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Immobilization of Leachable Toxic Soil Pollutants by Using Oxidative Enzymes†

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Screening of leachable toxic chemicals in a horseradish peroxidase-H_2O_2 immobilization system established that immobilization was promising for most phenolic pollutants but not for benzoic acid, 2,6-dinitrocresol, or dibutyl phthalate. The treatment did not mobilize inherently nonmobile pollutants such as anilines and benzo[*a*]pyrene. In a separate study, an extracellular laccase in the culture filtrate of *Geotrichum candidum* was selected from five fungal enzymes evaluated as a cost-effective substitute for horseradish peroxidase. This enzyme was used in demonstrating the immobilization and subsequent fate of ^{14}C-labeled 4-methylphenol and 2,4-dichlorophenol in soil columns. When applied to Lakewood sand, 98.1% of 4-methylphenol was leached through with distilled water. Two days after immobilization treatment with the *G. candidum* culture filtrate, only 9.1% of the added 4-methylphenol was leached with the same volume of water. Of the more refractory test pollutant 2,4-dichlorophenol, 91.6% had leached at time zero and 48.5% had leached 1 day after the immobilization treatment. However, 2 weeks after immobilization, only 12.0% of the 2,4-dichlorophenol was leached compared with 61.7% from the control column that received no immobilization treatment. No remobilization of the bound pollutants was detected during 3- and 4-week incubation periods. Enzymatic immobilization of phenolic contaminants in soil appears to be a promising technique for the reduction of groundwater pollution by such substances.

The most hazardous and least tractable aspect of chemically contaminated land sites is the resulting contamination of groundwater with leachable toxic waste components (12). Traditional remedies, such as excavation and removal of the contaminated soil to a secure chemical landfill and bioremediation of the contaminated groundwater, both suffer from various technical difficulties or exorbitant cost.

In the carbon cycle of the soil, highly biodegradable organic compounds are converted to CO_2 and microbial biomass, but the less biodegradable products of decay processes, such as phenolics from lignin, are stabilized as soil organic matter (14). For conversion into nonleachable humic polymers, the precursors need to be activated by autooxidation, microbial enzymes, or free radicals of other chemical compounds. The role of microbial enzymes in oxidative coupling reactions affecting the fate of natural and xenobiotic aromatic compounds in soil was reviewed by Sjoblad and Bollag (13) and Bollag (5). The polymeric products of such reactions are in general much less soluble in water than the parent monomers. Activation by microbial enzymes can also lead to direct incorporation of xenobiotic residues into soil organic matter (1, 3). Both polymerization and incorporation into soil organic matter were expected to reduce the mobility of leachable xenobiotic pollutants in soil. The enzymatically induced immobilization of xenobiotic pollutants that are not readily biodegraded in soil offers a cost-effective biotechnological alternative for aquifer protection against polluting chemical spills. This paper describes tests on the feasibility of this approach by measuring the immobilization on soil columns of several potential groundwater pollutants with commercially available horseradish peroxidase. In addition, we present more detailed studies on the immobilization and subsequent fate of the radiolabeled test pollutants 4-methylphenol and 2,4-dichlorophenol with a fungal exoenzyme selected for its reactivity with a wide range of phenolic pollutants (13).

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MATERIALS AND METHODS

Materials. The oxidase reagent 3,3-dimethoxybenzidine and the phenolic and nonphenolic test compounds were purchased from Aldrich Chemical Co. (Milwaukee, Wis.). Their specified purity was 98% or higher, and they were used without further purification. The uniformly ^{14}C-ring-labeled radiochemicals 2,4-dichlorophenol (2,4-DCP) and 4-methylphenol (4-MP) had a radiochemical purity, as determined by thin-layer chromatography (TLC), of 98 and 99%, respectively. Their specific activities were 2.9 and 2.1 mCi/mmol, respectively, and they were purchased from Pathfinder Laboratories (St. Louis, Mo.). Horseradish peroxidase (HRP) type II, 152 purpurogallin units per mg, was purchased from Sigma Chemical Co. A partially hydrolyzed humic extract was prepared by adding 2 g of milled *Sphagnum* peat moss, obtained in a local garden supply store, to 500 ml of 0.1 N NaOH and autoclaving it as 121°C for 30 min under an N_2 atmosphere. After cooling, the pH was adjusted to 7.0 with H_2SO_4. Undissolved material was removed by filtration, and the resulting solution contained 1 mg (dry weight) of humic acid per ml, as determined by precipitation at pH 1.0.

The test soil, Lakewood sand, was collected in the Lebanon State Forest, N.J. Because of its very high permeability, it represents a "worst-case" soil in terms of leaching of chemical spills. As determined by the hydrometer procedure (11), its composition was 90% sand, 4% silt, and 6% clay. Organic matter, by ignition, was 1.3%; the pH was 4.5.

Geotrichum candidum (ATCC 26195), originally isolated from soil (6), was obtained from the American Type Culture Collection and grown for extracellular enzyme production on potato extract medium (Difco) supplemented with 1% glucose (pH 5.5) by the mat replacement technique. Roux bottles (1,000 ml) containing 100 ml of a spore suspension of

TABLE 1. Conditions and retention times for GC analysis of test compounds

Compound	Column (RSL)	Temp (°C)[a]			Retention time (min)
		Injector	Oven	Detector	
Benzo[*a*]pyrene	300	250	250	300	4.61
4-Chloroaniline	150	150	90	250	2.00
3-Chlorobenzoate	300	150	100	250	1.46
4-Chlorophenol	300	150	120	250	1.03
Dibutyl phthalate	150	200	180	250	1.79
2,6-Dinitrocresol	150	150	150	250	1.66
4-MP	150	100	95	250	0.84
Pentachlorophenol	300	150	150	250	1.92

[a] All temperatures were isothermal.

G. candidum were incubated in stationary culture at 27°C. On day 20, the spent culture medium was decanted. Aseptically, fresh medium was poured under the thick fungal mat, and incubation was continued for a 12-day period, when a second batch of enzyme was harvested from the same fungal mat. The spent culture medium was filtered through Whatman no. 1 filter paper and used as an enzyme source either immediately or after storage at −15°C.

Initial screening for immobilization. Testing was performed in two stages. For stage I, each test compound (50 mg) was dissolved in 10 ml of H_2O. Phenolics and benzoic acid were solubilized as their sodium salts. Control solutions were left standing for 2 h at 20°C with no enzyme addition and then extracted and analyzed as described below. To identical solutions, the immobilization treatment consisting of 50 U of HRP, 0.3% H_2O_2, and 10 mg of humic acid hydrolysate in 10 ml of distilled H_2O was applied. The humic hydrolysate was included to provide a polymeric matrix to which the oxidized compounds would bind. After a 2-h reaction period at 20°C, any precipitate was removed by filtration. The pH of the clarified solution was adjusted to favor extraction into a nonpolar solvent, and the unreacted residue was recovered by extraction into three portions of CH_2Cl_2. The solvent solutions were combined, concentrated in a rotary evaporator at 42°C, and analyzed by gas chromatography (GC). When removal from solution indicated reactivity of a test compound with the HRP immobilization system, the experiment was repeated on soil columns prepared by packing 100 g (dry weight) of Lakewood sand into a glass column (2 by 60 cm). The soil was brought to 50% of its water-holding capacity. Dissolved in 5 ml of H_2O, a 50-mg quantity of the test compound was applied to the top of the column. Subsequently, the soil column was leached with 500 ml of distilled water, the leachate was collected and extracted into methylene chloride, and the test compound was quantified by GC. These controls defined the leachability of the test compound without the immobilization treatment. For immobilization experiments, soil columns were prepared identically, but application of the test compound was followed by 10 ml of the previously described immobilization mixture applied to the top of the soil column. After a 2-h reaction period at 20°C, the columns were leached as above and the leachate was analyzed by GC for residual test compound. The retention times of the test compounds are listed in Table 1.

Because of its very low water solubility, benzo[*a*]pyrene was tested only in soil and with a slightly modified procedure. Benzo[*a*]pyrene was applied in 1 ml of CH_2Cl_2 to 2 g of dry Lakewood sand. The solvent was evaporated, and the treated soil was loaded on top of 8 g of untreated Lakewood sand. After immobilization as described above, this smaller soil column was leached with a proportionally smaller amount (100 ml) of H_2O. Recovery and measurement of benzo[*a*]pyrene in the leachate was performed as above.

Immobilization with *G. candidum* culture filtrate. The *G. candidum* experiments were also performed on columns of Lakewood sand. The experimental arrangement was similar to that described above except that the glass columns contained 70 g of soil (dry weight) adjusted to 50% of its water-holding capacity. The radiolabeled test compounds 2,4-DCP and 4-MP were added to separate columns at 5 mg, 8×10^5 dpm each, in 5 ml of H_2O. Use of the radiolabeled compounds allowed more complete residue analysis and recovery studies, since "bound" residues could be analyzed not only by removal from solution but also by direct measurement after incineration of the soil and trapping of the released $^{14}CO_2$.

After the test compounds were applied to the top of the soil columns, they were allowed to infiltrate. The immobilization mixture, applied subsequently, consisted of 15 ml of *G. candidum* culture filtrate, containing 425 U of enzyme per ml. In the case of 2,4-DCP, 4.5 mg of ferulic acid (equimolar to 2,4-DCP) was added simultaneously with the culture filtrate. Of the 15 ml of immobilization mixture, 5 ml was injected by syringe to a depth of 3 to 4 cm below the soil surface, i.e., below the contaminated-soil zone. The remaining 10 ml was applied to the top of the column. Control columns were treated similarly but with boiled culture filtrate. Columns were leached with 200 ml of distilled H_2O at various times. The leachates were acidified to pH 1.0 with H_2SO_4 and, after 1 h, were centrifuged at 4,000 × *g* for 30 min. The pellet and the supernatant were counted separately for radioactivity. Subsequently, the supernatant was extracted by three 25-ml portions of methylene chloride. The combined solvent extracts and the residual aqueous phase were counted for radioactivity separately.

The leached soil was mixed with a sufficient amount of anhydrous Na_2SO_4 for drying and Soxhlet-extracted first with 100 ml of acetone and subsequently with 100 ml of benzene for 3 h each. These extracts were counted for radioactivity. The extracted soil-Na_2SO_4 mixture was finely ground, and 1-g samples were oxidized with a model B 306 Packard Tri-Carb sample oxidizer. The evolved $^{14}CO_2$ was trapped and counted, and the value was listed as soil-bound residue.

A partial characterization of the radioactivity in the methylene chloride extract of the acidified aqueous column leachate and in the solvent extracts of the previously leached soil columns was performed by thin-layer chromatography (TLC) and GC. For TLC, the solvent extracts were applied to silica gel Redi Plates (20 by 20 cm; Fisher Scientific, Springfield, N.J.) and developed by using benzene–*p*-dioxane–acetic acid (90:25:4). Autoradiograms of the developed plates were prepared by 3-week exposure to X-ray film (XAR-5; Eastman Kodak, Rochester, N.Y.).

The amount of radioactivity migrating with R_fs identical to those of the 4-MP and 2,4-DCP standards was quantified by scraping and liquid scintillation counting of the silica gel associated with the spots. When the autoradiogram indicated spots differing from 4-MP and 2,4-DCP, a quantitative GC analysis of the solvent extracts was also performed.

Columns incubated prior to leaching for more than 1 day were closed off with a two-hole stopper flushing head. The headspace was flushed in 2- to 3-day intervals through a trap system (10), and any $^{14}CO_2$ released was counted. Thus, in addition to immobilization, the subsequent fate of the immo-

TABLE 2. Screening of model pollutants for reactivity in HRP immobilization systems

Test compound	Mean recovery (%) ± SD (n = 3)	
	Without treatment	With treatment
3-Chlorobenzoate[a]	100 ± 5	102 ± 4
Dibutyl phthalate[a]	95 ± 8	95 ± 2
2,6-Dinitrocresol[a]	96 ± 2	105 ± 5
4-Chlorophenol[b]	85 ± 10	65 ± 3
4-MP[b]	90 ± 3	12 ± 8
Pentachlorophenol[b,c]	82 ± 3	67 ± 4
Benzo[*a*]pyrene[b]	0.005	0.007
4-Chloroaniline[b]	2.20	0.05

[a] Recovery from solution.

[b] Recovery from soil columns. For these compounds, recovery data from solution are not presented.

[c] For oxidative condensation of pentachlorophenol, addition of an equimolar amount of ferulic acid was necessary.

bilized pollutants was also measured in terms of mineralization, volatilization, leachability after incubation for various time periods, solvent extractability, and association with a soil organic matter fraction (humic acid).

Analytical. GC analysis was performed with a Hewlett-Packard (Avondale, Pa.) model 5890 instrument with flame ionization detector and model 3392A integrator. The fused silica macrobore capillary columns (10 m by 0.53 mm) were purchased from Alltech Associates (Avondale, Pa.) and had bonded 1.2-μm liquid phases of either polydimethylsiloxane (RSL-150) or polyphenylmethylsiloxane (RSL-300). Oxygen-free N_2 at 10 ml/min was used as the carrier gas. The specific conditions for analysis and the retention times for each test compound are listed in Table 1.

Radioactivity was measured with a Beta Track model 6895 liquid scintillation counter (TM Analytic, Elk Grove Village, Ill.). Oxosol ^{14}C (National Diagnostics, Somerville, N.J.) counting fluid was used in trapping $^{14}CO_2$. All aqueous and solvent fractions were counted in Scintiverse E (Fisher Scientific). All counts were corrected for background and quenching by the external standard ratio method.

Oxidative enzyme activity was measured spectrophotometrically with 3,3′-dimethoxybenzidine (2). One unit of enzyme activity was defined as the amount causing a 0.001 OD unit per minute increase at 460 nm and 25°C.

RESULTS AND DISCUSSION

Initial screening for immobilization. Prior to committing the necessary time for the selection and optimization of an immobilization procedure, we found it prudent to conduct a rapid screen on a wide range of potential groundwater pollutants (Table 2). For this screening, we used a commercially available oxidative enzyme, HRP. The test compounds were selected to include an aromatic hydrocarbon, benzo[*a*]pyrene, as well as aromatic compounds bearing phenolic hydroxyl (4-methylphenol, 4-chlorophenol, pentachlorophenol, 2,6-dinitrocresol), carboxyl (3-chlorobenzoate), ester (dibutyl phthalate), and amino (4-chloroaniline) groups. In addition, some of these compounds were also chloro-, nitro-, or methyl-substituted. 3-Chlorobenzoate, dibutyl phthalate, and 2,6-dinitrocresol showed no detectable reactivity in the HRP solution test system and were not tested on soil columns. Addition of ferulic acid (3 mg ml^{-1}), a compound highly reactive with HRP, did not enhance removal of these compounds from solution. Such copolymerization of pollutants nonreactive to HRP was observed for 4,4′-di- and 2,4,5-trichlorobiphenyls (10 and 3 μg ml^{-1}) in the presence of reactive phenol (400 μg ml^{-1}), as reported by Klibanov et al. (8). Our attempts to coprecipitate 3-chlorobenzoate, dibutyl phthalate, and 2,6-dinitrocresol were performed at substantially higher concentrations than might be expected to result from accidental spills (5 mg ml^{-1} for the test compounds). The phenolic pollutants 4-chlorophenol, 4-methylphenol, and pentachlorophenol showed reactivity in solution and were subsequently also tested for immobilization on soil columns. 4-Methylphenol was immobilized effectively, the chlorophenols to a lesser degree. 4-Chloroaniline and benzo[*a*]pyrene had inherently low mobility in soil, the former because of its cationic nature, the latter because of its very low solubility in water (9). These two compounds were included in the test to ascertain that treatment with oxidative enzymes would not have the undesired side effect of mobilizing pollutants that normally have a low tendency to leach. No mobilization of these compounds was observed. The survey led us to conclude that the outlined immobilization treatment was promising for most phenolic pollutants but not for any of the other model compounds tested. Considering the strong electron-withdrawing effects of the nitro-substituents, the failure of 2,6-dinitrocresol to react was not surprising (13).

Immobilization with *G. candidum* culture filtrate. The *G. candidum* culture filtrate was selected as the most effective immobilizing agent for phenolic pollutants after it was compared with enzymes of several fungal and bacterial isolates and stock cultures (M. J. R. Shannon and R. Bartha, *in* Y.-C. Wu, ed., *Physicochemical and Biological Detoxification of Hazardous Wastes*, Hazardous Substance Management Research Center, Newark, N.J.). Based on substrate specificity and inhibitor studies, the dominant oxidative enzyme produced in the culture filtrate under the specified conditions was characterized as a laccase.

Table 3 shows the results of an experiment on soil columns contaminated with 5 mg of 4-MP. At time zero, virtually all the applied 4-MP was recovered as unchanged 4-MP from the leachate of the control columns, and as verified by GC, after 2 days, over 90% of the 4-MP was unaltered. The amount of 4-MP leaching from the control columns decreased with time to 71% on the final day of the experiment. These decreases were balanced by 4-MP incorporation into the soil-bound fraction. The relatively small amount of radioactivity associated with the humic precipitates and solvent extracts remained constant throughout the incubation.

In sharp contrast, from the leachate of treated columns, only 9.1% of the added 4-MP was recovered on day 2, and immobilization increased further with incubation time. An additional 4.1% of the added radioactivity was present in the leachate as a humic complex which was precipitated after acidification of the leachate. When the supernatant was partitioned with CH_2Cl_2 and the solvent extract was analyzed by GC and TLC, essentially all the remaining radioactivity was found to consist of intact 4-MP.

Most of the 4-MP immobilized on the column by the treatment remained solvent extractable. However, GC and TLC analysis of the solvent extracts recovered only trace amounts of intact 4-MP. Most of the radioactivity on the TLC plates formed an elongated spot, indicating the formation of polymeric and essentially water-insoluble products. The solvent extract of the control soil contained similarly low amounts of intact 4-MP but only trace amounts of polymeric products.

TABLE 3. Immobilization and fate of 4-MP in Lakewood sand

Day	% of added ^{14}C											
	Untreated						Treated with *G. candidum* culture filtrate					
	$^{14}CO_2$ evolved[a]	4-MP in leachate	Humic precipitate	Solvent extract	Soil bound	Total recovery	$^{14}CO_2$ evolved	4-MP in leachate	Humic precipitate	Solvent extract	Soil bound	Total recovery
0		98.1	<0.1	0.9		99.0						
2	ND	90.2	6.2	4.1	3.6	104.1	ND	9.1	4.1	64.9	17.7	99.5
4	0.5	88.6	8.1	5.3	2.9	105.4	0.1	7.0	3.7	74.0	16.1	100.9
12	1.1	81.5	5.6	5.5	8.1	101.8	0.3	7.7	2.4	69.5	13.3	93.2
21	1.7	70.7	5.2	3.7	19.5	100.8	0.4	6.8	2.3	58.6	10.0	78.1

[a] $^{14}CO_2$ evolution figures are cumulative. ND, Not determined.

In treated columns, the smaller portion of the immobilized radioactivity became solvent inextractable. This radioactivity represented 4-MP bound, most likely covalently, to the insoluble humic polymers of soil (1, 3). During 28 days of incubation, there was no evidence of remobilization of bound 4-MP. The mineralization rate of the bound and polymerized 4-MP residues during the incubation period was modest, amounting to a cumulative 0.4% of the radioactivity applied. This was less than the $^{14}CO_2$ evolution from columns with no immobilization treatment (1.7%). It is not surprising that 4-MP is mineralized at a higher rate than its polymeric condensation products. Based on these results, the immobilization treatment appears very promising for prevention of groundwater pollution by 4-MP and similar reactive phenolic compounds.

For comparison, Table 4 shows the results of an experiment conducted with 2,4-DCP, a much less reactive and thus more difficult candidate for immobilization treatment. At time zero, over 91% of the applied 2,4-DCP was leached from the untreated control column. GC and TLC analyses identified this radioactivity to represent only intact 2,4-DCP. During 3 weeks of incubation, leachable radioactivity decreased to 63%. As revealed by GC and TLC analysis, only half of this radioactivity represented free 2,4-DCP; the other half represented water-soluble but partially polymerized products or complexes of 2,4-DCP. Humic complexes in the leachate amounted to 3 to 4% of the radioactivity in the leachate from day 1 to the end of the experiment. The radioactivity disappearing form the leachate with time was converted largely to solvent-extractable polymeric material and, to a smaller extent, to soil-bound residue.

The immobilization treatment reduced 2,4-DCP leaching by day 1 to slightly more than half of the amount from the control column. GC and TLC analysis indicated that the leached radioactivity was intact 2,4-DCP. With additional incubation time, free 2,4-DCP in the leachate decreased to 10% of the applied amount while the acid-precipitated humic complex increased from 5.3 to 34.6%. The larger portion of the immobilized 2,4-DCP remained solvent extractable. Only trace amounts of free 2,4-DCP were present in the solvent extracts of the treated column, and similar trace amounts were seen in the solvent extracts of the untreated control columns. Most of the immobilized radioactivity was present in the form of solvent-extractable but water-insoluble polymeric products. The nature of oligomers and polymers formed from 2,4-DCP and 4-MP by oxidative enzymes was reviewed by Sjoblad and Bollag (13) and Bollag (5). Beyond using TLC and autoradiography to confirm their presence, we made no attempt to analyze these products further. There appears to be a trend for some of the solvent-extractable material to shift to inextractable soil-bound material with prolonged incubation. Cumulative $^{14}CO_2$ evolution from 2,4-DCP during 4 weeks of incubation in untreated soil columns was low (1.3%) and decreased further to 0.7% in treated soil columns. No evidence for remobilization of immobilized material was evident during the 4-week incubation period.

The use of HRP for removal of phenols and anilines from industrial wastewaters was previously demonstrated by Klibanov et al. (9). Our use of oxidative enzymes for immobilization of phenolic pollutants in soil for the prevention of groundwater pollution develops this idea further. The results obtained with the enzymatic immobilization of 4-MP and 2,4-DCP are encouraging and illustrate that groundwater pollution by phenolic substances could be controlled in this manner. Prior to the practical application of this technique, there is a need to maximize oxidative enzyme production through genetic and physiological means. It will also be necessary to perfect enzyme storage and field application and to demonstrate by appropriate tests a strong and lasting reduction in the toxicity of the phenolic compounds through polymerization and binding.

TABLE 4. Immobilization and fate of 2,4-DCP in Lakewood sand

Day	% of added ^{14}C											
	Untreated						Treated with *G. candidum* culture filtrate					
	$^{14}CO_2$ evolved[a]	2,4-DCP in leachate	Humic precipitate	Solvent extract	Soil bound	Total recovery	$^{14}CO_2$ evolved	2,4-DCP in leachate	Humic precipitate	Solvent extract	Soil bound	Total recovery
0		91.6	0.7	5.2		97.5						
1	ND	79.2	4.0	12.5	0.5	96.2	ND	48.5	5.3	26.9	8.5	89.2
14	0.3	61.7	3.4	16.7	2.7	84.7	0.4	11.6	34.5	27.8	14.7	89.0
21	0.8	63.0	3.8	17.7	2.2	87.4	0.6	16.2	27.4	22.8	19.5	86.5
28	1.3	ND	6.1	19.3	3.1	ND	0.7	10.0	34.6	23.1	17.9	86.3

[a] $^{14}CO_2$ evolution figures are cumulative. ND, Not determined.

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