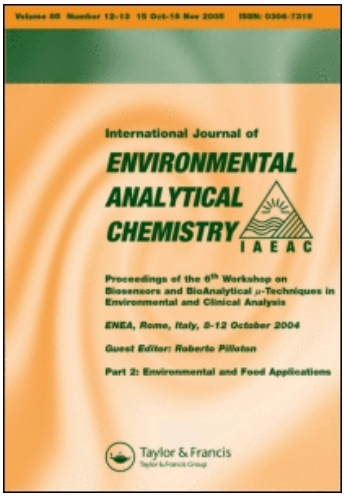

International Journal of Environmental Analytical Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713640455

A Model for Enzymatic Binding of Pollutants in the Soil

Jean-Marc Bollag[a]; Wendy B. Bollag[a]
[a] Laboratory of Soil Biochemistry, The Pennsylvania State University, University Park, PA, USA

To cite this Article Bollag, Jean-Marc and Bollag, Wendy B.(1990) 'A Model for Enzymatic Binding of Pollutants in the Soil', International Journal of Environmental Analytical Chemistry, 39: 2, 147 — 157
To link to this Article: DOI: 10.1080/03067319008027691
URL: http://dx.doi.org/10.1080/03067319008027691

Intern. J. Environ. Anal. Chem., Vol. 39, pp. 147–157
Reprints available directly from the publisher
Photocopying permitted by license only

A MODEL FOR ENZYMATIC BINDING OF POLLUTANTS IN THE SOIL

JEAN-MARC BOLLAG and WENDY B. BOLLAG

Laboratory of Soil Biochemistry, The Pennsylvania State University, University Park, PA 16802,USA

(*Received 27 February 1989; in final form 30 May 1989*)

Pollutants released into the environment may be bound to humic acid through abiotic or biologic processes whereby the formation of bound residues usually results in detoxification of the pollutant. Therefore, enhancing the binding of xenobiotic chemicals to humic material can serve as a means to reduce toxicity as well as migration of toxic compounds in the environment.

Complex formation can occur by an oxidative coupling reaction leading to oligomeric and polymeric products. We intensively investigated the effect of phenoloxidases (peroxidases, tyrosinases, and laccases) on the binding of substituted phenols and aromatic amines to humus monomers as well as to humic substances. Based on a large number of isolated and identified cross-coupling products, we are able to elucidate the sites and mechanisms of binding and also to determine the efficiency of some coupling reactions.

Copolymerization largely depends on the chemical reactivity of the substrates involved. Certain phenolic humus constituents, such as guaiacol or ferulic acid, are highly reactive in the presence of phenoloxidases. When one of these compounds was incubated together with a phenoloxidase with less or even non-reactive phenols, anilines or other chemicals, a synergistic reaction took place, resulting in increased formation of bound residues of these compounds.

KEYWORDS: Detoxification, phenoloxidases, peroxidases, laccases, humic substances, copolymerization

INTRODUCTION

Although few would question the importance of pesticides in the success of modern agriculture, the widespread use of these compounds generates a potentially hard to solve and unenviable problem—their removal from the environment. Because of the toxicity of these pesticides, their potential accumulation in soil, ground water, surface water and the plants grown in contaminated soil has become an issue of great concern. Conventional clean-up methods, although effective, are cumbersome and cost-inefficient. However, current research into alternative detoxification methods suggests that enzyme-catalyzed polymerization and/or binding of pesticides to soil residues may represent an efficient means of removing certain compounds from soil and water.

Our discussion will center on three important questions:

1. Do enzymes derived from soil microorganisms catalyze the polymerization and/or binding of xenobiotics such as pesticides to soil constituents?

2. What is the mechanism of the enzyme-catalyzed attachment of the bound pesticide residues?
3. Are these bound residues stable and less toxic than the free parent molecules and how does this relate to the fate of xenobiotics in soil and water?

By addressing these three topics we hope to answer the much broader and perhaps more significant question of whether microbial enzyme-catalyzed polymerization and binding of xenobiotics to soil constituents represents a feasible alternative method for the detoxification of pollutants in the environment.

Do Enzymes Derived from Soil Microorganisms Catalyze the Polymerization and Binding of Xenobiotics to Soil Constituents?

In the last several years we have intensively investigated a class of microbial enzymes, the phenoloxidases, for their ability to catalyze the polymerization and/or binding of numerous pesticides to humic constituents. Our interest in these enzymes initially resulted from the observation that a member of this class of enzymes, namely a laccase from the fungus *Rhizoctonia praticola*, was able to elicit the oxidative polymerization of various phenolic and naphtholic compounds.[1] This ability of a phenoloxidase to catalyze such polymerization reactions suggested its potential use for the detoxification of contaminated waters, as polymerization often results in precipitation of the products,[2] which can then be easily removed by filtration.

Moreover, although in these initial experiments a single xenobiotic was polymerized, subsequent experiments demonstrated that the inclusion of phenolic humus constituents, such as syringic acid, vanillic acid or vanillin, resulted in the enzyme-induced formation of various cross-coupling products. Indeed, further experiments have indicated that a wide variety of xenobiotics can become cross-coupled to naturally-occurring humic monomers by the action of phenoloxidases. These xenobiotics include phenols such as various mono-, di-, and tri-substituted chlorophenols[3] and 2,6-xylenol,[4] and anilines such as 4-chloroaniline, 3,4-dichloroaniline, and 2,6-diethylaniline.[5]

That man-made pesticides could be cross-coupled with naturally-occurring humic monomers suggested the possibility that this oxidative coupling reaction could play a role in the covalent incorporation of xenobiotics into humic material. In fact, we have recently shown that phenoloxidases can catalyze the incorporation of xenobiotics into fulvic and humic acids.[6,7] Figure 1 shows the removal of ^{14}C-labelled-2,4-dichlorophenol (2,4-DCP) from an aqueous solution as a result of its enzyme-catalyzed polymerization and binding to fulvic acid. This effect required the addition of active enzyme; that is, less than 5% of the ^{14}C-2,4-DCP was removed in the absence of enzyme or in the presence of heat-inactivated (boiled) enzyme. Furthermore, although fulvic acid was not essential for the enzyme-induced removal of ^{14}C-2,4-DCP—since the phenoloxidase-catalyzed polymerization could occur in the absence of fulvic acid—the addition of fulvic acid increased removal by approximately 100% with all enzymes studied.

This enhanced removal of a xenobiotic is by no means unique to fulvic acid. In

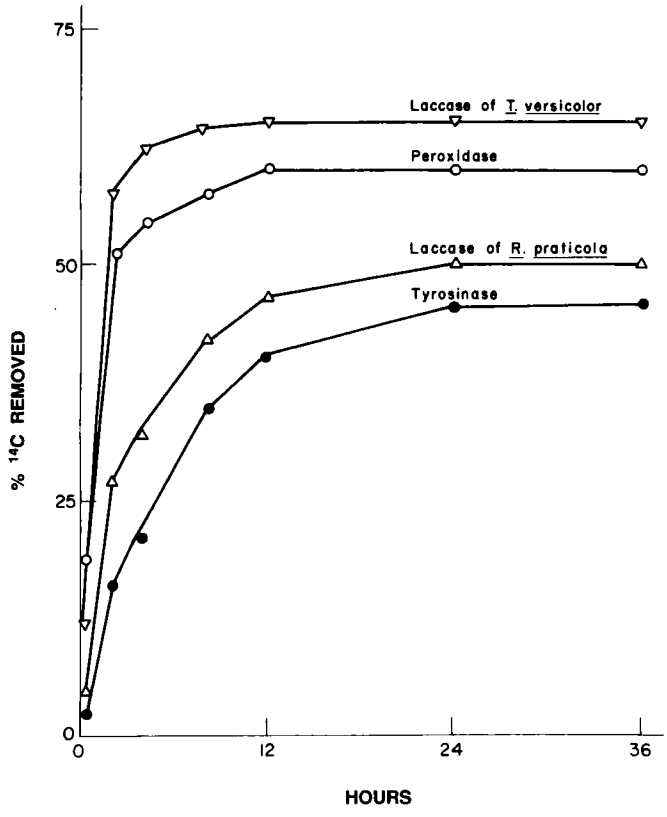

Figure 1 Removal of ^{14}C-2,4-dichlorophenol through binding to fulvic acid and polymerization in the presence of oxidoreductases.[6]

several studies we have found that the addition of a highly reactive humic monomer, such as syringic acid, to a phenoloxidase-containing medium can initiate the effective polymerization and/or binding of a molecule which by itself is only poorly transformed, if at all.[8] In Table 1, it is shown that certain substrates, e.g., syringic acid in the case of 2,4-DCP and ferulic or vanillic acid in the case of phenol, can more than double the amount of xenobiotic removed by the laccase of *R. praticola*. This fact may be potentially very important, as by the appropriate choice of substrate large quantities of xenobiotics could be efficiently removed from contaminated soil or water. Thus, there seems to be no shortage of examples of the enzyme-induced polymerization and binding of xenobiotics, and the coupling reaction catalyzed by phenoloxidases most likely represents an important mechanism for the incorporation of xenobiotics into humus.

What is the Mechanism of the Phenoloxidase-catalyzed Attachment of the Bound Pesticide Residues?

One of our prime research interests has been to elucidate the mechanism of the phenoloxidase-catalyzed polymerization and binding of pesticides to humic

Table 1 Removal of 2,4-dichlorophenol and phenol alone and in the presence of another phenolic substrate[8,a]

Substrate to be removed	*Substrate to enhance removal*	*Substrate removal μmol (%)*	
		Substrate to be removed	*Substrate to enhance removal*
2,4-Dichlorophenol	None	0.30(12%)	—
	Catechol	0.00(0%)	2.15(86%)
	Ferulic acid	0.42(17%)	2.50(100%)
	Guaiacol	0.30(12%)	2.45(98%)
	Syringic acid	0.80(32%)	2.48(99%)
	Vanillic acid	0.55(22%)	2.48(99%)
Phenol	None	0.12(5%)	—
	2,6-Dimethylphenol	0.02(1%)	2.50(100%)
	Ferulic acid	0.30(12%)	2.50(100%)
	Guaiacol	0.12(5%)	2.40(96%)
	Syringic acid	0.00(0%)	2.48(99%)
	Vanillic acid	0.33(13%)	2.50(100%)

[a]Enzyme concentration was 0.5 DMP units/ml and the concentration of each substrate was 0.5 m*M* (total amount = 2.5 μmol).

materials. To do so, we incubate, under controlled conditions, the appropriate xenobiotic with the phenoloxidase of interest in the presence or absence of humic monomers. The resulting oligomers can then be isolated using various chromatographic techniques, such as thin-layer and high-performance-liquid chromatography, and their chemical compositions determined by a combination of nuclear magnetic resonance and mass spectroscopy. By this protocol we have identified several products of phenoloxidase-catalyzed reactions and have proposed a number of reaction pathways to explain our findings. The oligomerization of the naturally-occurring phenol, syringic acid, is shown in Figure 2. This reaction exemplifies the types of phenoloxidase-catalyzed reactions we have observed in our studies. Such quinonoid oligomers have also been demonstrated in reaction mixtures containing phenoloxidases incubated with guaiacol, halogenated phenols, 2,6-diethylaniline, 2,6-xylenol and phenylalanine ethyl esters.[9] It is thought that the enzyme-induced oxidation of naturally-occurring phenols to yield free-radical quinonoid structures is a common pathway in the phenoloxidase-catalyzed polymerization and binding of both naturally-occurring and man-made compounds. In the case of aromatic amines, oligomers are generated via nucleophilic or Michael additions of amino groups to this free radical through imine linkages. Thus, although the scheme presented in Figure 2 is specific for the oxidative oligomerization of syringic acid, it can serve as a model for the polymerization of naturally-occurring phenols to form humus materials and for the incorporation of xenobiotics into humus materials via copolymerization (cross-coupling) as illustrated in Figure 3.

Another common pathway illustrated in Figure 2 and 3 is the decarboxylation of a highly reactive compound such as syringic acid and the formation of a covalent bond at that site to generate phenolic oligomers such as Product II in Figure 2, 2,6-dimethoxy-4-(2′,6′-dimethoxy-4′-carboxyphenoxy) phenol, and the

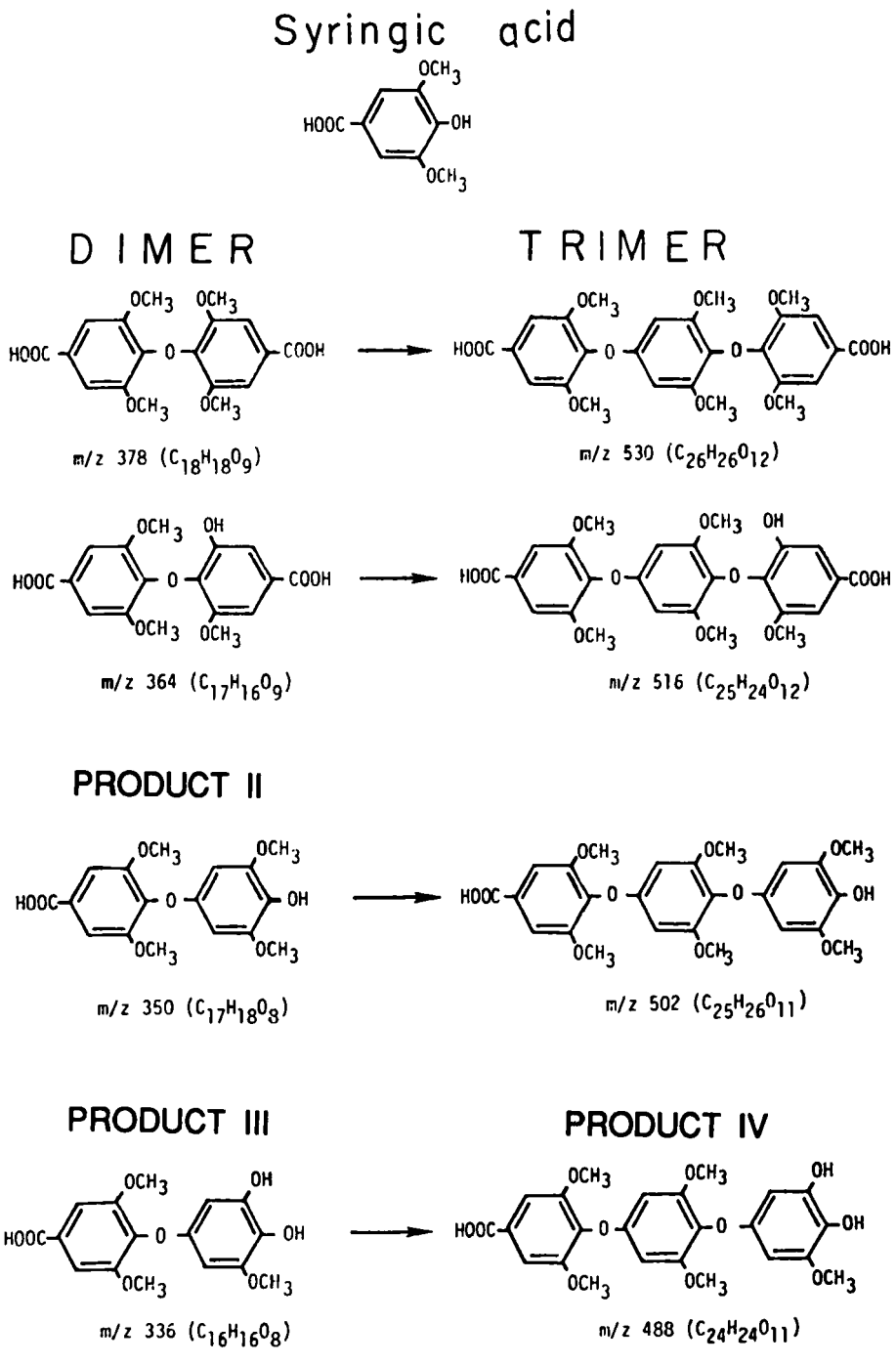

Figure 2 Proposed molecular structures of the formed dimers and the corresponding trimers of syringic acid.[20]

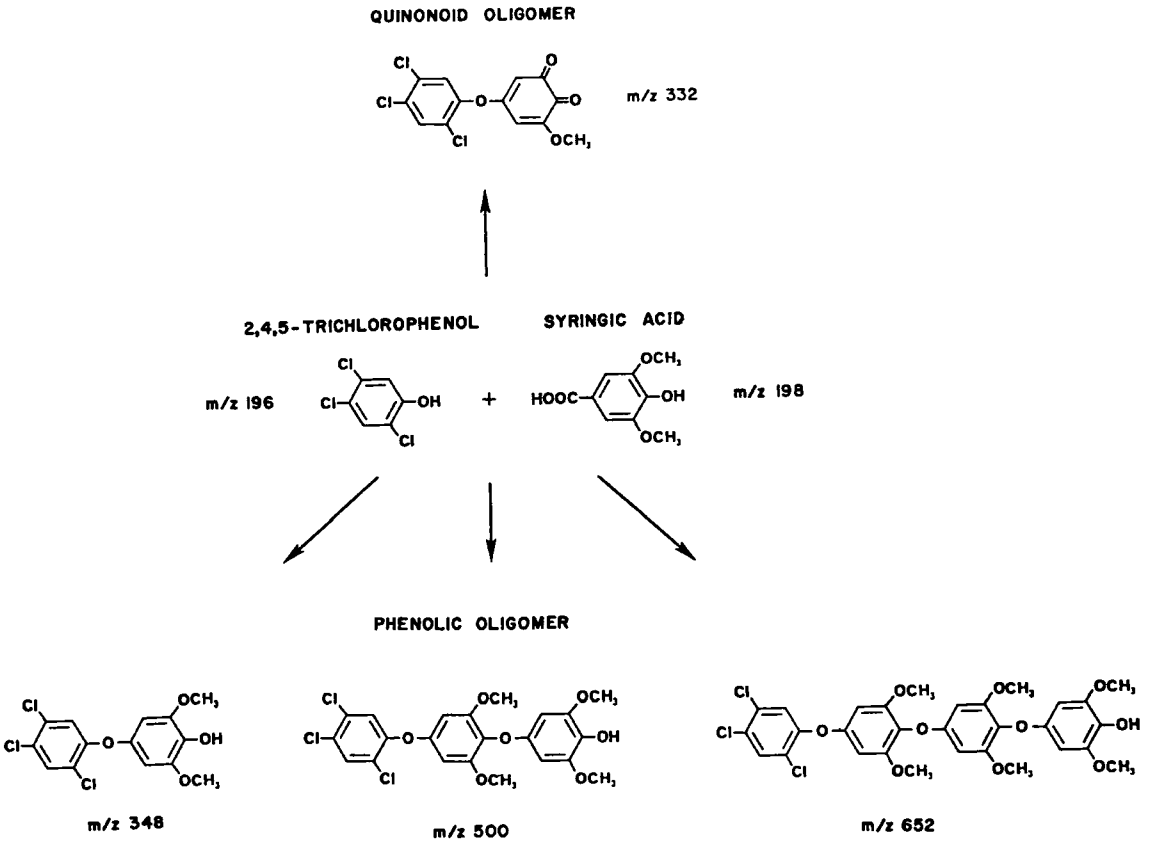

Figure 3 Proposed molecular structures of hybrid products of syringic acid and 2,4,-5-trichlorophenol.[3]

phenolic oligomers in Figure 3. A similar event also occurs in the enzymatic cross-coupling of syringic, vanillic, protacatechuic and gallic acids with a number of xenobiotics including 2,4-dichlorophenol; pentachlorophenol; 4-chloroaniline; 2,6-diethylaniline; and 3,4-dichloroaniline.[3,9,10] Thus, our results indicated that enzyme-catalyzed cross-coupling reactions following decarboxylation are a common mechanism of oligomer formation.

It is not clear, however, if dehalogenation is a typical result of phenoloxidase action on halogenated xenobiotics. In a study of cross-coupling between syringic acid and halogenated phenols, we found no evidence of dehalogenation, but in experiments with substituted anilines, dehalogenation did occur. This discrepancy makes apparent the need for further research into phenoloxidase-induced covalent binding of xenobiotics to naturally-occuring phenols, in order to understand more fully the mechanism of phenoloxidase activity and the role of these enzymes.

Are the Humus-bound Xenobiotic Residues Stable and Less Toxic Than the Free Parent Molecules?

The third and final topic we wish to address is the two-fold question of the toxicity of the xenobiotics: Is the bound residue less toxic than the parent molecule and could it subsequently be released by the activity of soil microorganisms? Both of these questions must be answered before the polymerization and/or binding of pesticides to humic materials can truly be considered as a potential candidate for the removal of contaminants from soil and water.

Theoretically, binding of a pesticide to humic acids, clays, or other materials would be expected to decrease its toxic effects in the environment.[11] Binding can reduce the amount of a compound available to interact with the biota,[12,13] and as the quantity of an available xenobiotic is reduced, toxicity also declines.

We have investigated this hypothesis using again the laccase from the fungus *Rhizoctonia praticola*.[14] In a fungal growth medium, the minimum concentration of a variety of phenolic pesticides which inhibited growth of *R. praticola* was incubated with an inoculum of the fungus in the presence and absence of a naturally-occurring phenol and added laccase. As shown in Table 2, fungal growth was inhibited by the methylphenols, *o*-cresol, *p*-cresol and 2,6-xylenol. However, the addition of laccase in the case of *p*-cresol and 2,6-xylenol and of laccase and syringic acid in the case of *o*-cresol allowed growth of the previously-inhibited *R. praticola*. This ability of the laccase to alter the toxicity of the methylphenols appeared to be related to the capacity of the enzyme to decrease the levels of the parent compound by transformation, polymerization and/or cross-coupling with syringic acid.

This detoxification capability of the laccase was not confined to methylphenols, as chlorophenols were also detoxified by the enzyme. Illustrated in Figure 4 is the growth of the fungus over time under various conditions. It is apparent that while the addition of either laccase or syringic acid alone could not overcome the inhibitory effect of 2,4-DCP on fungal growth, the presence of both the enzyme and the naturally-occurring phenol removed the growth inhibition by 2,4-DCP. We believe this phenomenon is related to the ability of syringic acid to enhance

Table 2 Detoxification of methylphenols by *Rhizoctonia* laccase in the presence or absence of syringic acid[14,a]

Medium treatment	*Concn (mM)[b] of remaining:*		*Days till*		*Final biomass ± SD[c]*
	Methylphenols	*SYR*	*First growth*	*Confluence*	
o-Cresol					
Alone	2.0	—	NG	NG	0
+LAC	1.51	—	NG	NG	0
+SYR	2.14	1.00	NG	NG	0
+SYR and LAC	0.2	0.01	5–7	9–10	641 ± 8
p-Cresol					
Alone	2.0	—	NG	NG	0
+LAC	0.85	—	5–6	11–13	633 ± 22
+SYR	1.95	0.99	NG	NG	0
+SYR and LAC	0.18	<0.01	7–8	12–15	644 ± 10
2,6-Xylenol					
Alone	1.0	—	NG	NG	0
+LAC	<0.01	—	3–4	8–9	631 ± 38
+SYR	0.88	1.01	NG	NG	0
+SYR and LAC	<0.01	0.06	3–4	8–9	609 ± 13
SYR					
Alone	—	ND	2	6–7	613 ± 32
+LAC	—	ND	3–4	8–9	662 ± 20
Ethanol control	—	—	2	6–7	622 ± 29
Control (no ethanol)	—	—	2	5–6	520 ± 16

[a]Abbreviations: SYR, syringic acid; LAC, laccase; ND, not determined; NG no growth. *Rhizoctonia* laccase, 1 DMP unit per ml.
[b]Determined by HPLC analysis (1 day after addition of phenols and laccase).
[c]Dry weight in milligrams.

cross-coupling of the xenobiotic, as previously described, and thus effectively remove it from solution. The resulting decrease in toxicity of the pesticide confirms the potential use of the phenoloxidase catalyzed polymerization and/or binding of xenobiotics to humic constituents as a means of pollution control.

The question persists, however, whether these pesticide residues, once bound, are stable to microbial action and remain associated with humus to maintain their decreased toxicity. If these bound xenobiotics were to be released at a later time, they would constitute a future hazard, with binding merely delaying their toxic effects. If, on the other hand, their subsequent release is limited, small amounts of the free pesticides could be mineralized by soil microorganisms to prevent any toxic accumulation. Indeed, in our experiments with synthetic humic acid polymers into which labelled ^{14}C-chlorophenols were incorporated, only small amounts of radioacitivity were released from the polymers by microbial action, and a relatively large proportion was subsequently mineralized to $^{14}CO_2$.[15] Of great importance was the fact that after 10 weeks more than three-quarters of the initially-bound radioactivity remained associated with the humic acid precipitate (79.0% of the initially-bound ^{14}C-4-chlorophenol; 77.2% of the ^{14}C-2,4-DCP; 83.8% of the ^{14}C-2,4,5-trichlorophenol; and 78.9% of the ^{14}C-pentachlorophenol). We also proposed that prolonged incubation would be unlikely to lead to significant

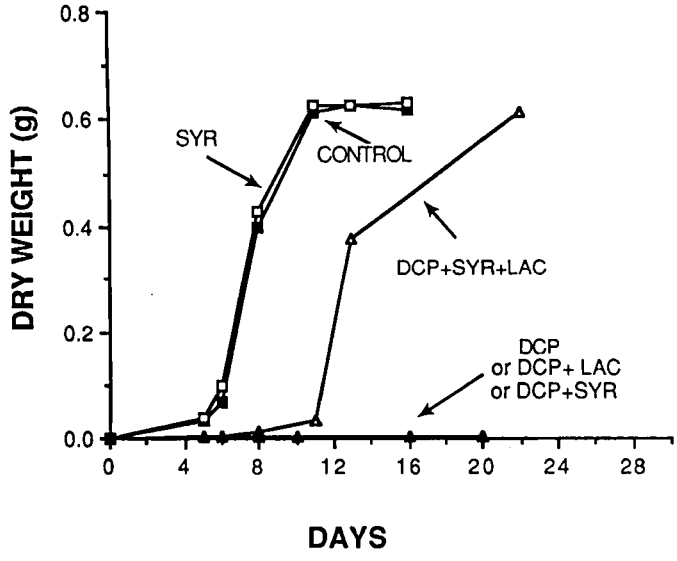

Figure 4 Growth of *Rhizoctonia praticola* in the presence of 2,4-dichlorophenol and 2,4-dichlorophenol cross-coupled with syringic acid. [The reagents used were 2,4-dichlorophenol (DCP), 0.2 mM; syringic acid (SYR), 0.5 mM; and laccase (LAC), 0.5 DMP units per ml. Points are averages of duplicate samples.][14]

further release, since it appears that only some "surface" fraction of the bound residue is releasable, while the remainder is bound to a "core" that is inaccessible to microorganisms. Thus, bound substances were only released to a very limited extent, and once released they were mineralized by microorganisms and abiotic factors. These results are in agreement with the findings of other laboratories.[16–18] Thus, to date, all available data indicate that release of bound residues is minimal, suggesting that once bound to humus, xenobiotics are unlikely to adversely affect the environment.

In this discussion we have given an informative overview of the phenoloxidase-catalyzed polymerization and binding of xenobiotics to humic constituents, using examples from our own laboratory. The demonstrated ability of phenoloxidases, for instance the laccase from *R. praticola*, to polymerize and cross-couple naturally-occurring phenols and pesticide residues has suggested that these enzymes are not only likely to be involved in the formation of humus but also may be useful as a method for detoxifying contaminated soil and water. The latter suggestion has also been supported by the capacity of highly reactive substrates, such as syringic acid, to enhance the enzyme-catalyzed binding of relatively less reactive or unreactive xenobiotics. The result is the covalent attachment of the xenobiotic to another xenobiotic molecule or to a naturally-occurring phenol via the phenoloxidase-induced formation of an oxidative free radical and subsequent attack by this free radical of another compound in the reaction mixture. As we have discussed, this covalent attachment has two consequences:

1. Decreasing the availability of the pesticide for interaction with the biota and thus decreasing its toxicity, and
2. Limiting the ability of the bound xenobiotic to be released by the activity of microorganisms.

Both observations of the enzyme-catalyzed binding confirm this method's potential use for the detoxification of pesticides.

Nevertheless, additional research is necessary before this phenoloxidase-induced polymerization and binding may be practically employed to decontaminate xenobiotics. For instance, it is not clear if free phenoloxidases can retain activity for the time period necessary and under the severe conditions associated with *in situ* decontamination for the complete binding and detoxification of a pesticide. Along this line we have begun investigations into the ability of phenoloxidases immobilized on various supports to catalyze the binding of xenobiotics and retain activity under severe *in situ* conditions. Results are promising and indicate that immobilized enzymes are indeed more stable to, for example, extremes of temperature yet remain active upon immobilization to appropriate supports.[19] Another issue requiring resolution is the releasability of these bound residues upon exposure to *in situ* conditions, even though all available data to date indicate that release is limited. And finally, investigations into the mechanism of phenoloxidase-catalyzed polymerization and binding may provide important information not only on the incorporation of xenobiotics into humus but also on the process of humification itself.

Acknowledgement

This work was supported in part by a research grant from the Environmental Protection Agency (R 811 518), a research grant from the U.S. Geological Survey (No. 14–08–0001–G1137) and by the Pennsylvania Agricultural Experiment Station (Journal series No. 8172).

References

1. R. D. Sjoblad, R. D. Minard and J.-M. Bollag, *Pestic. Biochem. Physiol.* **6,** 457 (1976).
2. A. M. Klibanov, T.-M. Tu and K. P. Scott, *Science* **221,** 259 (1983).
3. J.-M. Bollag and S.-Y. Liu, *Pestic. Biochem. Physiol.* **23,** 261 (1985).
4. S.-Y. Liu and J.-M. Bollag, *Water, Air and Soil Pollution* **25,** 97 (1985).
5. T. Hoff, S.-Y. Liu and J.-M. Bollag, *Appl. Environ. Microbiol.* **49,** 1040 (1985).
6. J. M. Sarkar, R. L. Malcolm and J.-M. Bollag, *Soil Sci. Soc. Am. J.* **52,** 688 (1988).
7. J. Dec and J.-M. Bollag, *Arch. Environ. Contam. Toxicol.* **19,** 534 (1990).
8. K. L. Shuttleworth and J.-M. Bollag, *Enzyme Microb. Technol.* **8,** 171 (1986).
9. J.-M. Bollag. In: *Aquatic and Terrestrial Humic Substances,* (R. F. Christman and E. T. Gjessing, eds.) (Ann Arbor Science Publishers, Inc., 1983).
10. J.-M. Bollag, R. D. Minard and S.-Y. Liu, *Environ. Sci. Technol.* **17,** 72 (1983).
11. D. J. Richards and W. K. Shieh, *Water Res.* **20,** 1077 (1986).
12. J. F. McCarthy and B. D. Jimenez, *Environ. Toxicol. Chem.* **4,** 511 (1985).
13. A. V. Ogram, R. E. Jessup, L. T. Ou and P. S. C. Rao, *Appl. Environ. Microbiol.* **49,** 582 (1985).
14. J.-M. Bollag, K. L. Shuttleworth and D. H. Anderson, *Appl. Environ. Microbiol.* **54,** 3086 (1988).
15. J. Dec and J.-M. Bollag, *Soil Sci. Soc. Am. J.* **52,** 1366 (1988).

16. T.-S. Hsu and R. Bartha, *Soil Sci.* **118,** 213 (1974).
17. T.-S. Hsu and R. Bartha, *J. Agric. Food Chem.* **24,** 118 (1976).
18. I. C. MacRae, *Soil Biol. Biochem.* **18,** 221 (1986).
19. J. M. Sarkar, A. Leonowicz and J.-M. Bollag, *Soil. Biol. Biochem.* **21,** 233 (1989).
20. S.-Y. Liu, R. D. Minard and J.-M. Bollag, *Soil Sci. Soc. Am. J.* **45,** 100 (1981).