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XENOBIOTIC CHEMICALS AND SOIL

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SUMMARY

EPA Grant Number: R 826646
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Immobilization phenomena occurring in soil are of great environmental importance because they reduce the bioavailability and degradation of organic xenobiotics. This research integrated several experimental approaches that, in recent years, have shown great potential for investigating soil-bound chemicals. The compounds under investigation were labeled with $^{14}\text{C}$ and $^{13}\text{C}$ or $^{15}\text{N}$ for analysis by radiocounting and $^{13}\text{C}$ or $^{15}\text{N}$ NMR spectroscopy. $^{19}\text{F}$ was also used to evaluate xenobiotic binding by NMR spectroscopy. In this project, the research focused on four topics: (1) immobilization of TNT in composted soil, (2) immobilization of chlorinated aromatics in various humic materials, (3) trifluralin binding to soil, and (4) release of sequestered and irreversibly bound xenobiotics during incubation with fresh soil.

A clay loam soil from Pennsylvania without a history of exposure to explosives was incubated with 5 g kg$^{-1}$ of $^{15}\text{N}$-labeled 2,4,6-trinitrotoluene (TNT) and 200 µCi kg$^{-1}$ of $^{14}\text{C}$-TNT for 3 days and then amended with compost at a 1:2 (mass/mass) soil to compost ratio. The compost was prepared by mixing 40% alfalfa hay, 40% grass hay, 10% spent mushroom compost, and 10% municipal biosolids. The mixture of soil and compost was inoculated with cattle manure, amended with glucose and starch, and incubated for 37 days under anoxic conditions. The anoxic incubation was followed by 26 days of forced aerobic incubation.

At the end of the aerobic growth phase, most of the radioactivity was associated with organic matter; only 8.7% could be extracted with water and methanol, but no TNT was present in the extracts as determined by high-performance liquid chromatography. The unextractable radioactivity was associated with humic acid (40.0±1.0 %), fulvic acid (14.3±1.4%), and humin (28.2±0.5%). Radioactive materials associated with humic acid and humin were analyzed by solid-state $^{15}\text{N}$-nuclear magnetic resonance (NMR) spectrometry. The NMR spectra indicated
that nitro groups of TNT had been reduced to amino groups; these were subsequently involved in the formation associations with soil organic matter consistent with covalent binding.

Chlorinated phenols and anilines are transformed and detoxified in soil through oxidative coupling reactions mediated by enzymes or metal oxides. The reactions may be influenced by humic constituents, such as syringaldehyde or catechol, which originate from lignin decomposition, and are also subject to oxidative coupling. In this study, the effect of humic constituents on xenobiotic transformation was evaluated \textit{in vitro} based on the determination of unreacted chlorophenols and chloroanilines. In experiments with peroxidase, laccase and birnessite ($\delta$-MnO$_2$), the transformation of most chlorophenols was considerably enhanced by the addition of syringaldehyde. Less enhancement was observed using 4-hydroxybenzoic acid, and the addition of catechol resulted in a reduction of most transformations. The opposite was observed in experiments with tyrosinase, in which case catechol caused considerable enhancement of chlorophenol transformation. The varying effect of catechol can be explained by different transformation mechanisms involving either $\alpha$-quinone coupling (with tyrosinase) or free radical coupling (with peroxidase, laccase, or birnessite). Regardless of the agent used to mediate the reactions, chloroanilines seemed to undergo nucleophilic addition to quinone oligomers that resulted from coupling of the humic constituents. Catechol, which readily forms quinones and quinone oligomers, was most efficient in enhancing these reactions.

Incubations of chlorinated phenols and anilines with oxidoreductive catalysts (peroxidase, laccase, tyrosinase, and birnessite) in the presence of humic acid led to oligomerization of the substrates or their binding to organic matter. The effect of humic acid on the overall transformation depended on the substrate, type of catalyst, the concentration and source of humic acid. At low humic acid concentrations (less than 10 mg/L), the transformation of 4-chlorophenol (4-CP) was enhanced, but at higher concentrations of humic acid (more than 10 mM), no further enhancement occurred. The transformation of 4-chloroaniline (4-CA) was only slightly affected after the addition of humic acid. In experiments with $^{14}$C-labeled substrates, 4-CP was mainly bound to humic acid and formed few oligomers, whereas 4-CA was largely subject to oligomerization with less binding to humic acid. Binding and oligomerization of 4-CP did not change with increasing concentration of humic acid, but with 4-CA, binding increased and oligomerization decreased. It appears that nucleophilic binding of 4-CA depended largely on the availability of carbonyl and quinone groups in humic acid and, therefore, the distribution
of the transformed substrate between oligomers and organic matter greatly depended on the source of humic acid.

Chlorinated phenols and anilines were transformed by oxidoreductive catalysts with release of chloride ions in both the absence and the presence of humic substances (syringaldehyde, catechol, and humic acid). Dehalogenation of these xenobiotics resulted from oxidative coupling reactions occurring at the chlorinated sites of the substrates. The effect of humic substances on dehalogenation depended on the mechanism of oxidative coupling. In a free-radical reaction mediated by peroxidase, laccase, or birnessite (δ-MnO$_2$), syringaldehyde enhanced the dehalogenation of most of the chlorinated phenols, but it did not enhance the dehalogenation of the chloroanilines. With catechol, which does not form free radicals, dehalogenation was reduced or remained the same for both the chlorophenols and the chloroanilines. However, in tyrosinase-mediated reactions controlled by nucleophilic addition, catechol enhanced the dehalogenation of most of the chlorophenols, whereas syringaldehyde had little effect. Humic acid in most cases enhanced the dehalogenation of the chlorophenols, but it had little effect on the dehalogenation of the chloroanilines. On a molar basis, changes in dehalogenation caused by humic substances were proportional to the respective changes in substrate transformation. Only syringaldehyde was capable of releasing disproportionately high amounts of chloride ions from chlorophenols, apparently as a result of multiple cross-couplings to one molecule of the substrate.

Trifluralin is a widely used herbicide for the control of broad leaf weeds in cotton, alfalfa and soybeans. Previous research indicated that in wet soils of high organic matter contents, trifluralin is likely to undergo binding and thus sustain significant losses in herbicidal activity. Bound residue formation may, at the same time, result in a delayed pollution problem. To evaluate trifluralin binding, experiments were undertaken, in which the $^{14}$C-labeled herbicide was incubated for 7 weeks with four soils of different organic matter contents under anaerobic metabolic conditions enhanced by flushing with N$_2$ gas. The presence of fluorine ($^{19}$F) in the trifluralin molecule was used to obtain structural information by $^{19}$F NMR spectroscopy. As determined by radiocounting, trifluralin binding ranged between 10 and 53% of the initial $^{14}$C depending on soil tested. NMR analyses of the methanol-extractable and unextractable $^{19}$F suggests that soil binding may largely involve reduced metabolites of the herbicide. Incubation of trifluralin with zero valent iron produced a product (Fe-TR) that was tentatively identified by
mass spectrometry as 2,6-diaminotrifluralin. This product and one of the standard metabolites (1,2-diaminotrifluralin or TR6) spontaneously formed covalent bonds with fulvic acid (FA), as indicated by the appearance of new resonances in the $^{19}$F NMR spectra taken periodically over a 3-week contact time. No unaltered Fe-TR or TR6 could be recovered by chloroform extraction of these complexes. At short contact times, TR6 and Fe-TR formed weak physical bonds with FA as the respective spin-spin relaxation times ($T_1$) decreased from 1300-1831 msec for TR6 or Fe-TR analyzed in the absence of FA to 150 – 410 msec for TR6/FA or Fe-TR/FA mixtures. In general, the results indicated that trifluralin immobilization involved a variety of mechanisms (covalent binding, adsorption, sequestration) and with time it became increasingly stable.

Concerns exist that pollutants incorporated into soil organic matter may be released upon exposure to microbial activity, hydrolytic conditions or other environmental factors that may disrupt the bonds formed. To test this possibility, various soil materials (whole soil, whole soil extracted with methanol, humic acid, or humin) containing free and/or bound residues of $^{14}$C-labeled xenobiotics [2,4-dichlorophenol (DCP), 2,4,6-trinitrotoluene (TNT), or cyprodinil] were mixed with fresh uncontaminated soils and incubated for 141 days under forced aeration. The incubations were monitored for evolution of $^{14}$CO$_2$ and volatile xenobiotics. Soil samples taken at specific times were extracted with methanol, and fractionated into fulvic acid, humic acid, and humin. The distribution of radioactivity in specific fractions was determined by LSC. The experiments showed some decrease in bound radiocarbon with time due to release. Small amounts of this radioactivity were found in methanol extracts. The release coincided with the evolution of $^{14}$CO$_2$ indicating the occurrence of microbial degradation. The amounts of radioactivity present in methanol extracts (2 to 25%) and $^{14}$CO$_2$ (5 to 40%) differed considerably depending on the compound under investigation. The results indicate that after incorporation into humic materials, the pollutant is practically indistinguishable from soil organic matter. It can be assumed that mineralization of the bound residue would occur at a rate similar to that of mineralization of natural humus. Even if some covalently bound molecules are released and become bioavailable, it will not occur in mass quantities to cause toxic effects.
DETERMINATION OF BINDING INTERACTIONS BETWEEN XENOBIOTIC CHEMICALS AND SOIL

CHAPTER 1. INTRODUCTION

1.1 Overview

The purpose of this project is to evaluate the mechanisms of pollutant immobilization in terrestrial systems. Previous studies indicated that organic pollutants may naturally bind to soil and become less bioavailable and toxic. These observations led to the idea that binding phenomena could be used as a means of detoxification. Xenobiotic compounds can be bound to soil through physical sorption or chemical reaction. From the environmental point of view, the latter is preferred because it frequently leads to the formation of strong covalent bonds that are difficult to disrupt by microbial activity or chemical treatment. Sorption may also lead to strong immobilization of xenobiotics. As indicated by recent studies, the rates of desorption are subject to reduction with the length of time that the xenobiotics reside in soil or are “aged”. Aging is related to adsorption phenomena occurring in remote microsites within the soil matrix. Xenobiotics can be sequestered in these sites following the diffusion through organic matter or intraparticle micropores. Sequestered compounds can be recovered, although with difficulty, by vigorous extraction of soil with organic solvents. From the standpoint of bioavailability, however, sequestration is believed to be irreversible as the sequestered chemicals do not desorb readily back into soil solution. Covalently bound chemicals cannot be recovered by exhaustive extraction with organic solvents. Many xenobiotics present in soil may undergo transformation to reactive metabolites that are more prone to covalent binding than the parent compounds. Thorough evaluation of the nature of binding is essential for determining the environmental significance of immobilized chemicals, i.e., for addressing the question whether, after binding, xenobiotics are safely “locked” in the soil matrix or can rather serve as a pool of toxic residues that may be released in the future.

This project integrated several experimental approaches that, in recent years, have shown great potential for investigating soil-bound chemicals. The compounds under investigation were labeled with $^{14}$C and $^{13}$C or $^{15}$N for analysis by radiocounting and $^{13}$C or $^{15}$N NMR spectroscopy. $^{19}$F was also used in NMR spectroscopy to evaluate xenobiotic binding. In this project period, the research focused on four topics: (1) immobilization of TNT in composted soil, (2)
immobilization of chlorinated aromatics in various humic materials, (3) trifluralin binding to soil, and (4) release of sequestered and irreversibly bound xenobiotics during incubation with fresh soil.

1.2 Formation of bound residues of TNT in composted soil

Large numbers of sites exist worldwide that are heavily contaminated with 2,4,6-trinitrotoluene (TNT) mainly due to the manufacture and testing of munitions (Spain, 1995). Owing to the electron withdrawing nature of its three nitro groups, TNT is very recalcitrant and resists degradation in natural soil and water systems (Rieger and Knackmuss, 1995). Microbial transformation of TNT to CO$_2$ has been observed in several studies (Fernando et al., 1990; Fiorella and Spain, 1997; French et al., 1998), but concentrations of TNT in these investigations were generally too low to simulate the actual conditions in polluted sites, where TNT concentrations can reach 1% (10,000 mg kg$^{-1}$ TNT) (Comfort et al., 1995).

Anaerobic/aerobic treatment has been shown to be a viable technique for remediation of TNT-polluted soils (Daun et al., 1998; Lenke et al., 1998). To date, the research has mainly focused on experiments involving anoxic soil slurries with subsequent exposure to oxygen to evaluate the corresponding transformation pathways. Relatively little is known, however, about transformation processes in soil amended with compost. Composting is one of the few bioremediation technologies to be applied for large-scale bioremediation of soil, as demonstrated by the U.S. Army for explosives contaminated soils (Roy F. Weston, Inc., 1993). Like soil slurry treatment, composting is aimed at the irreversible binding of reduced metabolites of TNT to organic matter. There was some concern about using this approach since composting is an oxidative process, and reducing conditions favor the conversion of nitro groups into amino groups. However, in the study of Canton et al. (1994), 74% of the radioactivity from $^{14}$C-labeled TNT was already bound on the first day of composting; this increased to 99% by day 90. Drzyzga et al. (1998) found that 84% of initially added radioactive TNT was immobilized in different soil fractions after nine weeks of anoxic and oxic treatment.

Amino and hydroxylamino groups of the reduced metabolites of TNT are known to interact with quinone moieties present in soil organic matter (Rieger and Knackmuss, 1995). The addition of compost to contaminated soil has the potential to add more reactive quinone groups.
to facilitate faster and more thorough immobilization of reduced metabolites of TNT. In addition, composting adds an active consortium of microorganisms and carbon for microbial metabolism.

In several previous studies, the interactions between specific xenobiotics and soil organic matter were investigated using chemicals labeled with stable isotopes, such as $^{13}$C or $^{15}$N. For instance, $^{15}$N label was used in research on binding of TNT (Achtnich et al., 1999; Knicker et al., 1999) and aniline (Thorn et al., 1996), and $^{13}$C was applied to investigate soil-bound residues of the fungicide cyprodinil (Dec et al., 1997a,b), and covalent binding of 2,4-dichlorophenol to humic acid (Bortiatynski et al., 1994; Hatcher et al., 1993). In the present study, TNT was labeled with $^{14}$C to allow investigation into the distribution of reduced metabolites in different soil fractions, and with $^{15}$N to elucidate the nature of the linkage causing the reduced metabolites of TNT to be irreversibly bound in the composted soil.

1.3 Oxidative coupling of aromatic xenobiotics

Chlorinated phenols and anilines may be introduced into the environment by accidental spills, illegal release of industrial and municipal wastewater, and excessive use of pesticides. As analogues of humic constituents, these pollutants can be incorporated into the soil organic matter or dissolved humic materials. The incorporation relies largely on the oxidative coupling reaction mediated by extra cellular phenoloxidases or metal oxides present in soil or sediments (Hsu and Bartha, 1974a; Parris, 1980; Sjoblad and Bollag, 1981; You et al., 1982; McBride, 1987; Lehmann et al., 1987; Simmons et al., 1989; Wang and Huang, 1991; Bollag, 1992; Weber et al., 1996). The reaction of oxidative coupling is of great environmental significance because in most cases it leads to the detoxification of xenobiotic substrates (Bollag, 1992).

In the presence of phenoloxidases or metal oxides, chlorinated phenols and anilines are first oxidized to free radicals or quinones. In the second stage, the oxidation products are subject to chemical coupling. If the reaction takes place in polluted aqueous environments, the oxidation products couple primarily to each other and precipitate out of solution in the form of non-toxic polymers (Klibanov et al., 1983; Simmons et al., 1989). In polluted soils, they couple mainly to humus resulting in the formation of covalent linkages (Bollag, 1992).
Both the aqueous and soil environments contain large amounts of natural phenols (e.g., ferulic acid, syringaldehyde, pyrogallol, hydroxybenzoic acid, or catechol) that originate from lignin decomposition and are major substrates for oxidative coupling reactions leading to the formation of humus (Stevenson, 1994). These humic constituents may influence the transformation of xenobiotics during oxidative coupling by competing for active sites on enzyme molecules or mineral surfaces, or by cross-coupling with the xenobiotic molecules (Bollag, 1992).

The use of enzymes and metal oxides that can stimulate binding or polymerization of the xenobiotic substrates is currently considered an alternative means of soil clean-up and water treatment (Berry and Boyd, 1985; McBride, 1987; Dec et al., 1990; Dec and Bollag, 1990; Roper et al., 1995). In view of this idea, knowledge related to the effect of humic constituents on the transformation of the pollutants during oxidative coupling is of great significance. There are indications that the enhanced transformation may result from high reactivity of humic constituents incubated with less reactive pollutants (Klibanov et al., 1983; Roper et al., 1995). Preliminary experiments for the present investigation indicated, however, that high reactivity alone does not guarantee enhancement unless combined with other factors. A reactive lignin derivative such as catechol, for instance, was found to greatly reduce the transformation of 2,4-dichlorophenol in the presence of peroxidase. In the presence of tyrosinase, however, the transformation of 2,4-dichlorophenol was considerably enhanced by the addition of catechol. These observations indicated that besides the reactivity of humic constituents, the transformation patterns of chlorinated phenols and anilines may depend on the mechanism of oxidative coupling, which may vary with the type of oxidizing agent and the chemical structure of compounds under investigation. To evaluate this hypothesis it was deemed necessary to test, in different combinations, a wide variety of reaction components (chlorinated pollutants, humic constituents, and oxidizing agents). It was expected that by generating a sufficient amount of data, it should be possible to identify different transformation patterns and assign them to specific mechanisms of oxidative coupling.

Xenobiotic phenols and anilines are represented in this study by six compounds from each group, substituted analogously with chlorine atoms. The investigation includes 16 randomly selected humic constituents that vary in the type and the number of substituents. Enzymes used in this study represent monophenol monooxygenases (i.e., laccases and tyrosinases) and
peroxidases. To oxidize phenols or anilines, these enzymes require either molecular oxygen (laccases and tyrosinases) or hydrogen peroxide (peroxidases) as electron acceptors. In the presence of laccases and peroxidases, the pollutants are oxidized to free radicals (Klibanov et al., 1983; Bollag, 1992). Free radicals are also generated in the presence of metal oxides (Wang and Huang, 1991), such as birnessite ($\delta$-MnO$_2$), which was included in this investigation. Reactions with tyrosinases, on the other hand, lead to ortho-hydroxylation of monophenols and oxidation of the resultant ortho-diphenols to ortho-quinones (Dec and Bollag, 1995). Using the selected enzymes and birnessite, the investigated compounds could be tested for all known mechanisms of oxidative coupling.

1.4 Binding interactions between aromatics and humic via oxidative coupling

Recent studies (Wang and Huang, 1991; Bollag, 1992; Nicell et al., 1993; Bollag et al., 1997) indicate that phenols and anilines can be incorporated into soil organic matter or dissolved humic materials after treatment with various oxidoreductases or metal oxides. Some humic constituents proved to enhance the incorporation processes (Park et al., 1998). As the products of lignin decomposition, humic constituents are aromatic in nature and possess a variety of functional groups that may be involved in the formation of covalent bonds between pollutants and the organic matter (Hsu and Bartha, 1974; Parris, 1980; You et al., 1982; Hatcher et al., 1993; Stevenson, 1994; Thorn et al., 1996; Weber et al., 1996)

Using electron spin resonance spectrometry (ESR), Suflita et al. (1981) demonstrated that fulvic acids contain stable free radicals that can contribute to the incorporation of xenobiotics. Based on experiments with chemically modified fulvic acids, Schnitzer and Skinner (1969) suggested that the formation of free radicals involves hydroxyl groups of phenolic moieties that are present in humic materials. According to Steelink and Tollin (1967), free radicals can also result from the quinone and amine components of humus. Schnitzer and Skinner (1969) estimated that one free radical occurs for as many as 440,000 carbon and 30,000 oxygen atoms of fulvic acid. At such a low concentration, the inherent free radicals can hardly contribute to the extensive binding observed in most studies (Wang and Huang, 1991; Bollag, 1992; Nicell et al., 1993; Bollag et al., 1997). There are indications that in the case of chlorinated phenols additional free radicals can be transferred to humic materials from phenoxy radicals generated by
enzymatic oxidation of the substrates (Dec and Bollag, 1994). As demonstrated by $^{13}$C NMR spectrometry (Hatcher et al., 1993), a free radical reaction between 2,4-dichlorophenol and humic acid resulted in the formation of carbon-carbon and oxygen-carbon linkages.

Anilines can covalently bind to quinone components of humic materials through 1,4-nucleophilic addition. Parris (1980) monitored such a reaction by infrared spectroscopy after 4-chloroaniline was stirred in methanol with 1,4-benzoquinone. According to Hsu and Bartha (1974), hydrolysis-resistant binding of aniline may occur when the amino group of aniline reacts with carboxyl and carbonyl groups of humic acids to form heterocyclic ring structures. Weber et al. (1996) demonstrated that binding of aniline to 1,4-benzoquinone was reduced by using hydrogen sulfide to block nucleophilic addition of aniline. Reduced binding of aniline was also observed after the reduction of carbonyl moieties in humic substances by sodium borohydride (Weber et al., 1996). The covalent binding of aniline compounds to carbonyl moieties and quinone components of humic substances was recently confirmed by $^{15}$N-NMR spectroscopy (Thorn et al., 1996).

Model studies on the transformation of chlorinated phenols and anilines by oxidoreductive catalysts in the presence of humic materials can provide considerable insight into the fate of these chemicals in terrestrial and aquatic systems (Berry and Boyd, 1985; Bollag, 1992). In a previous investigation, the phenolic and aniline compounds were incubated with monomer humic constituents, such as syringaldehyde, catechol, and 4-hydroxybenzoic acid, to determine the effects of the latter on pollutant transformation (Park et al., 1998). The results indicated that this effect depended primarily on the mechanisms of oxidative coupling, a reaction that controlled transformation. When one of the two possible transformation mechanisms (free radical coupling or $o$-quinone coupling) was common to both co-substrates, humic monomers usually enhanced the transformation of chlorinated compounds. If, however, the co-substrates were subject to different mechanisms of oxidative coupling, humic monomers had no effect or reduced the transformation. In this study, the pollutants were incubated with natural humic acids to further approximate the conditions in soil and aquatic environments.
1.5 Dehalogenation of aromatics during oxidative coupling to humic acid

Oxidoreductive enzymes such as laccase, peroxidase, and tyrosinase are considered to be important catalysts of oxidative coupling reactions occurring in terrestrial systems (Berry and Boyd, 1984). Metal oxides and clay minerals commonly present in soil also may mediate these reactions (Wang et al. 1978; Larson and Hufnagel 1980; Wang and Huang 1991). An important consequence of oxidative coupling is the formation of humus through polymerization of the phenolic products of lignin decomposition (Stevenson 1994; Ladd and Butler 1981). Some toxic xenobiotics, such as chlorinated phenols and anilines, may be involved in oxidative coupling that results in polymerization, cross-coupling with naturally occurring phenols, or binding to soil organic matter (Bollag, 1992).

Chlorinated phenols and anilines are subject to dehalogenation during these reactions. Hammel and Tardone (1988) detected dechlorinated metabolites of 2,4,6-trichlorophenol after it had been incubated with peroxidase from Phanerochaete chrysosporium. Dec and Bollag (1990; 1994) and Roy-Arcand and Archibald (1991) reported a release of chloride ions from chlorinated phenols during incubation with several oxidoreductases or birnessite ($\delta$-MnO$_2$). Additionally, oxidoreductase-mediated dehalogenation of chloroanilines was reported (Dec and Bollag 1995).

Chloride release associated with oxidative coupling is not to be confused with dehalogenation reactions that occur during microbial degradation of chlorinated aromatics, as was the case for pentachlorophenol that was degraded to 2,3,5,6-tetrachloro-2,5-cyclohexan-1,4-dione by the white rot fungus Phanerochaete chrysosporium (Mileski et al., 1988), or for the chlorophenoxyalkanoic herbicide mecoprop that was degraded to 2-methylphenol by a mixed culture of soil bacteria (Kelly et al., 1991). Neither is this type of chloride release to be confused with the reductive dehalogenation by aerobic or anaerobic microorganisms (Suflita et al., 1984; Kuhn and Suflita, 1989; Kohring et al., 1989).

According to recent findings (Dec and Bollag 1994; 1995), release of chloride ions from chlorinated phenols and anilines was a result of the coupling reaction, rather than catalyst activity. The catalysts only oxidized the substrates to quinone products or free radicals capable of coupling to each other or to soil organic matter. Chlorine atoms were released (in the form of ions) if they happened to be attached to carbon atoms engaged in coupling, especially those at
the ortho or para positions. Insufficient production of free radicals or quinones at unfavorable pH, low enzyme concentration, or short incubation time led to reductions in chloride release (Dec and Bollag 1995).

As determined in related studies (Park et al., 1999; Park et al., 2000a), humic substances had a considerable effect on the transformation of chlorinated phenols during oxidative coupling reactions. The reaction mixtures in the above mentioned investigations were monitored for the release of chloride ions from the tested chemicals, and the dehalogenation aspect is evaluated in a follow up paper (Park et al. 2000b).

1.6 Determination of soil-bound trifluralin by $^{19}$F NMR spectroscopy

Trifluralin ($\alpha$,\,$\alpha$,\,$\alpha$-trifluoro-2,6-dinitro-N,N-dipropyl-$p$-toluidine) is a dinitroaniline pre-emergence herbicide used for the control of broadleaf weeds for various crops including cotton, soybeans and alfalfa (Grover et al., 1997; USGS. 2002). It is among the top five herbicides produced annually in the U.S. (24,000 metric tons yr$^{-1}$) with $300$ million in annual sales (Grover et al., 1997) despite being a suspected carcinogen. Previous studies on trifluralin have included its fate and transport in soils (Golab et al., 1979; Matterre et al., 1998), the transformation under low redox or anoxic conditions (Parr and Smith, 1973; Willis et al., 1974; Tor et al., 2000), the co-metabolism in sewage systems (Jacobson et al., 1980), and trifluralin photodecomposition (Crosby and Lettis, 1973; Lettis and Crosby, 1974; Mabury and Crosby, 1995). In order to maintain the viability of the herbicide and prevent photodecomposition, it was recommended that trifluralin be injected under the soil surface (Grover et al., 1997; DOW Agrosciences, 2002) However, subsurface application may not necessarily prevent losses in herbicidal activity, since as demonstrated by Grover et al. (1997), in wet soils of high organic matter contents, reduced forms of trifluralin can become incorporated into soil organic matter. The incorporation, in turn, may evolve into a delayed pollution problem in case of future release of bound trifluralin residues under favorable conditions.

To date, relatively little has been done to investigate the mechanism of trifluralin binding despite the progress in determining other xenobiotic interactions with soil (Pignatello and Xing, 1996; Dec and Bollag, 1997, Sparks, 1999). Recent research in this area involved both $^{14}$C-labeled contaminants and compounds labeled with stable isotopes to facilitate the analyses of
xenobiotic/soil organic matter complexes by NMR techniques. In the studies of Strynar et al. (2002) using $^{15}$N-2,4,6-trinitrotoluene (TNT), NMR evidence suggested that reducing the compound to its amino derivatives under anoxic conditions led to the formation of very stable covalent bonds with soil and compost humic materials. Among other studies on binding interactions that involved xenobiotics labeled with stable isotopes were those using $^{15}$N-aniline (Thorn et al., 1996), $^{13}$C-2,4-dichlorophenol (Hatcher et al., 1993), and $^{13}$C-cyprodinil (Dec et al., 1997). Presently, $^{19}$F emerges as an additional element for research in this area. Benefits of using $^{19}$F over the use of other isotopes include lack of background $^{19}$F signal in soils, increased sensitivity and spectral range relative to $^{15}$N and $^{13}$C, and perhaps most important, no need to synthesize $^{19}$F-labeled xenobiotics as $^{19}$F is 100% abundant in fluorinated compounds and constitutes an elemental tracer. A limitation is that only F-containing xenobiotics may be investigated, though Chien et al. (1997) used atrazine derivatized with fluorine for their investigation.

Previously, $^{19}$F NMR has been used to study interactions between fluorinated xenobiotics, such as atrazine (Chien et al., 1997; Chien and Bleam, 1997) or 4’-fluoro-1’-acetonaphthone (Dixon et al., 1999), and various humic materials. Other studies used this technique to investigate fipronil degradation pathways (Ngim et al., 2000), characterize photolysis products of 3-trifluoromethyl-4-nitrophenol (Ellis and Mabury, 2000), detect the chlorodifluoroacetic acid ions (Martin et al., 2000), and evaluate sorption of hexafluorobenzene to soil organic matter (Kohl et al., 2000), sediments, polymers or activated carbon (Cornelissen et al., 2000). Mabury and Crosby (1995) appear to be the only researchers that investigated trifluralin in depth by $^{19}$F NMR, mainly to identify photodegradation products that could react with soil and form bound residues.

In this study, $^{19}$F NMR was applied to investigate the formation of soil-bound residues of trifluralin under anoxic metabolic conditions. The hypothesis was that, as was the case for TNT (Strynar et al., 2002), the nitro groups of the trifluralin molecule would be reduced to amino groups that would then react with soil organic matter. In an analogous manner, aromatic amines reacted with humates via carbonyl or quinone moieties (Paris, 1980). Based on previous reports (Golab et al., 1979; Grover et al., 1997), bound residues of trifluralin were assumed to retain the trifluoromethyl (CF$_3$) group. Also, no study to date has suggested mineralization of the CF$_3$.
group, at least in the short term (3 years). It is postulated that eventually the trifluoromethyl group could be oxidized to carboxylic acid, and bound metabolites could be mineralized following rupture of the aromatic ring (Golab et al., 1979).

### 1.7 Stability of soil-bound xenobiotics in fresh soil

Considerable efforts have been made in the recent past to enhance the understanding of xenobiotic interactions with soil. Investigations into the problem of soil-bound chemicals have covered a wide variety of compounds (e.g., pesticides, industrial pollutants, explosives etc.) and interactions, including sorption, sequestration and covalent binding (Barriuso and Koskinen, 1996; Piccolo et al., 1996; Luthy et., al., 1997; Xing and Pignatello, 1997; Dec and Bollag, 1997; Achtnich et al., 1999; Leuking et al., 2000; Zhu and Selim, 2000). Despite progress in clarifying the nature of pollutant binding, uncertainties linger about the stability and bioavailability of xenobiotics involved in different immobilization phenomena (Alexander, 2000).

Adsorption is thought to be the least stable immobilization mechanism, with complete desorption theoretically possible, although certain chemicals (e.g., diquat or paraquat) that are strongly adsorbed to soil surfaces through cation exchange have little or no desorption (Sparks, 1999). Xenobiotics retained by weak physical interactions, such as van der Waals forces or H-bonding can be released and become bioavailable. Therefore, adsorption of xenobiotics is generally not a useful decontamination mechanism.

Sequestration is sometimes referred to as slow sorption, because it involves a gradual diffusion of one material (xenobiotics molecules) within another (soil matrix) and requires long contact times (months, even years) to reach equilibrium. Given enough time and energy, sequestered compounds may be completely recovered from soil by extraction with organic solvents. However, under field conditions, sequestration is practically irreversible (years to decades), and is increasingly viewed as a potential mechanism for efficient immobilization of xenobiotics in the soil matrix.

Hatzinger and Alexander (1995) provided strong evidence that, even if aged in sterile soil, xenobiotics may undergo sequestration and with time become less and less susceptible to mineralization, which they interpreted as a decrease in bioavailability. Chung and Alexander (1998) found marked differences in the bioavailability of xenobiotics depending on the duration
of their residence in soil. In the study of Alexander and Alexander (2000), a 7-day aging of genotoxic xenobiotics, such as benzo(a)pyrene and 9,10-dimethyl-1,2-benzanthracene, resulted in reduced soil toxicity, but it did not result in the degradation of the compounds. Since more than 90% of the initially applied pollutants could be then recovered by organic solvent extraction, it was concluded that an extractable pollutant is not necessarily bioavailable.

Alexander (1995, 2000) pointed out that the reduced bioavailability of the sequestered xenobiotics is not taken into account in current methods for risk assessment. As a result, such methods probably overestimate the impact of toxic chemicals on the environment.

Covalent binding of xenobiotics to soil organic matter is another consequence of aging. Because xenobiotics involved in this form of binding cannot be recovered from soil by organic solvent extraction, covalent interactions have been thought to be more stable than sequestration. Extractability is operationally defined and may not be an entirely reliable factor in correlating the nature and the stability of immobilized compounds. Dec et al (1997b), for instance, determined that following exhaustive extraction of cyprodinil polluted soil with methanol and 0.5 M NaOH, additional amounts of free cyprodinil could be released from the humin fraction through silylation. It is possible that such hidden portions of free pollutants might have remained unaccounted for in other investigations and misinterpreted as covalently bound, rather than sequestered.

Previous investigations (with PAHs, nitroaromatics, chlorinated phenols, anilines, and cyprodinil and many other xenobiotics) addressed various aspects of xenobiotic binding, such as the role of microorganisms in bound residue formation (Kästner et al., 1999), the effect of environmental stress on the stability of binding (Eschenbach et al., 1998), the occurrence of binding under aerobic and anaerobic growth conditions (Strynar et al., 2002), immobilization kinetics (Weber et al., 1996), or the chemical nature of bound xenobiotics (Thorn et al., 1996; Dec et al., 1997a,b,c). However, the problem of the release of bound xenobiotics by soil microorganisms has received limited attention (Hsu and Bartha, 1974; Khan and Ivarson, 1981; Dec et al., 1990).

In this study, microbial release was investigated using whole soil or soil components (HA, humin) from previous investigations on the immobilization of 2,4,6-trinitrotoluene (Strynar et al., 2002), cyprodinil (Dec et al., 1997b) and 2,4-dichlorophenol (unpublished data). After mixing with fresh soil that served as a carrier of microbial activity, the samples were monitored
over time for the evolved $^{14}$CO$_2$, volatiles, extractable pollutants and the remaining bound residues.
CHAPTER 2. MATERIALS AND METHODS

2.1 Composting of TNT polluted soil

The uniformly labeled $^{14}$C-TNT was obtained from the U.S. Army Center for Health Promotion and Preventive Medicine (USACHPPM), Aberdeen Proving Ground, Maryland; its specific activity was 0.1 mCi mg$^{-1}$. The $^{15}$N-TNT of 96-97% purity and the label located in all three nitro groups was synthesized by Dr. Michael Major (USACHPPM). Compost consisted of 40% alfalfa hay, 40% grass hay, 10% spent mushroom compost, and 10% municipal biosolids compost by weight. The components were homogenized in a food processor prior to application and inoculated with methanogens from cattle manure. Fresh manure (1 kg) was collected from a local cattle farm, combined with 1 L of water (10% w/vol.) and placed in an air-tight vessel; 20 mL of this slurry was used as the inoculum.

Soil used for experimentation was a Hagerstown soil (fine, mixed, mesic Typic Halpudalf) collected from the A horizon at the Pennsylvania State University Agronomy Farm. Fifty grams of uncontaminated soil were thoroughly mixed with 0.25 g of $^{15}$N-TNT and 10 µCi of uniformly labeled $^{14}$C-TNT dissolved in methanol. This resulted in 5 g kg$^{-1}$$^{15}$N-TNT and 200 µCi $^{14}$C-TNT kg$^{-1}$ soil. After a 3-day incubation, the soil was split into two equal 25 g samples, each of which was placed in a stoppered 1-L Erlenmeyer flask and amended with compost at a 2:1 compost to soil ratio. The control sample consisted of 25 g soil and compost amendment with no added TNT. After thorough mixing, the flask was fitted with a gas vent as shown in Figure 1. Also added to the flask were: an anaerobic microbial consortium, glucose (20 mL of a 10% glucose solution w/v), and water (50 mL).

The incubation consisted of an anaerobic growth phase that lasted 37 days, followed by an aerobic growth phase that lasted 26 days. Prior to the first phase of incubation, the headspace was flushed with N$_2$ to create anoxic conditions. During the anaerobic growth phase of the experiment the compost was saturated with water due to glucose/starch solution additions.

Throughout the aerobic phase, the compost/soil mixture was exposed to forced aeration by exchanging the headspace with an air pump. The compost was mixed weekly and allowed to dry as a result of no moisture replenishment.
The headspace was sampled periodically during the anaerobic growth phase and analyzed for gas production (H₂, CO₂ and CH₄) on a gas chromatograph (Hewlett Packard Model 5890, Palo Alto, CA) equipped with a thermal conductivity (TCD) detector. The GC analysis was performed using a 60/80 Carboxen-1000 stainless steel column (4.6 m x 2.1 mm) from Supelco (Bellefonte, PA). The oven temperature was maintained at a gradient from 70°C (3 min.) to 220°C at 10°C/min. and held at 220°C for 15 minutes. The carrier gas was helium (28 mL/min), so was the reference gas (9 mL/min).

An inline scintillation cocktail trap, containing of 20 mL of scintillation cocktail (R.J. Harvey Carbon-14 Cocktail, Hillsdale, New Jersey) was used to monitor the evolution of ¹⁴CO₂ from the compost. The total output of gas production was monitored using the inverted graduated cylinder (1000 mL) as shown in Figure 1. Occasionally 10 mL of starch (2% w/v) solution or 10 mL of glucose (4% w/v) solution was added when gas production decreased.

2.2 Fractionation and analyses of composted TNT soil

On the completion of the aerobic growth phase, the composted soil was homogenized and processed by extraction with deionized water, followed by Soxhlet extraction with methanol, and by extraction with 0.5 M NaOH. The solids were weighed after each extraction step for mass balance calculations.

Water extractions were carried out by a 1-h shaking of 5.0 g samples with three consecutive portions of deionized water (50 mL). After centrifugation, the water extract was acidified to pH 0.95 to precipitate humic acid material, and then the supernatant was extracted three times with 25 mL of dichloromethane to extract free residues of TNT. Both the aqueous phase and the organic phase were analyzed by thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), and liquid scintillation counting (LSC). For analysis 1/3rd of the water extract (50 mL used) was lyophilized and redissolved in 10.0 mL of water. The dichloromethane extract was evaporated to dryness under N₂ gas and redissolved in 10.0 mL of dichloromethane.

Soxhlet extraction was carried out for 24 h using 100 mL of methanol. The entire methanol extract was condensed to 10 mL and analyzed by TLC, HPLC, and LSC.
Extraction with 0.5 M NaOH (50 mL) was carried out for 24 h with shaking. After centrifugation, the solids were rinsed 5 times with 10 mL of 0.1 M NaOH and analyzed by radiocounting (after combustion to $^{14}\text{CO}_2$) and solid-state NMR. The NaOH extract and washings were combined, acidified with 5 M HCl to pH 0.95, stored in a refrigerator overnight to facilitate the precipitation of humic acid, and centrifuged. The pellet of humic acid was rinsed 5 times with 0.1 M HCl, redissolved in 0.1 M NaOH and analyzed by LSC and liquid-state NMR. The washings were combined with the supernatant (fulvic acid fraction) for analysis by HPLC and LSC.

Prior to TLC analysis, the aqueous phase (10 mL) was frozen and freeze-dried under high vacuum. The dichloromethane and methanol extracts (10 mL) were evaporated to dryness under a light N$_2$ gas stream. The dry residues from organic extracts were redissolved in 0.25 mL of dichloromethane and methanol, respectively. The dry residue of water extract was redissolved in 0.25 ml of dimethylsulfoxide (DMSO). The entire volumes of the extracts were applied as 5-mm spots to a 0.25 mm silica gel 60 F254 precoated TLC plate (E. Merck, Darmstadt, Germany) and developed using an 85/15 toluene/methanol solvent system. A spot of $^{14}\text{C}$-labeled standard of TNT was used as a reference. Before and after the analysis, the plate was imaged on a Bioscan System 200 Imaging Scanner (Washington, D.C.). Imaging was conducted for 30 minutes on each TLC lane to maximize resolution.

The HPLC analysis was carried out using a Nucleosil C18 4.6 mm x 250 mm column with a 5 µm particle size (Sigma-Aldrich, St. Louis, MO) and a Security Guard C18 guard column 4.0mm L x 3.0mm ID (inner diameter) (Phenomenex, Torrance, CA). Samples were analyzed for TNT in the extracting solvent (water, methanol, dichloromethane) used. Mobile phase (50/50 methanol/water) was delivered at 0.5 mL/min using a Waters Alliance 2690 Separations Module (Milford, MA). Detectors included a Waters 2487 Dual Absorbance Detector operated at 254 nm, and a Raytest Ramona inline radioactivity detector (New Castle, DE). The detection limit for radioactivity was found to be 150 dpm 100 µL and for UV absorbance at 254 nm to be 0.5 µg TNT ml$^{-1}$. A standard curve for TNT was developed by serial dilution of a 10,000 µg mL$^{-1}$ TNT solution. Injections of 1, 10, 100 and 1000 µg TNT mL$^{-1}$ were used for standardization. A maximum injection volume (100 µL) was used for detection of TNT in any extracts. For
retention time comparisons, 10 µL of the 100 µg mL⁻¹ TNT solution was used. Chromatograms were stacked and normalized for maximum peak height.

The LSC analysis was performed on a Beta Trac 6895 liquid scintillation counter (Tracor Analytic, Elk Grove, IL) using Eco-Scint cocktail (National Diagnostics, Atlanta, GA). The extracted solids (0.1 g) were combusted in a R.J. Harvey OX600 Biological Oxidizer with ¹⁴CO₂ trapping in 15 mL of R.J. Harvey Carbon-14 Cocktail followed by LSC analysis.

Prior to NMR analysis humic acid was redissolved in 25 mL of 0.1 M NaOH and dialyzed for 48 h against deionized water using a Spectropore membrane with a 6000-8000 Da cutoff. Water was exchanged every 12 hours and constantly stirred using a magnetic bar. The dialyzed solution of humic acid was freeze-dried in a Labconco Lyph-Loc 6 freeze-drier and stored in a desiccator until analysis by NMR. The NaOH-extracted solids (designated as humin) also were subjected to freeze-drying prior to NMR analysis.

The liquid-state ¹⁵N-NMR spectrum of the free ¹⁵N-labeled TNT (50 mg) dissolved in 1 mL of d₄-methanol was obtained on a Bruker AMX-2-500 spectrometer (Billerica, MA) using a regular 5-mm NMR tube. The resonance frequency for ¹⁵N was 50.678 MHz. The solid-state ¹⁵N NMR spectra of humic acid and humin were obtained using a Chemagnetics CMX-300 spectrometer (Varian Inc., Palo Alto, CA) operating at 30.137 MHz for ¹⁵N. All samples were referenced using an external standard of ¹⁵N-glycine, set at 32.6 ppm versus liquid ammonia at 25°C (0 ppm). The cross-polarization with magic angle spin (CP-MAS) spinning pulse sequence was applied. The calibrated ¹⁵N pulse width was 6.2 µsec with a relaxation delay of 0.3 seconds. A spectral window of 30 kHz was employed to observe the range of the ¹⁵N signals. Between 300,000 and 400,000 scans were collected for each sample.

2.3 Oxidative coupling of aromatics

The compounds 2-, 3- and 4-chlorophenol (2-CP, 3-CP and 4-CP), 2,4- and 2,5-dichlorophenol (2,4- and 2,5-DCP), gallic acid, ferulic acid, protocatechuic acid and guaiacol were purchased from Sigma Chemical Co. (St. Louis, MO). 2-, 3,- and 4-Chloroaniline (2-, 3- and 4-CA), 2,4-dichloroaniline (2,4-DCA), 2,4,5-trichloroaniline (2,4,5-TCA), 4-hydroxybenzoic acid (4-HBA), vanillic acid and caffeic acid were bought from Aldrich Chemical Co. (Milwaukee, WI). 2,4,5-Trichlorophenol (2,4,5-TCP), syringaldehyde, vanillin and syringic
Acid were purchased from Fluka AG (Buchs, Switzerland). Catechol, pyrogallol and salicylic acid were obtained from Fisher Scientific Co. (Fair Lawn, NJ), and hydroquinone was bought from J.T. Baker (Phillipsburg, NJ). Uniformly labeled $^{14}$C-4-CP (98% purity and specific activity of $3.30 \times 10^8$ Bq/mM) and $^{14}$C-4-CA (98% purity and specific activity of $8.55 \times 10^8$ Bq/mM) were bought from Sigma Chemical Co. (St. Louis, MO).

Leonardite humic acid was extracted from humus powder (WP-80) provided by Humus Products of America (Richmond, TX) and Hagerstown humic acid was extracted from Hagerstown soil according to the method of Stevenson (1994). Suwannee River humic acid reference and soil humic acid standard were purchased from the International Humic Substances Society (IHSS, Denver, CO). Stock solutions (1,000 mg/L) were prepared for all humic acids in 0.2 M acetate buffer (pH 5.6).

The laccase (EC 1.10.3.2 p-phenol oxidase) isolated from the fungus Trametes villosa was obtained from Novo Nordisk (Danbury, CT). One unit of laccase activity is defined as the amount of enzyme that causes a change in absorbance at 468 nm of 1.0/min in 3.4 mL of a 1 mM solution of 2,6-dimethoxyphenol in citrate-phosphate buffer (pH 3.8).

Horseradish peroxidase with an RZ (Reinheitszahl) of 1.2 and an activity of 87 units/mg of solid was purchased from Sigma Chemical Co. (St. Louis, MO). The RZ value, which represents peroxidase purity, is the ratio of absorbency at 403 nm due to the hemin group to that at 275 nm caused by the protein. The activity is expressed in units defined as the amount of peroxidase needed to form 1.0 mg of purpurogallin from pyrogallol in 20 s at pH 6.0 and 20ºC.

Mushroom tyrosinase, with an activity of 4400 units/mg of solid, was obtained from Sigma Chemical Co. (St. Louis, MO). One unit of tyrosinase activity is defined as the amount of enzyme that causes an increase in absorbance at 280 nm of 0.001/min at pH 6.5 and 25ºC in a 3-mL reaction mixture containing L-tyrosine.

Birnessite (manganous manganite, $\delta$-MnO$_2$) was prepared according to the method of McKenzie (McKenzie, 1971).

Because of the large number of assays to be carried out simultaneously, the determination of reaction kinetics for each combination was not feasible. Therefore the effect of humic constituents on the extent of transformation was evaluated mostly on the basis of the overall disappearance of xenobiotics in the supernatant as determined by high-performance liquid chromatography (HPLC).
Unless specified otherwise, chlorinated phenols and anilines (0.3 mM) were dissolved in 0.2 M acetate buffer at pH 5.6, and 10-mL samples were incubated in triplicate with various enzymes and birnessite in the presence or absence of different humic constituents (0.3 mM). The incubations without humic constituents served as reference controls for calculating changes in transformation of the pollutants. Laccase, peroxidase, tyrosinase and birnessite were applied at concentrations of 1.5 units/mL, 0.15 units/mL, 40 units/mL and 0.5 mg/mL, respectively. Peroxidase was used along with hydrogen peroxide (0.5 mM H₂O₂) as an electron acceptor. The remaining enzymes and birnessite utilized the molecular oxygen present in the solution. After a 2-hour incubation with peroxidase and a 24-hour incubation with laccase, tyrosinase or birnessite, 0.17 ml of concentrated nitric acid was added to stop the reaction and, after centrifugation at 12,000 g, the reaction mixtures were analyzed by HPLC.

The kinetics experiments were carried out with laccase. Selected xenobiotics (4-CP and 4-CA) and humic constituents (syringaldehyde, catechol or 4-HBA) were incubated with the enzyme separately or in combinations with each other under the above specified conditions except that the reactions were stopped after 10, 20, 30, 60, 180, 600 or 1440 min. In these experiments, the disappearance of humic constituents was also monitored.

The dependence of substrate transformation on the concentration of humic constituents was also investigated using laccase, which was incubated with 4-CP or 4-CA (0.3 mM) in the presence of 0.01, 0.03, 0.05, 0.1, 0.3, 1.0 or 3.0 mM of syringaldehyde, 4-HBA, or catechol.

Before HPLC analysis, the supernatant was filtered through a 0.45-µm membrane filter (Millipore Corp., Milford, MA) and washed with water and then with methanol to a specific volume. The analysis was conducted on a Waters Associates (Milford, MA) HPLC system equipped with two Model 510 solvent delivery systems, a Model 717™plus autosampler, a Model 440 UV absorbance detector operated at 280 nm (chlorophenols and humic constituents) or 254 nm (chloroanilines), and a Supelcosil 15 cm x 4.6 mm LC-18DB column of 5-µm particle size with a LC-18DB guard column (Supelco, Bellefonte, PA). As in previous studies (Dec and Bollag, 1990; Berry and Boyd, 1985; Nonhebel and Walton, 1974), the mobile phases for analysis of chlorophenols and humic substituents were composed of an aqueous component A and a methanol component B, each containing 1% acetic acid (Dec and Bollag, 1990). For chloroanilines and 4-HBA, the aqueous component A and the methanol component B each contained 2% acetic acid and 0.018 M ammonium acetate (Simmons et al., 1989). The ratio of A
to B ranged from 15/85 to 75/25 depending on the compound under investigation. Retention
times for all analyzed compounds ranged from 4.5 to 7.5 min.

2.4 Oxidative coupling of aromatics in the presence of humic acid

Various chlorinated phenols and anilines (0.3 mM) were dissolved in acetate buffer at pH
5.6, and 5 mL samples were incubated with different catalysts in the presence or absence of
leonardite humic acid or humic acids from other sources (50 mg/L). All experiments were
conducted in triplicate. Unless otherwise stated, peroxidase, laccase, tyrosinase and birnessite
were applied at concentrations of 0.15 units/mL, 1.5 units/mL, 40 units/mL and 0.5 mg/mL,
respectively. After a 2-hr incubation with peroxidase and a 24-hr incubation with laccase,
tyrosinase or birnessite, reaction mixtures were acidified with 150 µL of concentrated HNO₃ to
stop the transformation and centrifuged at 10,000 g to separate the precipitated humic acid and
oligomer products. The effect of humic acid on the extent of transformation was evaluated on
the basis of the disappearance of xenobiotics in the supernatant as determined by high-
performance liquid chromatography (HPLC).

The distribution of reaction products was determined in experiments involving ¹⁴C-labeled 4-
CP and 4-CA that were incubated with leonardite humic acid in the presence of different
catalysts. After acidification and centrifugation of the reaction mixtures, the supernatants were
analyzed by HPLC and liquid scintillation counting for the remaining substrate and radioactive
oligomer products, respectively. The pellets consisting of precipitated humic acid and insoluble
oligomers were washed three times with 2 mL of water acidified with HCl (pH<1) and freeze-
dried. The dried material was redissolved in 1 mL of 0.5N NaOH and then extracted with
methylene chloride. Methylene chloride extracts were analyzed by HPLC for substrates
physically bound to humic acid, and by liquid scintillation counting for oligomer products
removed from humic acid by methylene chloride extraction. Aliquots of the extracted NaOH
solution were analyzed by radiocounting to determine the amount of substrate covalently bound
to humic acid.

The effect of the humic acid concentration on the transformation of 4-CP and 4-CA (0.3 mM)
was investigated using leonardite humic acid at concentrations of 0, 5, 10, 30 and 50 mg/L.
Peroxidase, laccase, tyrosinase and birnessite were applied at 0.15 units/mL, 1.5 units/mL, 4 units/mL (or 20 units/L for 4-CA) and 0.5 mg/mL, respectively.

After centrifuging, the supernatants were filtered through a 0.45µm nylon membrane filter (Millipore Corp., Milford, MA) and analyzed on a Waters HPLC system equipped with two Model 510 solvent delivery systems, a Model 717™ plus autosampler, a Model 480 UV absorbency detector operated at 280 nm and a Supelcosil 15 cm × 4.6 mm LC-18 DB column of 5 µm particle size with LC-18 DB guard column (Supelco, Bellefonte, PA). The mobile phase for analysis of chlorinated phenols was composed of an aqueous component A and methanol component B, each containing 1% acetic acid. For chlorinated anilines, the aqueous component A and the methanol component B each contained 2% acetic acid and 0.018 M ammonium acetate. The ratio of components A and B ranged from 15/85 to 75/25, depending on the substrate under investigation. The flow rate of the mobile phase was 0.9 mL/min. The retention times ranged from 4.5 to 7.5 min.

2.5 Dehalogenation of aromatics during oxidative coupling

Chlorinated phenols and anilines (0.3 mM) were dissolved in acetate buffer at pH 5.6, and triplicate 5- or 10-mL samples were incubated with various catalysts in the presence of syringaldehyde, catechol (0.3 mM), or leonardite humic acid (0.05 mg/mL). The catalysts laccase, peroxidase, tyrosinase, and birnessite were applied at concentrations of 1.5 units/mL, 0.15 unit/mL, 40 units/mL, and 0.5 mg/mL, respectively. Peroxidase was used along with hydrogen peroxide (0.5 mM H₂O₂) as an electron acceptor. The dependence of substrate transformation on the concentration of humic constituents was investigated using laccase, which was incubated with 4-CP or 4-CA in the presence of 0.01, 0.03, 0.05, 0.1, 0.3, or 1.0 mM of syringaldehyde or catechol. To investigate the effect of humic acid concentration on the disappearance of substrates incubated with oxidoreductive catalysts, 0, 5, 10, 30, and 50 mg/L of leonardite humic acid were used. Reaction mixtures without humic constituents or humic acid served as controls. After a 2-hour incubation with peroxidase and a 24-hour incubation with laccase, tyrosinase, or birnessite, concentrated nitric acid was added (0.15 mL) to stop transformation and the reaction mixtures were centrifuged at 12,000g. Time-course experiments using laccase incubated with 4-CP or 4-CA alone or in combination with syringaldehyde or
catechol were stopped, and the mixtures were centrifuged after 10, 20, or 30 min, and 1, 3, 10, or 24 hr.

The supernatants were analyzed by high-performance liquid chromatography (HPLC) for the remaining substrates as described previously (Park et al. 1999). Prior to determining the rates of dehalogenation, 2-mL aliquots of the supernatants were passed through a SEP-PAK C18 cartridge to filter out any soluble colored oligomers or humic substances that would interfere with the spectrometric measurements. The released chloride ions were determined according to Iwasaki et al. (Iwasaki et al. 1956). The filtrates were amended with 0.5 mL of a 0.25M ferric ammonium sulfate solution (12.05 g/100 mL of 9M HNO₃) and 0.5 mL of a saturated solution of mercury thiocyanate in ethanol. Absorption measurements were conducted after 10 min at 460 nm on a model 2000 spectrophotometer (Bausch and Lomb, Rochester, NY). Calibration curves were prepared using NaCl solutions.

### 2.6 Immobilization of trifluralin in soil

Unlabeled and ¹⁴C-labeled trifluralin were donated by Dow-Elanco (Indianapolis, IN). The same company donated five standards of reduced trifluralin derivatives as possible products of anaerobic incubation of trifluralin in soils. Two other possible products of trifluralin reduction were obtained by incubating 1 mg of trifluralin dissolved in 20:80 water:methanol with 50 mg of zero valent iron or zero valent iron with palladium, both donated by Dr. Thomas Mallouk, The Pennsylvania State University. After the reaction, the solution was passed through a 0.45 µm nylon filter and analyzed via LC-MS and ¹⁹F-NMR. The chemical names, structures of trifluralin and its transformation products are listed in Table 1.

Fifty-gram samples of four uncontaminated local soils (designated Pope, Hagerstown, Chagrin and Carlisle) were thoroughly mixed with 10 mg of trifluralin and 0.23 µCi of uniformly labeled ¹⁴C-trifluralin dissolved in methanol to result in an initial herbicide concentration of 200 mg kg⁻¹ and an initial radioactivity of 4.6 µCi kg⁻¹. Table 2 lists physiochemical properties of the soils. Soils were collected 1 week prior to use, and stored at 4°C. Soil characteristics were determined by The Pennsylvania State University Agricultural Analytical Testing service using standard soil testing procedures. The samples were distributed into 100-mL serum bottles, amended with an anaerobic microbial consortium from swine manure slurry (2.0 mL), glucose (3
mL of a 5% glucose solution w/v), flushed with N₂ gas to create anoxic conditions, crimp sealed with a butyl rubber stopper and incubated in darkness for 7 weeks at 25°C.

### 2.7 Fractionation and analyses of trifluralin soil-bound residues

On the completion of the incubation, soil samples were extracted by shaking for 24 h with 50 mL of methanol, centrifuged at 10,000 g for 1 hour, and rinsed 3 times by re-suspending in 10 mL of methanol and shaking for 1 hour. The supernatants were combined and the total extract was prepared for analysis by radiocounting, thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), mass spectrometry (MS), and ¹⁹F NMR spectroscopy. The solids were air-dried, extracted by a 24-hour shaking with N₂-purged 0.1 M NaOH (75 mL), centrifuged for 1 hour at 10,000 g, and rinsed five times with 30 mL of 0.1 M NaOH. The resulting humin fraction was analyzed by radiocounting (after combustion to ¹⁴CO₂ and trapping in scintillation cocktail). The NaOH extract (combined with the washings) was acidified with 5 M HCl to pH <1.0, stored overnight in a refrigerator to facilitate the precipitation of humic acid (HA), and centrifuged. The pellet of humic acid was rinsed 5 times with 0.1 M HCl, redissolved in 0.1 M NaOH, analyzed by radiocounting, and prepared for subsequent ¹⁹F liquid-state or solid state NMR analyses by dialysis against water using a 6-8 kDa membrane and freeze-drying. The washings were combined with the supernatant (fulvic acid fraction, FA) and also analyzed by radiocounting and ¹⁹F NMR.

Radiocounting was performed on a Beta Trac 6895 liquid scintillation counter (Tracor Analytic, Elk Grove, IL) using Eco-Scint cocktail (National Diagnostics, Atlanta, GA). The extracted solids (0.1 g) were prepared for radioactivity measurements by combustion in a R.J. Harvey OX600 Biological Oxidizer (Hillsdale, NJ) with ¹⁴CO₂ trapping in 15 mL of R.J. Harvey Carbon-14 Cocktail. Liquid samples (0.5 mL, methanol extracts, FA, HA) were analyzed in 15 mL of scintillation cocktail (Ecoscint, National Diagnostics, Atlanta, GA).

Prior to TLC analysis, aliquots of the methanol extracts (2.0 mL) were evaporated to dryness under a gentle stream of N₂, redissolved in 100 L of methanol, and applied as 5-mm spots to a 0.25 mm silica gel 60 F254 precoated TLC plates (E. Merck, Darmstadt, Germany). A spot of ¹⁴C-labeled standard of trifluralin was used as a reference for each TLC plate. The plates were developed using an 90/10 hexane/acetone solvent system according to Jacobson et al., 1980), then air-dried, and imaged on a Bioscan System 200 Imaging Scanner (Washington, D.C.).
values (retention factor) were determined by dividing the distance moved by the product spot by
the distance moved by the solvent front.

The MS analysis of the methanol extracts was performed on a Quattro-II mass spectrometer
(Micromass, Beverly, MA) that was coupled to a Shimadzu LC-10ADvp pump (Shimadzu
Instruments, Columbia, MD). The spectra were generated using atmospheric pressure chemical
ionization (APCI). Trifluralin transformation products were separated on a Waters Spherisorb
ODS1 4.6 mm x 250 mm column with a 5 µm particle size (Waters, Milford, MA) and a Security
Guard C18 guard column 4.0mm L x 3.0mm ID (inner diameter) (Phenomenex, Torrance, CA).
The mobile phase was delivered at 1.0 mL/min in a gradient mode that began at 55:45
acetonitrile:water and ramped to 100:0 acetonitrile:water over 10 minutes, followed by holding
for 5 minutes and then ramping back down to 55:45 acetonitrile:water over five minutes.

For 19F NMR analyses, methanol extracts (1.0 mL) were evaporated to dryness and
redissolved in d₄-methanol (0.5 mL), and fulvic acid samples (10-25 mg) were dissolved in 50%
solution of d₄-methanol in deionized water. Liquid state 19F NMR spectra were acquired on a
Bruker DPX-300 spectrometer operating at 282.13 MHz, typically with 64 – 1024 scans and a
relaxation delay of 0.5 – 1 second. All spectra were referenced to CFCl₃ with chemical shift set
to 0.0 ppm. Solid-state 19F NMR spectra for humic acid were obtained using a 11.7-T
Varian/Chemagnetics Infinity spectrometer operating at 470.081 MHz. All spectra were
referenced to sodium trifluoroacetate (-79.13 ppm). Solid trifluralin or trifluralin mixed with
control Chagrin HA with mortar and pestle were used as controls. Control HA from Chagrin soil
was extracted as above.

Fulvic acid isolated from a control sample of Chagrin soil was dissolved in a 50% solution of
d₄-methanol in deionized water and spiked with the reduced trifluralin products (TR6, Fe-TR or
Pd-Fe-TR) immediately before the 19F NMR analysis or spin-spin relaxation time measurements
(both on a Bruker DPX-300 spectrometer). The NMR spectra were taken at selected time
intervals from time zero up to 3 weeks. All spectra were referenced to CFCl₃ with chemical shift
set to 0 ppm. Typical spectra were acquired with 1024 scans and a relaxation delay of 1.0 sec.

Spin-spin relaxation time experiments were performed using TR6 and Fe-TR. The
compounds were analyzed with and without FA. The measurements were carried at 90° pulse =
10.85, 180° π pulse = 21.7, sw = 5.164 ppm, and o1p = -63.307 ppm, with 16 points used for
pulse delays (range 1 msec to 16 seconds) acquired with 2 scans. Relaxations were determined
using an inversion recovery for peaks that were clearly defined in the spectra. Multiple peaks were chosen in “broad resonances” that probably represented bound metabolites.

2.8 Microbial release of soil-bound xenobiotics incubated with fresh soil

Various soil materials (Table 3) containing bound residues of 2,4,6-trinitrotoluene (TNT), 2,4-dichlorophenol (DCP), or 4-cyclopropyl-6-methyl-N-phenyl-2-pyrimidinamine (cyprodinil) were placed in 125-mL conical flasks and mixed with a fresh local soil for incubation. Fresh soil was collected within 7 days of the start of experiments and stored at 4°C prior to use. One day before experimentation fresh soil was incubated at 27°C to stimulate microbial activity. Additionally, fresh soil was spiked with $^{14}$C-DCP immediately before incubation without mixing with other soil materials (Table 3).

Total mass of each combination (fresh soil plus test material) was 25.0 g, but the amounts of the polluted and fresh soil materials varied so that all samples received the same total carbon (65 mg) addition (Table 3). Prior to use, control samples (without $^{14}$C-xenobiotics) were analyzed for total carbon (TC) content using a Shimadzu TOC 5000A Total Organic Carbon Analyzer with a Shimadzu Solid Sample Module 5000A. Triplicate measurements were made for all test materials and compared to a standard curve of glucose (40% w/v). In addition, each flask was adjusted by distilled water addition to the water holding capacity of the fresh soil (20% WHC) prior to incubation.

Table 4 presents chemical structures of test chemicals, and Table 5 lists the characteristics of fresh Pope soil. The characteristics of the fresh Pope soil were determined by the Pennsylvania State University Agricultural Analytical Testing Services using common soil testing procedures. Table 6 shows the TC% for each tested material.

Each incubation flask was inserted into an air-flow system (Figure 2), which allowed for the scrubbing of CO$_2$ from the inlet air, maintaining constant soil moisture, and entrapment of volatiles (in 1 M H$_2$SO$_4$/ethylene glycol, 50/50 v/v, one trap) and $^{14}$CO$_2$ (in 0.5 M NaOH, three traps). Flasks were incubated at room temperature 25°C ± 2°C. The traps and soil samples (1 g) were removed for analysis at 0, 7, 14, 40, 85 and 141 days.

In a separate experiment, fresh moist soil (100 g) was amended with DCP (100 mg kg$^{-1}$) and placed in a 1-L conical flask for acclimation in the air flow system. On day 0, and then every 5
days until 40 days, 10-g sub-samples were transferred from the incubation flask into 125-mL conical flasks, spiked with \(^{14}\)C-DCP (0.043 \(\mu\)Ci), and incubated for 24 hours in air-flow systems. \(^{14}\)CO\(_2\) evolved and entrapped during that 24-hour period was quantified by liquid scintillation counting.

Materials containing only bound residues (\(^{14}\)C-DCP extracted soil and \(^{14}\)C-TNT extracted soil) were Soxhlet extracted for 24 hours with methanol. Unextracted materials containing bound residues (\(^{14}\)C-DCP unextracted soil and \(^{14}\)C-TNT unextracted soil) were not extracted prior to use. The \(^{14}\)C-DCP humin sample was previously extracted by soxhlet extraction as above, then extracted with 0.5 M NaOH to remove FA and HA fractions. The \(^{14}\)C-TNT HA fraction was isolated as described in Strynar et al., (2002). The \(^{14}\)C-phenyl and \(^{14}\)C-pyrimidyl labeled cyprodinil soil samples were extracted as described in Dec et al., (1997a).
CHAPTER 3. RESULTS

3.1 Fractionation of TNT polluted soil after composting

At the end of the incubation period, most (82.5%) of the TNT was associated with organic matter or the mineral fraction of soil; only 6.9% of the added radioactivity could be extracted with water (together with soluble organic matter) and another 1.8% with methanol (Table 7). Upon acidification and centrifugation of the water extract, 5.5% of the initial radioactivity remained in the supernatant, with 1.2% precipitating in humic acid-like material. Dichloromethane extraction of the acidified water fraction resulted in the extraction of only 0.2% of the initial radioactivity in that fraction. HPLC analysis of the water, methanol and dichloromethane extracts did not result in the detection of any free TNT (Figure 3).

Fractionation of the extracted solids revealed that unextractable radioactivity was associated with humic acid (40.0±1.0%), fulvic acid (14.3±1.4%), and humin (28.2±0.5%) (Table 7). No $^{14}$C-TNT was mineralized as indicated by the fact that no $^{14}$CO$_2$ was found in the inline scintillation trap. The recovery of original radioactivity was 91.2%. Humic acid and humin contained the largest amount of bound radioactivity and thus were analyzed by solid state NMR for bound $^{15}$N-TNT.

3.2 Analysis of TNT soil fractions

The radio imaging of the TLC plate suggested that no free $^{14}$C-TNT was detected in the aqueous phase and organic extracts originating from the composted soil (Figure 4). Following the TLC analysis, the R$_f$ of the $^{14}$C-TNT standard was 0.71; residual radioactivity was attributed to impurities remaining at the point of application. In contrast, all radioactivity for the methanol and water extracts remained at the starting line. The dichloromethane extract, in which only 0.2% of the initial radioactivity was present, showed no clear radioactive region on the TLC plate.

The $^{15}$N-NMR spectrum of the free TNT standard shows resonance peaks centered around 362 ppm and 368 ppm (Figure 5). These peaks can be attributed to two nitro groups at the ortho positions (368 ppm) and to one nitro group at the para position (362 ppm).
The $^{15}$N-NMR spectrum of the humic acid control showed background resonances centered at 30 ppm (corresponding to aliphatic amines) and at 120 ppm (attributed to amides, ureas, and tertiary amines) (Figure 6). The spectrum for the humic acid sample containing bound $^{15}$N-TNT, in addition to the same background signals, showed a large peak in the region of 60-90 ppm that can be attributed to the presence of protonated aromatic amines originating from TNT. A shoulder or broadening of the background resonances is also seen at 130 ppm; this can be attributed to amides and/or tertiary amines originating from the bound residue.

The $^{15}$N-NMR spectrum of the humin control showed one background signal at 30 ppm that is also seen in the spectrum of the sample containing bound $^{15}$N-TNT (Figure 7). The presence of a broad resonance in the range 40-80 ppm can be clearly distinguished in the spectrum of the sample, implying the presence of TNT residues incorporated into the humin fraction in the form of primary and secondary aromatic amines.

### 3.3. Oxidative coupling reactions between chlorinated aromatics and humic constituents

The transformation of 4-CP in experiments utilizing peroxidase varied with the type of humic constituent. As shown in Table 8, humic constituents incubated with peroxidase may be divided into three groups: (1) those that enhanced the transformation of 4-CP relative to control samples, which did not involve humic constituents, (2) those that had minimal effect, and (3) those that reduced the transformation. Experiments with other enzymes and birnessite showed somewhat different transformation patterns (data not shown). With laccase, for instance, humic constituents from the first group enhanced the transformation of 4-CP. Enhanced transformation, although to a lesser extent, was also observed for the second group of humic constituents, and essentially no effect or reduced transformation was shown with the third group of humic constituents. When tyrosinase was employed, 4-CP was readily transformed in the presence or absence of the co-substrates. With birnessite, only two co-substrates from the first group (syringaldehyde and 2,6-dimethoxyphenol) enhanced the transformation; the remaining co-substrates from this group reduced transformation. Co-substrates from the second group were of minimal effect and those from the third group reduced transformation by birnessite. Three humic constituents (syringaldehyde, 4-HBA and catechol) were selected as representatives of the above
listed groups of humic constituents for further investigation into their effects on the transformation of different chlorinated phenols and chloroanilines. Syringaldehyde represented humic constituents that enhanced 4-CP transformation, 4-HBA represented those that had minimal effect, and catechol was one of the humic constituents that reduced the transformation of 4-CP.

Figure 9 presents time-courses for the laccase-mediated transformation of 4-CP and 4-CA in the presence of the three selected humic constituents. The transformation of 4-CP in the reaction with syringaldehyde (100%) was not only greater than in reactions without humic constituents (34%), with HBA (49%), and with catechol (16%), but also much faster, approximating the maximum after only 1 hour (Figure 9). The slopes of the time-courses for 4-CP alone and 4-CP combined with 4-HBA or catechol indicated that maximum transformation had not been achieved within the 24-hour incubation period.

The transformation of 4-CA (Figure 9) was rapid during the initial phase of the reactions with syringaldehyde and catechol, reaching 69% and 72%, respectively, after 1 hour. Afterwards, 4-CA transformation steadily increased to reach 82% with syringaldehyde and 100% with catechol within 24 hours. The transformation of 4-CA alone and with 4-HBA was relatively slow, ranging from about 2% after 10 minutes to 20% and 32%, respectively, after 24 hours.

Figure 10 illustrates how 4-CP and 4-CA were affecting the transformation of humic constituents during the 24-hour incubations discussed above. The transformations of syringaldehyde and catechol incubated alone were very fast, approximating 90% and 100%, respectively, within 1-3 hours. Upon the addition of 4-CP or 4-CA, transformation of syringaldehyde increased slightly during the first hour (to about 97%). The time-course for catechol incubated with 4-CP was practically unchanged compared to that for catechol incubated alone, while in the presence of 4-CA, catechol transformation was reduced to about 60% after a 3-hour incubation and remained unchanged thereafter. Unlike the case for syringaldehyde and catechol, laccase-mediated transformation of 4-HBA was negligible (1% within 24 hours) and increased only slightly in the presence of 4-CP and 4-CA (to 4% and 2%, respectively).

With one exception, the transformation of 4-CP and 4-CA was enhanced by increasing concentrations of humic constituents (Figure 11). The exception was the decreased transformation of 4-CP (from about 50 to 8%) when the concentration of catechol increased from 0.1 to 3 mM. At lower concentrations, ranging from 0.01 to 0.1 mM, a slight increase in 4-CP
transformation was observed (from about 45 to 50%).

Transformation of both 4-CP and 4-CA increased quickly from about 40% to a maximum (99 and 82%, respectively) when the concentration of syringaldehyde was increased from 0.01 to 0.3 mM. In the same concentration range, a rapid increase was also observed in the transformation of 4-CA with catechol (from about 40 to 100%). No appreciable changes in the transformation rates were observed at syringaldehyde and catechol concentrations higher than 0.3 mM. Increasing the concentration of 4-HBA from 0.01 to 0.1 or 0.3 caused negligible increases (less than 10%) in the transformation of 4-CP and 4-CA. Faster transformation was observed at higher 4-HBA concentrations (0.3 to 3 mM), reaching about 65 and 60% for 4-CP and 4-CA, respectively.

As expected based on Figures 9 through 11, the peroxidase-, laccase-, or birnessite-mediated transformation of chlorophenols determined after incubation was in most cases greater in the presence of syringaldehyde than in its absence (Table 8). Exceptions call for the reduced transformation of 3-CP with peroxidase, and the unchanged transformation of 2,4-DCP with laccase. Considerably fewer enhanced and more reduced or unchanged chlorophenol transformations were observed in the presence of 4-HBA. In the presence of catechol, reduced transformation was observed in most of the reaction mixtures. The only exceptions were the enhanced transformation of 2-CP and unchanged transformations of 3-CP and 2,4-DCP, all with laccase.

Catechol combined with tyrosinase caused a greater transformation of chlorophenols than did other humic constituents (Table 9). Syringaldehyde combined with tyrosinase showed either negligible effect or decreased the transformation of the substrates. The addition of 4-HBA had practically no effect on tyrosinase-mediated reactions. The tyrosinase-mediated transformation of 4-CP was 100% regardless of humic constituent used.

The transformation of chloroanilines was in most cases enhanced by the addition of humic constituents (Table 10). This outcome is consistent with the results presented in Figures 9 through 11. The greatest enhancement was caused by catechol, not only when it was applied in combination with tyrosinase, as was the case with chlorophenols, but also in the presence of other enzymes and birnessite. The only exception was the reduced transformation of 2-CA in the reaction mediated by birnessite.

Syringaldehyde caused a considerable increase in the transformation of chloroanilines (by
more than 10%) only in reactions involving laccase and in the birnessite-mediated reaction with 2,4,5-TCA. The enhancements, however, were less than those observed for catechol. In the case of 2,4-DCA incubated with peroxidase, 4-CA incubated with tyrosinase, and 2-CA and 4-CA incubated with birnessite, the addition of syringaldehyde resulted in reduced transformation. In the remaining reactions, syringaldehyde either slightly enhanced transformation (by less than 10%) or had no effect (transformation of 2-CA, 3-CA, and 4-CA by peroxidase).

Most reactions involving 4-HBA resulted in a slight enhancement of chloroaniline transformation (by less than 10%). Only the transformations of 3-CA catalyzed by peroxidase and 4-CA catalyzed by laccase or tyrosinase were enhanced by more than 10%. In one reaction, 4-HBA caused a decrease in chloroaniline transformation (when combined with 2,4,5-TCA and birnessite), and in five other reactions, it had no effect (when combined with 2-CA and laccase or birnessite, with 4-CA and peroxidase or birnessite, and with 2,4,5-TCA and peroxidase).

As will be discussed later, the transformation patterns depended on the mechanisms of oxidative coupling, which deferred for different catalysts (Figure 12).

3.4 Oxidative coupling of aromatics in the presence of humic acid

Most substrates in the control samples (incubations without catalyst) were subject to some losses (especially 2-CP, 2,4-DCP, 2-CA and 4-CA), apparently as a result of volatilization or sorption to humic acid. Table 11 shows that with peroxidase, tyrosinase or birnessite, the transformation of chlorinated phenols was in most cases greater in the presence of humic acid than in its absence. As an exception, incubations of 2,4,5-TCP with tyrosinase resulted in either negligible (1.2% without humic acid) or no transformation (with humic acid). In the case of 4-CP, tyrosinase caused a 100% transformation both without and with humic acid. Using laccase, transformation of most chlorinated phenols was either reduced or unchanged after the addition of humic acid; however, in the case of 2-CP a 12% increase was observed.

The transformation of most chlorinated anilines incubated with peroxidase, laccase or tyrosinase remained mostly unchanged or was reduced after the addition of humic acid (Table 12). The transformation of 4-CA in the absence of humic acid was in general greater than that of other chloroanilines. With peroxidase and birnessite, the transformation of 4-CA was slightly increased in the presence of humic acid; however, with laccase and tyrosinase, humic acid
caused a reduction in 4-CA transformation. In the case of 2-CA, humic acid increased transformations mediated by all enzymes. An increase was also observed for 3-CA incubated with laccase and tyrosinase, but the transformation of 3-CA incubated with and without humic acid was relatively low. Similarly, negligible transformations were observed for 2,4-DCA and 2,4,5-DCP when enzymatic reactions were carried out both without and with humic acid (Table 12). With birnessite, all chloroanilines except for 2-CA were transformed to a greater extent in the presence of humic acid than in its absence. The almost complete transformation of 2-CA incubated alone with the catalyst was slightly reduced in reaction with humic acid.

Figure 13 shows changes in the transformation of 4-CP incubated with increasing concentration of humic acid. When incubation was carried out without catalysts (control samples), about 18% of 4-CP was removed from the supernatant probably due to sorption of the substrate on humic acid. In the presence of laccase, the transformation of 4-CP (calculated by subtracting the percentage of the remaining substrate from 100%) increased from 34% to 40.7% when the concentration of humic acid was increased from 0 mg/L to 10 mg/L, and then decreased to 25.7% when the concentration of humic acid was increased to 50 mg/L. A steady increase in the transformation of 4-CP was observed with increasing laccase activity (from 0.35 to 4.5 units/L), both in the presence and absence of humic acid (data not shown). Throughout the whole range of laccase activity, the transformations observed in the presence of 10 mg/L of humic acid (they increased from 20% to 90%) were 15 to 20% greater than those observed for 150 mg/L of humic acid (they increased from 5 to 70%).

With peroxidase, the transformation of 4-CP increased from 32.2% to 94.8% when the concentration of humic acid was increased from 0 and 30 mg/L, and no additional transformation occurred with further increases in humic acid concentration (Figure 13). In the case of tyrosinase, the transformation increased from 78.1% when 4-CP was incubated alone to 87.5% when it was incubated with 10 mg/L of humic acid; there was no further increase in transformation at higher concentrations of humic acid. Almost complete transformation of 4-CP was observed during incubations with birnessite and humic acid (5 mg/L and more).

The effect of increasing concentration of humic acid on the transformation of 4-CA is shown in Figure 14. In control samples (without catalyst), only 3.5% of 4-CA was removed, probably by sorption to humic acid (5 mg/L and more). With peroxidase, the transformation of 4-CA hardly changed when humic acid was added; it increased slightly at low humic acid
concentrations (up to 10 mg/L), but dropped back to the initial level when the concentration of humic acid was further increased. In the presence of laccase, the transformation of 4-CA decreased from 31.0% when it was incubated alone with the enzyme to about 22% after the addition of humic acid (5 mg/L or more). A decrease in the transformation was also observed for tyrosinase (from 19.4 to about 7%). 4-CA incubated with birnessite was almost completely transformed in the presence of humic acid (5 mg/L and more), whereas without humic acid the transformation was 85%.

The distribution of radioactivity after the experiments involving $^{14}$C-labeled 4-CP and 4-CA is presented in Tables 13 and 14. As determined in preliminary tests using $^{14}$C-labeled 4-CP or 4-CA, more than 90% of radioactivity present in oligomer products formed in the absence of humic acid could be recovered from the reaction mixture by extraction with methylene chloride (data not shown). In contrast, when methylene chloride was used for the extraction of the substrates incorporated in humic acid, the recoveries were negligible. This indicated that methylene chloride extraction can be useful in separating oligomer products from humic acid containing bound substrates.

When $^{14}$C-labeled 4-CP and 4-CA were incubated with humic acid in the absence of catalysts, most radioactivity represented unaltered substrates and was found in the supernatants extracted with methylene chloride. In experiments involving the catalysts, considerable amounts of radioactivity represented transformed 4-CP or 4-CA distributed between oligomer products and humic acid. In the case of 4-CP, the humic acid fraction contained much more transformed substrate than oligomer products (Table 13). In the case of 4-CA, however, more transformed substrate was detected in oligomer products than in humic acid (Table 14).

Changes in enzyme activity had little effect on the distribution of radioactivity (data not shown). In experiments with peroxidase, the percentage of the transformed 4-CP and 4-CA found in oligomer products (8% and 27%, respectively) and humic acid (75% and 15%, respectively) slightly increased with increasing enzyme activity, but the differences between the percents of radioactivity in the respective fractions remained practically the same.

Figure 15 presents the distribution of radioactivity at different concentrations of humic acid incubated with $^{14}$C-labeled substrates in the presence of peroxidase. In experiments with 4-CP, the distribution of radioactivity stayed essentially the same with increasing concentrations of humic acid. In the case of 4-CA, however, radioactivity found in oligomeric products decreased
significantly with increasing concentration of humic acid, whereas radioactivity detected in humic acid considerably increased.

The distribution of radioactivity for $^{14}$C-labeled 4-CP and 4-CA after incubation with peroxidase was also determined in experiments involving different humic acids (Table 15). In the case of 4-CP, the source of humic acid had little effect on the distribution of radioactivity between oligomer products and humic acid. With 4-CA, however, the distribution patterns varied considerably depending on the source of humic acid.

### 3.5 Dehalogenation of aromatics during oxidative coupling

Preliminary studies indicated that different humic constituents either enhanced, did not change, or reduced the dehalogenation of 4-CP in the presence of different oxidoreductases or birnessite (data not shown). The course of changes in dehalogenation caused by humic constituents coincided (with some exceptions) with that determined for the transformation of the substrate. Syringaldehyde was one of the most effective humic constituents in enhancing the dehalogenation of 4-CP, and catechol represented the humic constituents that considerably reduced the dehalogenation of 4-CP. Based on these results, these two chemicals were selected for experiments with other chlorinated substrates. In reactions involving syringaldehyde, dehalogenation of most chlorophenols (with the exception of 3-CP) was greatly enhanced in the presence of peroxidase, laccase, or birnessite (Table 16). The amount of chloride ions released from 1 mole of a given chlorophenol was at least twice that for chlorophenols incubated alone. For all other combinations of substrates, cosubstrates, and catalysts, changes in dehalogenation of the substrates were proportional to changes in their transformation. Little or no dehalogenation was observed for 3-CP either in the presence or in the absence of cosubstrates. With catechol, dehalogenation (and the corresponding transformation) was in most cases reduced or did not change significantly; only 2-CP dehalogenation was increased by catechol (in the presence of laccase). With tyrosinase, the addition of catechol led to a greater dehalogenation of chlorinated phenols than did the addition of syringaldehyde; the latter either slightly enhanced or reduced the dehalogenation of chlorophenols. Unlike the case for other catalysts, 3-CP was considerably dehalogenated in the presence of tyrosinase.
Chloroanilines in general were poorly dechlorinated when incubated alone with all of the catalysts. Chloroaniline transformation was generally enhanced by syringaldehyde or catechol; but the release of chloride ions from the substrates in most cases was reduced or remained unchanged (Table 17).

Time-course experiments using laccase (Figure 16) indicated that dehalogenation of 4-CP in the presence of syringaldehyde (95% after a 3-hr incubation) not only was greater, but also was much faster than in the presence of catechol (5% after 24 hr) and without cosubstrates (15% after 24 hr). Changes in the dehalogenation of 4-CA with time were negligible despite increasing transformation of the substrate.

Dehalogenation of 4-CP by laccase increased from 20% to 100% with increasing concentrations of syringaldehyde (from 0.01 to 1 mM), and decreased from 25.3% to 4.3% with increasing concentrations of catechol (from 0.01 to 1 mM) (Figure 17). There were almost no changes in the dehalogenation of 4-CA at increasing concentrations of syringaldehyde and catechol (Figure 17) despite increasing transformation of the substrate (from 40 and 41% to 80 and 90%, respectively).

Humic acid (0.05 mg/mL) enhanced dehalogenation of most chlorophenols (by 1.7 to 27%) in the presence of peroxidase and birnessite (Table 17). With tyrosinase, chlorophenol dehalogenation either was enhanced (by 7, 13, and 18% for 2-CP, 2,4-DCP, and 3-CP, respectively) or remained the same, and with laccase it remained the same or was reduced (by 5 and 10% for 2,4,5-TCP and 2,4-DCP, respectively). Changes in dehalogenation were proportional to the respective changes in substrate transformation.

Humic acid had little effect on the dehalogenation of chloroanilines, although in certain cases significant changes in substrate transformation were observed (Table 17). Chloroanilines only in the presence of birnessite were dehalogenated at relatively increased rates (7.6 to 36.2%) compared to the rates of the nonamended substrates (0.0 to 20.0%). Another exception was 4-CA which, in the presence of peroxidase, was dehalogenated at 17.1% when incubated alone and at 24.3% when incubated with humic acid. As in the case of the chlorophenols, changes in chloroaniline dehalogenation were proportional to the respective changes in substrate transformation.

In the presence of peroxidase and birnessite, dehalogenation of 4-CP increased from 10 and 15% to 30 and 45%, respectively, with increasing concentrations of humic acid (from 0 to 10
ppm), but did not increase further at higher humic acid concentrations (from 10 to 50 ppm) (Figure 18). The increase in 4-CP dehalogenation was proportional to the increase in substrate transformation. With laccase and tyrosinase neither transformation nor dehalogenation of 4-CP was subject to significant changes. For 4-CA, only with birnessite was there a significant increase in the substrate dehalogenation (from 20% to 38%) when the concentration of humic acid was increased from 0 to 10 ppm; no further increase in dehalogenation was observed at higher concentrations of humic acid (from 10 to 50 ppm) (Figure 18). With the other catalysts, dehalogenation essentially remained unchanged in the presence of humic acid.

### 3.6 Immobilization of trifluralin in soil

At the end of the 7-week incubation period, the recoveries of the added $^{14}$C ranged from 89.8% for Pope soil to 96.8% for Chagrin soil (Figure 19). The majority of extractable radioactivity was found in the methanol-extractable fraction. The Pope soil showed the highest percentage of extractable radioactivity (80.9%), with little radioactivity present in the HA, FA and humin fractions (9.0% combined). The remaining three soils (Hagerstown, Chagrin and Carlisle) showed significant amounts of unextractable radioactivity, ranging from 31.1% in Hagerstown soil to 53.1% in Carlisle soil. In most instances the percentage of bound radioactivity decreased in the following order: humin>HA>FA (5-35%, 2-15%, and 4-6%, respectively).

TLC analysis of the methanol extracts showed three major radioactive peaks, one of which represented unaltered herbicide, as indicated by its $R_f$ value (0.9) that matched that for the $^{14}$C-trifluralin standard (Figure 20). The Pope soil extract showed only the trifluralin peak. No trifluralin peak, however, was generated by the Chagrin soil extract that, on the other hand, generated two peaks at $R_f$ 0.0 and 0.5, representing transformation products. Methanol extracts from the remaining soils (Carlisle and Hagerstown) showed three peaks at $R_f$ 0.0, 0.5 and 0.9 for transformation products and residual trifluralin, respectively.

The $^{19}$F liquid state NMR spectra of the soil methanol extracts are shown in Figure 21. The chemical shift for trifluralin, relative to CFCl$_3$ (0 ppm), was -61.994 ppm (Figure 22). The Pope soil $^{19}$F-NMR spectrum showed only one peak (-61.989 ppm) that represented unaltered herbicide (Figure 21A). Resonances for residual trifluralin also appeared in the spectra for
Hagerstown soil (-61.995 ppm, Figure 21B) and Carlisle soil (-61.990 ppm, Figure 21D), and the spectrum for the Chagrin soil did not show a trifluralin resonance (Figure 21C). All spectra, except that for Pope soil, showed additional resonances that represented trifluralin metabolites. Several common resonances appeared at -60.868 to -60.874, -62.703 to -62.710, and -60.699 to -60.706 ppm (Figure 21B-D). However, none of these resonances matched the $^{19}$F peaks generated by the five metabolite standards from Dow-Elanco (Table 1 and Figure 22).

The $^{19}$F solid state NMR spectra of humic acids isolated (after incubation) from the Chagrin and Carlisle soils are presented in Figure 23 along with the spectra of trifluralin and the mixture of trifluralin with a control humic acid (from Chagrin soil). Both the Carlisle and Chagrin samples showed clear fluorine signals in the form of large broad resonances at -60.8 and -61.2 ppm, respectively. The signals were centered between several much smaller signals that might be spinning side-bands. A chloroform-extracted FA fraction from the Chagrin soil analyzed by liquid state $^{19}$F showed two broad resonances at -60.598 and -61.135 ppm (Figure 24).

Figure 22 shows $^{19}$F liquid state NMR spectra for the reduction products formed by incubating trifluralin with zero valent iron or zero valent iron with palladium. As tentatively determined by mass spectrometry, the reaction with zero valent iron resulted in the formation of 2,6-diamino trifluralin (Fe-TR) (m/z 276.2) with a $^{19}$F signal at -62.653 ppm (Figure 25). This signal did not match any of the $^{19}$F resonances collected for methanol extracts from the incubated soils, probably because Fe-TR-like products were involved in bound residue formation. In the case of the palladium zero valent iron, however, a product was formed with a chemical shift at -60.879 ppm that closely matched $^{19}$F signals shown in the spectra of methanol extracts from Hagerstown, Chagrin, and Carlisle soils (-60.874, -60.870, and -60.868 ppm, respectively) (Figure 21). Using mass spectrometry, this product (Pa-Fe-TR) (m/z 276.2) was tentatively identified as a 2,6-diamino derivative of trifluralin with an intramolecular hydrogen bond between two of the three nitrogen atoms (see chemical structure in Figure 25).

When Fe-TR was mixed with FA, an $^{19}$F signal at -62.676 ppm gradually disappeared over a 3-week contact time, giving rise to an emergence and a gradual increase in the size of a broad signal at -61.150 (Figure 26A). Similarly, a gradual disappearance of an $^{19}$F signal (at -61.450 ppm) and a gradual buildup of a new $^{19}$F signal (at -60.215 ppm) was observed for the Dow-Elanco standard 2,6-diamino trifluralin (TR6) when the latter was mixed with FA (Figure 26B).
In the absence of FA, spin-spin relaxation times for both Fe-TR (1192 msec) and TR6 (1831 msec) were much greater than those for Fe-TR (151.9 msec) or TR6 (248 to 410 msec) mixed with FA (Figure 27A and C). Chloroform extracts from FA showed $^{19}$F signals at -60.166 to -61.347 ppm (Figure 28) and at -60.269 to -60.856 ppm (Figure 29), but did not generate any signals corresponding to free Fe-TR (Figure 3.23) or TR6 (Figure 29). The extracted solutions showed some changes in their $^{19}$F NMR features as compared to $^{19}$F signals collected before chloroform extraction (Figure 28 and 29). The changes included reduced sizes of the major peaks and, in the case of the Fe-TR extract, the appearance of new signals (-60.789 and -61.525 ppm) on either side of the main peak.

### 3.7 Microbial release of soil-bound xenobiotics incubated with fresh soil

No TNT or its transformation products were extracted with methanol from $^{14}$C-TNT polluted test materials during the 141-day incubation with fresh soil (Figure 30). On the other hand, all of the three incubated materials (unextracted soil, methanol-extracted soil, and humic acid) showed a considerable evolution of $^{14}$CO$_2$. The percentage of $^{14}$CO$_2$ evolved from unextracted soil (58% of the initial radioactivity) was almost three times greater than that evolved from the methanol-extracted soil (20%) and humic acid (20%). In all instances an increase in $^{14}$CO$_2$ evolved corresponded to a decrease in bound TNT. No volatilization of TNT residues was observed for any of the incubated soil materials. The small amount of $^{14}$C-TNT-related radioactivity extracted with methanol from the composted soil prior to incubation (1.8%, data not shown) was closely associated with methanol-soluble humic material (Strynar et al., 2002).

Fractionation of TNT soil materials before and after incubation (i.e., on day 0 and day 141, respectively) showed, in general, low amounts of radioactivity in the methanol fraction (0 to 2.7% of the initial radioactivity), and considerable amounts of radioactivity in FA (13.5 to 28.4%), HA (35.4 to 73.4%), and humin (3.7 to 47.3%) (Table 18). Bound radioactivity present in the FA fraction after incubation decreased in all instances (by 6.2%, 8.9% and 6.7%) for unextracted soil, extracted soil and in fresh soil mixed with TNT-humic acid, respectively, compared to that present before incubation. The same pattern was observed for the HA fraction, although at much higher percentage of bound radioactivity. In the humin fraction, bound radioactivity increased in each of the soil materials (by 13.8 to 43.6%).
The incubation of DCP soils with fresh soil did not result in any increase in methanol-extractable radioactivity as compared to that determined on day 0 (Figure 31 and Table 18). In fact, in the methanol-extracted soil, a decrease was observed (from 7.8% of extracted radioactivity on day 0 to 0% on days 15 through 140). For the unextracted soil (14.9% of extracted radioactivity on day 0) the percentage of extractable radioactivity did not change throughout the rest of incubation period. In the humin fraction (11.8% of extracted radioactivity on day 0) methanol extractable radioactivity was reduced to 5% and remained fairly constant until the end of the incubation (5.3% on day 141). Little volatilization of DCP residues was detected for any of the incubated soil materials. In each of the incubated soils, the percentage of \(^{14}\)CO\(_2\) evolution was very low by day 14, then jumped quickly to 35-40% by day 40, and maintained this level afterwards. In all cases, the increase in \(^{14}\)CO\(_2\) evolved corresponded to a reduction in bound DCP.

The patterns of \(^{14}\)CO\(_2\) production and pollutant extractability in fresh soil spiked with \(^{14}\)C-DCP immediately before incubation differed considerably from those determined for mixed soil materials (Figure 31). The amount of \(^{14}\)CO\(_2\) evolved from fresh soil steadily increased from 0% of the initial radioactivity on day 0 to 25% on day 141. Bound residues showed a quick increase (from 18% to 60%) during the first 14 days of incubation, and then remained unchanged for the rest of the experiment. This increase corresponded with a rapid decrease of radioactivity in methanol extracts (from 84.0% on day 0 to 35.0% on day 14) (Table 18). Afterwards extractable radioactivity continued to decrease, but at a much slower rate (to 22.1% by day 141).

The amounts of radioactivity found in fractionated soil materials (not including fresh soil) ranged from 0 to 15.0% (of the initial radioactivity) for methanol extracts, from 11.0 to 27.9% for FA, from 18.6 to 39.3% for HA, and from 27.6 to 60.6% for humin (Table 18). After incubation, HA and humin fractions of all three soil materials showed increased amounts of bound radioactivity (by 2.0-2.5% and 8.2-19.7%, respectively) as compared to those found before incubation. The FA radioactivity decreased (by 4.5-14.4%) in all three soil materials. The methanol-extractable radioactivity was reduced in the humin soil and extracted soil (by 6.5 and 7.8%, respectively), and remained unchanged in the unextracted soil (about 15%).

For comparison, in the case of fresh soil, which was spiked with \(^{14}\)C-DCP immediately before incubation, FA, HA, and humin fractions analyzed after incubation showed increased radio activities (by 7.7, 21.0 and 33.2%, respectively) as compared to those analyzed before
incubation (Table 18). The methanol fraction showed a 61.9% decrease in the amount of extractable radioactivity.

Both TNT and DCP samples showed a 14-day lag period with little $^{14}\text{CO}_2$ evolution, followed by a marked increase in $^{14}\text{CO}_2$ evolved between days 14 and 40 that probably resulted from a corresponding increase in the activity of soil microorganisms (Figures 30 and 31). The experiment using soil samples taken at 5-day intervals from an acclimation flask and incubated for 24 hrs with $^{14}\text{C}-\text{DCP}$ has verified this hypothesis. As shown in Figure 32, $^{14}\text{CO}_2$ evolved from the acclimated samples taken between day 0 and day 20 ranged between 0.47 and 0.75 % of the added $^{14}\text{C}-\text{DCP}$. This relatively steady mineralization pattern was followed by a sharp rise in $^{14}\text{CO}_2$ production on day 25 (1.79%) with a gradual decrease to below the initial level (0.29%) by day 40.

Little $^{14}\text{CO}_2$ was evolved from the methanol-extracted soil with bound residues of $^{14}\text{C}$-cyprodinil (4.8 – 5.4%) during the 141 day incubation with fresh soil (Figure 33). A relatively large percentage of the initial radioactivity (about 20%) was recovered (by methanol extraction) on day 0; this amount remained practically unchanged (15-20%) throughout the rest of the experiment. The percentage of bound residues also did not show significant change throughout the experiment. Differences in the position of $^{14}\text{C}$ labeling (in the phenyl or pyrimidyl rings) had little effect on radiocarbon-based estimates of extractability or mineralization.

The recovery of radioactivity in different soil fractions ranged from 16.2 to 20.8% for methanol extracts, from 7.5 to 13.5% for FA, from 19.6 to 22.0% for HA, and from 44.0 to 53.1% for humin (Table 18). Bound radioactivity in FA and HA fraction changed little throughout the experiment. A slight decrease in humin-bound radioactivity (by 2.5%) was observed for $^{14}\text{C}$-phenyl labeled cyprodinil, which corresponded with a 4.4% increase in methanol extractable radioactivity. On the other hand, a 7.2% increase in humin radioactivity was observed for the pyrimidyl label, which corresponded with a 3.9% decrease in methanol extractability.
CHAPTER 4. DISCUSSION

4.1 Composting of TNT polluted soil

Use of compost in the anaerobic/aerobic treatment of soil polluted with $^{15}$N-TNT added large amounts of organic matter for binding of the reduced TNT metabolites. Complete reduction of the three nitro groups in the TNT requires strict anoxic conditions ($E_h \leq -200$ mV) and is facilitated by the addition of easily utilized carbonaceous substrates (Rieger and Knackmuss, 1995). According to Daun et al. (1998), strong binding of reduced TNT metabolites can occur via hydroxylamino or amino groups formed in early stages of TNT transformation (Figure 8). Hydroxylaminodinitrotoluene is very reactive towards clay and humic acid, and can be completely immobilized in these materials after 30 minutes (Daun et al., 1998). It is thus likely that in the presence of humic material, hydroxylamino intermediates would readily bind to soil despite the presence of intact nitro groups. This view is supported by $^{15}$N-NMR analysis that shows linkages formed via a single amine group in both the humic acid and humin fractions (Achtnich et al., 1999a). Binding involving at least two reduced groups would lead to the most stable bound residue. There are indications that partially reduced TNT molecules can be subject to further reduction after initial attachment to humic material, but it would require a prolonged anoxic treatment (Achtnich et al., 1999b).

Figures 6 and 7 are in agreement with the results of Achtnich et al. (1999a): after sufficient anaerobic growth treatment a signal from aromatic nitro groups completely disappeared and a signal corresponding to aromatic amines appeared. In this study, 37 days of anaerobic incubation were followed by 26 days of aerobic incubation compared with 83 days of anaerobic incubation in the study of Achtnich et al. (1999a). Despite the much shorter anaerobic growth phase, the corresponding NMR spectra are very similar to those of Achtnich et al. (1999a). The dramatic difference between chemical shifts for the nitrogen atom before and after the incubation is a strong indication of the bound nature of TNT residues in humic acid and humin. $^{15}$N NMR alone only indicates reduction of nitro groups to amino groups. However, when taken with the fact that NMR spectra showed broad resonances, and water and solvent extraction did not result in any free TNT, the logical conclusion is that TNT was reduced and covalently bound to soil organic matter.
TNT reduced metabolites can bind to clay and humic acid through various mechanisms (Daun et al., 1998). Binding to minerals may occur via hydrophobic interactions or the formation of coplanar electron-acceptor complexes, where oxygen ligands at the external siloxane surfaces of clays are electron donors and the π-orbitals of TNT serve as electron acceptors (Haderlein and Schwarzenbach, 1993; Haderlein et al., 1996). Immobilization in humic material may involve nucleophilic addition of the amino groups to carbonyl functions or to aromatic carbons of quinoidal structures (Daun et al., 1998; Thorn et al., 1996). The above binding interactions may occur during the aerobic phase of the decontamination method investigated in this study.

The fact that 8.7% of the radioactivity was extractable in water and methanol may be partly explained by the nature of the compost used. Most of the extracted radioactivity was found in the water extract. Based on visual inspection, the amount of organic matter extracted with water from the soil/compost mixture was fairly high compared to that extracted from soil alone (data not shown). Twenty percent of the compost mixture was made up of previously composted materials (spent mushroom and municipal biosolids that apparently were the source for this water-soluble organic matter). The fact that, after water extraction, only 1.8% of radioactivity was extracted with methanol demonstrates the irreversible nature of the bound residue. It is noteworthy that no free TNT or any of its reduced metabolites was found by HPLC analysis in water and methanol extracts (Figure 3). The HPLC chromatogram showed only a large peak of unidentified polar compounds that were eluted very quickly from the column. It is quite possible that the peak represented low molecular weight, fulvic acid-like polymer products. It is feasible that reduced metabolites of TNT may be associated with humic material that is extractable by water or methanol. Likewise, no free TNT or any of the reduced metabolites was present in the dichloromethane extract as determined by HPLC. The inline radioactivity detector did not show any 14C associated with the water- or methanol-soluble organic matter. Apparently, the concentration of bound TNT residues was below the sensitivity limit of the detector. TLC investigation of extracted fractions likewise showed the absence of free TNT (Figure 4). Some radioactivity was shown to be extracted by water or methanol; however, during TLC analysis, the radioactive region was not mobile suggesting association with low molecular weight organic matter.
Knowledge of functional groups present in fulvic acid, humic acid and humin may help to explain the pattern of $^{14}$C distribution into the different fractions of soil organic matter. For instance, if the primary mode of covalent binding of reduced TNT is via amino-quinone linkages, it is possible that an increased quinone content will be the cause of preferential binding to a specific humic fraction. According to Stevenson (1994), the quinone content of humic acids is generally higher than that of fulvic acids. However, the quinone content of humic acid and fulvic acid was not determined in this study. Also, little is known about the composition of humin beyond the fact that it consists of organic, mostly humic, material insoluble in alkali, and is closely associated with inorganic soil components. Noncovalent sequestration in the humin fraction may contribute to the overall immobilization of pollutants in the soil matrix. Xenobiotic compounds can also be sequestered in the fulvic and humic acid fractions. Sequestration of xenobiotics may involve various mechanisms, such as physical entrapment in nanometer-size voids within humic polymers, slow diffusion through organic matter to high-energy adsorption sites, or residence in micropores preventing the access to microorganisms (Alexander, 1999).

Some concern exists as to the stability of the covalent linkage between the reduced TNT metabolites and soil organic matter (Achtnich et al., 2000). In considering this problem one must address the question of whether or not organic pollutants incorporated into soil organic matter will be released upon exposure to microbial activity, hydrolytic conditions or other environmental factors that may disrupt the bonds formed. After incorporation of the partially or fully reduced TNT into humic materials, the pollutant may be indistinguishable from soil organic matter. This is based on the unextractable nature of the TNT and the formation of broad $^{15}$N resonances in HA and humin. Thus, it can be assumed that mineralization of the bound residue would occur at a rate similar to that of mineralization of natural humus. It can be expected that even if some covalently bound molecules are released and become bioavailable, it will not occur in mass quantities to cause toxic effects. Experiments are underway to determine the microbial degradation and mineralization of TNT residues bound to fulvic acid, humic acid and humin during the course of this study.
4.2 Oxidative coupling between chlorinated aromatics and humic constituents

Results obtained in this study strongly confirm the hypothesis that the impact of humic constituents on the transformation of chlorophenols and chloroanilines depends primarily on which of the known mechanisms of oxidative coupling are involved in a specific reaction. Previous research indicated that substrates that are readily transformed by oxidoreductive enzymes and birnessite should enhance the transformation of less reactive chemicals (Klibanov et al., 1983; Roper et al., 1995). For this reason, syringaldehyde and catechol, which are very reactive (Figure 10) due to the presence of the electron-donating methoxy group or another hydroxy group, were both expected to enhance the transformation of chlorophenols. In experiments with peroxidase, laccase and birnessite, syringaldehyde increased transformation; catechol, however, had an adverse effect on the transformation (Figure 9, Tables 8 and 9). On the other hand, in the presence of tyrosinase, catechol caused a considerable enhancement of chlorophenol transformation, whereas syringaldehyde had negligible effect. This and other differences cannot be explained without considering the mechanism of transformation.

Transformation reactions mediated by oxidoreductases or birnessite involve two stages: (1) enzyme- or birnessite-mediated oxidation of the substrates (xenobiotic chemicals or humic constituents), and (2) chemical coupling of the oxidation products. The transformation mechanisms may differ depending on molecular structure of the substrates and the type of the oxidizing agent (Figure 12). With peroxidase, laccase or birnessite most substrates (including xenobiotic phenols, phenolic humic constituents, and anilines) are oxidized in the first stage to free radicals (Bollag, 1992, Dec and Bollag, 1995, Liu et al., 1981, Simmons et al., 1988). Hydroxylated phenols, such as catechol, are oxidized by the same agents to ortho-quinones. Catechol molecules may occur as phenoxide anions that readily couple to ortho-quinones through nucleophilic substitution (Nonhebel and Walton, 1974). Ortho-quinones are also generated (via ortho-hydroxylation of phenols and anilines) in the presence of tyrosinase (Dec and Bollag, 1995).

In the second stage of one-substrate reactions, the oxidized compound undergoes coupling to itself. When co-substrates are present, however, cross-coupling may occur between different oxidation products. It can be expected that in order for cross-coupling to prevail, considerable amounts of the co-substrates must undergo the same transformation mechanism. This
requirement, however, was not always met. For instance, when 4-CP and catechol were incubated together with laccase, the former was oxidized with the formation of free radicals, whereas the latter formed phenoxide anions and o-quinones (Figure 12) (Nonhebel and Walton, 1974). In addition, catechol was transformed at a much greater rate than 4-CP (Figures 9 and 10), causing competitive inhibition of the transformation of the latter. Because the oxidation products of 4-CP and catechol differed in their oxidized forms and the rates of formation, they were not capable of efficient cross-coupling, especially at high concentrations of catechol (Figure 7). Apparently, phenoxide anions from catechol were predominately involved in nucleophilic coupling to o-quinones, whereas free radicals originating from 4-CP coupled mostly to each other. The overall result of this incompatibility was the reduced transformation of 4-CP relative to the control (Figures 9 and 11, Table 9). For the same reason, reduced transformation was observed for other chlorophenols incubated with catechol in the presence of laccase, peroxidase, or birnessite. Additionally, minimal effect or reduced transformation of 4-CP was determined for hydroquinone, phloroglucinol, gallic acid, protocatechuic acid and caffeic acid, which, like catechol, have two or more hydroxyl groups and are rapidly transformed (Table 8).

In contrast, during the laccase-mediated reaction of 4-CP with syringaldehyde as a co-substrate, both compounds were oxidized to free radicals (Figure 12). According to Dordick et al. (1992) and Fossey et al. (1995), free radicals can propagate through radical transfer. Apparently, the relatively slow formation of 4-CP free radicals was enhanced by radical transfer from the rapidly formed free radicals of syringaldehyde. Since the respective oxidation products represented the same oxidized form, they could readily couple to each other. As a result of this cross-coupling, the rate of 4-CP transformation increased from 34% (incubation of 4-CP without syringaldehyde) to 100% (incubation of 4-CP with syringaldehyde) (Figures 9 and 11). Similarly, enhanced transformation was observed for other chlorophenols incubated with syringaldehyde in combination with laccase, peroxidase, or birnessite (Table 9).

With tyrosinase catechol proved to be more effective than syringaldehyde in enhancing the transformation of chlorophenols (Table 9). This outcome can again be attributed to differences in the reaction mechanisms. Laccase, peroxidase and birnessite all oxidize chlorophenols to free radicals (Klibanov et al., 1983; Dec and Bollag, 1995). With tyrosinase, chlorophenols first undergo o-hydroxylation and then are further oxidized to the respective o-quinones (Figure 12) (Dec and Bollag, 1995). Catechol in the presence of tyrosinase is directly oxidized to o-quinone.
Since, in the present study, both components of the reaction mixture assumed the same oxidized form, they were capable of efficient cross-coupling (through nucleophilic substitution), thereby enhancing the transformation rate of the chlorophenols. In the case of syringaldehyde, the tyrosinase-mediated $o$-hydroxylation and $o$-quinone formation were apparently hindered by the presence of the two methoxy groups at the $o$-positions, and, consequently, the transformation of most chlorophenols was either reduced or unaffected (Table 9).

Contrary to the observations made for chlorophenols, transformation of chloroanilines was greatly enhanced by catechol in the presence of peroxidase, laccase, or birnessite (Figure 9, Table 10). Previous research has demonstrated that chlorinated anilines are nucleophiles and can undergo nucleophilic addition to quinone components and carbonyl groups of humic substances even in the absence of enzymes or birnessite (Parris, 1980; You et al., 1982; Hsu and Bartha, 1974a; Weber et al., 1996). In experiments with laccase, peroxidase or birnessite, chloroanilines apparently underwent nucleophilic addition to $o$-quinone molecules and quinone oligomers resulting from the oxidation and polymerization of catechol. As indicated in Figure 12, catechol was oxidized to $o$-quinone and formed quinone oligomers regardless of the oxidizing agent used; therefore, enhanced transformation was observed in the presence of both tyrosinase and other mediating agents. On the other hand, a reduction was found in the laccase-mediated transformation of catechol in the presence of 4-CA (Figure 10), because the phenoxide anion generated from catechol had to compete with 4-CA for $o$-quinone. This observation supports the notion that 4-CA undergoes cross-coupling to $o$-quinone generated from catechol.

With some exceptions, either insignificant or minor enhancements of chloroaniline transformation were observed in reactions involving syringaldehyde and 4-HBA (Table 10). In general, anilines are less reactive than phenols when incubated alone with oxidoreductases. Berry and Boyd (1984) determined that the rate constants for the peroxidase-mediated transformation of chloroanilines were lower at least by one order of magnitude than those determined for the respective chlorophenols. Apparently fewer free radicals per enzyme unit were generated during incubation with chloroanilines than with chlorophenols. In mixed reactions, humic constituents, which were oxidized to free radicals much faster than chlorinated anilines, seemed primarily to undergo coupling to themselves, with limited cross-coupling to the sparse chloroaniline free radicals (Simmons et al., 1989; Tatsumi et al., 1994). On the other hand, unlike chlorophenols, the unoxidized chloroanilines are known to undergo nucleophilic
addition to quinone oligomers originating from the free radical coupling of humic co-substrates (Simmons et al., 1989). For that reason, in some cases the transformation of chloroanilines in the presence of syringaldehyde (e.g., 4-CA incubated with laccase) and 4-HBA (e.g., 3-CA incubated with horseradish peroxidase) was considerably enhanced despite the slow production of chloroaniline free radicals (Figures 9 and 11, Table 10).

4-HBA incubated with laccase was transformed to a limited extent (Figure 10), therefore only negligible amounts of the unoxidized 4-CA could undergo nucleophilic addition to quinone oligomers originating from this humic constituent; little enhancement was also observed for the transformation of 4-CP. As in the case of chlorophenols, the enhancement of the tyrosinase-mediated transformation of chloroanilines in the presence of syringaldehyde was negligible (reduced transformation was even observed for 4-CA) due to the presence of the two methoxy groups at the o-position of the co-substrate molecule, which interfered with o-hydroxylation. Negligible enhancement of chloroaniline transformation (and a reduction in the case of 2,4-DCA) was also observed for peroxidase combined with syringaldehyde, apparently due to the competitive transformation of the latter. Slightly higher enhancement was observed with the less reactive and, therefore, not competitive 4-HBA (Table 10).

To summarize, the outcome of the two-substrate reaction can be predicted in many cases on the basis of knowledge regarding the mechanism of transformation. The enhancement of chloroaniline and chlorophenol transformation by humic constituents is only possible if the mechanism is common to both co-substrates, whether it involves nucleophilic addition or free radical coupling. Frequent instances of no enhancement or even reduced transformation despite the same mechanism of coupling appear to indicate the involvement of additional controlling factors (e.g., steric hindrance or competitive transformation), rather than a contradiction of the general principle.

4.3 Oxidative coupling of aromatics in the presence of humic acid

The effect of humic acid on the transformation of chlorinated phenols and anilines determined in this study differed considerably from the effect of monomer humic constituents, such as syringaldehyde, catechol, and 4-hydroxybenzoic acid, determined in previous research (Park et al., 1998). For instance, 4-hydroxybenzoic acid as a non-reactive compound had
practically no effect on the transformation of chlorinated substrates. In the presence of the very reactive syringaldehyde, transformation of chlorophenols was considerably enhanced during incubations with peroxidase, laccase, and birnessite, but the addition of catechol, which is also very reactive, resulted in a reduction of transformation. The opposite was observed in experiments with tyrosinase, in which case catechol caused considerable enhancement of chlorophenol transformation. Catechol was also more efficient than syringaldehyde in enhancing the transformation of chloroanilines in the presence of all catalysts. The varying effects of syringaldehyde and catechol resulted from the fact that they were transformed by different mechanisms involving either nucleophilic addition (catechol) or free radical coupling (syringaldehyde) (Park et al., 1999).

Humic acid combines in its structure a variety of components that can either enhance, reduce or have no effect on the transformation of xenobiotic substrates. Therefore, it is not surprising that the effect of humic acid on the transformation of chlorinated phenols and anilines was less pronounced than the effect of monomer humic constituents (Park et al., 1998). Frequently, strong enhancements in substrate transformation observed for monomeric humic constituents, such as syringaldehyde or catechol, were considerably diminished in reactions with humic acid (Tables 11 and 12). On the other hand, humic acid considerably enhanced many transformation reactions that were either unaffected, slowed down or only slightly enhanced by humic monomers.

Humic acid, with its various functional groups and substituents, is regarded as an important factor in detoxification processes occurring in soil, especially those controlled by an oxidative coupling reaction. Previous studies indicated that humic substances can have an inhibitory effect on enzymes involved in oxidative coupling (Mato et al., 1972; Vaughan and Malcolm, 1979; Berry and Boyd, 1985; Sarkar and Bollag, 1987). According to Sarkar and Bollag (1987), oxidoreductases are inhibited because humic acids have the ability to complex metal ions in the active sites of enzymes. In addition, substrates can be adsorbed to humic acids and become less available to enzymatic activity. Vaughan and Ord (1982) suggested that humic acid may reduce the concentration of free radicals generated during enzymatic oxidation of phenolic compounds.

In this study, humic acid in many cases enhanced the transformation of chlorinated pollutants (Tables 11 and 12). The enhancements may be related to the observation that in the absence of humic acid, the chlorinated compounds were subject exclusively to oligomerization, whereas in
the presence of humic acid they also underwent binding (Tables 13 and 14). Apparently, the inhibitory effect of oligomer products that are known to adsorb on the active sites of enzymes (Nakamoto and Machida, 1992) was greater than that of humic acid with the incorporated substrates. Considerable enhancements of substrate transformation were observed especially in the presence of birnessite, which appeared to be less susceptible to inhibition by the reaction products or humic acid than were enzymes.

Berry and Boyd (1984) demonstrated that peroxidase transforms 4-CP ten times faster than 4-CA. It seems that in peroxidase-mediated reactions with humic acid, the radical transfer from the slowly generated free radicals of 4-CA to humic acid was less efficient than in the case of 4-CP and, therefore, only 22.7% of $^{14}$C-labeled 4-CA underwent binding to humic acid as compared to 25.1% found in oligomer products (Table 14). Similar distribution pattern was observed for other catalysts, in the presence of which 4-CA coupled preferably to itself to form oligomer products. Except for the reaction with birnessite, the major portion of the initially applied 4-CA remained unreacted (Table 14). In the case of 4-CP, most of the applied substrate was bound to humic acid (Table 13), probably due to efficient radical transfer from rapidly generated free radicals of the substrate.

The relatively slow transformation of 4-CA by peroxidase was probably responsible for changes in the distribution of radioactivity between oligomer products and humic acid when $^{14}$C-labeled 4-CA was incubated with humic acid from different sources (Table 15). No such changes were observed with 4-CP which, in the presence of peroxidase, could readily generate free radicals in each humic acid and indiscriminately undergo extensive binding with little oligomerization (Steelink and Tollin, 1967; Bollag et al., 1980; Dec and Bollag, 1990; Roper et al., 1995). In the case of 4-CA, the extent of binding to different humic acids was probably dependent on the availability of carbonyl groups or quinone moieties to which 4-CA, as a nucleophile, could couple through nucleophilic addition (Hsu and Bartha, 1974; Parris, 1980; You et al., 1982; Simmons et al., 1989; Tatsumi et al., 1994; Thorn et al., 1996; Weber et al., 1996). This conclusion however, remains to be confirmed in further studies.

Based on the data presented in Table 13, it appears that in soil environments treated with oxidoreductive catalysts, chlorinated phenols should be transformed mainly through binding to humic acid. Binding may also be a major transformation pathway in aquatic systems containing sufficient concentrations of dissolved humus (e.g., 50 mg/L as in the present investigation).
According to Table 14, chloroanilines should largely undergo oligomerization with less binding. Figure 15, however, indicates that chloroaniline binding may greatly increase at high concentrations of humic acid as a result of covalent binding through nucleophilic addition to carbonyl and quinone components of the organic matter. It can be expected that with long incubation times (several days, rather than 24 hrs as in this study), additional amounts of chloroanilines may be subject to binding controlled by nucleophilic addition (Thorn et al., 1996).

4.4. Dehalogenation of aromatics during oxidative coupling

Previous studies on the transformation of chlorinated phenols and anilines by oxidoreductive catalysts demonstrated that the substrates were dehalogenated as a result of free-radical coupling or nucleophilic addition (Dec and Bollag 1994; 1995). Chloride ions were released from the aromatic carbons occupying the ortho or para positions that hosted the unpaired electron, or from the quinone carbons that were subject to a nucleophilic attack by phenoxide anions. This study demonstrated that phenolic products of lignin decomposition can be involved indirectly in dehalogenation through cross-coupling with chlorinated substrates. Dehalogenation also may result from oxidative coupling of chlorinated phenols and anilines to humic acid.

The addition of syringaldehyde enhanced the dehalogenation of all chlorophenols in the presence of peroxidase, laccase, and birnessite. The enhancement was a direct consequence of the increased transformation of the substrates as they cross-coupled with syringaldehyde. As shown previously (Park et al. 1999), cross-coupling was possible because both the chlorophenols and the syringaldehyde were transformed by the same, free radical mechanism. On a molar basis, at least twice as many chloride ions were released in the presence of syringaldehyde than in its absence. This could happen only if two or more molecules of syringaldehyde reacted with one molecule of chlorophenol, thus increasing the probability of coupling at the chlorinated site. In contrast, neither transformation nor dehalogenation of chlorophenols was enhanced to any extent in the presence of tyrosinase, because syringaldehyde could not be effectively oxidized to o-quinone as a result of a steric hindrance caused by the presence of two methoxy groups in the ortho positions.

Unlike the case for chlorophenols (Table 16), syringaldehyde had little effect on the dehalogenation of chloroanilines (Table 17). Simmons et al. (1987) determined that the
formation of N-N linkages provided a predominant pathway for free-radical coupling of the chloroanilines to themselves. Additionally, N-C and C-C couplings occurred at the ortho or para positions. Apparently, N-C and C-C bonds also can be formed when chloroanilines undergo cross-coupling. As already stated, only reactions involving chlorine-substituted carbons can result in dehalogenation; chloride ions are unlikely to be released when free radicals of chloroanilines undergo coupling or cross-coupling via the nitrogen atom. Also, no chloride ions can be released during nucleophilic addition of chloroanilines to quinone moieties of oligomer products, because, as shown previously (Parris 1980; Thorn et al. 1996), these interactions occur exclusively via the nitrogen atom.

With catechol, dehalogenation of chlorophenols either was reduced or remained unchanged except for the enhancements observed in the presence of tyrosinase (Table 16). As determined by Park et al. (1999), catechol was oxidized to o-quinone in the presence of all of the catalysts whereas chlorophenols were oxidized to o-quinones only by tyrosinase. Therefore, only in the presence of tyrosinase could the chlorophenols efficiently cross-couple with catechol (through nucleophilic addition) and result in enhanced dehalogenation. With the other catalysts, cross-coupling was restricted because the chlorophenols were oxidized to free radicals that were more likely to couple to themselves than engage in cross-coupling with o-quinone. In the case of the chloroanilines, catechol significantly enhanced the transformation of the substrates in the presence of all catalysts, but as a result of cross-coupling via the nitrogen atom (Thorn et al. 1996), dehalogenation either was reduced or remained the same.

Humic acid enhanced chlorophenol dehalogenation in the presence of all of the catalysts except for laccase, which seemed to be incapable of generating sufficient oxidation products to enhance binding (Table 16). Chloroaniline dehalogenation was enhanced by humic acid only in the presence of birnessite; with the other catalysts it remained essentially unchanged. Birnessite apparently generated enough free radicals to enhance binding and dehalogenation. The differences between the dehalogenation patterns of chlorophenols and chloroanilines seem to result from the following circumstances. Depending on the catalyst, binding of chlorophenols to humic acid can be controlled by only one of two possible mechanisms: free radical coupling or nucleophilic addition (Dec and Bollag 1995; Park et al. 1999). In contrast, chloroanilines may undergo free-radical coupling and nucleophilic addition simultaneously. On the other hand, they are oxidized to free radicals to a lesser extent than are chlorophenols (Berry and Boyd 1984).
Chloroaniline molecules that do not oxidize to free radicals are free to engage in binding through nucleophilic addition. This reaction, however, is less likely to result in dehalogenation than in free-radical coupling, and therefore chlorophenols involved in binding to humic acid are, in general, more susceptible to dehalogenation than are chloroanilines.

In view of this research, it is clear that humic substances may modify, to a certain extent, the dehalogenation patterns of chlorinated phenols and anilines. The effects of humic substances on chloride release resulted from their participation in oxidative coupling; therefore, the underlying mechanisms of dehalogenation did not change compared to reactions involving only the chlorinated substrates.

4.5 Immobilization of trifluralin in soil

The soils under investigation differed significantly in their ability to transform and immobilize trifluralin under anaerobic incubations. Based on radio-scanning of the TLC plates (Figure 20), most of trifluralin incubated with Pope soil (80.9% of the initial $^{14}$C) remained unaltered, and only 9% occurred in the bound form at the completion of the 7-week incubation (Figure 19). In contrast, the Chagrin soil showed no free trifluralin (Figure 20), but it produced considerable amounts of methanol-extractable transformation products (58%) and bound material (40%) (Figure 19). The differences were probably a result of different soil properties (Table 2). Carlisle soil that had the highest organic matter content (7.4%), clay content (35.2%), and cation exchange capacity (19.9 meq/100 g) showed the greatest amount of bound residues (53%) despite the presence of some unaltered herbicide (Figure 20). In contrast, Pope soil, which had less organic matter (3.3%), more sand (55.9%), less clay (18.7%) and smaller cation exchange capacity (9.8 meq/100g) than Carlisle soil, was the least effective in trifluralin transformation and binding (Figures 19 and 20). Chagrin soil with its relatively low organic matter content (2.8%) and high pH (7.6) was the most effective in trifluralin transformation (probably due to increased microbial activity as compared to other soils), but not as effective in binding as Carlisle soil.

The $^{19}$F liquid state NMR analysis confirmed the presence of only unaltered trifluralin (with chemical shift at -61.989 ppm) in the methanol extract from Pope soil (Figure 21A), and the absence of trifluralin (no corresponding peak in the NMR spectrum) in the Chagrin soil extract (Figure 21C). Trifluralin signals for Hagerstown and Carlisle soils (-61.995 and -61.990 ppm,
respectively) were considerably reduced relative to that for Pope soil (Figure 21B and D), confirming the outcome of the TLC analysis.

Methanol-extractable transformation products with \( R_f \) values of 0 and 0.51, 0.56, or 0.61 (as determined by radio-scanning of the TLC plates; Figure 20) generated corresponding signals in the NMR spectra. These signals represented at least 14 different compounds (Figure 21). The signals were very narrow and well resolved, indicating that they represented free trifluralin metabolites, rather than trifluralin metabolites complexed with methanol-soluble components of soil organic matter. Golab et al. (1979) reported 31 extractable trifluralin metabolites formed in soil under field conditions, 28 of which were identified. The identified products belonged to four categories: (1) products with the dealkylated amino group and/or reduced nitro groups, (2) heterocyclic benzimidazole products, (3) azoxy and azo dimeric products, and (4) miscellaneous products of oxidation/hydroxylation (Golab et al., 1979). The latter category is probably not represented by any of the \( ^{19}F \) signals collected in this study for methanol extracts, because the experiments were carried out under anoxic conditions. The anaerobic incubation in the study of Golab et al. (1979), created by flooding the soil, resulted in the formation of three major products, including compounds with one or two nitro groups reduced to amino groups, and a heterocyclic benzimidazole (Golab et al, 1979). In the study of Golab et al., (1979) the same compounds were also formed, although in smaller amounts, under aerobic growth conditions, and it was believed that some of these compounds were involved in the formation of bound residues.

The chemical shifts of standard metabolites (listed as TR2, TR6, TR15, 4-ABT and 2-NITRO in Table 1, Figure 22) did not match any of the resonances shown by the methanol extracts. This indicates that, if formed, some of these compounds underwent covalent binding and thus could not be extracted.

Humic acid samples from Carlisle and Chagrin soils, analyzed by \( ^{19}F \) solid state NMR, generated large, broad resonances at -60.8 and -60.2 ppm (Figure 23) that demonstrate xenobiotic associations with organic matter and are consistent with covalent binding. Large, broad resonances (at -60.598 and -61.135 ppm) were also generated by reduced trifluralin products as well that demonstrate xenobiotic-humic associations consistent with covalent binding to fulvic acid from the Chagrin soil as analyzed by \( ^{19}F \) liquid state NMR (Figure 24). The presence of two major resonances, rather than one, as was the case for humic acid, indicated
that the fulvic acid-bound residues of trifluralin exist in two distinctly different chemical environments, or underwent binding via two distinctly different reduced metabolites of trifluralin. It is also possible the resolution of liquid state $^{19}$F NMR is superior to solid state $^{19}$F NMR in resolving these broad resonances.

No free trifluralin products could contribute to these signals, because all free material was removed during the 48-hour dialysis of humic acids or by chloroform extraction of fulvic acid. Furthermore, free xenobiotics usually generate sharp and well-resolved NMR peaks (Dec et al., 1997), similar to the signal generated by the trifluralin standard (-63.4 ppm) that was mixed with humic acid shortly before the analysis (Figure 23). A slight broadening (by paramagnetic radicals) of this signal as compared to the trifluralin peak collected in the absence of humic acid, and the appearance of the semi-symmetrical side-bands next to this signal suggested weak interactions between the herbicide molecule and humic acid (e.g., van der Waals forces or hydrogen bonding).

At long contact times (weeks to months rather than hours), a given compound may chemically react with different functional groups present in the complex molecule of humic acid, and generate a large number of overlapping resonances that appear on the NMR spectra as one broad resonance (Thorn et al., 1996; Dec et al, 1997). The several smaller broad resonances that appeared in the humic acid spectra (Figure 23) resembled the side-bands shown by the mixture of trifluralin with the control humic acid, but they probably represented trifluralin metabolites involved in covalent binding, rather than weak interactions. This is based on speculation, however, extended dialysis and acid/base treatment should have removed all metabolites held by weak interactions leaving only covalently bound metabolites.

Experiments involving reactions between reduced trifluralin products (Fe-TR and TR6) and fulvic acid (Figure 26) supported the hypothesis that the broad resonances generated by solid and liquid soil fractions resulted from covalent binding of amino metabolites of trifluralin, probably through nucleophilic addition to quinone moieties of humic or fulvic acids. As already mentioned, Fe-TR was obtained by incubating trifluralin with zero valent iron. It showed $^{19}$F resonances (Figure 25) that did not match any of the $^{19}$F resonances generated by the methanol extracts (Figure 21), which suggests that Fe-TR-like products (2,6-diamines) might be involved in bound residue formation. On the other hand, the reaction with the palladium zero valent iron produced a 2,6-diamine derivative that had an intramolecular hydrogen bond between two of the
three nitrogen atoms (Figure 25). The methanol extracts showed the presence of trifluralin products with $^{19}$F resonances matching those of Pd-Fe-TR, which suggested that the intramolecular hydrogen bonding made 2,6-diamine derivatives unreactive and thus not capable of binding. It is quite possible that the only difference between the two produced trifluralin metabolites (Figure 25) is a pH effect where the Fe-Pd-TR is simply protonated. In the soil both could be produced. The difference appears to be the Fe-TR metabolite reacts with soil organic matter whereas the Fe-Pd-TR does not, potentially due to protonation. During anaerobic metabolism, acidic products abound which could lead to reduction in pH and potential exists for protonation of reduced trifluralin metabolites inhibiting reactivity.

The gradual reduction in the size of Fe-TR and TR6 resonances (at -62.446, -62.635, -62.676 ppm and at -61.450 ppm, respectively) and the gradual buildup of new broad signals (at -61.150 ppm, and at -60.215, -60.414, -60.441, and -61.796, respectively) during the 3-week incubation of Fe-TR and TR6 with fulvic acid (Figure 26) supported the hypothesis that 2,6-diamine products of trifluralin metabolism are bound to humic material with time. Aromatic amines have been shown to react quickly but reversibly with soil organic matter via imine bonds, and slower but irreversibly via nucleophilic addition of aromatic amines to soil organic matter quinione moieties (Parris, 1980). The unextractable nature of the FA complexes, formation of broad $^{19}$F resonances and growth of the resonance with time all suggest the formation of covalent bonds.

The reduced spin-spin relaxation times ($T_1$) of Fe-TR and TR6 that were complexed with fulvic acid as compared with those determined for Fe-TR and TR6 analyzed in the absence of humic acid (Figure 27) are another confirmation of strong covalent interactions between di-amino derivatives of trifluralin and soil organic matter. Measuring spin-spin ($T_1$) or spin-matrix ($T_2$) relaxation times has been used in the past to examine xenobiotic interactions with humic materials.

Nanny et al. (1997), for instance, used $^{13}$C NMR $T_1$ relaxation to investigate noncovalent interactions between acenaphthenone and fulvic acid samples in aqueous solutions. Their approach was based on the assumption that nuclei of xenobiotics associated with humic material through either strong linkages (covalent binding) or weak linkages (hydrogen bonding, sorption, sequestration) should relax faster than those of free xenobiotics. Depending on the concentration of the reaction components, the $T_1$ of acenaphthenone adsorbed to fulvic acid was 25 sec, whereas the $T_1$ of free acenaphthenone ranged between 15 and 25 sec. In this study, the $T_1$
measured for Fe-TR or TR6 in the absence of fulvic acid were 1192 – 1831 msec, and they were reduced to 152 – 410 msec when fulvic acid was added (Figure 27). From this observation we can conclude that interactions occurred between FA and reduced metabolites of trifluralin.

Dixon et al. (1999) used $^{19}$F NMR $T_1$ and $T_2$ relaxation measurements to investigate interactions between 4’-fluoro-1’-acetonaphthone and Suwannee River fulvic acid (SRFA). Addition of 4.5 mg SRFA mL$^{-1}$ reduced $T_1$ relaxation times from 1.781 to 0.555 sec, with observed broadening of resonances. However, Dixon et al. (1999) attributed interactions to sorption either by hydrophobic interactions or hydrogen bonding. In this investigation, similar reduction in $T_1$ relaxation measurements are reported (Figure 27).

After mixing with fulvic acid, the Fe-TR signal moved from -62.007 ppm ($T_1$ 1192 msec) to -61.188 ppm ($T_1$ 152 msec), and showed considerable line broadening (Figure 27), demonstrating xenobiotic-humic associations consistent with covalent binding. However, line broadening could be simply due to heterogeneity of environments in which the Fe-TR is immobilized. In the case of TR6, however, the initial signals at -61.510 ppm remained in the spectrum (at -61.438 ppm) even after the addition of fulvic acid (Figure 27). This peak decreased and considerably broadened with time as new broad resonances appeared maximizing at -60.213 and -61.793 ppm (Figure 27). It was expected that $T_1$ measurements for these new peaks would be different than those for the remainder of the original peak; however, all relaxation times were in a narrow range of 248.21 to 337.72 msec. This illustrated the fact that based on $T_1$ measurements, it is not always possible to attribute the NMR signals to either covalent or non-covalent binding interactions.

In an attempt to verify the covalent nature of bonds, the Fe-TR and TR6 complexes with fulvic acid (identified based on $T_1$ relaxation measurements) were extracted with chloroform and again analyzed by $^{19}$F NMR (Figures 28 and 29). Chloroform extracts showed no $^{19}$F signals for free Fe-TR (at -62.007 ppm) or TR6 (at -61.486 ppm), suggesting the covalent nature of associations between these compounds and fulvic acid. The chemical shifts ranging from -60.166 to -61.347 ppm (for Fe-TR chloroform extract) and from -60.269 to -60.856 ppm (for TR6 chloroform extract), can probably be attributed to Fe-TR and TR6 associated with chloroform-soluble fulvic acid components, as these resonances were in the same range as newly formed broad resonances.
The chloroform extraction of the Fe-TR/FA (Fe-TR metabolite incubated with FA) resulted in a reduction of the peak at -61.188 ppm, and the appearance of two new peaks at -60.789 and -61.525 ppm (Figure 28). The non-symmetrical distances between the new peaks and the main resonance at -61.191 ppm (0.402 and 0.334 ppm) indicated that these signals were not spinning side-bands, and probably reflected changes in the structural arrangement of the chemically bound Fe-TR molecules after the removal of the chloroform-soluble fulvic acid complex.

As a result of chloroform extraction of the TR6/fulvic acid complex, the large sharp peak at -61.441 ppm decreased somewhat (more in height, rather than in size or peak area); and the center of the peak at -60.215 ppm shifted to -60.249 ppm (Figure 29). In view of this relatively minor modification, the latter peak probably represented reduced trifluralin molecules that were covalently attached to fulvic acid. The peak at -61.441 ppm probably represents the unaltered TR6 molecules that probably were retained by strong electrostatic attraction between the negative charges of fulvic acid and the positive charges of the protonated amino groups, and, therefore, resisted chloroform extraction.

In general, broad resonances generated by the fulvic acid complexes (Figure 28 and 29) appeared in the same regions of the spectra as those collected for the fulvic acid fraction of the incubated soil (Figure 24). These and other NMR data (lack of extractable amino metabolites, broad $^{19}\text{F}$ resonances in FA and HA from trifluralin/soil incubations) collected in this study can be considered a strong confirmation of the hypothesis that trifluralin binding in soil proceeds through the formation of amino derivatives that in turn interacted with soil organic matter. The differences in the location of $^{19}\text{F}$ signals in the NMR spectra could represent varying numbers and positions of amino groups in different transformation products.

The $^{19}\text{F}$ NMR technique shows great potential for investigating the fate of important fluorinated pollutants in soil environments. If combined with a sufficient number of reference standards and traditional approaches (e.g., using $^{14}\text{C}$-labeled xenobiotics), it can quickly provide reliable information on both transformation pathways and the mechanisms of binding. Various aspects of using this techniques in environmental studies are discussed in a review paper that is now under preparation.
4.6. Microbial release of soil-bound xenobiotics incubated with fresh soil

The analyses of different fractions before and after incubation with fresh soil showed no trends indicating release of the immobilized contaminants in terms of free residues present in methanol extracts. Incubations involving soil samples with $^{14}$C-labeled TNT and DCP resulted in a considerable decrease in the percentage of bound radioactivity, but mainly due to the release in the form of $^{14}$CO$_2$. The extent of $^{14}$CO$_2$ evolution differed depending on the tested soil material.

The experiments for TNT and DCP release involved soil samples that were either extracted or unextracted with methanol prior to incubation with fresh soil (Figures 30 and 31, respectively). In the case of DCP samples, methanol extraction did not alter $^{14}$CO$_2$ evolved, but it did alter the percentage of extractable and bound $^{14}$C. For TNT samples, the methanol-extracted soil showed a marked reduction of $^{14}$CO$_2$ evolution (by about 20%) as compared to unextracted soil; this reduction corresponded with an increase in the percentage of binding (Figure 30).

Previous studies (Strynar et al. 2002) indicated that a fraction of TNT residue in composted soil was associated with low molecular weight humic materials that represented a labile portion of organic matter. Biodegradation of this labile organic matter during incubation of unextracted soil probably resulted in a simultaneous biodegradation of the bound TNT residue (Figure 30). The extraction of soil with methanol prior to incubation apparently removed this labile soil components together with bound $^{14}$C and thus reduced the pool of biodegradable radiocarbon. In support of this is the fact that $^{14}$CO$_2$ evolution and bound $^{14}$C determined for methanol-extracted soil were in good agreement with those determined for the HA material that was free of any labile organic fractions and contained only bound residues of TNT (Figure 30). Since no increase in methanol-extractable $^{14}$C resulted from incubating any of the TNT soil materials, evolution of $^{14}$CO$_2$ must have originated from $^{14}$C carbon which, despite being bound to HA and other non-labile organic matter fractions, became available to soil microorganisms.

In the case of DCP, methanol-extracted soil showed decreased contents of extractable $^{14}$C as compared to unextracted soil, which did not seem to influence either $^{14}$CO$_2$ evolution or the percentage of bound $^{14}$C (Figure 31). This trend suggests that the evolved $^{14}$CO$_2$ originated from
bound $^{14}$C, rather than from methanol-extractable $^{14}$C, and that the latter served as a pool for making up for the losses of bound $^{14}$C due to $^{14}$CO$_2$ production.

The humin fraction incubated with fresh soil yielded methanol-extractable $^{14}$C between that of the extracted and unextracted soils, with $^{14}$CO$_2$ evolution that appeared to be higher than that for either extracted or unextracted soil (Figure 31). This confirms the conclusion that bound residues of DCP were mineralized more readily than free residues of DCP. Another evidence to support this is a previous finding that $^{14}$C-DCP incorporated into a humic acid and exposed to soil microorganisms generated 4.5 to 7 times more $^{14}$CO$_2$ than did free DCP under the same experimental conditions (Dec et al., 1990). It is possible that free DCP had a toxic or inhibitory effect on microbial metabolism, and that in the absence of freely available DCP, microbial activity thrived on both organic matter and the associated xenobiotic $^{14}$C.

All noticeable increases in $^{14}$CO$_2$ evolution from TNT and DCP samples occurred in the period between 14 and 40 days of incubation (Figure 30 and 31). Before and after this period, the changes in $^{14}$CO$_2$ evolution were negligible. The initial lack of $^{14}$CO$_2$ evolution resembled patterns observed in many biodegradation studies, in which many xenobiotics showed an initial acclimation or lag phase of no or slow mineralization, followed by a phase of rapid mineralization that in turn was followed by a decline in the rate of mineralization (Alexander, 1994). It is believed that this mineralization pattern is a reflection of the changing size of microbial populations responsible for xenobiotic breakdown. To test this hypothesis, a separate soil sample was mixed with DCP and acclimated under air for 40 days. Sub-samples taken periodically from this soil for a 24-h incubation (after spiking with $^{14}$C-DCP) showed a typical pattern of slow, then rapid, and then declining $^{14}$CO$_2$ evolution indicative of corresponding changes in the density of microbial population (Figure 32). The peak of microbial activity, as measured by $^{14}$CO$_2$ evolution, occurred between 20 and 40 days of acclimation, which closely corresponded with the period of increased $^{14}$CO$_2$ production in soil samples containing bound residues of TNT and DCP (Figures 30 and 31).

In previous studies (Dec et al., 1997), soil samples with bound residues of $^{14}$C-cyprodinil were extracted 4 times with methanol. This, however, did not remove all of the extractable $^{14}$C, because much of it was retained through sequesteration and required more time and energy to be completely recovered (Dec and Bollag, 1997). Sequestration may be the reason why about 20% of $^{14}$C recovered in this study at each incubation period was found in the methanol fraction.
(Figure 33). However, neither bound nor extractable $^{14}$C was affected by incubation with fresh soil. By the end of incubation, only 4.8 – 5.4 % was evolved as $^{14}$CO$_2$, indicating that neither sequestered nor chemically bound residues were readily accessible to soil microorganisms.

In the study on soil-bound residue formation using $^{14}$C-cyprodinil (Dec 1997a), $^{14}$CO$_2$ evolution ranged from 1.7 to 24.7% of the initial radioactivity, depending on the soil origin, cyprodinil concentration, (1.5 to 500 mg kg$^{-1}$) and the location of the $^{14}$C label (phenyl vs. pyrimidyl rings). The percentages of $^{14}$CO$_2$ evolved from the phenyl ring were about 3 times greater than those evolved from the pyrimidyl ring. However, bound residues of $^{14}$C-cyprodinil incubated with fresh soil did not differ in $^{14}$CO$_2$ evolution depending on the label location. The formation of soil-bound residues of cyprodinil involves at least three pathways: (1) sequestration of the unaltered compound, (2) cleavage of the cyprodinil molecule between the phenyl and pyrimidyl rings, followed by chemical binding of the separated moieties, (3) sequestration of the cleavage moieties and other biodegradation products (Dec et al., 1997). The limited $^{14}$CO$_2$ evolution (4.8 – 5.4 %) from bound residues of cyprodinil during incubation with fresh soil indicated that soil microorganisms had little access to both sequestered and chemically bound residues of cyprodinil, and that the immobilized phenyl moieties were less bioavailable than the pyrimidyl moieties based on $^{14}$CO$_2$ evolution. It is also possible that cyprodinil (a fungicide) had an inhibitory effect on an important microbial population in this soil. However, Dec et al. (1997a) found 500 mg kg$^{-1}$ not to be inhibitory toward microbial degradation of cyprodinil, and concentrations in these experiments were about 10 fold lower in whole soil (Table 3).

Fractionation of TNT soil materials revealed that incubation with fresh soil caused some changes in the distribution of extractable bound radioactivity (Table 18). After incubation, methanol extracts and FA and HA fractions showed in most cases reduced percentages of both extractable and bound $^{14}$C. In contrast, bound $^{14}$C in the humin fraction of all tested soil materials increased considerably as a result of incubation, which may be considered proof for stabilization of the immobilized compound. Percent decreases in the FA and HA fraction, very well correspond with percent increases in the humin fraction (Table 18).

As was the case for TNT samples, fractionation of DCP soil materials revealed changes in the distribution of extractable bound radioactivity as a result of incubation (Table 18). In methanol extracts and FA fractions, the percentage of $^{14}$C decreased, whereas the HA and humin fractions showed increased percentages of bound $^{14}$C, which may be construed as a stabilization
effect. In the case of cypordinil (Table 18), the fractionation patterns essentially did not change as a result of incubation, indicating a stable nature of bound residues.

The distribution of any xenobiotics in soils and sediments is heterogeneous in nature. Sampling to get representative samples is problematic in both laboratory and field experiments. Variability in total recovered radioactivity for each treatment reflected this. This investigation was conducted to evaluate trends with time, rather than absolute distribution of xenobiotics in soil. There was a spread of total recovered radioactivity within treatments and between treatments. Evolved radioactivity ($^{14}$CO$_2$ and volatile) is cumulative with time for the whole soil, whereas the remaining fractions (methanol extractable, FA, HA and humin) are determined from samples taken and calculated to represent the whole soil. Sampling from a “hotspot” can dramatically affect the total radioactivity recovered. However, by treating the fractions data as a percentage of the total radioactivity recovered, the effect of sampling error is minimized. It is for this reason why in most instances, comparisons of day 0 and day 141 results are used, or general trends are indicated.

To summarize, the results of this study did not confirm fears that soil-bound xenobiotics may be susceptible to microbial release leading to a delayed pollution problem. Trends from the incubation with fresh soil did not indicate any increase in methanol-extractable residues of $^{14}$C-TNT, $^{14}$C-DCP or $^{14}$C-cypordinil. The distribution of bound $^{14}$C in TNT and DCP samples shifted in favor of the humin fraction (in cypordinil samples it essentially remained unchanged), which may be viewed as further stabilization of the immobilized compounds. Mineralization of bound residues was the only release pathway that seemed to correspond with decreasing TNT or DCP binding (in the case of cypordinil, mineralization was negligible). The evolution of $^{14}$CO$_2$ following the exposure of bound chemicals to soil microorganisms strongly indicated that bound xenobiotics may be bioavailable, but their microbial release does not necessarily constitute a threat to the environment.
CHAPTER 5. CONCLUSIONS

The investigation of interactions between xenobiotic compounds and humic material is complex. In studying these interactions, application of $^{14}$C-labeled, stable isotope-labeled ($^{15}$N) or natural element tracer ($^{19}$F) xenobiotics appears to be the most logical approach. Xenobiotics were selected to represent broad classes of compounds typically found in contaminated soil. Application of particular xenobiotics in this investigation resulted from known contamination problems (TNT, trifluralin, cyprodinil, DCP and other chlorinated aromatics and) or practical reasons (cyprodinil).

Two mechanisms were involved in the covalent binding of these selected xenobiotics to soil organic matter. These mechanisms were: (1) nucleophilic addition of aromatic amines to quinone moieties in soil organic matter (TNT, trifluralin and the pyrimidyl moiety of cyprodinil) and (2) C-C and C-O (ester and ether) attachment of phenolic free radicals to soil organic matter (DCP and phenyl moiety of cyprodinil).

$^{15}$N and $^{19}$F NMR spectra demonstrated xenobiotic associations with organic matter and are consistent with covalent binding (TNT, trifluralin). However, proof of this mechanism can not be determined from NMR data alone. NMR data must be combined with other observations (lack of extractable reduced metabolites, appearance of broad $^{15}$N and $^{19}$F NMR resonances, interaction of model compounds with humic material) and previous literature to draw the logical conclusion that nucleophilic addition is the likely immobilization mechanism for xenobiotics containing nitro groups. The finding that phenolics covalently bind via free radical formation and subsequent C-C and C-O linkages relies largely on previously published literature (DCP and the phenyl moiety of cyprodinil). A limitation in this investigation is direct spectroscopic evidence for the formation of the hypothesized linkages, though the data suggest these linkages formed. It is possible that undisputed evidence for the formation of these linkages could be determined by using $^{13}$C/$^{15}$N or $^1$H/$^{13}$C 2D NMR.

The stability of xenobiotics immobilized in soil is paramount to determination of environmental risk. Determination of mechanisms of immobilization of xenobiotics (covalent binding or sequestration) is academic, as long as the mechanism is stable. Stability, in this sense, includes two components: contact time and bioavailability. An immobilized xenobiotic can be
seen as stable, if it is held for a long period of time (years to decades or longer) without release of free compound. In addition, an immobilized xenobiotic can be seen as stable if: (1) the compound is not bioavailable, or (2) if the compound is bioavailable to microorganisms but does not result in free compound release.

The incubation of soil or humic material with previously immobilized $^{14}$C-labeled xenobiotics (TNT, DCP, cyprodinil) in fresh soil indicates these compounds are held in a stable manner based on estimates of extractability and mineralization as determined by radiocarbon. Furthermore, results indicate that even xenobiotics covalently bound in soil organic matter or sequestered in soil are bioavailable, as indicated by $^{14}$CO$_2$ release. However, in no instance did exposure to microbial activity result in the increase in methanol extractable xenobiotics. In fact, with time extractable radioactivity decreased in most cases and radioactivity increased in the humin fraction in all cases. These findings support the conclusion that the selected xenobiotics are held via stable covalent linkages or sequestration in soil organic/mineral matter and are not easily released.

Though these findings support the hypothesis that bound xenobiotics are immobilized in soil by covalent binding or sequestration and are stable, interpretation is limited by xenobiotics selected. Future work could be more meaningful if a larger set of xenobiotics, soils and environmental conditions were evaluated to assess immobilization and stability. Application of $^{14}$C and stable isotope-labeled xenobiotics still appears to be the most practical approach to this end. However, prolonged experiments under field conditions (containment to prevent off-site movement with exposure to field conditions) may be a necessary step to enable lab-scale to field-scale extrapolations. In addition, as mentioned, 2D NMR could show without a doubt that the hypothesized mechanisms of immobilization occur and the stability could be investigated more closely. Exposure of immobilized xenobiotics in soils to selected microorganisms and conditions for longer times scales (months to years) could prove the stability of xenobiotics immobilized in soil without question.
ACKNOWLEDGEMENTS

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REFERENCES


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Table 1. Chemicals used for experiments.

<table>
<thead>
<tr>
<th>Chemical Structure</th>
<th>Chemical Name</th>
<th>Common Name</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Chemical 1" /></td>
<td>α,α,α-Trifluoro-2,6-dinitro-N,N-dipropyl-p-toluidine</td>
<td>Trifluralin</td>
</tr>
<tr>
<td><img src="image2.png" alt="Chemical 2" /></td>
<td>α,α,α- Trifluoro-2,6-dinitro-N-(N-propyl)-p-toluidine</td>
<td>TR2</td>
</tr>
<tr>
<td><img src="image3.png" alt="Chemical 3" /></td>
<td>1,2-Benzenediamine,3-nitro-5-(trifluoromethyl)</td>
<td>TR6</td>
</tr>
<tr>
<td><img src="image4.png" alt="Chemical 4" /></td>
<td>1H-Benzimidazole, 2-ethyl-4-nitro-6-(trifluoromethyl)</td>
<td>TR15</td>
</tr>
<tr>
<td><img src="image5.png" alt="Chemical 5" /></td>
<td>4-Aminobenzotrifluoride</td>
<td>4-ABT</td>
</tr>
<tr>
<td><img src="image6.png" alt="Chemical 6" /></td>
<td>N,N-Di-N-propyl-2-nitro-α,α- trifluoro-p-toluidine</td>
<td>2-NITRO</td>
</tr>
<tr>
<td><img src="image7.png" alt="Chemical 7" /></td>
<td>α,α,α-Trifluoro-2,6-diamino-N,N-dipropyl-p-toluidine</td>
<td>Fe-TR</td>
</tr>
<tr>
<td><img src="image8.png" alt="Chemical 8" /></td>
<td>α,α,α-Trifluoro-2,6-dinitro-N,N-dipropyl-p-toluidine</td>
<td>Pd-Fe-TR</td>
</tr>
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Table 2. Physiochemical properties of soil.

<table>
<thead>
<tr>
<th></th>
<th>Pope</th>
<th>Hagerstown</th>
<th>Chagrin</th>
<th>Carlisle</th>
</tr>
</thead>
<tbody>
<tr>
<td>OM %</td>
<td>3.3</td>
<td>2.7</td>
<td>2.8</td>
<td>7.4</td>
</tr>
<tr>
<td>Sand %</td>
<td>55.9</td>
<td>29.9</td>
<td>27.7</td>
<td>35.9</td>
</tr>
<tr>
<td>Silt %</td>
<td>25.4</td>
<td>46.2</td>
<td>38.0</td>
<td>28.9</td>
</tr>
<tr>
<td>Clay %</td>
<td>18.7</td>
<td>24.0</td>
<td>34.2</td>
<td>35.2</td>
</tr>
<tr>
<td>Texture</td>
<td>Sandy Loam</td>
<td>Loam</td>
<td>Clay Loam</td>
<td>Clay Loam</td>
</tr>
<tr>
<td>CEC (meq/100g)</td>
<td>9.8</td>
<td>9.2</td>
<td>17.0</td>
<td>19.9</td>
</tr>
<tr>
<td>N %</td>
<td>0.21</td>
<td>0.15</td>
<td>0.15</td>
<td>0.31</td>
</tr>
<tr>
<td>pH</td>
<td>5.1</td>
<td>6.2</td>
<td>7.6</td>
<td>5.1</td>
</tr>
<tr>
<td>Class.</td>
<td>Fluventic Dystrochrept</td>
<td>Typic Fluventic</td>
<td>Dystric Typic Medisaprist</td>
<td>Eutrochrept</td>
</tr>
</tbody>
</table>
Table 3. Samples of soil or soil components containing “bound” $^{14}$C-labeled xenobiotics used for this investigation.

<table>
<thead>
<tr>
<th>Test compound</th>
<th>Test soil material</th>
<th>Mass test soil material (g)</th>
<th>Mass fresh soil (g)</th>
<th>Total radioactivity ($\mu$Ci)</th>
<th>Concentration in whole soil (ppm)</th>
<th>Concentration in test soil material (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{14}$C-TNT</td>
<td>Unextracted soil</td>
<td>0.22</td>
<td>24.78</td>
<td>0.017</td>
<td>8.53</td>
<td>969.09</td>
</tr>
<tr>
<td></td>
<td>Extracted soil</td>
<td>0.22</td>
<td>24.78</td>
<td>0.017</td>
<td>8.80</td>
<td>999.45</td>
</tr>
<tr>
<td></td>
<td>Humic acid</td>
<td>0.15</td>
<td>24.85</td>
<td>0.047</td>
<td>24.21</td>
<td>4035.07</td>
</tr>
<tr>
<td>$^{14}$C-DCP</td>
<td>Fresh soil</td>
<td>-----</td>
<td>100</td>
<td>8.52</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Unextracted soil</td>
<td>1.60</td>
<td>23.40</td>
<td>0.042</td>
<td>5.65</td>
<td>88.28</td>
</tr>
<tr>
<td></td>
<td>Extracted soil</td>
<td>1.60</td>
<td>23.40</td>
<td>0.030</td>
<td>4.04</td>
<td>63.16</td>
</tr>
<tr>
<td></td>
<td>Humin</td>
<td>3.28</td>
<td>21.72</td>
<td>0.028</td>
<td>3.70</td>
<td>28.16</td>
</tr>
<tr>
<td>$^{14}$C-cyprodinil</td>
<td>Extracted soil phenyl label</td>
<td>5.44</td>
<td>19.56</td>
<td>0.298</td>
<td>59.6</td>
<td>273.9</td>
</tr>
<tr>
<td></td>
<td>Extracted soil pyrimidyl label</td>
<td>5.44</td>
<td>19.56</td>
<td>0.247</td>
<td>49.4</td>
<td>227.0</td>
</tr>
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</table>
Table 4. Chemical structures of test chemicals

<table>
<thead>
<tr>
<th>Chemical Structure</th>
<th>Chemical Formula</th>
<th>Common Name</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="Chemical Structure" /></td>
<td>2,4-Dichlorophenol</td>
<td>DCP</td>
</tr>
<tr>
<td><img src="image2" alt="Chemical Structure" /></td>
<td>4-Cyclopropyl6-methyl-N-phenyl-2-pyrimidinamine</td>
<td>Cyprodinil</td>
</tr>
<tr>
<td><img src="image3" alt="Chemical Structure" /></td>
<td>2,4,6-Trinitrotoluene</td>
<td>TNT</td>
</tr>
</tbody>
</table>

* = indicates uniformly ring labeled with $^{14}$C  
• = indicates $^{14}$C labeling at the C-2 position of the pyrimidyl ring
Table 5. Physio-chemical properties of fresh Pope soil.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
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</thead>
<tbody>
<tr>
<td>Sand (%)</td>
<td>55.9</td>
</tr>
<tr>
<td>Silt (%)</td>
<td>25.4</td>
</tr>
<tr>
<td>Clay (%)</td>
<td>18.7</td>
</tr>
<tr>
<td>pH</td>
<td>5.1</td>
</tr>
<tr>
<td>Organic matter (%)</td>
<td>3.3</td>
</tr>
<tr>
<td>N (%)</td>
<td>0.21</td>
</tr>
<tr>
<td>C/N ratio</td>
<td>7.86</td>
</tr>
<tr>
<td>CEC (cmol charge/kg)</td>
<td>9.8</td>
</tr>
<tr>
<td>Textural Class</td>
<td>sandy loam</td>
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<tr>
<td>Soil Classification</td>
<td>Fluventic Dystrochrept</td>
</tr>
</tbody>
</table>
Table 6. Total Carbon determination for control test materials used for incubations.

<table>
<thead>
<tr>
<th>Test compound</th>
<th>Test soil material</th>
<th>Average TC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unextracted soil</td>
<td>29.5 ± 0.4</td>
</tr>
<tr>
<td>14C-TNT</td>
<td>Extracted soil</td>
<td>29.5 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>Humic acid</td>
<td>44.2 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>Fresh soil</td>
<td>4.07 ± 0.10</td>
</tr>
<tr>
<td>14C-DCP</td>
<td>Unextracted soil</td>
<td>4.07 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>Extracted soil</td>
<td>4.07 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>Humin</td>
<td>1.98 ± 0.03</td>
</tr>
<tr>
<td>14C-cyprodinil</td>
<td>Extracted soil phenyl label</td>
<td>1.19 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>Extracted soil pyrimidyl label</td>
<td>1.19 ± 0.01</td>
</tr>
</tbody>
</table>
Table 7. Fractionation of soil/compost mixture after incubation with 20 μCi kg\(^{-1}\) \(^{14}\)C-labeled TNT. Reported values are average radioactivity values for duplicate samples followed by the standard deviation.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>% of Initial Radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Water extraction</strong></td>
<td></td>
</tr>
<tr>
<td>Humic-like precipitate</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>Dichloromethane extract</td>
<td>0.2 ± 0.0</td>
</tr>
<tr>
<td>Water after dichloromethane extraction</td>
<td>5.5 ± 0.8</td>
</tr>
<tr>
<td><strong>Methanol extraction</strong></td>
<td>1.8 ± 0.7</td>
</tr>
<tr>
<td><strong>NaOH extraction</strong></td>
<td></td>
</tr>
<tr>
<td>Humic Acid</td>
<td>40.0 ± 1.0</td>
</tr>
<tr>
<td>Fulvic Acid</td>
<td>14.3 ± 1.4</td>
</tr>
<tr>
<td>Humin</td>
<td>28.2 ± 0.5</td>
</tr>
<tr>
<td><strong>Total Recovered</strong></td>
<td>91.2 ± 1.6</td>
</tr>
</tbody>
</table>
Table 8. Transformation of 4-chlorophenol incubated with peroxidase in the presence of various humic constituents

<table>
<thead>
<tr>
<th>Humic constituent</th>
<th>Molecular structure ¹</th>
<th>Transformation of 4-Chlorophenol (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (Control)</td>
<td></td>
<td>32.3 ± 2.5</td>
</tr>
<tr>
<td>Enhanced Transformation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Syringaldehyde</td>
<td>2(OCH₃), 4(CHO), 6(OCH₃)</td>
<td>82.0 ± 3.1</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>2(OCH₃), 4(COOH-CH=CH-)</td>
<td>71.8 ± 8.7</td>
</tr>
<tr>
<td>Guaiacol</td>
<td>2(OCH₃)</td>
<td>56.1 ± 0.9</td>
</tr>
<tr>
<td>Vanillic acid</td>
<td>2(OCH₃), 4(COOH)</td>
<td>62.8 ± 3.8</td>
</tr>
<tr>
<td>2,6-Dimethoxyphenol</td>
<td>2(OCH₃), 6(OCH₃)</td>
<td>73.0 ± 4.9</td>
</tr>
<tr>
<td>Vanillin</td>
<td>2(OCH₃), 4(CHO)</td>
<td>74.2 ± 6.6</td>
</tr>
<tr>
<td>Phloroglucinol</td>
<td>3(OH), 5(OH)</td>
<td>47.9 ± 2.9</td>
</tr>
<tr>
<td>Reduced Transformation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Catechol</td>
<td>2(OH)</td>
<td>11.1 ± 0.7</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>2(OH), 4(COOH), 6(OH)</td>
<td>16.3 ± 1.7</td>
</tr>
<tr>
<td>Hydroquinone</td>
<td>4(OH)</td>
<td>21.9 ± 0.8</td>
</tr>
<tr>
<td>4-Methoxyphenol</td>
<td>4(OCH₃)</td>
<td>7.2 ± 1.2</td>
</tr>
</tbody>
</table>

¹ The numbers preceding the substituents refer to the position on the aromatic ring of phenol.
Table 9. Transformation of chlorophenols incubated with peroxidase, laccase, tyrosinase and birnessite in the presence of humic constituents.

<table>
<thead>
<tr>
<th></th>
<th>Transformation of chlorophenols (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2-CP</td>
</tr>
<tr>
<td><strong>Peroxidase</strong></td>
<td></td>
</tr>
<tr>
<td>Substrate alone</td>
<td>22.9 ± 1.7</td>
</tr>
<tr>
<td>Substrate + Syringaldehyde</td>
<td>62.5 ± 0.6</td>
</tr>
<tr>
<td>+ 4-HBA</td>
<td>34.7 ± 2.8</td>
</tr>
<tr>
<td>+ Catechol</td>
<td>18.5 ± 2.2</td>
</tr>
<tr>
<td><strong>Laccase</strong></td>
<td></td>
</tr>
<tr>
<td>Substrate alone</td>
<td>43.1 ± 1.6</td>
</tr>
<tr>
<td>Substrate + Syringaldehyde</td>
<td>95.2 ± 0.2</td>
</tr>
<tr>
<td>+ 4-HBA</td>
<td>79.7 ± 0.3</td>
</tr>
<tr>
<td>+ Catechol</td>
<td>66.0 ± 0.8</td>
</tr>
<tr>
<td><strong>Tyrosinase</strong></td>
<td></td>
</tr>
<tr>
<td>Substrate alone</td>
<td>11.4 ± 2.2</td>
</tr>
<tr>
<td>Substrate + Syringaldehyde</td>
<td>6.2 ± 0.2</td>
</tr>
<tr>
<td>+ 4-HBA</td>
<td>13.3 ± 2.1</td>
</tr>
<tr>
<td>+ Catechol</td>
<td>35.4 ± 1.5</td>
</tr>
<tr>
<td><strong>Birnessite</strong></td>
<td></td>
</tr>
<tr>
<td>Substrate alone</td>
<td>62.0 ± 0.6</td>
</tr>
<tr>
<td>Substrate + Syringaldehyde</td>
<td>75.1 ± 3.3</td>
</tr>
<tr>
<td>+ 4-HBA</td>
<td>58.6 ± 2.1</td>
</tr>
<tr>
<td>+ Catechol</td>
<td>20.8 ± 1.4</td>
</tr>
</tbody>
</table>
Table 10. Transformation of chloroanilines incubated with peroxidase, laccase, tyrosinase and birnessite in the presence of humic constituents.

<table>
<thead>
<tr>
<th></th>
<th>Transformation of chloroanilines ( %)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2-Chloroaniline</td>
</tr>
<tr>
<td><strong>Peroxidase</strong></td>
<td></td>
</tr>
<tr>
<td>Substrate alone</td>
<td>4.4 ± 4.7</td>
</tr>
<tr>
<td>Substrate + Syringaldehyde</td>
<td>6.0 ± 4.1</td>
</tr>
<tr>
<td>+ 4-HBA</td>
<td>12.3 ± 0.1</td>
</tr>
<tr>
<td>+ Catechol</td>
<td>17.8 ± 1.9</td>
</tr>
<tr>
<td><strong>Laccase</strong></td>
<td></td>
</tr>
<tr>
<td>Substrate alone</td>
<td>9.4 ± 0.1</td>
</tr>
<tr>
<td>Substrate + Syringaldehyde</td>
<td>23.0 ± 3.6</td>
</tr>
<tr>
<td>+ 4-HBA</td>
<td>10.0 ± 0.2</td>
</tr>
<tr>
<td>+ Catechol</td>
<td>63.0 ± 0.7</td>
</tr>
<tr>
<td><strong>Tyrosinase</strong></td>
<td></td>
</tr>
<tr>
<td>Substrate alone</td>
<td>10.1 ± 0.9</td>
</tr>
<tr>
<td>Substrate + Syringaldehyde</td>
<td>12.5 ± 0.3</td>
</tr>
<tr>
<td>+ 4-HBA</td>
<td>12.6 ± 0.4</td>
</tr>
<tr>
<td>+ Catechol</td>
<td>53.9 ± 0.2</td>
</tr>
<tr>
<td><strong>Birnessite</strong></td>
<td></td>
</tr>
<tr>
<td>Substrate alone</td>
<td>99.6 ± 0.8</td>
</tr>
<tr>
<td>Substrate + Syringaldehyde</td>
<td>53.4 ± 1.8</td>
</tr>
<tr>
<td>+ 4-HBA</td>
<td>97.4 ± 2.4</td>
</tr>
<tr>
<td>+ Catechol</td>
<td>73.2 ± 2.9</td>
</tr>
</tbody>
</table>
Table 11. Transformation of chlorinated phenols incubated with oxidoreductive catalysts in the absence or presence of leonardite humic acid (50 mg/L).

<table>
<thead>
<tr>
<th>Catalyst</th>
<th>Humic acid (mg/L)</th>
<th>Transformation of chlorophenols (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>2-Chlorophenol</th>
<th>3-Chlorophenol</th>
<th>4-Chlorophenol</th>
<th>4-Dichlorophenol</th>
<th>2,4,5-Trichlorophenol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without Catalyst</td>
<td>50</td>
<td>16.0</td>
<td>5.4</td>
<td>3.6</td>
<td>28.5</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>Peroxidase</td>
<td>0</td>
<td>22.9</td>
<td>11.6</td>
<td>32.2</td>
<td>75.1</td>
<td>42.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>94.9</td>
<td>75.0</td>
<td>98.9</td>
<td>99.3</td>
<td>98.0</td>
<td></td>
</tr>
<tr>
<td>Laccase</td>
<td>0</td>
<td>43.1</td>
<td>8.1</td>
<td>34.0</td>
<td>95.9</td>
<td>27.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>54.9</td>
<td>6.7</td>
<td>30.1</td>
<td>92.0</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>Tyrosinase</td>
<td>0</td>
<td>11.4</td>
<td>32.2</td>
<td>100.0</td>
<td>20.4</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>41.5</td>
<td>68.8</td>
<td>100.0</td>
<td>66.4</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>Birnessite</td>
<td>0</td>
<td>62.0</td>
<td>32.8</td>
<td>45.4</td>
<td>77.6</td>
<td>37.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>98.0</td>
<td>85.1</td>
<td>98.2</td>
<td>100.0</td>
<td>95.3</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>The SD for transformation of chlorophenols (%) ranged between 0.2 and 7.9
Table 12. Transformation of chlorinated anilines incubated with oxidoreductive catalysts in the absence or presence of leonardite humic acid (50 mg/L).

<table>
<thead>
<tr>
<th>Catalyst</th>
<th>Humic acid (mg/L)</th>
<th>Transformation of chloroanilines (%)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2-Chloroaniline</td>
</tr>
<tr>
<td>Without Catalyst</td>
<td>50</td>
<td>21.6</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>0</td>
<td>4.4</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>20.7</td>
</tr>
<tr>
<td>Laccase</td>
<td>0</td>
<td>9.4</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>23.5</td>
</tr>
<tr>
<td>Tyrosinase</td>
<td>0</td>
<td>10.1</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>23.5</td>
</tr>
<tr>
<td>Birnessite</td>
<td>0</td>
<td>99.6</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>97.0</td>
</tr>
</tbody>
</table>

<sup>a</sup>The SD for transformation of chloroanilines (%) ranged between 0.1 and 6.0
Table 13. Distribution of radioactivity when $^{14}$C-4-chlorophenol (4-CP) was incubated with oxidoreductive catalysts in the presence of humic acid (50 mg/L).

<table>
<thead>
<tr>
<th>Catalyst</th>
<th>Radioactivity (%)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Supematant</td>
<td>Precipitate</td>
</tr>
<tr>
<td></td>
<td>Remaining 4-CP$^b$</td>
<td>Oligomeric Products$^c$</td>
</tr>
<tr>
<td>Without enzyme</td>
<td>92.2</td>
<td>0.0</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>0.0</td>
<td>5.4</td>
</tr>
<tr>
<td>Laccase</td>
<td>67.2</td>
<td>2.5</td>
</tr>
<tr>
<td>Tyrosinase</td>
<td>0.0</td>
<td>27.6</td>
</tr>
<tr>
<td>Birnessite</td>
<td>2.5</td>
<td>19.8</td>
</tr>
</tbody>
</table>

$^a$ The SD for radioactivity(%) of each fraction ranged between 0.0 and 2.0
$^b$ Remaining 4-CP in supernatant and CH$_2$Cl$_2$ extract of precipitate were measured by HPLC
$^c$ Percentage radioactivity in the oligomeric products was calculated by subtraction of percentage radioactivity in remaining 4-CP from percentage of total radioactivity in supernatant or CH$_2$Cl$_2$ extract
Table 14. Distribution of radioactivity when $^{14}$C-4-chloroaniline (4-CA) was incubated with oxidoreductive catalysts in the presence of leonardite humic acid (50 mg/L).

<table>
<thead>
<tr>
<th>Catalyst</th>
<th>Supernatant</th>
<th></th>
<th>Precipitate</th>
<th>Humic Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Remaining 4-CA&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Oligomeric Products&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Remaining 4-CA&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Oligomeric Products&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Without enzyme</td>
<td>87.5</td>
<td>0.7</td>
<td>0.0</td>
<td>4.4</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>41.5</td>
<td>8.1</td>
<td>5.7</td>
<td>17.0</td>
</tr>
<tr>
<td>Laccase</td>
<td>82.3</td>
<td>2.0</td>
<td>0.6</td>
<td>4.4</td>
</tr>
<tr>
<td>Tyrosinase</td>
<td>59.8</td>
<td>13.4</td>
<td>14.7</td>
<td>0.0</td>
</tr>
<tr>
<td>Birnessite</td>
<td>7.1</td>
<td>24.9</td>
<td>6.3</td>
<td>31.7</td>
</tr>
</tbody>
</table>

<sup>a</sup>The SD for radioactivity(%) of each fraction ranged between 0.0 and 5.7.
<sup>b</sup>Remaining 4-CA in supernatant and CH$_2$Cl$_2$ extract of precipitate were measured by HPLC.
<sup>c</sup>Percentage radioactivity in the oligomeric products was calculated by subtraction of percentage radioactivity in remaining 4-CA from percentage of total radioactivity in supernatant or CH$_2$Cl$_2$ extract.
Table 15. Distribution of radioactivity when $^{14}$C-4-chlorophenol (4-CP) and $^{14}$C-4-chloroaniline (4-CA) were incubated with peroxidase (0.08 units/ml) in the presence of humic acid (50 mg/L) extracted from different sources.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Humic Acid $^b$</th>
<th>Radioactivity (%)$^a$</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Supernatant</td>
<td>Precipitate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Remaining Substrate$^c$</td>
<td>Oligomeric Products</td>
</tr>
<tr>
<td>4-CP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LHA</td>
<td>0.0</td>
<td>5.4</td>
<td>1.1</td>
</tr>
<tr>
<td>SHAS</td>
<td>0.0</td>
<td>8.7</td>
<td>0.0</td>
</tr>
<tr>
<td>SRHA</td>
<td>0.0</td>
<td>14.0</td>
<td>0.0</td>
</tr>
<tr>
<td>HTHA</td>
<td>0.0</td>
<td>9.7</td>
<td>0.0</td>
</tr>
<tr>
<td>4-CA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LHA</td>
<td>41.5</td>
<td>10.1</td>
<td>5.7</td>
</tr>
<tr>
<td>SHAS</td>
<td>25.5</td>
<td>26.0</td>
<td>5.6</td>
</tr>
<tr>
<td>SRHA</td>
<td>24.0</td>
<td>16.9</td>
<td>8.3</td>
</tr>
<tr>
<td>HTHA</td>
<td>31.3</td>
<td>25.8</td>
<td>4.2</td>
</tr>
</tbody>
</table>

$^a$ The SD for radioactivity(%) of each fraction ranged between 0.0 and 8.3.

$^b$ Humic acid

LHA = Leonardite humic acid extracted from leonardite humus

SHAS = IHSS Soil humic acid standard

SRHA = IHSS Suwannee River humic acid reference

HTHA = Humic acid extracted from Hagerstown soil

$^c$ Remaining substrate in supernatant and CH$_2$Cl$_2$ extract of precipitate were measured by HPLC.

$^d$ Percentage radioactivity in the oligomeric products was calculated by subtraction of percentage radioactivity in remaining substrate from percentage of total radioactivity in supernatant or CH$_2$Cl$_2$ extract.
Table 16. Dechlorination (% Cl) and transformation (% Tr) of chlorophenols in the presence of humic substances.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Catalyst</th>
<th>Cosubstrate</th>
<th>2-CP</th>
<th>3-CP</th>
<th>4-CP</th>
<th>2,4-DCP</th>
<th>2,4,5-TCP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>% Cl</td>
<td>% Tr</td>
<td>% Cl</td>
<td>% Tr</td>
<td>% Cl</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>None</td>
<td>1.3</td>
<td>22.9</td>
<td>0.0</td>
<td>11.6</td>
<td>9.0</td>
</tr>
<tr>
<td></td>
<td>Syringaldehyde</td>
<td>29.5</td>
<td>62.5</td>
<td>0.3</td>
<td>9.1</td>
<td>69.1</td>
</tr>
<tr>
<td></td>
<td>Catechol</td>
<td>0.0</td>
<td>18.5</td>
<td>0.0</td>
<td>7.9</td>
<td>5.3</td>
</tr>
<tr>
<td></td>
<td>Humic acid</td>
<td>12.0</td>
<td>94.9</td>
<td>1.7</td>
<td>75.0</td>
<td>36.1</td>
</tr>
<tr>
<td>Laccase</td>
<td>None</td>
<td>3.0</td>
<td>43.1</td>
<td>0.2</td>
<td>8.1</td>
<td>13.2</td>
</tr>
<tr>
<td></td>
<td>Syringaldehyde</td>
<td>44.4</td>
<td>95.2</td>
<td>0.4</td>
<td>32.5</td>
<td>90.9</td>
</tr>
<tr>
<td></td>
<td>Catechol</td>
<td>5.6</td>
<td>66.0</td>
<td>0.0</td>
<td>7.4</td>
<td>4.7</td>
</tr>
<tr>
<td></td>
<td>Humic acid</td>
<td>5.1</td>
<td>54.9</td>
<td>0.2</td>
<td>6.7</td>
<td>12.5</td>
</tr>
<tr>
<td>Tyrosinase</td>
<td>None</td>
<td>1.1</td>
<td>11.4</td>
<td>16.2</td>
<td>32.2</td>
<td>59.7</td>
</tr>
<tr>
<td></td>
<td>Syringaldehyde</td>
<td>0.3</td>
<td>6.2</td>
<td>20.3</td>
<td>32.6</td>
<td>75.6</td>
</tr>
<tr>
<td></td>
<td>Catechol</td>
<td>10.5</td>
<td>35.4</td>
<td>34.5</td>
<td>47.9</td>
<td>70.9</td>
</tr>
<tr>
<td></td>
<td>Humic acid</td>
<td>8.0</td>
<td>41.5</td>
<td>34.9</td>
<td>68.8</td>
<td>59.1</td>
</tr>
<tr>
<td>Birnessite</td>
<td>None</td>
<td>8.7</td>
<td>62.0</td>
<td>0.5</td>
<td>32.8</td>
<td>14.9</td>
</tr>
<tr>
<td></td>
<td>Syringaldehyde</td>
<td>35.3</td>
<td>75.1</td>
<td>1.5</td>
<td>57.0</td>
<td>87.7</td>
</tr>
<tr>
<td></td>
<td>Catechol</td>
<td>0.6</td>
<td>20.8</td>
<td>0.6</td>
<td>16.0</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>Humic acid</td>
<td>16.1</td>
<td>98.0</td>
<td>2.8</td>
<td>85.1</td>
<td>41.6</td>
</tr>
</tbody>
</table>

\textsuperscript{a} The SD for % Cl ranged between 0.0 and 4.3 and the SD for % Tr ranged between 0.1 and 9.7.
Table 17. Dechlorination (% Cl) and transformation (% Tr) of chloroanilines in the presence of humic substances.

<table>
<thead>
<tr>
<th>Catalyst</th>
<th>Cosubstrate</th>
<th>2-CA</th>
<th></th>
<th>2,4-DCA</th>
<th></th>
<th>2,4,5-TCA</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>% Cl</td>
<td>% Tr</td>
<td>% Cl</td>
<td>% Tr</td>
<td>% Cl</td>
<td>% Tr</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>None</td>
<td>0.6</td>
<td>4.4</td>
<td>0.0</td>
<td>28.8</td>
<td>17.1</td>
<td>78.3</td>
</tr>
<tr>
<td></td>
<td>Syringaldehyde</td>
<td>0.0</td>
<td>6.0</td>
<td>0.0</td>
<td>30.4</td>
<td>0.9</td>
<td>76.7</td>
</tr>
<tr>
<td></td>
<td>Catechol</td>
<td>1.1</td>
<td>17.8</td>
<td>0.0</td>
<td>76.9</td>
<td>0.7</td>
<td>83.1</td>
</tr>
<tr>
<td></td>
<td>Humic Acid</td>
<td>0.4</td>
<td>20.7</td>
<td>0.0</td>
<td>5.1</td>
<td>24.3</td>
<td>80.1</td>
</tr>
<tr>
<td>Laccase</td>
<td>None</td>
<td>0.8</td>
<td>9.4</td>
<td>0.3</td>
<td>0.8</td>
<td>1.5</td>
<td>31.0</td>
</tr>
<tr>
<td></td>
<td>Syringaldehyde</td>
<td>1.1</td>
<td>23.0</td>
<td>0.3</td>
<td>59.6</td>
<td>1.0</td>
<td>83.6</td>
</tr>
<tr>
<td></td>
<td>Catechol</td>
<td>0.3</td>
<td>63.0</td>
<td>0.3</td>
<td>65.8</td>
<td>0.3</td>
<td>98.6</td>
</tr>
<tr>
<td></td>
<td>Humic Acid</td>
<td>2.8</td>
<td>23.5</td>
<td>0.2</td>
<td>5.1</td>
<td>1.4</td>
<td>22.5</td>
</tr>
<tr>
<td>Tyrosinase</td>
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<td>10.1</td>
<td>0.8</td>
<td>4.0</td>
<td>9.5</td>
<td>39.1</td>
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<td>Syringaldehyde</td>
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<td>0.7</td>
<td>8.3</td>
<td>0.5</td>
<td>5.4</td>
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<td>Catechol</td>
<td>0.8</td>
<td>53.9</td>
<td>1.0</td>
<td>86.6</td>
<td>0.5</td>
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<td>Humic Acid</td>
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<td>0.4</td>
<td>6.2</td>
<td>6.7</td>
<td>36.0</td>
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<td>97.0</td>
<td>8.2</td>
<td>93.6</td>
<td>36.2</td>
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*a The SD for % Cl ranged between 0.0 and 6.2 and the SD for % Tr ranged between 0.1 and 6.1.
Table 18. Fractionation of soil materials containing bound residues of $^{14}$C-TNT, $^{14}$C-DCP and $^{14}$C-cyprodinil before and after a 141-day incubation with fresh soil.

<table>
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<th>Test compound</th>
<th>Test soil material</th>
<th>Days of incubation</th>
<th>% of radioactivity in:</th>
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<td>Methanol extract</td>
<td>Fulvic acid</td>
<td>Humic acid</td>
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<td>1.1</td>
<td>27.5</td>
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<td></td>
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<td>21.3</td>
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<tr>
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<td>27.9</td>
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<td>141</td>
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<td>cyprodinil</td>
<td>phenyl label</td>
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<td>20.1</td>
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<td>pyrimidyl label</td>
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<td>16.2</td>
<td>12.6</td>
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Figure 1. Experimental setup for anaerobic incubation of $^{15}$N- and $^{14}$C-labeled TNT in soil mixed with the compost.
Figure 2. Experimental set-up for monitoring bound residues of $^{14}$C-labeled test compounds during incubation with fresh soil.
Figure 3. HPLC chromatograms of TNT standard and the liquid solutions resulting from the fractionation of soil incubated with $^{15}$N- and $^{14}$C-labeled TNT.
Figure 4. Thin-layer chromatograms of various $^{14}$C labeled TNT compost extracts and standards.
Figure 5. Liquid-state $^{15}$N-NMR full spectra of the $^{15}$N-labeled TNT standard (in box expanded region (380 – 350 ppm) to show the $^{15}$N peaks).
Figure 6. Solid-state $^{15}$N-NMR spectra of humic acid isolated from the soil/compost mixture after incubation without (control) and with $^{15}$N-TNT.
Figure 7. Solid-state $^{15}$N-NMR spectra of the humin fraction originating from the soil/compost mixture incubated without (control) and with $^{15}$N-TNT.
Figure 8. Anaerobic transformation of 2,4,6-trinitrotoluene (TNT) to 2,4,6-triaminotoluene (TAT) (from Daun et al., 1998).
Figure 9. Kinetics of the transformation of 4-chlorophenol (0.3 mM) and 4-chloroaniline (0.3 mM) incubated at pH 5.6 with humic constituents (0.3 mM) in the presence of laccase (1.5 units/mL).
Figure 10. Kinetics of the transformation of humic constituents (0.3 mM) incubated at pH 5.6 with 4-chlorophenol (0.3 mM) or 4-chloroaniline (0.3 mM) in the presence of laccase (1.5 units/mL).
Figure 11. Effect of increased concentrations of humic constituents on the transformation of 4-chlorophenol (0.3 mM) and 4-chloroaniline (0.3 mM) incubated for 24 hours with laccase (1.5 units/mL, pH 5.6).
Figure 12. Oxidation pathways of chlorinated substrates and humic constituents in the presence of various enzymes and birnessite.
Figure 13. Effect of humic acid concentration on the transformation of 4-chlorophenol (0.3 mM) incubated with oxidoreductive catalysts. (Some error bars are invisible as error deviation is very small.) (Incubation time: 2 hours for peroxidase; 24 hours for the other catalysts.)
Figure 14. Effect of humic acid concentration on the transformation of 4-chloroaniline (0.3 mM) incubated with oxidoreductive catalysts. (Some error bars are invisible as error deviation is very small.) (Incubation time: 2 hours for peroxidase; 24 hours for the other catalysts.)
Figure 15. Effect of humic acid concentration on the distribution of radioactivity after incubation of $^{14}$C-labeled 4-chlorophenol and 4-chloroaniline with humic acid in the presence of peroxidase. (Some error bars are invisible as error deviation is very small.)
Figure 16. Effect of incubation time on the dehalogenation of 4-chlorophenol and 4-chloroaniline by laccase in the presence of humic constituents.
Figure 17. Effect of the concentration of humic constituents on the dehalogenation of 4-chlorophenol and 4-chloroaniline in the presence of laccase.
Figure 18. Effect of the concentration of humic acid on the dehalogenation of 4-chlorophenol and 4-chloroaniline in the presence of various oxidoreductases or birnessite.
Figure 19. Fractionation of $^{14}$C-trifluralin after a 7-week anaerobic incubation. Standard error for each fraction ranged from 0.074% to 1.77%.
Figure 20. Radio-scanning of Thin Layer Chromatography (TLC) plates.
Figure 21. $^{19}$FNMR liquid-state spectra of methanol extracts from soils incubated with trifluralin: (A) Pope soil, (B) Hagerstown soil, (C) Chagrin soil, (D) Carlisle soil.
Figure 22. $^{19}$FNMR liquid-state spectra of trifluralin and five metabolite standards.
Figure 23. $^{19}$FNMR solid-state spectra of trifluralin, trifluralin mixed with control HA and HA from two soils with bound residues of trifluralin.
Figure 24. $^{19}$FNMR liquid-state spectrum of Chagrin fulvic acid with bound metabolites of trifluralin.
Figure 25. $^{19}$FNMR liquid-state spectra of trifluralin reduction products formed by reacting with zero valent Fe or Fe-Pd.
Figure 26. $^{19}$FNMR liquid-state spectra of (A) Fe-TR metabolite and (B) TR6 metabolite involved in binding to Chagrin fulvic acid.
Figure 27. $^{19}$FNMR liquid-state T1 relaxation experiments of (A) Fe-TR metabolite, (B) Fe-TR with Chagrin FA, (C) TR6 metabolite, (D) TR6 metabolite with Chagrin FA.
Figure 28. $^{19}$FNMR liquid-state spectra of Fe-TR/FA chloroform extraction experiment.
Figure 29. $^{19}$FNMR liquid-state spectra of TR6/FA chloroform extraction experiment.
Figure 30. Extractability and mineralization of bound residues of $^{14}$C-TNT incubated for 141 days with fresh soil.
Figure 31. Extractability and mineralization of bound residues of $^{14}$C-DCP and fresh $^{14}$C DCP incubated for 141 days with fresh soil.
Figure 32. Evolution of $^{14}$CO$_2$ from $^{14}$C-DCP incubated for 24 hours in fresh soil acclimated for different periods of time with DCP.
Figure 33. Extractability and mineralization of bound residues of $^{14}$C-cyprodinil incubated for 141 days with fresh soil.
LIST OF PUBLICATIONS

(RESULTING FROM THE EPA-SUPPORTED GRANT: R 826646-01-0)

Referenced journals, book volumes, and symposia proceedings


Papers presented at scientific meetings


