triazine concentrations (Shao *et al.*, 1995). Absorbance at 270 nm (A_{270}) was measured, and values were compared with a standard curve generated using known concentrations of atrazine. Enzyme activity tests were performed at five temperatures between 0°C and 24°C on three batches of cross-linked enzyme 7, 8 and 9 months after cross-linking. The 7-month-old cells were the same ones used in the field remediation study, while the 8- and 9-month-old cells were from separate 1 l batch reactions. Samples were stored: (i) frozen as a 20% suspension in neutralized cross-linking media; (ii) at room temperature as a 20% suspension in neutralized cross-linking media; (iii) at room temperature as a 20%

suspension in 30 mM phosphate buffer, pH 7.2; or (iv) as a moistened cell pellet.

Spill site soil

The spill occurred in spring 1997 in South Dakota when a 950 l tank of 0.5 kg l^{-1} atrazine suspension fell off a truck and burst open. The spill was contained by excavating and covering 26 m^3 of contaminated soil with 6 ml plastic sheeting. As shown in Fig. 3, distribution of atrazine in the soil after 18 months of on-site storage was non-uniform. The silty-loam soil, an Eakin–Ethan complex (USDA, 1995), had 3.6% (w/w) organic matter, pH 7.4, and an electrical conductivity of 5.1 mmho cm^{-1} (1:1 slurry). Soil analysis was performed by the Soils Testing Laboratory at the University of Minnesota. The soil was very low in phosphorous, with a Bray's *P*-value of 1 p.p.m. Total nitrogen was 0.68% (w/w) (6800 p.p.m.), and was partly a result of the atrazine content itself, which was estimated to add $\approx$ 2400 p.p.m. nitrogen to the soil.

Microbial mechanism for atrazine degradation

Bench studies were performed on atrazine-contaminated soil to assess the extent to which disappearance of atrazine in this soil is the result of microbial action versus surface-catalysed hydrolysis. A 300 g aliquot of the spill-contaminated soil was sieved through a 2 mm screen, moistened to $\approx$ 20% of dry weight, and divided into six 250 ml sterile Erlenmeyer culture flasks. One set of triplicates was sterilized by autoclaving the soil for 1 h per day at 121°C on each of three subsequent days, after which the soil moisture level was restored to 20% using sterile water. The other set of triplicates remained untreated. Samples were incubated at 27°C, and soil sterility was verified at each sampling point using LB and minimal media plate assays as described below. The sterile technique was used to obtain aliquots for atrazine analysis, and atrazine levels were determined by soil extraction and gas chromatography analysis as described below.

Biostimulation bench-top experiments

Bench studies were performed at 27°C; 45 g aliquots of spill-contaminated soil were incubated with various nutritional supplements in sterile 250 ml Erlenmeyer flasks to assess the extent to which soil amendments would stimulate microorganisms present in the soil to metabolize atrazine (biostimulation). Preliminary experiments screened the 13 potential biostimulation agents shown in Table 1. Soil used in these experiments was contaminated with atrazine from an accidental spill. Initial atrazine concentration in the soil aliquots was determined individually, and ranged from 3400 p.p.m. to 6700 p.p.m.; the efficacy of the supplements was evaluated by comparing the decline in atrazine after 19 days. This experiment was followed by a statistically rigorous three-factor central composite experiment (Box *et al*., 1978). This experimental method is a subset of factorial statistical designs that, when coupled with multiple regression analysis to calculate the response surface, allows quantitative optimization of parameter levels to test simultaneously and rigorously the effects of multiple experimental parameters on atrazine degradation. The three variables were chosen for testing based on the results of the preliminary experiments, combined with previous results showing that pH affects atrazine degradation rate (Mattan, 1998). Carbon as a 50:50 mixture of dextrose–citrate was added to soil in the range of 0–25 000 p.p.m. (0–1.16 g carbon per 45 g soil), phosphate was added in the range of 0–600 p.p.m. as 12 mM sodium phosphate buffer, and change in soil pH was attempted by adjusting the pH of the added phosphate buffer in the range 6.5–8.5. Sixteen samples were augmented as shown in Table 2. The experimental protocol was the same as the preliminary experiment, with the following differences: soil was homogenized so that the initial atrazine concentration in the soil aliquots was uniform at 4500 p.p.m., and the incubation period was reduced to 13 days. The results were analysed for significance using the MACANOVA statistical software package developed at the University of Minnesota.

Field study experimental protocol

Selection of field-scale treatment protocols was guided by the results of the initial laboratory-scale experiments. Four treatment protocols were set up in triplicate, as shown in Table 3. Treatments consisted of: (i) 0.75 m^3 control plots containing only moistened soil; (ii) 0.75 m^3 biostimulation plots augmented with 300 p.p.m. phosphate in the form of triple superphosphate fertilizer; (iii) 0.38 m^3 bioaugmentation plots receiving AtzA enzyme in the form of 0.5% (w/w) killed recombinant *E. coli* cells; and (iv) 0.38 m^3 plots receiving a combination of phosphate plus cells. A Bobcat skid loader was used to separate 7 m^3 of the most highly contaminated portions of soil from the total volume of 26 m^3 contaminated with atrazine. This portion was homogenized as well as possible using the Bobcat skid loader to mix and combine repeatedly the soil on a large tarpaulin. This same method was used to mix treatments homogeneously into appropriate subportions of this soil. Nine 0.75 m^3 treatment bins (0.3 m $\times$ 1.2 m $\times$ 2.4 m), constructed from 1.3-cm-thick plywood lined with 6 ml polyethylene, were designed to contain contaminated soil and treatments fully.

Sampling

Individual samples consisted of 50 ml of soil taken from multiple places from within each treatment plot. Samples were obtained in triplicate from each treatment plot, resulting in nine individual data points at each time point for each of the four treatment protocols. Time points for sample acquisition were time 0 (22 September 1998), 1 week (30 September 1998), 4 weeks (21 October 1998), 8.5 weeks (21 November

Table 3. Treatments used in field-scale bioremediation studies.

	Treatment protocol
Control	Moistened soil
Biostimulation	300 p.p.m. phosphorus (triple superphosphate)
Bioaugmentation	0.5% (w/w) killed cross-linked recombinant cells
Combination	300 p.p.m. phosphorus + 0.5% cells

1998) and 12 weeks (16 December 1998). Samples were immediately frozen on dry ice and shipped overnight to the University of Minnesota for analysis. On receipt, samples were stored at −15°C until analysis could be performed.

Analytical methods

Plate assays. Modified R-minimal medium (Eaton and Ribbons, 1982; Selifonova *et al*., 1993) plates containing 500 p.p.m. atrazine as the sole nitrogen source were used to determine whether atrazine-metabolizing microorganisms were present in the spill-contaminated soil. These plates were opaque with the suspension of small atrazine particles in clear agar. A zone of clearing surrounding the colonies indicated degradation of atrazine by bacteria (deSouza *et al*., 1995). Plates used to grow recombinant strains contained 30 $\mu g\,ml^{-1}$ chloramphenicol.

Atrazine extraction. Aliquots of soil weighing between 5 and 10 g were taken from the homogenized soil samples for atrazine analysis, and the remainder of the sample was refrozen at −15°C. Atrazine concentration was assessed using a simplified procedure based on a methanol (MeOH) extraction procedure for determining p.p.b. concentrations of atrazine in soil (Koskinen *et al*., 1991). The soil aliquots were shaken at 250 r.p.m. with 20 ml of water and 25 ml of dichloromethane (DCM) for a minimum of 2 h on a reciprocating shaker. Tubes were centrifuged at 3000 r.p.m. for 15 min. The DCM layer was pipetted into a glass vial and dried by adding a small amount of anhydrous sodium sulphate. Between 20 μl and 50 μl of the DCM layer was added to 1 ml of HPLC-grade DCM for gas chromatography. Final dilutions for each sample were calculated individually. The accuracy of the simplified procedure was tested by comparing the atrazine extracted from homogenized soil samples using the MeOH extraction procedure (Koskinen *et al*., 1991) with that extracted from the same samples using the simplified procedure ($n = 72$). At high atrazine concentrations (greater than 1400 p.p.m.), the two extraction techniques produced statistically similar results (P-value $= 0.9$).

Gas chromatography. Gas chromatography was performed using a Hewlett-Packard 6890 GC system equipped with a flame ionization detector and interfaced to an HP 79994A Chemstation. The HP capillary column used was 30 m long, 320 μm in diameter and contained a 0.25 μm film of 5% phenyl methyl siloxane. Temperature was ramped over the 15 min run period from 50°C to 300°C. Injection volume was 2 μl. The chromatograph was operated in a constant pressure mode at 1295 mmHg (25 psi), with constant gas composition of 30 $ml\,min^{-1}$ hydrogen, 350 $ml\,min^{-1}$ air and 25 $ml\,min^{-1}$ make-up gas, which was either ultrapure helium or nitrogen. Calibration standards were run with each set of samples. Atrazine retention time was 9.1 min with ultrapure He make-up gas or 8.9 min with N_2 make-up gas.

Soil temperature. The soil temperature at 10 cm depth was obtained from the Chamberlain-National Weather Service reporting station number a391619, elevation 445 km, latitude 43.73° N and longitude 99.32° W. This weather station is less than 65 km from the spill site, elevation 490 km.

Statistical analysis. Data were tested for statistical significance using the analysis of variance package included in Microsoft Excel 98.

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