Chapter 19

Enhancing Biodegradation for Detoxification of Herbicide Waste in Soil

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Pesticides in soil at high concentrations have been found to be unusually resistant to normal biodegradative processes. Microbial systems have been proposed as cost effective techniques suitable for cleanup of pesticide waste. In an attempt to enhance the detoxification in soil of high concentrations of the herbicide alachlor, experiments were designed to test the effects of several factors on alachlor persistence: soil dilution, concentration, formulation, nutrient amendments, and microbial inoculation. Alachlor in soil from a waste site degraded faster when diluted with uncontaminated soil by 90% than when diluted by 10 or 50%. Alachlor was metabolized into water-soluble compounds in soil at a concentration of 10 ppm but not at 1000 ppm. Amendment of soil with ground corn or soybean stubble stimulated the biodegradation of alachlor at a concentration of 100 ppm but not at 1000 ppm. When alachlor was present as a sole carbon source, several bacterial isolates partially detoxified concentrations of 10 ppm but not 100 ppm. Fungal isolates could cometabolically degrade 100 ppm alachlor in pure culture; however, inoculation of soil with an alachlor-degrading fungus alone did not enhance degradation.

The use of pesticides has received intense scrutiny for generations. The concern about residues in food and potential adverse health effects predates the advent of synthetic organic pesticides in the late 1940's (1).

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Since the passage of the Resource Conservation Recovery Act (1976), much concern has focused on pesticide waste, which is inevitably generated by agricultural and urban sectors alike (2-4). In Illinois, agrochemical retail facilities have been particularly problematic. These facilities provide farmers with a variety of services including the custom application of fertilizers and pesticides. Many chemicals are handled at one loading location where spillage is common, resulting in the accumulation of high concentrations of hazardous chemicals. Rinsing of equipment and of empty containers also produces pesticide contaminated discharges that can move off-site as runoff if not handled properly. Very similar conditions of spillage and rinseout may occur on individual farms, especially when the same site is used repeatedly for loading and cleanup. The Illinois Environmental Protection Agency has documented problems of high level pesticide contamination at various agrochemical retail facilities around the state, and the Illinois Department of Public Health has frequently detected unusually high concentrations of pesticides in on-site wells and nearby community wells (5).

Biodegradable pesticides can be extremely persistent when present in the soil at unusually high concentrations (6-10), which contributes to an increased risk of surface and ground water contamination. When a business has a major contamination incident, state or federal regulatory agencies can order a cleanup, but such action is more difficult for private farms and residences. The nature of the cleanup is more problematic. Contaminated soil is excavated and removed to a "secure" landfill. The end result is perhaps a cleaner site, but the waste has not been detoxified.

Cleanup technologies have been proposed for on-site and/or off-site destruction of pesticide wastes (4). The most reliable technologies focus on detoxification of liquid wastes. Destruction of wastes in soil is more difficult and expensive, especially for small businesses and individual homeowners.

New approaches that are consistent with the ubiquity of pesticide waste and mindful of the costs are needed for cleanup in situ, especially when soil has been highly contaminated by past disposal practices (4). Decontamination by microbial systems, which is suitable for meeting those needs, is becoming a feasible technology to clean up waste (11-13). Several strategies have been used for the development of microbial decontamination systems (i.e., bioremediation systems): (1) pretreatment of contaminants with various reagents to produce degradates more amenable to microbial mineralization; (2) treatment of wastes with microbial enzymes; (3) enrichment of wastes to stimulate indigenous microorganisms

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(biostimulation [14]); (4) inoculation of wastes with adapted microorganisms (bioaugmentation [14]).

Recent research with pesticide wastewater has shown that prior treatment with ozone alone (15) or UV light and ozone (16,17) facilitated the microbial metabolism of the resulting degradation products. Similarly, exposure of chlorinated phenols to UV light and hydrogen peroxide allowed microbial mineralization to proceed (18). These emerging technologies, although promising for wastewater, are not easily applicable to treatment of contaminated soil.

A second strategy that might prove useful for decontaminating pesticide wastes in soil has been the addition of hydrolytic enzymes derived from microorganisms (19,20). Organophosphorus insecticides such as parathion and diazinon are most susceptible to decontamination by enzymes. Some success has been realized with immobilization of degradative enzymes on inert surfaces through which wastewater is passed and detoxified.

The third strategy, biostimulation, stimulates degradative activities of resident microflora by enriching the environment with nutrient amendments or by changing the physical characteristics of the environment. For example, degradation of organic solvents and petroleum products can be enhanced in groundwater by addition of oxygen and nutrients (21). Chlorinated pesticides such as toxaphene can be partially decontaminated by addition of nutrients to soils maintained under anaerobic conditions (22). Recently, the degradation of a variety of pesticide classes, including phenoxyacetate, dinitroaniline, and triazine herbicides and organophosphorus insecticides was enhanced in highly contaminated soil by organic, nutrient, and mineral amendments (23). Degradation of pentachlorophenol in anaerobic (flooded) soil was enhanced by the addition of anaerobic sewage sludge (24). DDT degraded significantly faster in flooded soil amended with rice straw (25).

The bioaugmentation strategy involves the selection of adapted microbial strains that metabolize a pesticide as a carbon or nutrient source. Under these circumstances, the rate of degradation is enhanced compared to the rate normally observed in soil or water. The development of an adapted microbial strain usually begins with the enrichment and isolation of pesticide-degraders from the contaminated environment. Enrichment is relatively easy with compounds that are used as carbon or nutrient sources. Compounds that are not readily utilizable, however, require novel approaches to enrich and isolate potential degraders, which may be manipulated to enhance their degradative capabilities.

Recently, contaminant-degrading strains of microorganisms have been constructed by recruiting into a single organism the genes coding for degradative enzymes

(26,27). The success of this technique has hinged upon the knowledge that many xenobiotic-degrading genes are resident on extrachromosomal pieces of DNA called plasmids (28). Pesticide degradation plasmids were first described for 2,4-D and MCPA (29-31). Plasmids are also known to code for enzymes degrading 2,4,5-T (13) and the OP insecticide diazinon (32). Several copies of a specific plasmid occur within an individual cell, and plasmids can be transferred from a donor cell to a recipient cell. Thus, the degradative potential of a microorganism can be quickly amplified in the population. Furthermore, plasmid exchange can occur between species, although it occurs more readily within a species.

By taking advantage of bacterial transformation and conjugation, researchers have constructed strains possessing an entire pathway for xenobiotic mineralization by culturing in chemostats two or more strains possessing complementary parts of the pathway. For example, microbial inocula from hazardous waste sites were placed in a chemostat with microbial strains possessing known plasmids for aromatic hydrocarbon and chlorobenzoate metabolism (27). After cultivation with 2,4,5-T as a sole carbon source for 8 to 10 months, an isolate was produced that could utilize 2,4,5-T. Success in breeding a microbial strain with the capability of metabolizing chlorobenzene has been obtained using a three-stage chemostat system with two known isolates having complementary capabilities of chlorobenzoate metabolism (33).

Our interest in microbial systems for decontamination of pesticide waste evolved from our attempts to remediate highly contaminated soil at an agrochemical retail facility in Piatt County, IL (34). Soil containing high levels of alachlor (2-chloro-2',6'-diethyl-N-[methoxymethyl] acetanilide) (24,000 ppm in the top 7.5 cm of one location) was excavated and land-applied to corn and soybean plots in an effort to stimulate natural biodegradative mechanisms. Other contaminants included metolachlor (2-chloro-N-[2-ethyl-6-methylphenyl]-N-[2-methoxy-1-methylethyl] acetamide), atrazine (2-chloro-4-[ethylamino]-6-[isopropylamino]-S-triazine), trifluralin (α,α,α-trifluro-2,6-dinitro-N-N-dipropyl-p-toluidine), and nitrogen fertilizer. In corn and soybean plots receiving contaminated soil, alachlor and metolachlor persistence was significantly greater than in plots freshly treated with herbicide sprays comprised of equivalent concentrations (10). Soil that had been left in piles on the waste site seemed to have a reduced microbial population and a depressed enzyme activity that may have accounted for the persistence of the herbicides. In laboratory experiments, simulated spills of alachlor (10,000 ppm soil) reduced microbial bioactivity, and the pesticide did not degrade after one year (10).

In an effort to enhance detoxification of high concentrations of alachlor, we designed studies to test the effects of soil dilution, concentration, formulation, and nutrient amendments on persistence. Additionally, we developed a protocol to enrich, isolate, and screen bacteria and fungi for enhanced capabilities of alachlor degradation. Our studies represent the intital stages of the biostimulation and bioaugmentation strategies for waste cleanup.

Materials and Methods

Soil used in the alachlor persistence studies and in the enrichments for alachlor-degrading organisms was derived from two sources: a waste site at an agrochemical facility in Piatt Co., IL and a soybean plot near the waste site that was divided into replicated blocks for a land application study (34). The soil type was a mixture of Ipava silt loam (fine, montmorillonitic, mesic, Aquic Argiudolls) and Sable silty clay loam (fine silty mixed, mesic, Typic Haplaquolls) with pH 5.4-5.5. The soil at the waste site had been excavated and stored in piles which were sampled as needed for laboratory studies (waste-pile soil). Untreated check plots in the soybean field served as sources of uncontaminated soil (CHECK soil). Soils were stored at 2-4°C and passed through a 3-mm screen before use.

Soil Dilution Experiment. Waste-pile soil was mixed with CHECK soil in large plastic bags to produce dilutions of 0, 10, 50, and 90%, which yielded mean alachlor concentrations of 46.8, 47.4, 29.4, and 6.52 ppm, respectively. Thirty-gram portions (oven-dry weight) of soil were dispensed into 250 mL Erlenmeyer flasks and adjusted to 30% moisture (w/w). The flasks were covered with Parafilm and incubated at 25°C. Immediately after mixing, and at periodic intervals during the next 42 days, triplicate flasks were removed for extraction of alachlor. Activity of dehydrogenase soil enzyme was assayed (35) in soils simultaneously incubated in companion flasks.

Effect of Concentration and Formulation. Moist CHECK soil (26.4% moisture, 30 g oven-dry weight) was treated with technical grade alachlor (prepared in acetone) or an emulsifiable concentrate formulation (Lasso 4EC, 45.1% a.i., prepared in water) to yield application rates of 10, 100, and 1000 ppm soil. Stock solutions of alachlor were prepared by mixing the appropropiate amount of either the technical grade or emulsifiable alachlor with 2.6 μCi of uniformly ring-labelled ^{14}C alachlor (Monsanto Co., specific activity=13.74 mg/mCi, radiochemical purity=95%).

One hundred microliters of stock solutions were applied to the soil. After sitting for approximately 6

hours under a fume hood, the flasks were swirled by hand to mix the soil and closed off with rubber stoppers from which hung plastic center wells containing 0.5 mL of 2 N KOH, which served as traps for $^{14}CO_2$. One set of treated soils was capped immediately to determine if $^{14}CO_2$ was evolved during the 6-hour aeration period. A set of untreated soils served as controls to correct for background radioactivity. A third set of soils was treated with 80 μg of uniformly ring-labelled ^{14}C-glucose to ensure that CO_2 was being trapped in the center wells.

Soils were extracted on the same day as application and after 28 days of incubation at 25°C. Every 2-3 days during the interim, flasks were opened and the center wells were removed and placed directly in liquid scintillation cocktail (Biosafe II) for determination of radioactivity. Soils were extracted twice by stirring with ethyl acetate followed by a third extraction with a 1:1:1 mixture of hexane/acetone/methanol. After the last extraction, the soil was filtered through glass microfiber filter paper. The solvents were combined and evaporated to dryness under vacuum at 35°C.

After evaporation, the extract was partitioned between water and methylene chloride (1:1). The water phase was reextracted with CH_2Cl_2. The aqueous and organic phases were evaporated to dryness and made to a 2 mL volume with acetone and methanol, respectively. Five hundred microliters of each phase were counted by liquid scintillation spectrometry. Parent alachlor was determined in the partitioned extracts by GLC.

Enrichment and Isolation of Potential Alachlor-Degrading Microorganisms. The protocol for enriching, isolating, and screening alachlor-degrading microorganisms is summarized in Figure 1. A modified soil perfusion system (36) and soils from a simulated alachlor spill containing 10,000 ppm alachlor served as primary enrichments for selecting potential degraders. Inocula from the primary sources were used to further enrich for alachlor-degraders in chemostat and batch shake flasks (Figure 1, secondary enrichment). The perfusing medium contained mineral salts medium (MSM), alachlor (100 mg/L), dextrose (500 mg/L), and 100 mg/L of yeast extract, chloroacetate, benzoic acid and p-chloroaniline. The mineral salts medium was composed of (g per L final concentration): $MgSO_4 \cdot 7H_2O$ (0.2 g); NaCl (0.1 g); $CaCl_2.2H_2O$ (0.1 g); KNO_3 (0.5 g), and K_2HPO_4 (1.0 g). The source of soil was a soybean plot to which alachlor-contaminated soil had been land-applied.

The chemostat vessel was a modified 500-mL Virtis fermentor with a teflon impeller. The medium consisted of 350 mL MSM containing alachlor (100 mg/L), glucose (100 mg/L), and yeast extract (50 mg/L). The chemostat was inoculated with 20 mL of soil perfusate, the inoculum was allowed to grow to stationary phase as a batch culture,

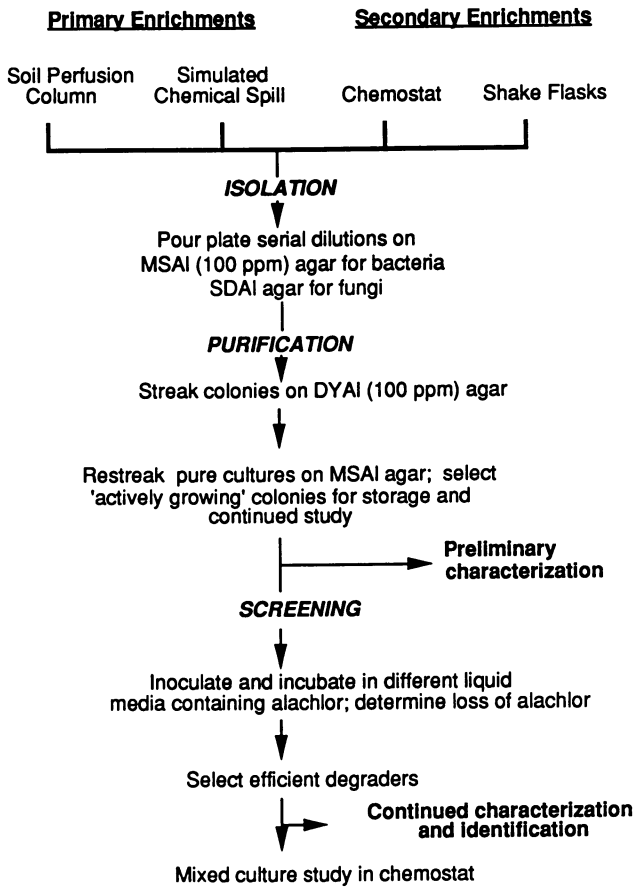

Figure 1. Protocol for enrichment, isolation, and study of microbial cultures.

and then fresh medium was pumped in at the rate of 0.1 mL/min. The chemostat was sampled periodically for microbial isolation and chemical assay of alachlor.

Batch culture systems consisted of 125-mL Erlenmeyer flasks containing 50 mL MSM and 100 mg/L alachlor. The flasks were inoculated directly with 0.5 g of soil from simulated chemical spill experiments or with 1 mL aliquots of soil suspensions (1 g soil/10 mL H_2O). The flasks were incubated on a rotary shaker at 25°C for up to 4 weeks with periodic sampling for microbial isolation.

Cultures from the enrichment systems were subsampled periodically and plated either on agar containing MSM plus alachlor (MSAl) to isolate bacteria or on Sabaroud dextrose with alachlor (SDAl) to isolate fungi (Figure 1, isolation step). The agar plates were incubated at 25°C and examined periodically for microbial growth. Bacterial colonies that formed on MSAl agar were purified by streaking on dextrose-yeast extract-alachlor (DYAl) agar (Figure 1, purification step). Purified bacterial colonies were restreaked on MSAl and isolates that grew were stored in DYAl for further characterization. Fungi were purified by hyphal tipping on SDAl. All pure isolates were maintained on their respective agar preparations and stored at 5°C.

Screening Potential Alachlor-Degrading Bacteria. Twenty-eight bacterial isolates were preliminarily screened for the ability to degrade alachlor cometabolically or as a sole carbon source. From this test, nine isolates were selected for further screening. First, the bacteria were inoculated into 20 mL of filter-sterilized MSAl containing glucose (100 mg/L) and yeast extract (500 mg/L). After incubation for 24 h on a rotary shaker, 0.2 mL of culture was inoculated into test tubes containing 5 mL of one of the following filter-sterilized media: (1) alachlor (10 or 100 ppm) in MSM (MS-Al); (2) alachlor (10 or 100 ppm) in MSM + yeast extract (50 mg/L) + dextrose (0.1 or 1.0 g/L) (YAl-D or YAl-D+); (3) alachlor (10 or 100 ppm) in soil extract + dextrose (0.0, 0.1 or 1.0 g/L) (SEA1, SEAl-D or SEAl-D+).

Soil extract was prepared by autoclaving a mixture of 1 kg CHECK soil and 1.5 L of H_2O for 30 min at 121°C. After cooling at 5°C overnight, the soil suspension was filtered through glass microfiber filters and centrifuged at 5523 x g for 5 min. The extract was buffered at pH 6.8 using K_2HPO_4 (0.5 g/L).

After inoculation, duplicate cultures were incubated in the dark without agitation at 25°C for 10 days. Four-mL aliquots of the cultures were extracted twice with ethyl acetate for analysis of alachlor. Controls consisted of uninoculated media.

Screening Potential Alachlor-Degrading Fungi. Two fungal isolates (CCF-1 and CCF-2) were tested in batch shake-flask cultures in a medium (PYAl) containing (g/L): peptone (1.0), yeast extract (3.0), dextrose (20.0), K_2HPO_4 (1.0), $CaSO_4$ (0.014) and alachlor (0.1). Inocula were prepared by growing two isolates in PYAl. After 5 days, growth was harvested by centrifugation, and the fungal pellets were aseptically macerated. One-mL aliquots of the homogenized cultures were inoculated into 50 mL PYAl or MSAl. Controls included alachlor-fortified, uninoculated flasks and inoculated flasks containing peptone-yeast extract medium without alachlor. At intervals of 3, 7, and 14 days, cultures were filtered and washed; the filtrate and washings were combined and extracted twice with ethyl acetate for alachlor analysis. Chloride released from alachlor was analyzed in part of the filtrate by using a modified ferricyanide colormetric method (37).

Effect of Nutrient Amendments and Microbial Inoculum. Batches of CHECK soil (2-mm mesh, 25% moisture) were mixed with ground (2-mm mesh) corn (CS) or soybean (SB) stubble at a rate of 20 g/kg oven-dried soil. Thirty grams of amended soils were weighed into flasks; half of the flasks containing SB were treated with a stock solution of NH_4NO_3 at a rate of 1 mg N/g (SB+N). Aliquots of formulated alachlor (Lasso 4EC) were pipetted on soil to yield concentrations of 100 or 1000 ppm. About 1-2 hours after pesticide treatment, half of the flasks assigned to each treatment were inoculated with fungal isolate CCF1 (0.5 mg fungal units/0.28 mL/g oven-dry soil), which had been blended in phosphate-buffered water (pH 7) containing alachlor (100 ppm). Unamended, uninoculated soils fortified with alachlor were controls. All flasks were closed with Parafilm and incubated at 25°C. Once a week the flasks were opened for aeration. On days 0, 14, 28, and 56, flasks were removed for extraction and analysis of alachlor, soil dehydrogenase (35), and soil esterase. (38).

Extraction and Analysis of Alachlor. Soil was slurried with 12 mL of water and extracted twice by stirring with 50 mL of ethyl acetate for 45 minutes. The solvent was decanted after each extraction and concentrated on a steam bath. The extract was rediluted with ethyl acetate and analyzed by GLC with nitrogen-phosphorus specific detection. Residues were separated on a 90-cm x 0.2 mm i.d. glass column packed with 5% Apiezon + 0.125% DEGS maintained isothermally at 190°C. Injector and detector were held at 250°C, and gas flow rates were adjusted as needed to obtain maximum sensitivity and resolution. Residues were quantitated by the method of external standards.

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Results and Discussion

Effects of Soil Dilution. In previous studies at an agrochemical facility contaminated with herbicide waste, alachlor had not totally degraded after two years in soil that had been excavated and stored in piles on the ground (10). Land application has been studied as a method for stimulating biodegradation by diluting the soil on cropped land (34). Under laboratory conditions, alachlor degraded slower in waste-pile soil than it did in soil diluted by 90% (w/w) (Table I). Seventy-percent of the alachlor was detoxified in 42 days compared to ≤30% in waste-pile soil diluted up to 50%. The initial concentration of alachlor in soil diluted by 90% was 6.5 ppm, which would be similar to the concentration in a 15-cm depth of soil after a typical application of alachlor at a rate 3.36 kg AI/ha The typical half-life of alachlor in field soil ranges from 2-4 weeks (39), but high concentrations typical of waste are very persistent (40).

The activity of soil dehydrogenase at the beginning of the soil dilution experiment in 0, 10, 50, and 90% diluted waste-pile soil was 18, 0, 65, and 138%, respectively, of the activity in CHECK soil. After 21 days, soil dehydrogenase was still inhibited in the 0 and 10% diluted waste-pile soil. The inhibition of enzyme activity suggests that high concentrations of alachlor may be toxic, but microbial bioactivity can be restored if contaminated soil is diluted enough.

Table I. Percentage Recovery of Alachlor in Diluted and Undiluted Waste-Pile Soil

	% of Initial PPM Recovered at Indicated % Dilution			
Days	0	10	50	90
5	122	106	123	98
10	112	98	128	79
21	135	78	102	39
42	76	71	106	30

Effect of Concentration and Formulation. Other research has shown that high concentrations of pesticides are very persistent compared to normally applied levels (8,9). Furthermore, high concentrations can reduce microbial populations, which may explain the slower rate of biodegradation (8). Although high concentrations of alachlor seemed to reduce microbial bioactivity, we were unsure if the effect was due to alachlor itself or additives in the formulation; therefore, we conducted a study to look at the interaction of concentration and

formulation (technical vs. emulsifiable concentrate) on alachlor degradation.

Six hours after application, recovery of ^{14}C in each phase as a percentage of the intially added amount was not significantly different among the various concentrations

Table II. Effect of Concentration and Formulation on Metabolism of Alachlor in Soil at 28 Days Following Application

		% of Initially Added Alachlor	
Concentration	Phase	Technical	Emulsifiable
10 ppm	^{14}C organic	25.2	23.3
	^{14}C aqueous	15.2	9.5
	$^{14}CO_2$	0.4	0.5
	^{14}C unextracted	59.2	66.7
	alachlor organic	23.5	25.5
	alachlor aqueous	0.0	0.0
100 ppm	^{14}C organic	66.9	67.2
	^{14}C aqueous	7.6	5.0
	$^{14}CO_2$	0.2	0.2
	^{14}C unextracted	25.3	27.6
	alachlor organic	69.2	76.2
	alachlor aqueous	0.1	0.0
1000 ppm	^{14}C organic	113.4	102.6
	^{14}C aqueous	0.9	1.2
	$^{14}CO_2$	0.2	0.2
	^{14}C unextracted	0.0	0.0
	alachlor organic	131.4	119.2
	alachlor aqueous	0.0	0.2

or formulations. The small percentage of ^{14}C detected in the aqueous phase (<2.5%) could have been an artifact from the partitioning process. No $^{14}CO_2$ was lost in the 6 hours allowed for aeration of the flasks containing the emulsificable concentrate formulation.

Twenty-eight days after application, cumulative $^{14}CO_2$ in the 10 ppm treatments was higher than in the 100 and 1000 ppm treatments but still averaged less than 0.5% of the added radioactivity (Table II). Recovery of radiolabel in the organic phase was significantly lower in the 10 ppm treatment than in the 100 or 1000 ppm treatment, but for any one concentration there was no significant difference between technical and emulsifiable concentrate formulations. The complete recovery of both parent alachlor and ^{14}C in the organic phase of the 1000 ppm treatment indicated that alachlor was not

significantly degraded in the 1000 ppm treatment. Alachlor degraded more slowly at 100 ppm than at 10 ppm.

Partitioning of radioactivity between the organic and aqueous phase showed a nearly logarithmic increase in the ratio of ^{14}C in each phase from 10 ppm to 100 ppm regardless of formulation (Table II). In the aqueous and organic phases of the 10 ppm treatments, the absence of parent alachlor in the GLC analysis showed that the two-fold difference in ^{14}C between the two phases was in part due to production of water soluble metabolites. The 10 ppm treatment contained nearly twice as much water soluble radiolabel as the 100 ppm treatment.

Alachlor has been shown to degrade in soil by microbial cometabolism rather than by mineralization (41), and CO_2 was not produced after introduction of radiolabelled alachlor to microbial suspensions (41,42). An early investigation of alachlor metabolism by a pure culture of fungus showed significant production of 2,6-diethylaniline, which could be further metabolized (42). More recent research, however, has shown that alachlor is dechlorinated in soil followed by conjugation with glutathione (J. Malik, Monsanto Agricultural Products Co., personal communication). The conjugates are then further metabolized to yield sulfonic and oxanilic acid derivatives of alachlor (39), which would be very water soluble.

Screening of Bacteria for Degradation of Alachlor. When alachlor was present as a sole carbon source in MSM (MS-Al), three isolates, C1, SA3-2, and SA3-3, degraded 59, 45, and 27%, respectively, of a 10 ppm dose after 10 days of incubation (Table III). At a dose of 100 ppm, however, no bacterial isolate could degrade alachlor as a sole carbon source (Table IV). Isolate C1 very actively detoxified 10 ppm alachlor in the presence of additional nutrients but seemed poisoned by 100 ppm alachlor (Table III,IV). Isolates CCII, C4, and SA3-1 degraded 20-27% of 100 ppm alachlor in media containing dextrose plus soil extract or yeast extract. In other research, Novick et al. (41) were unable to isolate alachlor mineralizers after soil inocula were treated with 1 and 100 ppm alachlor. In a study with the closely related herbicide metolachlor (43), mineralizing organisms could not be isolated and it was noted that actinomycetes could not tolerate concentrations $\geq$200 ppm. These data suggest microbial toxicity at high concentrations of alachlor and metolachlor.

Screening of Fungi for Alachlor Degradation. Fungal isolates CCF-1 (tentatively identified as *Fusarium* sp.) and CCF-2 degraded more than 70% and 50%, respectively of a 100 ppm dose of alachlor after 14 days of incubation in peptone-yeast extract medium (PYAl). About 18% of the

chlorine in alachlor was released as chloride in both cultures. Neither isolate degraded alachlor when present as a sole carbon source in MSM. These data show that efforts to develop microbial systems for detoxification of waste need to focus on fungi in addition to bacteria. Indeed, a white rot fungus has been recently reported to degrade several organochlorine compounds (44).

Table III. Percentage Alachlor Removed from Solution after 10 Days of Incubation with 10 ppm Alachlor in Indicated Media[a]

Isolate	MS-Al	YAl-D	YAl-D+	SEAl	SEAl-D	SEAl-D+
SA3-1	0.0	0.0	6.8	-	-	-
SA3-2	45.4	21.1	0.0	23.2	25.4	25.4
SA3-3	26.5	0.0	0.0	0.0	0.0	0.0
SA4	0.0	12.4	16.7	0.0	0.0	10.3
2A1	0.0	13.8	0.0	0.0	0.0	1.5
C1	58.5	47.4	46.5	38.2	40.7	48.0
C2	15.3	16.7	26.5	14.7	2.9	0.0
C4	13.4	12.2	56.7	12.5	23.3	2.8
CCII	12.2	6.1	30.2	12.5	57.9	12.5

[a] See text for culture media codes.

Table IV. Percentage Alachlor Removed from Solution after 10 Days of Incubation with 100 ppm Alachlor in Indicated Media[a]

Isolate	MS-Al	YAl-D	YAl-D+	SEAl	SEAl-D	SEAL-D+
SA3-1	0.0	23.0	0.0	0.0	0.0	0.0
SA3-2	0.0	0.0	0.0	0.0	0.0	5.0
SA3-3	0.0	6.5	0.0	0.0	0.0	12.3
SA4	0.0	0.9	11.5	1.8	18.2	17.7
2A1	0.0	16.2	10.8	0.0	12.2	14.3
C1	0.0	0.0	0.0	5.9	0.0	0.0
C2	0.0	16.5	0.0	9.0	0.0	9.6
C4	0.0	2.2	15.6	22.7	20.0	22.3
CCII	5.0	21.0	21.3	16.5	27.4	20.8

[a] See text for culture media codes.

Effect of Nutrient Amendments and Microbial Inoculum. Degradation of a 100 ppm dose of alachlor was enhanced when the soil was amended with ground corn and soybean stubble (Table V). After 56 days of incubation about 94 and 83% of the alachlor were degraded in CS and SB+N-amended soils respectively, compared to about 40% degradation in the unamended soils. In soils amended with SB alone, degradation was slower than in soils with CS and

SB+N but was still significantly higher than degradation in the unamended soil.

The rationale for choosing the organic amendments was based on a hypothesis that increasing the carbon to nitrogen (C:N) ratio in soil would stimulate microbial activity and cause a depletion of soil N, and thereby induce microbial attack of less readily available N sources like alachlor. The order of C:N ratio in the amendments was CS < SB+N < SB. According to the hypothesis, the order of degradation rate should have been

Table V. Degradation of 100 ppm Alachlor in Soils Amended with Corn and Soybean Stubble and Incubated with Fungal Isolate CCF-1[a]

Soil Treatment		Percentage Alachlor Recovered [a] Days of Incubation		
Amendment	Inoculation	14	28	56
none	no	97 A	63 A	59 A
corn	no	56 B	24 C	6 C
soybean	no	68 B	42 B	36 B
soybean+N	no	65 B	26 C	17 C
none	yes	84 A	63 A	57 A
corn	yes	38 C	23 C	4 C
soybean	yes	55 B	37 B	28 B
soybean+N	yes	53 B	22 C	3 C

[a] Means followed by the same letter within an inoculation group are not significantly different according to Duncan's multiple range test (p=0.05).

SB > SB+N > CS. In fact, the more rapid degradation of alachlor in CS- and SB+N-amended soil coincided better with indicators of microbial activity than C:N ratio. For example, soil dehydrogenase activity and soil esterase in CS- and SB+N-amended soils was usually higher than in unamended and SB-amended soils (Figure 2). Although dehydrogenase in SB-amended soils was lower than in unamended soils, soil esterase was significantly higher in SB-amended soils. Soil enzyme activity is indicative of microbial metabolic activity but is not necessarily correlated with capacity to degrade xenobiotics; the data do show, however, that stimulation of enzyme activity was coincidental with a rapid loss of alachlor.

On the basis of efficiency of alachlor removal from solution, fungal isolate CCF-1 was chosen for soil inoculation. CCF-1 alone did not enhance the degradation of alachlor, but the combination of inoculum and amendment, compared to organic amendments alone, slightly enhanced degradation. This additional enhancement was more pronounced in the SB- and SB+N-amended soils than in the CS-amended soils. To determine whether the isolate is

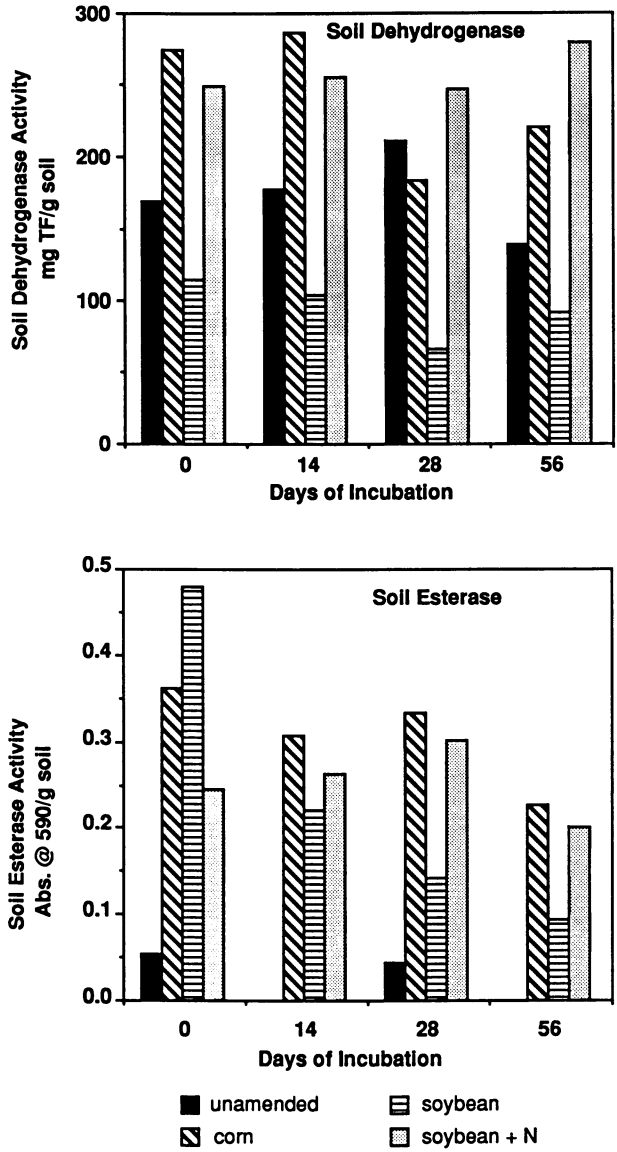

Figure 2. Soil enzyme activity in soils treated with alachlor at a rate 100 ppm, amended with corn, soybean, or soybean+N, and inoculated with fungal isolate CCF-1.

useful in the field, increased dosages of CCF-1 would have to be tested to overcome interspecific competition from other microorganisms. The research of Barles et al.(45) and Kilbane et al. (46) shows that microbial inoculation of soil can be successful if a compound is mineralizable, but whether cometabolizing organisms can compete successfully remains to be seen.

After 56 days of incubation, degradation of 1000 ppm alachlor did not exceed 35% in any soil treatment (Table VI). About 33% of the applied alachlor was degraded in the inoculated, CS-amended soil after 28 days, but no further degradation was observed thereafter. In nearly every two-way comparison, alachlor degraded significantly faster in the 100 ppm treatments than in the 1000 ppm treatments. Coincidentally, soil dehydrogenase activity in all 1000 ppm treatments was severely depressed.

The soil amendment experiments demonstrate that alachlor is degraded by cometabolism rather than mineralization in the soil. In other words, alachlor can be partially degraded in the soil (i.e., detoxified), but its metabolism does not result in procurement of energy or nutrients for growth. By supplying extra nutrients, indigenous microorganisms that have an incidental ability to detoxify a chemical can proliferate, which coincidentally causes a decline in the observed concentration of the chemical. For example, after addition of various organic amendments to soil maintained under flooded (or anaerobic conditions), other researchers have reported enhanced biodegradation of recalcitrant organochlorine pesticides such as pentachlorophenol (24), DDT (25) and toxaphene (47). Under aerobic conditions, glucose and wheat straw have enhanced the biodegradation of the asymetrical triazine herbicide metribuzin, but

Table VI. Degradation of 1000 ppm Alachlor in Soils Amended with Corn and Soybean Stubble and Incubated with Fungal Isolate CCF-1

Soil Treatment		Percentage Alachlor Recovered[a] Days of Incubation		
Amendment	Inoculation	14	28	56
none	no	96 BC	81 A	87 A
corn	no	87 C	73 A	83 A
soybean	no	116 A	79 A	84 A
soybean+N	no	105 A	99 A	88 A
none	yes	89 A	102 A	86 A
corn	yes	79 A	67 C	67 A
soybean	yes	77 A	82 BC	75 A
soybean+N	yes	96 A	89 AB	77 A

[a] Means followed by the same letter within an inoculation group are not significantly different according to Duncan's multiple range test (p=0.05).

alfalfa residue slowed biodegradation (48). This latter research suggests caution is called for when using amendments and mineral nutrients or altering environmental conditions. Recently, Winterlin et al.(23) showed that the degradation of a number of pesticides could be enhanced by certain amendments or environmental conditions, but some conditions actually inhibited degradation. The process of pesticide degradation by cometabolism underscores the need to understand the types of environmental manipulations that are necessary to optimize microbial performance.

In conclusion, enhanced biodegradation is a desirable phenomenon to be exploited for detoxification of pesticide waste. Degradation of compounds that are not mineralizable can still be enhanced if appropriate nutrients and environmental conditions are present. Thus, microbial systems for waste disposal should not be limited by cometabolic nutritional strategies but will require more intensive study of the biochemical ecology of microorganisms.

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