DECONTAMINATING SOIL WITH ENZYMES

An In Situ Method Using Phenolic and Anilinic Compounds

JEAN-MARC BOLLAG
The Pennsylvania State University
University Park, PA 16802

Xenobiotics are man-made compounds often introduced into the environment at concentrations that cause undesirable effects. They can be transformed as a result of biotic and abiotic processes, leading to changes in their chemical state and ultimately in their toxicity and reactivity. Ideally, xenobiotics are transformed into carbon dioxide, water, and mineral elements. However, many of them are converted to intermediate products that can be as toxic as or more so than their parent compounds. This article focuses on the complexation of organic xenobiotics with humic material in aquatic or terrestrial environments.

Since the development of pesticides in the early 1940s, large quantities of organic chemicals have been applied to the environment in an attempt to destroy disease-carrying and crop-damaging organisms or to control weeds. As time has passed, we have become increasingly aware of the potential side-effects incurred by the constant use of these chemicals. Many pesticides and other xenobiotics have proven to be highly persistent compounds that are resistant to natural transformation or degradation processes. As a result, a significant portion of xenobiotics remains in the environment for prolonged periods of time.

A variety of physicochemical techniques are available for the cleanup of contaminated soils. However, many of these processes involve large-scale excavation or long-term treatment of polluted soil. Furthermore, many of these approaches are quite costly and do not lead to total decontamination. Consequently, there is a growing need to develop alternative methods for

the in situ decontamination of soil environments.

One detoxification method currently being studied involves the binding of phenolic and anilinic chemicals to humic material. Because many man-made compounds resemble naturally occurring humic acid precursors, these compounds can be incorporated into humus during the humification process (formation of humus).

The binding of xenobiotics to humus is believed to decrease the amount of material available to interact with the biota, thus reducing the toxicity of the parent compound. Binding may also inhibit the movement of xenobiotics via leaching, thus preventing the contamination of aquatic environments. The incorporation of xenobiotics into humus is a naturally occurring process and can be enhanced by adding extracellular enzymes or abiotic catalysts or by altering the environment's physicochemical conditions.

The information presented here focuses on the mechanism by which halogenated phenols and anilines are bound to humic material and the chemical nature of these bound residues. Data on the toxicity and ultimate stability of bound chemicals is also presented. Our studies indicate that enzymatic coupling is a promising method for detoxification of environments contaminated with xenobiotics.

Xenobiotic and humic reactions

The binding of xenobiotics to humic substances constitutes one of the major reactions by which these chemicals are transformed in nature. Xenobiotics interact with colloids through several mechanisms, and a number of reviews describe the possible interactions (*1, 2*).

Adsorption occurs primarily as a consequence of the attraction between the solid surface of soil and the solution or vapor phase of the xenobiotic. Phenolic compounds adsorb to soil colloids via several mechanisms, including van der Waals forces, charge-transfer complexation, hydrogen bonding, and hydrophobic interactions (*3*). The nature and strength of adsorption depend largely on the chemical class or structure of the molecule. Unless covalent bonds have been formed, adsorption is a reversible process because of its physicochemical nature, and a percentage of xenobiotics bound via adsorption processes remains available to interact with the biota.

Nevertheless, there is abundant evidence to suggest that the longer adsorbed residues remain in soil, the more resistant they become to extraction and degradation (*4*). This resistance may result from a redistribution of the pollutant from weaker to stronger binding sites, or from a slow incorporation of the pollutant into humus.

The most persistent complexes result from the covalent binding of xenobiotics to humic material. These complexes, often referred to as the "bound residues" of soil, are highly resistant to acid and base hydrolysis, thermal treatment, and microbial degradation (*5–8*). Oxidative coupling, one of the most important chemical reactions in the humification process, leads to the incorporation of naturally occurring humic acid precursors and synthetic phenolic compounds into soil humus via covalent linkages.

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The oxidative coupling of phenols is a free-radical process involving the loss of an electron and a proton from phenol. This loss results in the formation of a resonance-stabilized free radical. Two free radicals can undergo coupling in a variety of ways in the reaction of 2,4-dichlorophenol with syringic acid, a humus monomer, as illustrated in Figure 1. Phenolic reactants are linked through C–C and C–O bonds; aromatic amines form C–N and N–N linkages (*9*).

Oxidative coupling is mediated by a number of biological and abiotic catalysts, including microbial enzymes, inorganic chemicals (e.g., oxides or oxyhydroxides of Al, Fe, and Mn), and clay minerals (*10–12*). Coupling reactions can also occur spontaneously in the presence of oxygen at alkaline pH (*13*). Spontaneous reactions frequently lead to the incorporation of nonphenolic compounds into humic polymers. Amino acids, amino sugars, and peptides can react with both polyphenolic and quinone compounds to become incorporated into humus by way of spontaneous coupling reactions.

Many soil microorganisms produce extracellular oxidoreductases capable of catalyzing the coupling of aromatic compounds. These enzymes are classified as either peroxidases or polyphenol oxidases. Peroxidases are produced by plants and microorganisms and catalyze a variety of reactions, including the polymerization and depolymerization of lignin (*14*). All peroxidases contain an iron porphyrin ring and require the presence of peroxides (e.g., hydrogen peroxide) for activity. In particular, horseradish peroxidase catalyzes the polymerization of a wide range of phenolic and anilinic compounds. The use of horseradish peroxidase in the detoxification of industrial wastewater has been examined (*15, 16*).

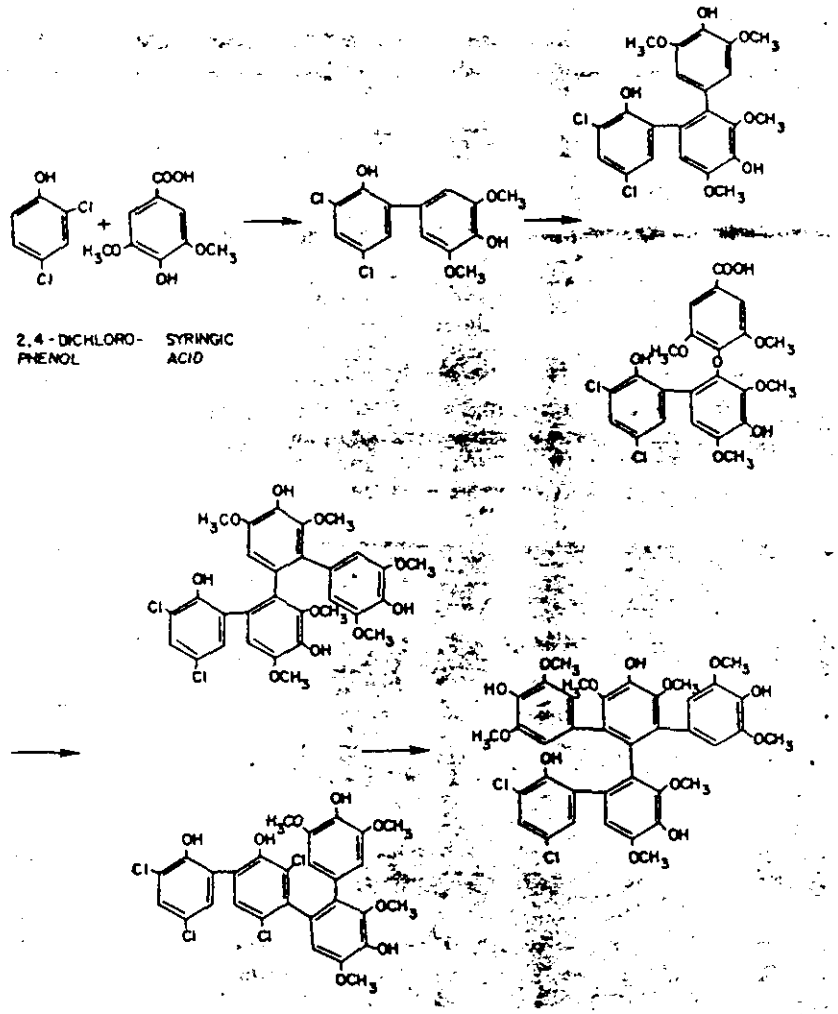
Polyphenol oxidases are divided into two subclasses: laccases and tyrosinases. Both enzyme groups require bimolecular oxygen, but no coenzyme, for activity. However, these enzymes differ in the mechanism by which they oxidize phenols. Tyrosinases form an *o*-diphenol from the parent compound and subsequently release an oxidized, usually highly reactive, *o*-quinone (*10*). In an alkaline environment, the quinone products slowly polymerize through autoxidative processes. Laccases, however, oxidize phenolic compounds to form their corresponding anionic free radicals. Laccases may prove to be the most useful of the oxidoreductases because they produce these very reactive radicals and because, unlike peroxidases, they do not require the presence of hydrogen peroxide.

Oxidative couplings with humus

As previously mentioned, many phenolic chemicals and their degra-

FIGURE 1

Scheme of oxidative coupling products[a]



[a] Resulting from the combined incubation of syringic acid, a humic acid monomer, and 2,4-dichlorophenol with a laccase from *Rhizoctonia praticola*. (Adapted with permission from Reference 19. Copyright 1980, Soil Science Society of Agronomy.)

dation products resemble naturally occurring humic acid precursors. Consequently, these synthetic compounds are often incorporated into humus during the humification process. Several studies illustrate the incorporation of pesticides into humic material. Mathur and Morley (*17*) demonstrated the incorporation of the insecticide methoxychlor [1,1,1-trichloro-2,2-di-(4-methoxyphenol)ethane] into a humic acid analog. Wolf and Martin (*18*) observed that 2,4-D (2,4-dichlorophenoxyacetic acid) and chlorpropham (isopropyl 3-chlorophenyl carbamate) are incorporated into humic acid polymers.

The products formed during cross-coupling of pollutants and humic acids are highly heterogeneous and complex and therefore are quite difficult to identify. To elucidate the mechanism by which xenobiotics are incorporated into humus and to identify the products formed, workers in our laboratory studied the binding process through the use of model systems. Laccases isolated from *Trametes versicolor*, *Rhizoctonia praticola*, or other fungi were incubated with various chlorinated phenols or aromatic amines in the presence of specific phenolic humic constituents. The hybrid products formed were isolated by thin-layer chromatography or by high-performance liquid chromatography and then characterized by mass and nuclear magnetic resonance spectroscopy (*9*). The compound 2,4-dichlorophenol (2,4-DCP), a degradation product of the herbicide 2,4-D, coupled with humic-derived compounds such as orcinol, syringic acid, vanillic acid, and vanillin (*19*) (Table 1).

In subsequent experiments, phenols containing one to five chlorines (4-chlorophenol, 2,4- and 2,6-dichlorophenol, 2,3,6- and 2,4,5-trichlorophenol, 2,3,5,6-tetrachlorophenol, and pentachlorophenol) were cross-coupled with syringic acid by the laccase from the fungus *Rhizoctonia praticola* (*20*). Two types of cross-coupling products were formed: quinonoid oligomers, consisting of chlorophenols linked by ether bonds to orthoquinoline products of syringic acid; and phenolic oligomers, consisting of chlorophenols bound by ether linkages to decarboxylated products of syringic acid.

The cross-coupling products formed are presented in Table 2. The laccase from *R. praticola* has also cross-coupled aromatic amines (substituted anilines) to humic monomers (*21*). Cross-coupling products of 4-chloroaniline and guaiacol are presented in Figure 2.

To study the coupling reaction, we examined the incorporation of phenols into humus under conditions approximating the natural soil habitat (*22*). The coupling of ^{14}C-labeled-2,4-DCP to stream fulvic acid occurred over wide pH and temperature ranges and was catalyzed by several enzymes. Furthermore, because the fulvic-acid-associated radioactivity was not released upon extensive washing with an organic solvent, it appeared that the phenol was indeed incorporated into the humic material.

Cheng et al. (*23*) also found that 2,4-DCP becomes gradually incorporated into soil organic matter. Other phenols, too, show binding to humic material. Martin et al. (*24*) demonstrated the incorporation of ^{14}C-ring-labeled catechol into fulvic and humic acids. Thus the enzymatic coupling of phenols to soil humic substances seems to be a general phenomenon in soils.

Biological consequences of binding

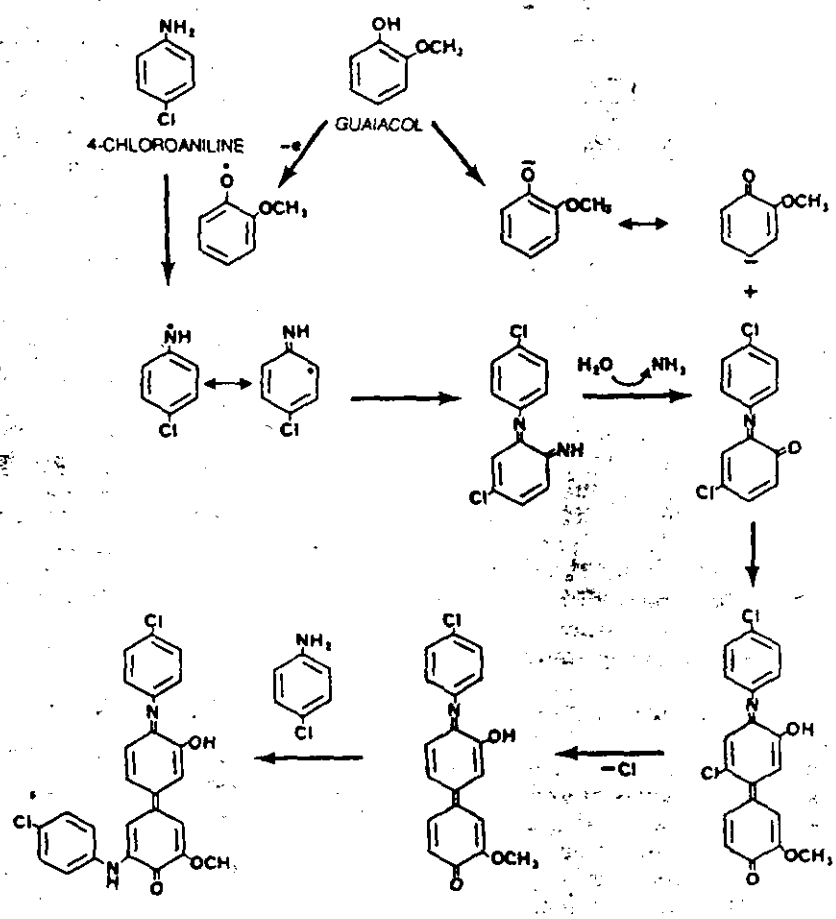
Soil microbiologists are concerned about the possible adverse effects of xenobiotics on the growth and activity of soil organisms. For example, xenobiotics may alter cellular morphology or inhibit biochemical reactions. There is also great concern that the leaching of pollutants from soil may lead to the contamination of drinking water. However, most soil biologists believe that binding pollutants to soil essentially serves to immobilize and detoxify hazardous compounds. Binding decreases the availability of the pesticide to interact with the biota and thus reduces its toxicity. In addition, incorporat-

TABLE 1
Formation of cross-coupling products of phenolic humus constituents and 2,4-dichlorophenol[a]

Substrates	Cross-coupling products (*m/z* value of molecular ion) Dimer	Trimer	Tetramer	Pentamer
2,4-Dichlorophenol + orcinol	284			
2,4-Dichlorophenol + vanillic acid	284	444 450		
2,4-Dichlorophenol + vanillin	312			
2,4-Dichlorophenol + syringic acid	314	466 510	618 626	770

[a] Incubated in the presence of a laccase from *Rhizoctonia praticola*.

FIGURE 2
Pathway for reaction products formed by incubation of 4-chloroaniline and guaiacol in the presence of a laccase of the fungus *Rhizoctonia praticola*



Adapted with permission from Reference 21.

ing xenobiotics into soil humus greatly reduces the movement of the chemicals through leaching and reduces the contamination of groundwater and other aquatic systems.

The ability of the laccase from the fungus *R. praticola* to transform and therefore detoxify phenolic compounds has been demonstrated (*25*). A toxic amount of phenolic compound was added to a medium containing naturally occurring phenols in the presence or absence of laccase. The medium was inoculated with *R. praticola*, and growth of the fungus was monitored. In the absence of laccase, the phenolic compounds inhibited growth; however, laccase reversed this inhibitory effect. Moreover, soluble synthetic humic acids inhibited growth of *R. praticola*, whereas polymerized phenols that formed large precipitates were not inhibitory. Therefore, the effectiveness of the detoxification procedure may depend on actual physical removal, via precipitation, of the polymerized phenols.

Before the binding of xenobiotics to humus can be used for decontamination, it will be necessary to investigate the stability of the bound complexes. If large quantities of the pollutant were released at a future time, formation of these compounds would pose a delayed environmental hazard. However, several investigators have demonstrated the stability of humus-bound xenobiotics. In one study, 3,4-dichloroaniline was applied to a German soil, and 46% of the compound remained bound to the soil two years after treatment (*26*). In a separate study, 83% of ^{14}C-labeled atrazine remained associated with the soil after nine years; 50% of this residue represented bound material (*27*).

The activity of microorganisms is believed to be the primary factor responsible for the release of bound residues. To study the release of bound pesticides, ^{14}C-labeled-*catechol* and *mono-*, *di-*, *tri-*, and pentachlorophenols bound to humic acid polymers were incubated with microbial soil populations and the release of radioactive compounds into the medium was monitored (*28*). This study demonstrated that insignificant quantities of ^{14}C-labeled compounds were released over a 13-week incubation period. Furthermore, this release was accompanied by a simultaneous mineralization of the bound material to $^{14}CO_2$.

As might be expected, the release of bound xenobiotics differs with the type of binding. It appears that a "surface" fraction of bound residue can be released, whereas the remainder is covalently bound to a "core" portion that is less accessible to microbial degradation. Overall, available data indicate that the microbial release of bound xenobiotics occurs extremely slowly. Once released, the xenobiotics can be mineralized or reincorporated into humus. Consequently, released residues should not accumulate and should not pose a delayed health hazard.

Factors affecting the process

Given that the incorporation of xenobiotics into humus eliminates or reduces their toxicity, future research should focus on methods for improving the binding process. The

TABLE 2
Formation of cross-coupling products of syringic acid and chlorophenols[a]

Reaction of syringic acid with:	Phenolic oligomers			
	Dimer	Trimer	Tetramer	Pentamer
4-Chlorophenol	280[b]	432	584	736
	$C_{14}H_{13}O_4Cl$[c]	$C_{22}H_{21}O_7Cl$	$C_{30}H_{29}O_{10}Cl$	$C_{38}H_{37}O_{13}Cl$
2,4-Dichlorophenol	314	466	618	770
	$C_{14}H_{12}O_4Cl_2$	$C_{22}H_{20}O_7Cl_2$	$C_{30}H_{28}O_{10}Cl_2$	$C_{38}H_{36}O_{13}Cl_2$
2,6-Dichlorophenol	314	466	618	770
	$C_{14}H_{12}O_4Cl_2$	$C_{22}H_{20}O_7Cl_2$	$C_{30}H_{28}O_{10}Cl_2$	$C_{38}H_{36}O_{13}Cl_2$
4-Bromo-2-chlorophenol	358	510	—	—
	$C_{14}H_{12}O_4BrCl$	$C_{22}H_{20}O_7BrCl$		
2,4,5-Trichlorophenol	348	500	652	804
	$C_{14}H_{11}O_4Cl_3$	$C_{22}H_{19}O_7Cl_3$	$C_{30}H_{27}O_{10}Cl_3$	$C_{38}H_{35}O_{13}Cl_3$
2,3,5,6-Tetrachlorophenol	382	534	686	838
	$C_{14}H_{10}O_4Cl_4$	$C_{22}H_{18}O_7Cl_4$	$C_{30}H_{26}O_{10}Cl_4$	$C_{38}H_{34}O_{13}Cl_4$
Pentachlorophenol	416	568	720	872
	$C_{14}H_9O_4Cl_5$	$C_{22}H_{17}O_7Cl_5$	$C_{30}H_{25}O_{10}Cl_5$	$C_{38}H_{33}O_{13}Cl_5$

[a] Incubated in the presence of a laccase from *Rhizoctonia praticola*.
[b] *m/z* value of molecular ion.
[c] Molecular composition based on number of chlorine atoms and *m/z* value.

binding of xenobiotics to humus depends on many environmental factors that vary with season, climate, soil type, and agricultural practices. Because of this variability, it is necessary to evaluate the incorporation of each xenobiotic into humus under specific sets of environmental conditions. For example, the pH of the environment has played an important role in determining the efficiency of polymerization (*29*). The phenoloxidases of many soil fungi have a pH optimum in the range of 4–7. Thus, in calcareous or acid soils, the activity of these enzymes may be reduced.

In addition, the pH optimum has depended on the chemical structure of the substrate. The position and type of substituent group also influences the polymerization process (*29, 30*). In general, transformation efficiency decreases with increasing molecular weight of the substituent group (e.g., methylphenols are transformed most efficiently, followed by methoxyphenols, chlorophenols, and bromophenols). Furthermore, the transformation efficiency of phenols decreases with increasing numbers of chlorine atoms. Because the oxidative coupling process is influenced by a wide variety of factors, and because these factors vary with type of substrate and the enzyme employed, caution must be exercised in interpreting data and extrapolating findings to the natural environment.

The use of cross-coupling techniques for soil decontamination purposes may be hindered by the relative inertness of some phenolic compounds to enzymatic action. However, the reactivity of some inert compounds can be enhanced by the addition of highly reactive substrates to reaction mixtures containing laccase. For instance, in the presence of either guaiacol or ferulic acid (both phenolic chemicals), the removal of phenol was found to be enhanced by more than twofold (*30*). Fahraeus and Ljunggren have reported that the rate of oxidation of *p*-cresol by the laccase of the fungus *Trametes versicolor* is significantly enhanced by the addition of catechol (*31*). Thus the applicability of the cross-coupling technique can be broadened by the use of copolymerizing agents.

The efficiency of enzymatic coupling can also be enhanced through the use of enzymes bound to solid supports. Sarkar et al. (*32*) found that the immobilization of laccase on soil supports increases the thermostability of the enzyme, its resistance to degradation by proteases, and its half-life. For example, laccase immobilized to kaolinite or soil removed 2,4-dichlorophenol as efficiently as did the free enzyme but retained its activity for a much longer time period. Furthermore, the immobilized enzyme could be recovered from the reaction solution and reused to transform substrate, with minimal loss of activity (*33*). Immobilized enzymes may prove more economical because they are biochemically more stable and can be used repeatedly to detoxify xenobiotics.

Aim research at binding process

The incorporation of xenobiotics and their derivatives into humus occurs readily in nature. It has been suggested that this process can be exploited to immobilize and detoxify hazardous compounds. The binding of pollutants to humus has several important consequences: the amount of compound available to interact with the biota is reduced; the complexed products are less toxic than their parent compounds; and insoluble precipitates are frequently formed, thus reducing the movement of chemicals through leaching.

The use of enzymatic coupling for detoxification has met with concern about the ultimate fate of bound pesticide residues. All available data indicate that after xenobiotics are incorporated into soil, they are released only minimally and gradually. The gradual release should not pose a delayed health hazard, because the slowly released compounds can be mineralized to CO_2 or immobilized again by natural humification processes.

Before this technique can be applied, extensive research is required to analyze the availability, accumulation, and toxicity of bound residues in nature. Researchers should continue to focus on the development of new methods for maximizing the binding process (e.g., through the use of immobilized enzymes or abiotic catalysts, or the addition of copolymerizing agents).

***Jean-Marc Bollag** is professor of soil microbiology in the College of Agricultural Sciences and the Environmental Resources Research Institute at the Pennsylvania State University, University Park, PA. He is also co-director of the Center of Bioremediation and Detoxification. He received his Ph.D. from the University of Basel, Switzerland. His research is in the microbial transformation of xenobiotics and the use of microbial and plant enzymes for detoxification of contaminated environments.*

Current evidence indicates that the enzymatic or abiotic incorporation of xenobiotics into humus is an efficient, cost-effective method for detoxifying hazardous pollutants.

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