Biodegradation of 2,4-dichlorophenoxyacetic acid (2,4-D) and pentachlorophenol (PCP) in soils by Ronald Allen Doughten

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Soils Montana State University
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Abstract:
Contamination of soil and water by organic pollutants is a widespread problem that has resulted in a great deal of public concern. 2,4-dichlorophenoxyacetic acid (2,4-D) and pentachlorophenol (PCP) are representative members of a large class of chlorinated pesticides. Furthermore, both compounds are of local concern in the state of Montana. Contaminant degradation in soils generally occurs by the action of microorganisms. However, biodegradation is often limited by chemical sorption to soil organic matter (SOM). To overcome this limitation, surfactants have successfully been used to solubilize contaminants from soil. The impact of SOM on 2,4-D sorption, availability, and degradation was investigated in 31 soils with a range of organic matter content. Biodegradation was measured as the accumulation of 14CO2 produced from 14C-2,4-D treated soils and evaluated against sorption to SOM. Surfactant influences on solubility and degradation were examined in 14C-PCP labeled soils inoculated with white-rot fungi. The extent of 2,4-D degradation was negatively correlated with SOM, illustrating the influence of sorption on bioavailability and degradation. However, the relationship to the partition coefficient, Kd, was much weaker, suggesting that mass transfer and slow desorption may be the dominant mechanisms controlling contaminant bioavailability in soils. The extent of PCP degradation, measured as the accumulation of 14CO2 from 14C-PCP treated soils was unrelated to contaminant solubility. The addition of 25 mg Tween 80 g soil-1 resulted in enhanced PCP degradation (26%) relative to a surfactant-free control (17%) despite an overall decrease in the aqueous solubility.

Fungal activity measured as biomass and respiration over the same time period increased, suggesting a physiological response rather than a chemical response to surfactant addition. We suggest that contaminant sorption to SOM strongly influences degradation, but mass transfer from the sorbed state controls bioavailability. Furthermore, the addition of surfactants at sub-critical micelle concentrations (CMC) can result in increased degradation without increasing overall solubility, and may therefore represent an effective strategy for the bioremediation of organic-contaminated soils.
BIODEGRADATION OF 2,4-DICHLOROPHENOXYACETIC ACID (2,4-D) AND PENTACHLOROPHENOL (PCP) IN SOILS

by

Ronald Allen Doughten

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Soils

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APPROVAL

of a thesis submitted by

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This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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Signature  E. M. A. Ortega
Date  15 April 1997
This thesis is dedicated to my father, Randall Doughten, who was taken from my life at far too early of an age. His influence and memory I carry with me always.
VITA

The author, Ronald Allen Doughten was born to Mr. and Mrs. Randall Doughten on January 23, 1971, in Havre, Montana. He graduated from Havre High School, Havre, Montana in 1989 with honors. In 1994, Ronald received a Bachelor’s degree in Chemistry from Montana State University. He graduated with highest honors. In August of 1994, he began working towards a Master of Science degree at Montana State University-Bozeman under the direction of Dr. William P. Inskeep.
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Contamination of soil and water by organic pollutants is a widespread problem that has resulted in a great deal of public concern. 2,4-dichlorophenoxyacetic acid (2,4-D) and pentachlorophenol (PCP) are representative members of a large class of chlorinated pesticides. Furthermore, both compounds are of local concern in the state of Montana. Contaminant degradation in soils generally occurs by the action of microorganisms. However, biodegradation is often limited by chemical sorption to soil organic matter (SOM). To overcome this limitation, surfactants have successfully been used to solubilize contaminants from soil. The impact of SOM on 2,4-D sorption, availability, and degradation was investigated in 31 soils with a range of organic matter content. Biodegradation was measured as the accumulation of $^{14}$CO$_2$ produced from $^{14}$C-2,4-D treated soils and evaluated against sorption to SOM. Surfactant influences on solubility and degradation were examined in $^{14}$C-PCP labeled soils inoculated with white-rot fungi. The extent of 2,4-D degradation was negatively correlated with SOM, illustrating the influence of sorption on bioavailability and degradation. However, the relationship to the partition coefficient, $K_d$, was much weaker, suggesting that mass transfer and slow desorption may be the dominant mechanisms controlling contaminant bioavailability in soils. The extent of PCP degradation, measured as the accumulation of $^{14}$CO$_2$ from $^{14}$C-PCP treated soils was unrelated to contaminant solubility. The addition of 25 mg Tween 80 g soil$^{-1}$ resulted in enhanced PCP degradation (26%) relative to a surfactant-free control (17%) despite an overall decrease in the aqueous solubility. Fungal activity measured as biomass and respiration over the same time period increased, suggesting a physiological response rather than a chemical response to surfactant addition. We suggest that contaminant sorption to SOM strongly influences degradation, but mass transfer from the sorbed state controls bioavailability. Furthermore, the addition of surfactants at sub-critical micelle concentrations (CMC) can result in increased degradation without increasing overall solubility, and may therefore represent an effective strategy for the bioremediation of organic-contaminated soils.
CHAPTER I

INTRODUCTION

"As man proceeds toward his announced goal of the conquest of nature, he has written a depressing record of destruction directed not only against the earth he inhabits but against the life that shares it with him." (Carson, 1962)

The Need for Contaminant Remediation

The introduction of organic chemicals into the environment has resulted in a great deal of public concern on local, national, and international levels. In the United States alone, approximately 80 billion pounds of hazardous wastes are produced annually. As recently as 15 years ago, the United States Environmental Protection Agency (US EPA) estimated that only 10% of these wastes were disposed of safely (Epstein et al., 1982). Synthetic chemicals in soil, water, and air have been indicted as causes of illness among exposed plants and animals. A classic example was the widespread use of the pesticide DDT during the 1940s and 1950s. DDT was found to bioaccumulate in the tissues of organisms and was passed through the food chain, resulting in the death of aquatic life, fish, and birds (including the bald eagle). Because of its toxicity to many non-target species including humans, DDT use was banned in the US in 1972. Although awareness of the impact of hazardous materials in the environment has risen, historical use and practices have led to large-scale pollution problems. As of 1993, the US EPA managed
the remediation of 1500-2000 sites under the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA or Superfund program), 1500-3000 sites under the Resource Conservation and Recovery Act (RCRA), and 295,000 underground storage tanks. In addition, 7300 site cleanups were under the administration of the US Department of Defense and 19,000 sites under the US Department of Energy.

On a local level, the US EPA recognizes nine national superfund priority sites in the state of Montana (Figure 1; US EPA, 1995). Four of these sites are predominantly contaminated by organic pollutants, including diesel fuel, creosote, and PCP.

![Figure 1. Locations of the nine EPA National Priority Sites in Montana. The shaded labels are those contaminated with organic chemicals; the others are contaminated with inorganics.](image-url)
In addition to these higher profile point sources, the application of pesticides can result in non-point source soil and groundwater contamination. Well testing programs around the state of Montana between 1984 and 1988 revealed the presence of seven different pesticide residues in regional groundwaters (DeLuca, 1989).

Fate of Organic Chemicals in the Environment

Once introduced into the environment, several factors influence the fate of an organic chemical (Figure 2). Surface processes such as runoff,
photodecomposition, and volatilization can initially reduce the amount of contaminant entering the soil and groundwater system. Uptake by plants and animals in the soil results in biological accumulation and/or detoxification. Soil transport processes associated with water movement through leaching or capillary flow (bottom of diagram) influence the movement of organic chemicals, particularly soluble compounds, and are major factors in assessing groundwater pollution potential. Abiotic chemical interactions between the soil and contaminant can result in chemical breakdown and detoxification. Other chemical processes such as sorption to clays, oxides, or soil organic matter (SOM) can strongly influence chemical mobility, persistence, and availability to soil microorganisms. Finally, biological degradation (biodegradation) by soil bacteria and fungi is the primary pathway of organic chemical breakdown and detoxification in soil.

**Strategies for Remediation of Contaminated Sites**

**Traditional Strategies to Remediation**

Based upon these soil processes, several strategies have been used for the treatment of hazardous chemicals in the environment: (1) Physical treatment (e.g. adsorption, filtration, insoluble phase extraction) alters the physical state of a hazardous material and requires further action to actually remove the contaminant. (2) Chemical treatment is used to detoxify a waste to a less toxic form and includes such strategies as stabilization, solidification, and encapsulation. Chemical alteration can result in the formation of hazardous by-products that require further remediation. (3) Thermal
treatment involves the combustion of hazardous materials. Incineration costs are often high; gas emissions and solid residues may require further disposal as hazardous wastes. (4) Removal and burial comprises the physical removal of a contaminated medium (i.e. soil, water) and relocation to a landfill or other holding facility. Hazardous waste removal does not detoxify the medium, and disposal in landfills often results in further contamination of soil and groundwater (Alexander, 1994; Fernando and Aust, 1994; Thayer, 1991).

**Bioremediation**

An alternate strategy to remediation of organic contaminants that has received great amounts of attention recently is bioremediation, or the use of biological agents (primarily microorganisms) to degrade soil and water contaminants. For purposes of remediation, biodegradation can be defined as the biologically mediated alteration of a contaminant from a more toxic form to a less toxic form. Microorganisms are the primary agents responsible for the breakdown of organic molecules in the environment, ultimately resulting in the production of harmless end products such as carbon dioxide and water. Through a biologically mediated redox process, aerobic organisms use organic compounds as a carbon and energy source and O$_2$ as an electron acceptor (Figure 3).

Under the appropriate conditions, microorganisms have successfully been used to remediate hazardous waste sites. Probably the best known example of the large scale use of bioremediation technologies was the effort to clean shorelines impacted by the 1989 *Exxon Valdez* oil spill in Prince William Sound, Alaska. The application of an oleophilic
fertilizer, consisting of oleic acid, urea, and lauryl phosphate, to oil-contaminated shores

stimulated degradation by indigenous microorganisms. A five-fold increase in rates of biodegradation over untreated sites was observed. The use of bioremediation resulted in greater cleanup than physical methods and was conducted at a cost significantly below the $1 million per day expended on soil washing (Pritchard and Costa, 1991).

Most attempts at bioremediation in the US have used two primary approaches: (1) the stimulation of indigenous microorganisms through fertilization or (2) the seeding of organisms into the environment. Stimulation of indigenous microorganisms involves furnishing organisms with needed resources that limit degradation of a specific contaminant or a suite of contaminants. The initial steps in the aerobic degradation of hydrocarbons by bacteria and fungi involve substrate oxidation, a process requiring the presence of molecular oxygen. In surface soils and the vadose zone, aerobic conditions

Figure 3. Aerobic metabolism of a substrate to cellular components and CO₂ by an attached bacterium.
normally prevail and can be maintained by adequate surface drainage or a periodic raising and lowering of the groundwater. Oxygen concentrations in contaminated aquifers, however, are very low (O$_2$ concentration at saturation in water is 8 ppm). The addition of hydrogen peroxide (H$_2$O$_2$) in stabilized, time release formulations has been used to boost O$_2$ concentrations in subsurface waters (Atlas and Barth, 1992). A second approach to stimulating microbial activity involves nutrient addition, the process used in the Alaska oil-spill. The introduction of carbon-rich compounds into a soil or water environment (as occurs in an oil spill) results in a high C to N ratio that is unfavorable for microbial degradation. The addition of N, P, or other limiting nutrients has successfully stimulated bioremediation of contaminants by indigenous organisms (Atlas, 1995; Atlas and Bartha, 1992).

Seeding is the purposeful introduction of organisms into the environment for the sole purpose of increasing the rate and/or extent of hydrocarbon degradation above that capable by native populations. Numbers of organisms capable of degrading specific compounds have been isolated from the environment. Grosser et al. (1991, 1995) demonstrated a significant increase in polyaromatic hydrocarbon (PAH) degradation in contaminated soils by the reintroduction of pyrene degrading microorganisms isolated from the site. Pyrene degradation by indigenous organisms was approximately 1% in 2 d; seeding resulted in 55% degradation in 2 d. Likewise, the addition of chlorobenzoate-degrading bacteria to polychlorinated biphenyl (PCB) contaminated soils resulted in degradation of 25% of the contaminant as compared to only 3% in uninoculated soils (Hickey et al., 1993).
Despite the successes of some bioremediation strategies, several key limitations make widespread application difficult and uncertain. Bacteria possess specific enzymes which are not capable of remediation of complex chemical mixtures, as are commonly found at contaminated sites. Maintenance of mixed microbial populations capable of degrading these mixtures is often difficult or impossible with current knowledge. Moreover, many seeded organisms are unable to compete with indigenous species, and the populations required to achieve enhanced degradation cannot be maintained. Furthermore, many of the most recalcitrant and toxic compounds are often degraded only through cometabolism, whereby another C-source is required to maintain microbial growth. The introduction of co-substrates into natural environments has met with limited success as interactions with other organisms in the environment are complex (Atlas, 1995; Alexander, 1994; Atlas and Bartha, 1992; Thayer, 1991).

Attempts to overcome biological limitations to biodegradation in natural environments have focused on two approaches. One approach has been to genetically engineer strains of bacteria that are competitive in soil environments and have greater degradation capabilities. In 1981, the first patent for a genetically engineered organism with contaminant degrading capabilities was granted in the U.S. However, attempts to modify organisms to produce strains with that can degrade specific pollutants in situ have met with little success. Furthermore, environmental regulations in most countries restrict the release of genetically engineered organisms into the environment (Atlas, 1995).

Another approach has been to use other naturally occurring organisms with enzymatic systems capable of degrading a suite of environmental pollutants. One such
genus of organisms are the white-rot fungi. One species, *Phanerochaete chrysosporium*, uses a non-specific extracellular enzyme system to degrade lignin and has the capabilities to degrade environmental pollutants as well (Barr and Aust, 1994). The properties of white-rot fungi and their applicability to bioremediation will be discussed in further detail in Chapter 3.

**Chemical Sorption Limitations to Bioavailability and Biodegradation**

**Chemical Sorption to Soil**

A major limitation to successful bioremediation is reduced substrate bioavailability due to chemical sorption. Chemical sorption in soils includes both adsorption, the retention of a molecule on the surface of a solid material, and partitioning.

![Figure 4. Sorption in soil involves both adsorption to surfaces and partitioning into the bulk solid.](image-url)
the retention of solute within the bulk solid (Figure 4). Adsorption occurs primarily to the mineral, clay, and oxide surfaces in soils; partitioning, especially of nonpolar organic chemicals (NOCs), occurs primarily in the organic fraction of soil. Sorption of NOCs is strongly influenced by two factors—the hydrophobicity of the compound and the amount of organic carbon present in the soil.

Chemical hydrophobicity may be expressed in terms of an octanol-water partition coefficient ($K_{ow}$), or the ratio of the chemical concentration found in octanol to the concentration present in water (in an octanol-water system):

$$K_{ow} = \frac{S_o}{S_w}$$

where $S_o$ (mg L$^{-1}$) is the chemical concentration in octanol and $S_w$ (mg L$^{-1}$) is the chemical concentration in water. The higher the $K_{ow}$, the more hydrophobic the chemical, the lower the aqueous solubility, and the greater the amount of chemical sorption that will occur (Chiou, 1989).

SOM is a chemically complex structure resulting from the decomposition and synthesis that occurs during the degradation of organic materials, primarily plant tissues (Brady, 1990); SOM is principally composed of C, H, and O. The surface of an organic matter aggregate is hydrophilic and consists of negatively charged hydroxyl, carboxylic, and phenolic functional groups. The internal structure is predominantly hydrophobic and is held together by covalent bonds, van der Waal forces, and dipole interactions. The quantity of SOM present directly affects the amount of NOC sorption that occurs in a soil (Chiou, 1989).
Chemical sorption to soils is expressed as a partition coefficient \( (K_d) \) that relates the amount of chemical in the sorbed phase to the concentration in the aqueous phase:

\[
K_d = \frac{C_{\text{sorbed}}}{C_{\text{aqueous}}}
\]

where \( C_{\text{sorbed}} \) is the concentration of sorbed compound (mg kg\(^{-1}\)) and \( C_{\text{aqueous}} \) is the aqueous concentration of compound in equilibrium with the sorbed phase (mg L\(^{-1}\)). Since the primary sorptive phase in soil is organic matter, the partition coefficient is commonly normalized to organic matter, \( K_{\text{om}} \):

\[
K_{\text{om}} = \frac{K_d}{X_{\text{som}}}
\]

where \( X_{\text{som}} \) is the fraction of the soil composed of organic matter. To account for structural and chemical variations in SOM, the partition coefficient can also be expressed in terms of soil organic carbon, \( (K_{\text{oc}}) \):

\[
K_{\text{oc}} = \frac{K_d}{X_{\text{oc}}}
\]

where \( X_{\text{oc}} \) is the fraction of organic carbon in the soil. Note that in all three expressions, larger partition coefficients correspond to greater sorption to soil (Chiou, 1989).

Mechanisms of Sorption to Soil

Two theories exist as to how organic molecules are sorbed to soil. The first view maintains that the process is governed by sorption to discrete sites on solid surfaces located in micropores within the soil matrix. The second theory holds that NOCs partition into the physical matrix of SOM and are distributed throughout the volume (Alexander, 1994). Sorption governed by the first mechanism is limited by a fixed
number of sorption sites and exhibits a plateau effect at high concentrations. Chiou (1989), however, presented strong evidence in favor of the partitioning mechanism. He noted that NOC isotherms showed strong linearity over a large range of concentrations and that sorption was strongly dependent on the quantity of SOM present. Most likely, both mechanisms contribute to NOC sorption to SOM (Pignatello and Xing, 1996). The dominating mechanism, however, will influence the thermodynamics and kinetics of contaminant removal from the sorbed state.

Sorption of NOCs to soil generally occurs as a two step process. First, a period of rapid, reversible NOC sorption to the soil occurs, which is driven by surface sorption. This is followed by a second period of slow sorption whereby the contaminant partitions into the solid matrix. Over long contact times, the period of slow sorption can result in $K_d$ values 30% greater than those obtained after a short equilibration time (Pignatello and Xing, 1996). Historically contaminated (aged) soils where NOCs have been present for months or years can be enriched in the slow fraction (Fu et al., 1994; Pignatello, 1989); consequently, aged chemicals are observed to be highly resistant to degradation as compared to freshly added chemicals (Hatzinger and Alexander, 1995; Steinberg et al., 1987; Fu et al., 1994). Poor understanding of sorption and desorption kinetics and their impact on bioavailability can result in poor planning of bioremediation schemes.

**Sorption and Bioavailability**

Reduced bioavailability due to contaminant sorption does not necessarily preclude degradation, but does severely limit the rate and extent of contaminant transformation. Several factors may be responsible for reduced degradation rates of sorbed compounds by
soil organisms: (1) desorption kinetics are slow enough that mass-transfer essentially limits degradation; (2) sorbed compounds cannot enter the cell and be acted on by intracellular enzymes; (3) extracellular enzymes are subject to sorption and lose catalytic activity; (4) sorbed compounds may be less available and accessible to extracellular enzymatic attack; (5) additional growth factors and nutrients may be sorbed and less available for microbial growth and activity; (6) near surface conditions may be depleted in nutrients or have a lowered pH, making it unfavorable for microbial growth and survival; and (7) the microbes themselves are attached and consequently have limited access to the contaminant molecules (Alexander, 1994).

Much discussion has centered on the availability of sorbed molecules for microbial degradation. One line of reasoning contends that organisms use only chemicals that are in solution; consequently, degradation is rate-limited by desorption kinetics. Furthermore, some microorganisms excrete extracellular metabolites that facilitate desorption and degradation. Guerin and Boyd (1992) examined the degradation of naphthalene sorbed to soil using two naphthalene-degrading organisms and observed both processes. One organism utilized naphthalene at the rate the compound desorbed, and degradation was limited by substrate solubility. The rate and extent of naphthalene degradation by a second organism, however, exceeded predictions made by desorption kinetics alone, indicating that the organism was enhancing desorption rates or was utilizing sorbed substrate. Another theory suggests that bacteria attached to surfaces degrade sorbed substrates and that the proximity between organism and substrate is responsible for degradation. Harms and Zehnder (1995) observed that the bioavailability
and degradation of 3-chlorodibenzofuran sorbed to teflon increased with bacterial attachment to the solid surface, suggesting that organism attachment facilitated contaminant availability.

The Role of Surfactants in Remediation

Surfactant Basics

Surfactants (SURFace ACTive AgeNTS) have received considerable attention for their potential to increase the aqueous solubility of sorbed contaminants and potentially enhance bioavailability in soil environments. Surfactants are amphipathic molecules, containing both hydrophilic and hydrophobic moieties; they tend to migrate to surfaces and interfaces or form new molecular surfaces by forming micelles. In aqueous solution, surfactants cause a reduction in surface and interfacial tensions by the interaction between the surfactant and the H-bonding of water molecules. These properties have led to widespread production and usage of surfactants as solubilizing agents in a variety of applications.

Surfactants can be classified into four categories based upon the charge of their polar moiety: (1) cationic, containing a positive charge, (2) anionic, containing a negative charge, (3) zwitterionic, containing both a positive and negative charge, and (4) nonionic, containing no charged functional groups (Figure 5). An important parameter of surfactants is the hydrophile-lipophile balance number (HLB). This parameter is a ratio between the molecular weight of the polar portion of the molecule and the molecular
weight of the entire structure; low HLB numbers are indicative of surfactants with high hydrophobicity (Rosen, 1989).

A primary solution characteristic of surfactants is their ability to form aggregates called micelles. Micelles are a spherical arrangement of surfactant monomers formed by the association of the hydrophobic groups, creating a nonpolar pseudophase in a hydrophilic shell. Surfactants with low HLB numbers form micelles with large hydrophobic cavities as compared to the polar exterior. The concentration at which micelles form is known as the critical micelle concentration (CMC). At concentrations below the CMC, surfactants exist in solution primarily as individual monomers; surfactant monomers also associate with surfaces, forming structures known as hemimicelles. As the concentration of monomers increases up to the CMC, the surface

<table>
<thead>
<tr>
<th>Nonionic</th>
<th>Anionic</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{HO(C}_2\text{H}_4\text{O)}_x\text{OH} )</td>
<td>( \text{CH}_3\text{-(CH}<em>2\text{)}</em>{10}\text{-CH}_2\text{-O-SO}_3^- )</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cationic</th>
<th>Zwitterionic</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{N-(C}_2\text{H}_4\text{O)}_x\text{H}_2^+ )</td>
<td>( \text{H}_2\text{N-CH}_2\text{-CH}_2\text{-COO}^- )</td>
</tr>
</tbody>
</table>

Figure 5. Surfactants can be classified into four groups based upon the charge of the polar group—nonionic, anionic, cationic, and zwitterionic—containing no charge, a negative charge, a positive charge, and both positive and negative charges, respectively.
tension of the solution decreases. At concentrations above the CMC, a constant monomer concentration is maintained in solution by equilibrium with the micelles and hemimicelles, and the surface tension of the solution becomes relatively constant.

Another characteristic of surfactants is their ability, under certain conditions, to form emulsions between two immiscible liquids. A sudden drop in interfacial tensions that can accompany the addition of surfactants in a two-phase liquid system results in the formation of fine droplets of one liquid in another (e.g. oil in water) (Rosen, 1989; Rouse et al., 1994). Emulsions can play an important role in the degradation of immiscible liquids (i.e. oil in water) by increasing the surface area of the immiscible phase.

Solubility Enhancement of NOCs in Aqueous Solutions

The aqueous solubility of NOCs can be enhanced by the addition of surfactants above the CMC. The hydrophobic core of a micelle acts as a partitioning phase for compounds with low water solubility. Kile and Chiou (1989) observed enhanced solubility of both 1,1-bis(p-chlorophenyl)-2,2,2,-trichloroethane (DDT) and trichlorobenzene (TCB) in six commercial surfactant solutions above the CMC. The solubility enhancement of the more insoluble compound, DDT, was more pronounced than that observed for TCB. The effect was also seen to vary with surfactant structure. With the nonionic surfactants studied, the solubility of NOCs increased proportionally with the length of the nonpolar chain content. Moreover, the solubility of DDT also increased at concentrations below the CMC—a phenomenon that has been observed for only compounds with extremely low water solubility—presumably due to interactions
between DDT and the surfactant monomers. However, increases in NOC solubility are normally directly related to micelle concentration. A linear relationship was observed between naphthalene, phenanthrene, and pyrene solubility and surfactant concentration in aqueous solution (Edwards et al., 1991). Furthermore, surfactants increase the rate of NOC solubilization. Grimberg et al. (1995) found that the rate of phenanthrene dissolution from solid to aqueous phase increased with increasing surfactant concentration.

Solubility Enhancement of NOCs in Soil Systems

In soil systems, surfactants have been successfully used to solubilize sorbed contaminants (Figure 6). For example, the soil-water partition coefficient ($K_d$) for anthracene was reduced by two orders magnitude with a 1% volume addition of phenylethoxylate surfactant (Liu et al., 1991). Increased solubility can be used to positively influence contaminant transport during soil washing or flushing. Tetrachloroethylene (TCE) and dodecane were successfully flushed from soil columns by the use of surfactant solutions (Pennell et al., 1994; Pennell et al., 1993). However, the rate of contaminant transport for both compounds was rate-limited by desorption. Evidence suggests that desorption kinetics in the presence of surfactants are highly soil dependent. Deitsch and Smith (1995) examined the rate of TCE dissolution from both an organic and a mineral soil treated with 3000 mg L$^{-1}$ Triton X-100. Desorption from the organic soil was rate-limited whereas solubilization from the mineral soil could be approximated assuming instantaneous equilibrium. Regardless of soil type, desorption rates are higher with the addition of surfactant than in the absence of any solubilizing
agent. Deitsch and Smith (1995) observed increased contaminant desorption rates from an organic soil treated with surfactant relative to a no surfactant control.

Contaminant desorption from soils is hypothesized to occur by two mechanisms: (1) micellar solubilization that is thermodynamically driven by the concentration gradient between sorbed and soluble states or (2) sorption and penetration of surfactant molecules to SOM which causes the organic matrix to swell, permitting a greater flux of contaminant from the sorbed to aqueous phase. By use of a radial diffusion model, Yeom et al. (1996) showed that surfactant enhanced PAH release from an organic rich soil-tar was driven by increased matrix diffusivities and not from partitioning to the micellar
pseudophase, suggesting that the influence of surfactants on desorption is due to the second mechanism.

Surfactant introduction into soil-water systems is more complex than pure water systems. Surfactants sorb to the soil, and the quantity of surfactant required to achieve CMC can be significantly higher than that in a pure aqueous system. Liu et al. (1991), using a soil to solution ratio of 1:8, required a surfactant addition that was 40 times greater than the CMC in pure water in order to achieve solubilization of sorbed anthracene. Furthermore, surfactant sorption can actually result in decreased solubility of NOCs. Sorption of Triton X-100 to a sandy soil resulted in increased soil-solution partition coefficients (Kd) for three chlorinated hydrocarbon compounds at surfactant concentrations below the CMC. Once the CMC was achieved, the apparent water solubility for all three compounds increased (Sun et al., 1995). Surfactant sorption is of major consequence in soil systems and needs to be considered in surfactant-aided remediation strategies.

Biodegradation of Organic Contaminants in Soils Treated with Surfactants

The use of surfactants in bioremediation strategies has met with mixed success. A few studies have shown that surfactants can solubilize contaminants and result in enhanced degradation by microorganisms (Thibault et al., 1996; Aronstein and Paterek, 1995; Tiehm, 1995). For instance, Guerin and Jones (1988) noted that phenanthrene mineralization by *Mycobacterium* was enhanced by the use of Tween surfactants above the CMC. The mechanism of contaminant degradation in micelles is unknown, however,
it has been suggested that (i) degradation occurs only after the compound has exited from
the micelle or that (ii) cell-micelle contact (and possible fusion between cell membrane
and micelle) may be responsible (Tiehm, 1995).

Contaminant degradation with surfactants at sub-CMC levels has also been
reported. Aronstein et al. (1991) noted that the degradation of sorbed phenanthrene and
biphenyl occurred in soils treated with surfactants below the CMC. They suggested that
surfactants facilitated mass-transport from the sorbed state and resulted in increased
degradation although contaminant solubility was not enhanced.

In contrast, many studies have shown that the use of surfactants inhibits
mineralization of NOCs in soil systems. Some researchers have suggested that NOCs
partitioned in micelles are unavailable to degrading bacteria (Guha and Jaffé, 1996; Laha
and Luthy, 1991). Others have noted a physiological effect of surfactants on degrading
organisms. Laha and Luthy (1992) reported that the mineralization of phenanthrene was
completely inhibited at surfactant concentrations above the CMC, but commenced after
dilution of surfactant concentrations to sub-CMC levels. Roch and Alexander (1995),
however, noted surfactant toxicity to a phenanthrene degrading *Pseudomonas* species at
concentrations below the CMC. Tiehm (1994) reported that surfactant toxicity to PAH
degrading bacteria was related to surfactant structure; surfactant toxicity decreased with
increasing hydrophilicity. Another theory for the failure of surfactants to aid in
degradation is the preferential use of surfactant as a substrate to the degrading organisms.
The exact mechanisms responsible for the inhibition of contaminant degradation in the
presence of surfactants are not clear. However, it is likely a number of factors contribute
and that degradation rates of NOCs are dependent on both the properties of the surfactant and organism alike.

**Biosurfactants**

It has been recognized that microorganisms produce surfactants (biosurfactants) as a mechanism for increasing accessibility to bound substrates (Rouse et al., 1994; Déziel et al., 1996; Van Dyke et al., 1993). It has been suggested that biologically-produced surfactants may be more effective in remediation strategies for several reasons: (1) unlike many synthetic surfactants, biosurfactants are degraded by microorganisms and may stimulate activity; (2) biosurfactants may be less toxic to organisms; and (3) biosurfactants may be more effective for specific degradation processes in natural environments.

Biosurfactant-producing bacteria are common in soil environments. Déziel et al. (1996) isolated 23 bacteria from a petroleum-contaminated soil that produced biosurfactants while growing on mineral salt agar using naphthalene and phenanthrene as sole C substrates. One of those isolated organisms, a strain of *Pseudomonas aeruginosa*, increased the water solubility of naphthalene when grown in liquid media. Biosurfactants have been used to increase the solubility and transport of contaminants in soils. Cyclodextrins, cyclic oligosaccharides produced by the enzymatic degradation of starch by bacteria, have shown solubilization potential. A cyclodextrin derivative, hydroxypropyl-β-cyclodextrin (HCPD), increased the solubility of six NOCs in solution, with the greatest solubility enhancements occurring with the more insoluble compounds (Wang and Brusseau, 1993). HPCD was also successfully used to flush NOCs through
two soils (Brusseau et al., 1994); a 10 g L\(^{-1}\) HPCD solution was calculated to be 100 times more effective at facilitating pyrene transport through a soil column than water alone.

Bioremediation strategies involving biosurfactants have been well illustrated by research conducted using rhamnolipids. Rhamnolipids produced by *Pseudomonas aeruginosa* strain UG2 (isolated from an oil-contaminated soil) are produced in relatively large quantities and effectively solubilize NOCs in pure solution as well as in soil (Van Dyke et al., 1993; Scheibenbogen et al., 1994). The effectiveness of the rhamnolipids to solubilize NOCs in soils was affected by soil type, contaminant aging, and biosurfactant sorption to soil (Van Dyke et al., 1993).

One remediation approach is to introduce the biosurfactant directly into contaminated soil. Providenti et al. (1995) introduced partially-purified rhamnolipids into a phenanthrene contaminated soil and observed increased mineralization by indigenous microorganisms. When the biosurfactant was introduced with a phenanthrene-degrading organism, mineralization was inhibited, perhaps due to preferential use of the surfactant by the degrading species.

A second remediation strategy is to introduce surfactant-producing organisms into the environment. In the same study by Providenti et al. (1995), inoculation with *Pseudomonas aeruginosa* UG2 alone did not improve mineralization. However, when the soil was inoculated with both the surfactant-producing organism as well as a phenanthrene-degrading organism, enhanced mineralization occurred. It was suggested that inoculation with biosurfactant-producing bacteria alone resulted in the proliferation
of organisms that preferentially used the rhamnolipids as a C-source and therefore
displaced the phenanthrene utilizing species, resulting in decreased degradation.

In summary, the use of biosurfactants in bioremediation is in its infancy. It is
clear that interactions among biosurfactants, organisms, soils, and pollutants are complex
and not completely understood. Further research to clarify surfactant-microbial
interactions may provide answers needed to evaluate the potential of biosurfactants in
bioremediation.

Objectives

Given the extent and number of sites contaminated with NOCs, there is a need to
better define the processes affecting contaminant degradation in soils. Of specific interest
locally is the remediation of two compounds of environmental concern around the state of
Montana, 2,4-dichlorophenoxyacetic acid (2,4-D) and pentachlorophenol (PCP). The
current research focused on the degradation of these compounds in Montana soils.

For 2,4-D the primary objectives were to: (1) compare degradation rates in 31
soils from around the state with widely different SOM contents, (2) determine the
applicability of three kinetic models for describing observed degradation, and (3)
investigate the relationships between 2,4-D sorption to SOM, bioavailability, and
biodegradation rates.

With respect to PCP, the objectives were to: (1) compare the abilities of
indigenous bacteria and white-rot fungi to degrade PCP in contaminated soil, (2)
investigate the effect of surfactant type (i.e. synthetic or naturally-derived) on degradation
by white-rot fungi, and (3) examine the potential mechanisms involved in surfactant-aided PCP degradation by white-rot fungi.

A common goal in both these studies was to examine the impact of contaminant availability on the degradation of environmentally significant pollutants by bacteria and fungi in soil.
CHAPTER 2

BIODEGRADATION OF 2,4-DICHLOROPHENOXYACETIC ACID (2,4-D) IN THIRTY-ONE AGRICULTURAL SOILS

2,4-dichlorophenoxyacetic acid (2,4-D) is a widely used herbicide for the control of broadleaf weeds and is a representative member of a large class of chlorinated pesticides. Although it is generally assumed that 2,4-D degradation rates are fairly rapid in surface soils (half-life: 7-10 d), 2,4-D has been detected at low concentrations in groundwaters around the state of Montana. The primary goal of the current study was to determine relationships among 2,4-D degradation rate parameters, soil organic C (SOC) contents, and 2,4-D sorption coefficients using mineral soils with a wide range in SOC contents. The degradation of $^{14}$C-2,4-D was monitored in batch vessels under aerobic conditions in 31 agricultural soils collected in Montana and treated with 2,4-D concentrations representative of field application rates. Three kinetic models, including first-order, modified first-order, and logistic, were fitted to the observed degradation curves. The modified first-order kinetic model (containing two fitted parameters, the rate constant, $k$, and the maximum extent of degradation, $P_{\text{max}}$) best described the observed production of $^{14}$CO$_2$ from $^{14}$C-2,4-D in all soils. The degradation of 2,4-D across all soils was characterized by short lag phases (less than 1 d), a period of maximum degradation
rate, and a wide range in the extents of degradation ($P_{\text{max}}$ values ranging from 0.2-0.8). Fitted rate constants were not correlated with SOC contents, nor with 2,4-D sorption coefficients, indicating that the extent of 2,4-D degradation may be limited by sorption to the soil organic matter (SOM) phase. Estimates of the total number of soil microorganisms culturable on non-selective media did not correlate strongly with fitted rate constants or values of $P_{\text{max}}$. This study suggests that rate constants taken by themselves (as if often the case for first-order modeling applications) are poor predictors of the fate of 2,4-D in soils due to the fact that extents of degradation are often limited by sorption to the SOM phase.

Introduction

2,4-dichlorophenoxyacetic acid (2,4-D) was the first commercial herbicide introduced in the United States in 1945. In states such as Montana, where significant acreage is used for cereal grain production, 2,4-D remains one of the most commonly used herbicides for broadleaf weed control. Literature values of the half-life ($t_{1/2}$) and sorption coefficient ($K_{\text{oc}}$) of pesticides are often used as input data in fate and transport models (e.g. LEACHM, Wagenet and Hutson, 1987) for predicting groundwater contamination potential. 2,4-D normally exhibits low sorption coefficients ($K_{\text{oc}} = 2.00 \times 10^{-2}$ m$^3$kg$^{-1}$) and relatively short half-lives in soil ($t_{1/2} = 7.1$ days; Jury et al., 1987) and is predicted to be of low pollution potential with respect to many other commonly used pesticides (primarily due to rapid degradation rates). However, many well monitoring surveys, including testing by the Environmental Management Division of the Montana
Department of Agriculture, have identified 2,4-D as a common contaminant of regional groundwaters (DeLuca, 1989).

The degradation of 2,4-D in soils has been shown to depend on numerous soil and environmental properties, including soil moisture, temperature, microbial biomass, soil organic C, soil depth, pore water velocity, and 2,4-D concentrations (Sandmann et al., 1988). Soil bacteria and fungi have both been reported to degrade 2,4-D (Donnelly et al., 1993; Yadav and Reddy, 1993; Stott et al., 1983). Although more work has been conducted to characterize bacteria responsible for 2,4-D degradation in soil, soil degradation studies generally do not differentiate between fungal and bacterial degradation. Greer et al. (1990) found that the duration of lag phases and times required for complete degradation in soil were linearly dependent on the concentration of 2,4-D and bacterial population density. Other researchers have also shown that the length of the lag phase increases with increasing 2,4-D concentrations in soil (Veeh et al., 1996; Parker and Doxtander, 1983; Ou, 1978). Rates of 2,4-D degradation also vary with soil organic matter (SOM) contents (Ogram et al., 1985). Veeh et al. (1996) noted increased rates of 2,4-D degradation in surface soils with greater SOM than in low organic matter subsurface soils. However, Greer et al. (1992) found that microbial 2,4-D degradation in a high organic matter soil (14%) was significantly lower than in a low organic matter soil (1.6%). Ogram et al. (1985) used three mathematical models relating 2,4-D degradation to substrate availability and concluded that sorbed 2,4-D was unavailable for microbial degradation, which suggests that higher SOM contents should result in lower extents of mineralization.
Numerous kinetic models have been used to empirically describe degradation rates of 2,4-D in soils, including first-order, modified first-order, and logistic expressions. Although the simplicity of the first-order model is convenient for estimating half-life values and for coupling degradation kinetics to transport models, it often fails to describe experimentally determined degradation rates in soils (Alexander and Scow, 1989). Microbial growth models such as the Monod and logistic expressions have also been used to empirically describe 2,4-D degradation in soils (Veeh et al., 1996; Estrella et al., 1993). The logistic growth model is particularly robust (three fitted parameters) and is useful for describing the lag period often observed prior to the onset of maximum degradation rates and maximum extent of degradation. Although microbial growth models often adequately describe degradation rates of organic contaminants in soils (due primarily to a greater number of fitted parameters) they do not necessarily reflect the complex soil processes controlling degradation kinetics. For example, the presence of soil aggregates and SOM influences mass transfer rates of organic chemicals to the aqueous phase and plays an important role in the kinetics of microbial degradation. Models based on microbial growth and/or simple first-order kinetics do not account for these effects (Scow and Hutson, 1992). Scow and Alexander (1992) showed that both the rate and extent of phenol degradation were lower in the presence of aggregates, demonstrating the importance of mass-transfer and diffusion processes in soil kinetic models.

The objectives of the current study were to (1) determine the rates and extents of 2,4-D degradation in 31 agricultural soils exhibiting a large range in SOM contents, and (2) evaluate the relationship among SOM contents, 2,4-D sorption coefficients ($K_d$), and
degradation rate parameters obtained from several empirical models used to describe kinetics of 2,4-D degradation. Although we are aware that 2,4-D degradation rates have been extensively studied in soil (Sandmann et al., 1988; Ogram et al., 1985; Greer et al., 1992), one of the unique features of the current study was the large number of soils used (31), which exhibited a wide range in SOM contents.

Materials and Methods

Chemicals

$^{14}$C-uniformly ring-labeled and $^{14}$C-carboxyl-labeled 2,4-D were purchased from Sigma Chemical (St. Louis, MO). Radiopurity was verified by high-pressure liquid chromatography (HPLC) on a C-18 reverse phase column at 254 nm using acetonitrile-0.2% phosphoric acid (25:75) with a flow rate of 1.5 mL min$^{-1}$ ($R_f = 6.5$ min). $^{14}$C was analyzed with a Beckman 171 Radioisotope detector (Beckman Instruments, Inc., Fullerton, CA).

Soils

Thirty-one agricultural soils from across Montana (Table 1) were collected from the surface horizons (top 30 cm) and stored field moist at 4°C until use. The majority of these soils were collected as pairs from adjacent locations representing the same soil series, but differing in management history: one soil representing a native range site with presumably no previous application history of 2,4-D, the other from a crop-fallow site which had been cultivated for nearly 50 y and had possibly seen several applications of 2,4-D (Neill, 1994).
Table 1. Soil series, locations, land-use specifications, texture, pH, EC, and soil organic carbon for the 31 soils used in the study.

<table>
<thead>
<tr>
<th>Soil Code</th>
<th>Soil Series</th>
<th>Location</th>
<th>Land Use</th>
<th>Sand</th>
<th>Silt</th>
<th>Clay</th>
<th>Class</th>
<th>pH</th>
<th>EC*</th>
<th>C (%)</th>
<th>Organic Carbon (L kg⁻¹)</th>
<th>K₄</th>
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<td>AMST</td>
<td>Amsterdam</td>
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<td>52</td>
<td>33</td>
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<td>0.50</td>
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<td>28</td>
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Batch Biodegradation

Fifteen g of sieved, field-moist soil were placed in 25 mL screw-cap erlenmeyer flasks (in duplicate) equipped with a gas exchange system (Figure 7). $^{14}$C-carboxyl-labeled 2,4-D (0.12 μCi) was added to the soil, simulating an application of 0.2 kg ha$^{-1}$ incorporated to a depth of 15 cm. Soil moisture was adjusted to $\theta_m = 0.36$ (mass basis) using double-deionized (DD) water. Evolved $^{14}$CO$_2$ was trapped in 10 mL 0.5 M NaOH and analyzed every 12 to 48 h using liquid scintillation analysis (Tri-Carb Liquid Scintillation Analyzer, Packard Instrument Co.). Soils were not analyzed for metabolites since previous studies have indicated that accumulation of metabolites is less than 5-10%
of the added 2,4-D (Ou, 1978; Smith and Aubin, 1991) and considering that removal of the carboxyl group is the first step in the degradation pathway (Aislabie and Lloyd-Jones, 1995; Kunc and Rybářová, 1983). At the conclusion of each experiment, a soil subsample was analyzed for total $^{14}$C by oxidation at 800°C under an $O_2$ atmosphere for four min (Biological Oxidizer Model OM300, R.J. Harvey Instrument Corp., Hillsdale, NJ). Average total recoveries of duplicate treatments exceeded 90%.

**Batch Sorption**

The sorption of 2,4-D was measured in 25 mL polypropylene centrifuge tubes using 15 g soil and 15 mL DD water containing an equivalent $^{14}$C ring-labeled 2,4-D concentration as in the biodegradation experiments. Microbial activity was inhibited using 0.02% sodium azide, verified by the absence of $^{14}$CO$_2$ evolution and greater than 90% recovery of total 2,4-D added to sorption treatments. Samples were equilibrated for 48 h on a table shaker, centrifuged, the supernatant filtered through a 0.45 μm nylon filter, and analyzed for $^{14}$C by liquid scintillation. Blanks containing no soil were used to verify that no measurable sorption occurred to centrifuge tubes. Sorption was determined as the difference between total $^{14}$C added and $^{14}$C in the supernatant.

**Bacterial Plate Counts**

An estimate of total bacterial numbers was determined for six of the 31 soils (bracketing a wide range of SOC contents, 0.26-3.72%) by plating soil dilutions on Yeast Extract-Peptone-Glucose media consisting of 5 g peptone, 5 g yeast extract, 2 g glucose, 15 g bacto agar, and 1 L water. Ten g of soil was added to milk dilution bottles containing 90 mL of phosphate buffer (1.67 g KH$_2$PO$_4$, 1.35 g K$_2$HPO$_4$) adjusted to pH
7.0, and shaken on a table shaker for 1 h. Seven serial 1:10 dilutions were made and 100 μL plated in triplicate. The plates were incubated at 30°C for 10 d and colony forming units (CFUs) determined from the average of plates containing between 30 and 300 colonies.

Analytical Methods

Three kinetic models were evaluated for their ability to describe 2,4-D degradation (by non-linear regression). The three models were chosen for their simplicity and possible application for degradation subroutines in transport models.

First-order kinetics:

\[ \frac{C}{C_0} = 1 - e^{kt} \]

where \( C/C_0 \) is the fraction of total \(^{14}\text{C}-2,4\text{-D} \) evolved as \(^{14}\text{CO}_2 \), \( k \) is the first-order rate constant, and \( t \) is time.

Modified first-order kinetics (Ogram et al., 1985):

\[ P = P_{\text{max}} (1 - e^{kt}) \]

where \( P \) is the fraction of total \(^{14}\text{C}-2,4\text{-D} \) evolved as \(^{14}\text{CO}_2 \), \( P_{\text{max}} \) is the maximum extent of mineralization in terms of total \(^{14}\text{C} \) added, \( k \) is the rate constant, and \( t \) is time.

Logistic Equation (Characklis, 1990):

\[ X = \frac{X_0 e^{kt}}{1 - \left( \frac{X_o}{X_m} (1 - e^{kt}) \right)} \]

where \( X \) is the fraction of \(^{14}\text{C}-2,4\text{-D} \) evolved as \(^{14}\text{CO}_2 \), \( X_0 \) is the initial fraction of CO2 evolved, \( X_m \) is the maximum extent of mineralization, \( k \) is the rate constant, and \( t \) is time.
Results and Discussion

Variation in 2,4-D Degradation Among Soils

Degradation curves for 2,4-D are shown for six of the 31 soils (Figure 8) to illustrate the short lag phases, the period of maximum degradation rates, and the wide range in extents of 2,4-D mineralization (20-80%). Degradation curves for all soils were typified by relatively short lag phases (< 24 h), and there was no evidence that lag phases were longer in native range (NR) soils with limited prior exposure to 2,4-D (i.e. no significant acclimation period by microorganisms prior to the onset of degradation). The observed lag phases were shorter than those commonly reported in previous studies. The

Figure 8. Degradation curves showing the fraction of $^{14}$C-2,4-D evolved as $^{14}$CO$_2$ from six of the 31 soils. The organic carbon content (SOC, %) of the soil is shown adjacent to each curve.
length of the lag phase has been shown to increase with increasing initial 2,4-D concentrations (Greer et al., 1990; Parker and Doxtander, 1983; Ou, 1978); consequently, the short lag phases observed in this study may relate to the low initial 2,4-D concentrations (used to reflect typical 2,4-D application rates).

The first-order kinetic model (one fitted parameter) did not adequately describe 2,4-D degradation in most of the soils studied (Table 2) and resulted in particularly low $r^2$ values for soils exhibiting higher maximum degradation rates and lower extents of 2,4-D degradation (e.g. WILL; Table 2). First-order kinetics assume that all of the added 2,4-D is available for degradation. The aqueous 2,4-D concentration in soils with high SOM can be substantially less than that originally introduced, due to sorption. Consequently, the first-order model is limited in its capability to describe degradation in soils exhibiting large amounts of 2,4-D sorption. The modified first-order equation includes a second parameter ($P_{\text{max}}$ term) that empirically accounts for the limited availability of 2,4-D, and was more successful in describing the observed degradation kinetics. The logistic equation includes a third fitted parameter, which is useful in describing the lag phase commonly observed prior to the onset of maximum degradation rate. Since only very short lag phases were observed in the majority of the soils studied, the logistic equation did not provide a better description of 2,4-D degradation kinetics compared to the modified first-order model.

The logistic and modified first-order expressions resulted in comparable parameter estimates of the extent of 2,4-D degradation ($X_m$ and $P_{\text{max}}$) and degradation rate constants. First-order rate constants were significantly lower than those fitted with the modified first-order or logistic models, due to the fact that the first-order model does
Table 2. Comparisons among the three models used to describe observed 2,4-D degradation in the 31 soils studied.

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not compensate for extents of degradation that are less than one. Consequently, first-order rate constants would not be accurate predictors of the long-term fate of 2,4-D in these soils. Both the modified first-order and logistic models adequately fitted the extent of degradation (P_max) obtained from the plateau region of the 2,4-D degradation curves. The extent of degradation is probably a function of both the amount of 2,4-D initially available for degradation, and the amount of 2,4-D that diffuses to sites of microbial activity via mass transfer. Consequently, once the fairly labile 2,4-D is utilized, degradation rates become limited by slow mass transfer rates from the SOM phase (Pignatello and Xing, 1996). Scow and Hutson (1992) and Scow and Alexander (1992) have also shown that degradation rates of phenol were limited by diffusion rates out of well-characterized polyacrylamide gel-exclusion beads, indicating that mass transfer rates are important in describing degradation rate constants and maximum extents of substrate degradation.

Relationships Among 2,4-D Degradation, SOC, and 2,4-D Sorption

Soil organic carbon (SOC) has been shown to negatively correlate with degradation rates and extents of nonpolar organic chemicals in soil environments (Greer et al., 1992; Ogram et al., 1985; Gordon and Millero, 1985). It is well established that “aging” of organic contaminants in soils or in SOC phases further reduces effective desorption rates and bioavailability to degrading microorganisms (Pignatello and Xing, 1996). In the current study, fitted rate constants from neither the modified first-order nor the logistic models correlated well with SOC (r^2 values < 0.01; Figure 9). Further, when soils were grouped as native-range or crop-fallow, no correlation between degradation
rate constants and SOC was observed. However, fitted values of $P_{\text{max}}$ from the modified first-order model (or $X_m$ in the logistic model) were negatively correlated with SOC ($r^2 = \ldots$

Figure 9. Top figure: Comparison of fitted 2,4-D degradation rate constant (h$^{-1}$) from the modified first-order model to SOC. Bottom figure: Maximum extent of degradation fitted from the modified first-order model compared to SOC.
suggesting that sorption of 2,4-D in the SOC phase limited bioavailability. The relationship between extent of degradation and SOC was much stronger among the crop-fallow soils ($r^2 = 0.65$) than for native range soils ($r^2 = 0.48$). It is uncertain whether this is just coincidental or whether it may relate to changes in characteristics of the SOM phase after long-term cultivation. Most importantly, among all soils, no correlation was observed between fitted rate constants and values of $P_{\text{max}}$ (or $X_m$ in the logistic model; Figure 10), indicating that the rate constant taken by itself becomes a poor predictor of the extent of degradation and the long term fate of 2,4-D in soil. This has important consequences for modeling 2,4-D degradation rates in soils, in that rate constants are often used as the only parameter to describe disappearance of

Figure 10. Comparison of the degradation rate constant ($h^{-1}$) and $P_{\text{max}}$, values obtained from modified first-order model.
organic compounds; it is clear that this approach would grossly overestimate the microbial degradation of 2,4-D in the majority of soils studied.

We have already indicated that extents of degradation (P_max) were negatively correlated with SOC contents (Figure 9). As expected, the sorption coefficients (K_d) of 2,4-D across all soils was also correlated with SOC contents (r^2 = 0.61, the slope of this plot yields an average K_oc value of 400 L kg^{-1}; Figure 11). However, when the extent of degradation was plotted against soluble fractions of 2,4-D (calculated based upon K_d values obtained from batch experiments) present at the start of each experiment, only a weak positive relationship was observed (Figure 12). This plot is essentially the inverse of a plot of P_max vs K_d. Interestingly, the relationship between the soluble fraction of 2,4-
Figure 12. Maximum extent of $^{14}$C-2,4-D degradation plotted against soluble 2,4-D in solution at $t = 0$ (calculated from $K_d$). Solid line is the observed relationship between available and degraded 2,4-D.

D present at time 0 (from the $K_d$ values) and extents of degradation was considerably poorer than the relationship of $P_{\text{max}}$ vs SOC contents. Although we expect that $K_d$ values or soluble fraction of 2,4-D are indeed important in defining the extent of degradation, the $K_d$ parameter does not account for mass transfer rates of slow desorption, which may be the more important mechanistic explanation for the strong correlation between $P_{\text{max}}$ and SOC. Although $K_d$ values are reflective of SOC contents and probably mass transfer rates of 2,4-D out of the sorbing phase as well, they do a poorer job describing the variation in $P_{\text{max}}$ values than SOC contents. A more appropriate parameter for explaining extents of degradation across all soils may be the effective desorption rate constant (e.g. mass transfer rate). We would expect that this parameter would be highly correlated with
SOC contents, and may explain why we observed better correlation between SOC and extents of 2,4-D degradation.

Relationships between Degradation and Soil Bacteria Numbers

Veeh et al. (1996) showed that 2,4-D degradation rates were positively correlated with SOC among different soil depths of the same soil profile. It was suggested that higher OC contents were simply reflective of higher microbial numbers and activity, resulting in higher degradation rates for surface soils compared to subsoils (B and C horizons). Other investigations have shown that the size of the 2,4-D-degrading population affects degradation rate. For instance, Cullimore (1981) found that increased degradation rates corresponded to increases in bacteria numbers in five soils, determined through a most probable number technique. In the current study, CFU estimates for six of the studied soils showed a weak positive correlation with SOC as has been previously reported. However, no relationships were observed between bacterial plate counts and maximum extent of 2,4-D degradation or degradation rate constants (Figure 13). Relative microbial estimates in soils based upon bacterial growth on non-selective media appear to be a poor indicator for 2,4-D degradation rates.

Moisture Effects on 2,4-D Degradation

Parker and Doxtader (1983) reported variations in the microbial degradation of 2,4-D under different soil moisture tensions. They reported greater rates and extents of 2,4-D degradation in soils with lower soil moisture tension (or higher water contents). Each of the 31 soils in the current study was brought to the same moisture content
Figure 13. Relationship between fitted extent of 2,4-D degradation ($P_{\text{max}}$), rate constants, and SOC to the number of colony forming units of bacteria isolated on non-selective media six soils (bracketing the range in SOC contents observed for the soils studied).

gravimetrically. To insure that large variations in observed 2,4-D degradation were not attributable to moisture effects, two soils were treated at five moisture contents. The rate
and extent of 2,4-D degradation in both soils was relatively insensitive to moisture variations ranging from 20-40% (Figure 14) although small decreases in the extent of mineralization were observed at $\theta_m = 0.22$, where low water contents may have reduced

Figure 14. Effect of moisture content on the degradation of 14C-ul-2,4-D in two soils. Moisture content of 0.55 is above saturation; 0.36 is approximately field capacity.
2,4-D mass transfer rates to sites of microbial activity. Both the Brocko and Amsterdam Soils exhibited slower rates of degradation at $\theta_m = 0.55$ (hyper-saturated conditions), than under unsaturated conditions (Figure 14). However, the extent of 2,4-D mineralization under saturated conditions was not significantly affected after 40 d of incubation. In summary, small variations in soil water contents among soils would not be expected to be responsible for the differences in 2,4-D degradation observed in the current study.

**Anaerobic versus Aerobic Degradation**

Finally, a brief study on the impact of anaerobic conditions on ring cleavage and 2,4-D mineralization was undertaken. Reductive dechlorination of halogenated pesticides such as 2,4-D has been reported to occur under anaerobic conditions (Kuhn and Suflita, 1989). Dechlorination of aromatic rings can act as a detoxification step in pesticide degradation and help to facilitate ring cleavage. Figure 15 illustrates that the incubation of $^{14}$C-UL-ring labeled 2,4-D under a N$_2$ atmosphere showed almost no $^{14}$CO$_2$ production as compared with soils under aerobic conditions. When the system was changed to an aerobic environment, very little variation in extent of degradation was observed from that under aerobic conditions. Pretreatment of 2,4-D with incubation under N$_2$ for 1000 hours followed by aerobic conditions did not appear to appreciable change $^{14}$CO$_2$ production from uniformly ring labeled 2,4-D as compared to purely aerobic conditions (Figure 15).
Figure 15. Effect of soil preconditioning under anaerobic conditions on the mineralization of 2,4-D in Amsterdam and Brocko soils ($\theta_m = 0.46$). A: $^{14}$CO$_2$ evolution from $^{14}$C-ul-ring-labeled 2,4-D under aerobic conditions; B: $^{14}$CO$_2$ evolution from $^{14}$C-ul-ring-labeled 2,4-D preconditioned under anaerobic conditions, then exposed to aerobic conditions.
Summary

The biodegradation of 2,4-D showed wide variation among 31 agricultural soils. Modeling 2,4-D degradation with first-order kinetics yielded poor predictions of degradation behavior and pesticide half-life in soil. Accounting for pesticide availability through the use of the modified first-order equation or logistic equation yielded more accurate predictions of both the rate and extent of degradation. SOC appears to be the dominant influence on bioavailability and degradation, as sorbed 2,4-D becomes less available for microbial degradation. It has been suggested that soluble 2,4-D is most easily degraded; however, solubility is controlled by both the extent of sorption and the mass transfer rates of desorption from SOC. Most importantly, the use of $K_d$ values and degradation rate constants alone could not accurately predict the degradation and persistence of 2,4-D in these soils. Pesticide degradation in soil environments is governed by a number of soil and environmental parameters that make degradation predictions difficult and may require that soil specific evaluations be made on a site by site basis.
Pentachlorophenol (PCP) is a common and persistent contaminant of soils at wood-preserving facilities. White-rot fungi have been identified as possessing enzyme systems capable of degrading PCP; however, degradation is often limited by contaminant sorption to soils. Surfactants can be used to minimize this limitation by increasing the bioavailability of sorbed hydrophobic molecules in soils. _Phanerochaete chrysosporium_ and a non-_Phanerochaete_ fungus were evaluated for their abilities to degrade $^{14}$C-PCP applied to an agricultural soil amended with 5 surfactants (lignosulfonic acid, sodium salt; lignosulfonic acid, sodium salt, acetate; hydroxypropyl-$\beta$-cyclodextrin; Tween 80; and Triton X-100). Each surfactant was applied to the contaminated soil at 25 mg g$^{-1}$ soil, yielding additions of C as surfactant similar to native soil organic C contents. Mineralization was measured as the total amount of $^{14}$CO$_2$ recovered from soil columns flushed with air. In four weeks, the non-_Phanerochaete_ fungus mineralized a greater amount of PCP than either _P. chrysosporium_ or the indigenous microflora. The addition of Tween 80 at sub-CMC levels resulted in mineralization of 26% of the added PCP versus 17% in a surfactant-free system. Total extractable PCP decreased to less than 10%
for both treatments. Enhanced PCP mineralization by the non-*Phanerochaete* fungus in the presence of Tween 80 appeared to be due to increased fungal activity, as indicated by increased respiration rates and fungal biomass, and not due to solubility enhancement of the contaminant. The use of surfactants, such as Tween 80, in combination with white-rot fungi may represent a strategy for the bioremediation of residual PCP in contaminated soils.

**Introduction**

Pentachlorophenol (PCP) has been extensively used as a fungicide and insecticide for the preservation of wood. Its ability to uncouple oxidative phosphorylation has led to widespread usage in the environment as a pesticide. Large amounts of chlorophenols, PCP in particular, have been detected in soils, natural waters, sediments, plant tissues, and human urine. PCP concentrations in soils at sawmills and wood-preserving facilities have been reported at concentrations up to several thousand ppm, with the highest levels associated with dipping basins or storage areas (Boyd, 1989). PCP is extremely toxic to plants and has been linked to deaths in fish as well as livestock. Human exposure occurs primarily through the food chain and it has been estimated that humans are exposed to 16 μg day\(^{-1}\) (Etoxnet, 1997; Boyd et al., 1989; Hattemer-Frey and Travis, 1989). Commercial formulations often include other highly toxic chemicals such as dioxins, which are often more toxic than PCP itself. Because of its extreme toxicity, PCP use is being phased out in the US and is currently listed as a restricted use pesticide. However, due to historic use, chlorinated phenols are ubiquitous in the environment and the US
EPA has listed PCP along with five other chlorophenols as priority pollutants (Etoxnet, 1997; Hale et al., 1994).

PCP is a weak acid (pKₐ = 4.35), and its aqueous solubility varies as a function of pH. Its solubility is commonly reported to be 14 mg L⁻¹ at pH 7, but can range from 3 mg L⁻¹ at pH 3 to 10,000 mg L⁻¹ at pH 9 (Arcand et al., 1995). Both the protonated and dissociated forms sorb strongly to soil organic matter (SOM) through a partitioning process whereby the compound diffuses into the organic matrix (log Kₐc = 4.51). Incorporation into SOM by oxidative coupling (a covalent bonding process) results in immobilization, reduced desorption rates, and microbial resistance (Alexander, 1994; Boyd, 1989). The persistence of PCP in soil has been reported to range between 14 d and 5 y, depending on environmental conditions (Hale et al., 1994).

Microbial degradation is often the primary mechanism of PCP removal from soil. Microbial degradation can occur under both aerobic and anaerobic conditions. Although aerobic bacteria capable of degrading PCP have been isolated from the environment, aerobic degradation is slow (half-lives of 45 to >72 d have been reported; Etoxnet, 1997; Boyd, et al., 1989; Hale et al., 1994). Anaerobic organisms are more effective degrading PCP through a process of reductive dechlorination followed by ring cleavage (half-lives of 15 to 30 d have been reported; Etoxnet, 1997; Boyd, et al., 1989). Bioremediation of PCP contaminated sites has shown significant promise; however, an acclimated microbial population is a necessary precursor for both aerobic and anaerobic degradation to occur. A number of investigators have found that the addition of PCP degrading bacteria to contaminated soils resulted in increased rates of degradation. Edgehill and Finn (1983)
found that the addition of $10^6$ *Arthrobacter* cells capable of degrading PCP per g of dry soil reduced the half-life of the pesticide from two weeks to less than one day. In contrast, Mueller et al. (1991) found that indigenous soil microorganisms were ineffective at removing significant quantities of PCP from coal-tar creosote contaminated groundwater over two weeks. In general, PCP is toxic to most bacteria at levels far below those commonly found in contaminated soils and alternate remediation techniques have been investigated.

Recently, numerous studies have reported that white-rot fungi may be used for the biodegradation of PCP in soils. White-rot fungi degrade lignin, the structural component of wood, via a free-radical process that is non-selective and non-stereospecific (Barr and Aust, 1994; Fernando et al., 1994). Two major peroxidases have been identified in *Phanerochaete chrysosporium* that are involved in the degradation process: lignin peroxidase (LiP) and manganese-dependent peroxidase (MnP). These enzymes are generally expressed under lignolytic conditions where C, N, or S is limiting. The non-selective nature of these enzymes has led to the observation that *P. chrysosporium* can degrade a number of environmentally recalcitrant compounds, including: polychlorinated biphenyls (PCBs), chlorinated phenols (e.g. PCP), polyaromatic hydrocarbons (e.g. phenanthrene, anthracene), polychlorinated hydrocarbons (e.g. aldrin and lindane), chlorinated aromatics (e.g. DDT), and munitions wastes (e.g. TNT, RDX) (Fernando et al., 1994; Khindaria, 1995; Aust, 1990).

The mineralization and transformation of PCP by white-rot fungi has been well documented in both liquid culture and soils (Mileski et al., 1988; Lin et al., 1990; Lamar...
et al., 1990; Lamar et al., 1994; Brodkorb and Legge, 1992; Lamar et al., 1993; Lamar and Dietrich, 1990; Morgan et al., 1993). The first step in PCP degradation is methylation of PCP to pentachloroanisole (PCA), believed to be a detoxification mechanism. Recently, Leštan and Lamar (1996) developed a fungal inoculum for the bioaugmentation of contaminated soils that successfully introduces white-rot fungi into soil in alginate pellets. In field tests, up to 90% of the PCP was degraded using this process. The use of white-rot fungi provides several advantages to the degradation of highly toxic and recalcitrant compounds: (1) they are naturally occurring organisms; (2) they produce nonspecific extracellular enzymes; (3) they can degrade mixtures of compounds; (4) they require no prior conditioning to the contaminants; (5) they can be cultivated on inexpensive growth media; and (6) they can survive under nutrient limiting conditions.

Despite the promise of white-rot fungi to degrade environmental pollutants in situ, the bioavailability of nonpolar organic chemicals (NOCs) such as PCP may limit the extent and rate of degradation. It has been established that both synthetic and naturally-derived surfactants can increase the solubility of a wide range of NOCs in soils (Sun et al., 1995; Sun and Boyd; 1993; Pennell et al., 1993; Kile and Chiou, 1989; Pennell et al., 1994; Wang and Brusseau, 1993; Van Dyke et al., 1993; Brusseau et al., 1994). Solubility enhancement occurs due to partitioning of the NOC from the soil into surfactant micelles, a structural arrangement of surfactant monomers where apolar “tails” form a nonpolar cavity. The formation of micelles occurs when surfactant concentrations exceed the critical micelle concentration (CMC).
The use of biosurfactants and/or naturally-derived surfactants has received interest since they are potentially less toxic to microorganisms. Biosurfactants may also serve as a C source and stimulate biological activity. A recent review of the influence of surfactants on the microbial degradation of organic compounds (Rouse et al., 1994) concluded that deficiencies in knowledge concerning surfactant-aided degradation currently limit attempts to successfully incorporate surfactants into remediation activities. Moreover, the influence of surfactants on fungal growth and NOC degradation is less well documented. However, it has been shown that the addition of surfactants to cultures of *Phanerochaete chrysosporium* resulted in increased ligninase production, suggesting that surfactants may stimulate nonspecific enzymatic degradation (Leštan, et al., 1990; Ashter and Corrius, 1986).

The objectives of the current study were to: (1) compare the abilities of *Phanerochaete chrysosporium*, a non-*Phanerochaete* white-rot species, and indigenous organisms to mineralize PCP in PCP contaminated soil, (2) examine the effect of surfactant type on fungal degradation of PCP, (3) examine the influence of surfactant concentration on PCP mineralization, and (4) determine the relationship between chemical and biological effects of surfactants on fungal degradation of PCP.

**Materials and Methods**

**Chemicals**

$^{14}$C-uniformly ring-labeled pentachlorophenol (PCP) was purchased from Sigma Chemical (St. Louis, MO). Radiopurity was verified by high-pressure liquid.
chromatography (HPLC) on an Econosil C18 10 micron reverse phase column (Alltech Associates, Inc. Deerfield, IL) using acetonitrile-0.2% phosphoric acid (80:20) with a flow rate of 1.0 mL min⁻¹ (R_f = 6.6 min) attached to a Beckman 171 Radioisotope Detector (Beckman Instruments, Inc., Fullerton, CA). Unlabelled PCP was purchased from Sigma Chemical (St. Louis, MO) and a stock solution of 10 mg mL⁻¹ prepared in acetone.

**Soil**

An uncontaminated agricultural soil, the Amsterdam silt loam (Gallatin Co., MT; Table 3), was collected from the surface 15 cm, passed through a 2 mm sieve, and stored field moist at 4°C until use. Prior to use, soils requiring sterilization were autoclaved for 2 h, let rest 48 h, autoclaved for 2 h, and oven dried at 105°C for 24 h; unsterilized soils were air-dried for 48 h. The soil was treated with 2 μCi ¹⁴C-PCP kg soil⁻¹ and additional unlabeled PCP to yield a final concentration of 100 mg kg⁻¹, delivered by spraying a PCP-acetone solution uniformly to soil (20 μL g⁻¹). The ¹⁴C concentration in the soil was verified by oxidation of triplicate soil subsamples at 800°C under an O₂ atmosphere for four min using a Biological Oxidizer, Model OX300 (R.J. Harvey Instrument Co., Hillsdale, NJ), and analyzed using scintillation analysis on a Packard Tri-Carb Liquid Scintillation Analyzer, Model 2200CA (Meriden, CT).

<table>
<thead>
<tr>
<th>% Sand</th>
<th>% Silt</th>
<th>% Clay</th>
<th>Textural Classification</th>
<th>Organic C (%)</th>
<th>CEC (mmol charge kg⁻¹)</th>
<th>pH (1:1)</th>
<th>θ_m (1/3 bar)</th>
</tr>
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<tr>
<td>15</td>
<td>52</td>
<td>33</td>
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<td>1.80</td>
<td>0.21</td>
<td>6.9</td>
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</tr>
</tbody>
</table>
Surfactants

Nine surfactants [lignosulfonic acid, sodium salt; lignosulfonic acid, sodium salt, acetate; hydroxypropyl-β-cyclodextrin; Tween 20, 40, 60, 80, 85; and Triton X-100 (Figure 16)] were purchased from Sigma Chemical Co. (St. Louis, MO). Each surfactant was mixed with deionized water to achieve a concentration that would yield the appropriate surfactant concentration when the soil was brought to field capacity water content (mass basis, $\theta_m = 0.26$).

Figure 16. Structures of the nine surfactants used in the study. The chemical formulas for the Tween series are given as the R group.
Fungi

*Phanerochaete chrysosporium* and a non-*Phanerochaete* species of white-rot fungi were provided by Mycotech Corp. (Butte, MT) growing on a solid substrate. Cultures were stored at 4°C until use. Fungal viability and enzyme production were periodically checked by growing the fungi on malt agar and Poly R-478 indicator agar (Freitag, et al., 1992). Discolorization of the indicator medium signaled positive enzyme production by the cultures.

Batch Biodegradation

Thirty-five g of PCP-labeled soil were mixed with 0.7 g sawdust, 2.1 g solid nutrient supplement (containing 3.1% N and 0.4% P by mass in a slow release formulation), 1.7 g fungal inoculum, and brought to field capacity water content (i.e. 1/3 bar) with double-deionized water or a surfactant containing solution; all treatments were conducted in triplicate. The soil mixture was packed into 2.54 cm diameter by 6 cm length acrylic columns and constantly flushed with humidified compressed air (Figure 17). PCP mineralization was measured as the accumulation of \(^{14}\)CO\(_2\) in 10 mL 0.5M NaOH traps changed every two d for four weeks. At the conclusion of each experiment, soils were mixed and subsamples oxidized in triplicate at 800°C under an O\(_2\) atmosphere for four min using a biological oxidizer. Total \(^{14}\)C recoveries generally exceeded 90%. Soils were solvent-extracted and analyzed for total PCP by HPLC analysis. PCP aging and extractability in the Amsterdam soil was followed by extraction of sterile soils over
the same time period.

PCP extraction and HPLC analysis

Soils were solvent-extracted by mixing 5 g soil, 10 g anhydrous Na₂SO₄ (dried for 2 h at 105°C), one microspatula of Na₂S₂O₄, 9.75 mL 1:1 acetone:hexane, and 0.25 mL concentrated H₂SO₄ in 22 mL glass scintillation vials. Samples were shaken on a table shaker overnight then filtered through a 0.45 μm nylon filter. One mL subsamples were dried under N₂, 1 mL of 0.5 M NaOH added to the residue, and samples equilibrated by shaking overnight. Samples were diluted to a total volume of 5 mL by the addition of 4 mL of 80:20 acetonitrile-0.2% phosphoric acid and passed through a 0.2 μm nylon filter.
before HPLC analysis. PCP was analyzed by UV absorbance at 254 nm after passing though an Econosil C18 10 micron reverse phase column using 80:20 acetonitrile-0.2% phosphoric acid at 1.0 mL min\(^{-1}\) (R\(_f\) = 6.6 min.) Extraction recoveries of PCP from the Amsterdam soil were approximately 90%.

The original solvent extract was analyzed for total remaining \(^{14}\)C by liquid scintillation analysis. Metabolite production was followed in selected experiments using HPLC-radioisotope detection (Beckman 171 Radioisotope Detector). An aqueous extraction of selected soils was also conducted, followed by total \(^{14}\)C analysis using liquid scintillation.

**PCP Sorption and Solubility**

The sorption of Tween 80 to Amsterdam soil was measured in triplicate using batch systems containing 1 g soil and 15 mL of solution (0, 10, 50, 200, 500, 1000, 2000, 4000, 6000, 8000, 10000, and 20,000 mg Tween 80 L\(^{-1}\)) in glass Corex centrifuge tubes. The mixtures were equilibrated for 24 hrs on a table shaker, centrifuged, and surface tension measurements made on the solution using a Cenco-du Nouy Interfacial Tensiometer (No. 70545, Central Scientific Co., Franklin Park, IL). A standard curve relating surface tension to Tween 80 concentration was used to determine Tween 80 sorption to Amsterdam soil.

The sorption of PCP to Amsterdam soil was measured in batch systems (triplicate) using 1 g of contaminated soil, containing 10, 50, 100, 500, and 1000 mg kg\(^{-1}\) PCP (including 2 μCi \(^{14}\)C-label kg soil\(^{-1}\)), and 15 mLs of solution containing 0, 100, 1000, and
10000 mg L\(^{-1}\) Tween 80. The suspensions were equilibrated for 24 h, centrifuged, and 1 mL of supernatant analyzed for total \(^{14}\)C by scintillation analysis. The equilibrium pH values ranged from 6.8 to 7.1. Sorption of PCP was determined as the difference between the total PCP added and the PCP remaining in solution. \(K_d\) values were determined by linear regression using the relationship \(Q = K_d C\), where \(Q\) is the sorbed concentration (mg kg\(^{-1}\)) and \(C\) is the aqueous concentration (mg L\(^{-1}\)), through the measured points.

The solubility enhancement of PCP in the presence of Tween 80 and Amsterdam soil was determined in batch systems (triplicate). Each treatment contained 5 g contaminated soil (100 mg kg\(^{-1}\) PCP) and 10 mL of surfactant solution, yielding a final concentration of 0, 3.6, 7.3, 36.6, and 183.5 mg Tween 80 g soil\(^{-1}\). The equilibrium pH values ranged from 6.8 to 7.1. PCP solubility was determined by analyzing \(^{14}\)C-PCP in solution of 1 mL aliquots by scintillation analysis.

**Fungal Activity**

Fungal respiration was estimated by monitoring total CO\(_2\) production as a function of time from soil columns by titrating 7.5 mL of trap solution for total alkalinity with standardized HCl. Fungal biomass was estimated in selected soil columns by measuring the ergosterol content. (Ergosterol is a component of cell walls specific to fungi and has been used as a measure of fungal growth in solid substrates; Davis and Lamar, 1992). Five g soil samples were mixed with 20 mL methanol in 50 mL tubes and mixed for 3 h. After settling, 5 mL of supernatant was filtered through a 0.45 \(\mu\)m filter into clean tubes containing 0.3 g KOH. Tubes were capped and incubated at 75\(^{\circ}\)C for 90
min and then allowed to cool. Tubes were shaken vigorously after the addition of 10 mL water and 5 mL hexane. After clarification, 4 mL of supernatant was transferred to a small vial and dried under N\textsubscript{2}. Residue was redissolved in 1 mL of methanol and analyzed by HPLC at 282 nm after passing through a C18 reverse-phase column using methanol at a flow rate of 0.75 mL min\textsuperscript{-1}.

**Results and Discussion**

**Effects of Surfactants on PCP Mineralization**

Mineralization of PCP in the Amsterdam soil by *Phanerochaete chrysosporium* was less than 5% after 27 d with all of the surfactants studied (Figure 18). *P. chrysosporium* did not colonize the soil effectively and after 10 d, the columns were visibly colonized by a contaminant fungus, verified by incubation of soil samples on malt

![Figure 18. Mineralization of PCP in Amsterdam soil by *Phanerochaete chrysosporium* in the presence of 25 mg surfactant g soil\textsuperscript{1}.](image-url)
agar and Poly-R 478 media. Previous laboratory studies with *P. chrysosporium* have generally been conducted at elevated temperatures (30°C or greater), well above the 21-23°C used in our laboratory experiments or found in typical field conditions, and may account for the poor response observed in the current study as compared with other studies. Indigenous soil microorganisms mineralized nearly 10% of the added PCP over four weeks and proved to be more effective at PCP mineralization than *P. chrysosporium*.

The non-*Phanerochaete* species was much more effective at colonizing soil (as evident by visual observation of hyphal growth) and mineralized between 15-36% of the added PCP, depending upon surfactant treatment (Figure 19). Incubation of soil subsamples from these columns on malt agar and Poly R-478 media at the termination of

![Figure 19. Mineralization of PCP in Amsterdam soil by a non-*Phanerochaete* species in the presence of 25 mg surfactant g soil⁻¹.](image-url)
mineralization experiments confirmed that the predominant species growing in these columns was a white-rot fungus. In the absence of surfactants, the non-\textit{Phanerochaete} species clearly exceeded the ability of indigenous soil microorganisms to mineralize PCP.

Although the five tested surfactants had no effect on PCP mineralization in soils inoculated with \textit{P. chrysosporium}, PCP mineralization by the non-\textit{Phanerochaete} species was enhanced by the addition of 25 mg Tween 80 g soil$^{-1}$ (25-36%, range for 3 experiments) relative to a no surfactant control (15-23%, range for 3 experiments). The presence of other surfactants (25 mg g$^{-1}$ of LSA, LSAA, CD, and Triton X-100) either had no effect or resulted in slight inhibition of PCP mineralization compared to the no surfactant control (Figure 19). With the exception of Tween 80, none of the surfactants

![Figure 20](image_url)  
**Figure 20.** Fungal respiration estimated from total CO$_2$ produced from Amsterdam soil columns treated with a non-\textit{Phanerochaete} species and five surfactants.
resulted in significantly higher production of total CO$_2$ (an indicator of total fungal activity), nor was there any evidence that these surfactants inhibited fungal growth (Figure 20). Further, there was no evidence that the naturally derived surfactants (LSA, LSAA, or CD) yielded higher CO$_2$ production than Triton X-100 or Tween 80 (synthetic surfactants).

Measurement of the total PCP (solvent extractable) remaining in soil as a function of time in the presence and absence of surfactant did not reveal the identical trend as observed with evolution of $^{14}$CO$_2$ (Figure 21). Specifically, Tween 80 did not cause as rapid a decline in extractable PCP as was expected from mineralization of PCP, judged from $^{14}$CO$_2$ production. Further, solvent extractable PCP decreased to less than 10% of

![Graph](image-url)

Figure 21. Mineralization of PCP (closed symbols) in Amsterdam soil with and without the addition of 25 mg Tween 80 g soil$^{-1}$ by a non-Phanerochaete species, followed by solvent extraction of PCP (open symbols) remaining in the soil.
the initial PCP concentration in both the presence and absence of 25 mg Tween 80 g soil\(^{-1}\). Initially we were concerned that aging may have affected PCP recoveries; however, separate experiments showed the PCP recoveries remained greater than 95% after 4 wks in sterile soils. Although it is clear that the disappearance of total PCP, as measured using solvent extraction, was indeed due to fungal degradation, extraction efficiency and extraction reproducibility remain significant concerns. Consequently, we place more emphasis on the \(^{14}\)CO\(_2\) data as a reliable measure of PCP mineralization. Finally, no significant quantities of labeled nonpolar metabolites, including pentachloroanisole, were detected using radioisotope detection in either treatment (0 vs 25 mg Tween 80 g soil\(^{-1}\)) over the course of the study.

To test the relative effectiveness of the Tween family of surfactants for enhancing PCP mineralization in soil, five Tween surfactants (Tween 20, 40, 60, 80, and 85) were added to the Amsterdam soil at 25 mg surfactant g soil\(^{-1}\) in the presence of the non-
Phanerochaete species. After four weeks, only the Tween 80 treatment resulted in greater PCP mineralization compared to the no surfactant control (Figure 22). Tween 80 has been shown to increase ligninase production in \(P.\ chrysosporium\), and this may account for the increased mineralization of PCP observed in the current study (Leštan et at., 1990; Ashter and Corrieu, 1986).

Potential Mechanisms of Surfactant Enhanced PCP Mineralization

The addition of 25 mg Tween 80 g soil\(^{-1}\) to systems containing the non-
Phanerochaete white-rot species consistently resulted in enhanced mineralization of PCP.
The potential mechanisms of enhanced mineralization include (1) an increase in the solubility and subsequent bioavailability of PCP as a function of surfactant concentration, and (2) an increase in fungal activity perhaps causing an increase in production of enzymes responsible for PCP degradation. In order to test these potential mechanisms, measurements of PCP mineralization were coupled with measurements of PCP sorption and solubility enhancement and estimates of fungal activity over a range of Tween 80 concentrations.

Mineralization of PCP by the non-Phanerochaete species increased with increasing levels of Tween 80 up to 25 mg g soil\(^{-1}\) (Figure 23). As mentioned previously, the addition of 25 mg Tween 80 g soil\(^{-1}\) resulted in mineralization of 26% of the added
\(^{14}\)C-PCP to \(^{14}\)CO\(_2\) compared to only 17% in the absence of Tween 80. Higher concentrations of Tween 80 were not tested due to solubility limitations of surfactant in the quantity of solution required to bring the soil to field capacity.

It is well established that for micelle-forming surfactants, significant solubility enhancement of NOCs is not observed unless surfactant concentrations exceed the CMC. Batch experiments designed to measure the solubility of PCP in the presence of Amsterdam soil showed that solubility enhancement did not occur until Tween 80 concentrations exceeded approximately 40 mg g\(^{-1}\). In fact, PCP solubility was lower relative to a no surfactant system at total surfactant concentrations below 40 mg g\(^{-1}\) (Figure 24). Sun et al. (1995) observed a similar trend for the solubility characteristics of

![Figure 23. Mineralization of PCP by a non-Phanerochaete species in Amsterdam soil amended with 0, 0.05, 0.5, 1, 5, or 25 mg Tween 80 g soil\(^{-1}\).](image)

<table>
<thead>
<tr>
<th>Tween 80 concentration (mg g soil(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
</tr>
<tr>
<td>0.40</td>
</tr>
<tr>
<td>0.30</td>
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<td>0.20</td>
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Time (days)
DDT and PCB in soil as a function of added surfactant (Triton X-100) concentration. They concluded that sorbed surfactant monomers resulted in greater sorption at Triton X-100 concentrations below effective CMC values, and further demonstrated that solubility enhancement was not observed until sufficient surfactant had been added to exceed maximum sorption, followed by a subsequent drop in surface tension due to the formation of surfactant micelles.

![Figure 24. Aqueous concentration of PCP and aqueous phase surface tension from Amsterdam soil equilibrated for 24 h with varying concentrations of Tween 80.](image)

Using surface tension measurements of Tween 80 solutions in the absence and presence of soil, the effective Tween 80 concentration required to reach CMC in the Amsterdam soil was estimated at 37.5 mg g soil\(^{-1}\). The highest Tween 80 concentration
used in the mineralization experiments was 25 mg g soil\(^{-1}\), well below the concentration required to result in increased PCP solubility due to the partitioning of PCP into surfactant micelles. PCP sorption isotherms conducted in the presence of Tween 80 demonstrated a similar response (Figure 25). The partition coefficients, \(K_d\), of PCP to the

![Sorption isotherms of PCP to Amsterdam soil in the presence of Tween 80 at concentrations of 0, 1.5, 15, and 150 mg Tween 80 g soil\(^{-1}\). The resulting \(K_d\) values are 1.14, 2.05, 14.04, and \\
\(<1\ \text{L kg}^{-1}\), respectively.](image)

Amsterdam soil increased at Tween 80 concentrations of 1.5 and 15 mg g soil\(^{-1}\), both below the CMC, and dramatically decreased at 150 mg Tween 80 g soil\(^{-1}\), a concentration of total surfactant which resulted in solution concentrations exceeding the CMC of Tween 80. Both the solubility data and the sorption data for PCP as a function of Tween 80 concentration support the conclusion that enhanced fungal mineralization in the presence
of Tween 80 was not due to solubility enhancement of PCP. Aronstein et al. (1991) reported a similar response for phenanthrene degradation by bacteria in soils treated with surfactants below the CMC, and suggested that sub-CMC levels of surfactants increased the rate of desorption from soils without increasing contaminant solubility. Although degradation is still limited by contaminant solubility, the extent of degradation would increase if surfactant addition resulted in enhanced desorption rates.

An alternate explanation for the increased PCP mineralization observed in the presence of Tween 80 at concentrations below the CMC is the stimulation of fungal activity by the surfactant. Macur et al. (in preparation) noted increased hexadecane and phenanthrene mineralization by indigenous organisms in soils treated with Witconol SN-70 at concentrations below the CMC. Respiration by the non-Phanerochaete fungus, estimated by total CO₂ production, was greater after 10 d in soils treated with 25 mg Tween 80 g soil⁻¹ than in surfactant-free systems (Figure 26). Fungal biomass in Tween 80 treated soils, measured as ergosterol present in the soil, was also significantly higher at 10 d than in the surfactant-free soil (Figure 26). The increased respiration and biomass at 10 d corresponds to the period of most rapid PCP mineralization. Biomass decreased during the final 14 d of incubation, corresponding to decreased PCP mineralization and lower respiration rates. The increased PCP mineralization observed in the Tween 80 treated soils appears to be related to a stimulation of fungal growth and activity which occurred at Tween 80 concentrations below the CMC, and not as a result of PCP solubility enhancement. Additional experiments using surfactant concentrations up to 200 mg g⁻¹ soil would be warranted to determine if Tween 80 concentrations necessary to
achieve significant solubility enhancement of PCP would result in further increases in mineralization rates. However, surfactant additions at such high rates (20%, w/w) would likely not be economically feasible for the remediation of contaminated soils.

![Graph](image)

**Figure 26.** Total CO$_2$ production from Amsterdam soil in the presence of a non-*Phanerochaete* species and treated with or without 25 mg Tween 80 g soil$^{-1}$ (filled symbols). Also shown is the amount of ergosterol present in the soil over the same time period (open symbols).

**Summary**

A non-*Phanerochaete* white-rot species exhibited more aggressive soil colonization and mineralized PCP more effectively than did indigenous organisms or *Phanerochaete chrysosporium*. The rates and extents of PCP mineralization increased with increasing concentrations of Tween 80 (up to 25 mg Tween 80 g soil$^{-1}$), but were
unaffected or depressed by other synthetic or naturally-derived surfactants. At a concentration of 25 mg Tween 80 g soil$^{-1}$, approximately 25% of PCP was mineralized to CO$_2$ compared to 15% in the absence of a surfactant. In both cases, PCP concentrations decreased to less than 10% of the original concentration after four weeks. The increased mineralization of PCP was not a result of increased PCP solubility since it was shown that the addition of 25 mg Tween 80 g soil$^{-1}$ was insufficient to achieve aqueous concentrations greater than the CMC. In fact a 25 mg Tween 80 g soil$^{-1}$ application rate actually resulted in a small decrease in PCP solubility. The addition of Tween 80 resulted in increased fungal activity, as inferred from increased respiration rates and increased fungal biomass, based upon ergosterol measurements. Our results show that the use of surfactants such as Tween 80 at sub-CMC levels in combination with white-rot fungi may represent a strategy for enhancing the remediation of PCP in contaminated soils. Further, higher concentrations of surfactant should be tested to determine if increases in PCP solubility result in greater mineralization rates, and to determine if high surfactant concentrations result in inhibition of fungal growth.

Acknowledgements

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CHAPTER 4

SUMMARY

The availability of an organic molecule to microorganisms is of central importance in soil bioremediation. In the degradation of 2,4-D (Chapter 2), sorption to soil organic matter resulted in lowering the amount of pesticide available for degradation, resulting in greater quantities of 2,4-D persisting in higher organic matter soils over low organic matter soils. Although a weak relationship was observed between the partition coefficient and extent of degradation, it was evident that sorption and thermodynamic data alone are poor predictors of biodegradation. Bioavailability is controlled by mass transfer processes as well as chemical sorption. A more appropriate approach to predicting contaminant bioavailability and biodegradation would be through the use of mass transfer rates that describe the kinetics of soil desorption processes.

The introduction of the surfactant Tween 80 at sub-CMC levels into PCP contaminated soils resulted in increasing the total extent of mineralization (Chapter 3). However, the increase was not a result of increased PCP solubility since the surfactant addition actually resulted in a slight decrease in total aqueous concentrations. The enhanced degradation achieved with surfactant addition resulted from either (1) increased fungal activity or (2) increased contaminant mass transfer from the sorbed state. Regardless of the exact mechanism involved, the addition of surfactants at sub-CMC
concentrations can result in increased contaminant degradation, suggesting the utility of surfactants in bioremediation strategies.

Despite the promises that bioremediation offers for cleaning the environment, some key issues remain unanswered and limit widespread application of these technologies. It is clear that microorganisms are the primary agents responsible for contaminant degradation, however, poor understanding of microbial communities and their interactions limit our ability to assess bioremediation potential and shape remediation schemes. Furthermore, surfactants appear to facilitate greater contaminant degradation in some instances, but microbial responses are varied, suggesting the need for greater investigation into bioavailability and biodegradation enhancement through these chemical agents.

A fuller understanding of the physical, chemical, and microbiological issues involved in soil environments would provide useful information for assessment of bioremediation potential in contaminated areas. It is encouraging to recognize that as our knowledge of the environment continues to grow, we are being equipped with powerful tools to help clean our world of our past mistakes. If mankind has learned one lesson from his legacy of pollution, it is an increased level of environmental awareness. Hopefully, it is this collective wisdom, and not our follies of the past, that will be passed on to future generations. As we continue to look at relationships among our species and the planet and strive to understand the processes taking place in soils, we are really laying the groundwork for a brighter, cleaner future.
REFERENCES CITED


United States Environmental Protection Agency: Region VIII. 1995. EPA national superfund priority list sites: Montana. USEPA, Region VIII. Denver, CO.


APPENDICES
APPENDIX A

ADDITIONAL 2,4-D DATA
Figure 27. Degradation of 2,4-D in 31 agricultural soils collected from around Montana. Soils demonstrated a wide range in both rates and extents of degradation. Degradation curves are shown without data points for clarity.
Figure 28. Comparison of three kinetic models used to describe 2,4-D degradation in soil. The points are the observed data; the solid line is the fit from the first order model; the dashed line is the modified first-order model; and the dotted line is the logistic model. Also shown are $r^2$ values from non-linear regression analyses. The modified first-order and logistic equations comparably described the observed degradation curves. First-order kinetics provided poor fits.
Figure 29. Relationship between maximum extent of 2,4-D degradation and fitted rate constants (from modified first-order) to soil organic carbon for the crop-fallow and native range groups of soils. A linear regression between extent of degradation and SOC is shown for each set of soils. The relationship was stronger for the crop-fallow soils than for the native-range soils. No apparent relationship was evident between the rate constant and SOC.
Figure 30. Comparison of aerobic and anaerobic conditions on $^{14}$C-carboxyl-labeled 2,4-D degradation ($\theta_m = 0.46$). Top figure is $^{14}$CO$_2$ production from $^{14}$C-carboxyl-labeled 2,4-D under aerobic conditions. Bottom figure is $^{14}$CO$_2$ production from $^{14}$C-carboxyl-labeled 2,4-D preconditioned under anaerobic conditions then exposed to aerobic conditions. Total extent of degradation increased in the Amsterdam soil when incubated under anaerobic conditions.
APPENDIX B

ADDITIONAL PCP DATA
Table 4. Soil characteristics for three PCP contaminated soils used in PCP degradation studies.

<table>
<thead>
<tr>
<th>Soil</th>
<th>% Sand</th>
<th>% Silt</th>
<th>% Clay</th>
<th>Textural class</th>
<th>Organic C (%)</th>
<th>CEC (mmhos cm⁻¹)</th>
<th>pH (1:1)</th>
<th>Water content (1/3 bar)</th>
<th>PCP concentration (mg kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amsterdam</td>
<td>15</td>
<td>52</td>
<td>33</td>
<td>Silt loam</td>
<td>1.8</td>
<td>0.21</td>
<td>6.9</td>
<td>0.26</td>
<td>100</td>
</tr>
<tr>
<td>Libby Pole</td>
<td>43</td>
<td>43</td>
<td>14</td>
<td>Loam</td>
<td>2.0</td>
<td>0.33</td>
<td>NA</td>
<td>0.23</td>
<td>445</td>
</tr>
<tr>
<td>Montana Pole</td>
<td>79</td>
<td>13</td>
<td>9</td>
<td>Loamy sand</td>
<td>2.3</td>
<td>0.22</td>
<td>8.1</td>
<td>0.12</td>
<td>273</td>
</tr>
</tbody>
</table>
Figure 31. Degradation of PCP in Amsterdam soil by a non-
*Phanerochaete* white-rot fungi treated with surfactants at 25 mg g soil$^{-1}$. The naturally-derived surfactant lecithin and the synthetic surfactant Witconol SN-70 are compared with no surfactant and Tween 80 treatments for comparison. Both lecithin and Witconol were less effective than either no surfactant or Tween 80 at promoting PCP degradation.
Figure 32. Effect of soil mixing on PCP degradation and fungal respiration by a non-
*Phanerochaete* species. Soil was mixed after 28 days and moisture content readjusted to field
capacity. Mixing resulted in a very slight increase in PCP degradation and fungal respiration.
Figure 33. Degradation of PCP in the Libby Pole soil by indigenous organisms and a non-
*Phanerochaete* species (F600), both with and without the addition of 25 mg Tween 80 g soil$^{-1}$. None of the treatments showed considerable PCP mineralization up to 24 d. Increases in total mineralization after 24 d are accompanied by very large standard errors, indicating non-uniform variation among replications.
Figure 34. Degradation of PCP in the Montana Pole soil by a non-*Phanerochaete* white-rot fungus and the surfactant Tween 80 at five concentrations. Tween 80 had no effect on the degradation of PCP, perhaps due to toxicity effects. The Montana Pole soil contains high levels of heavy metals (e.g. Cu) and other toxic materials such as arsenic and may have interfered with fungal growth.
Figure 35. Degradation of PCP in Amsterdam soil by indigenous organisms, a non-Phanerochaete white-rot fungus (F600), and a fungus isolated from PCP-contaminated soils (tentatively identified as a *Penicillium* species). The non-Phanerochaete fungus was more effective at PCP degradation (17%) than the indigenous soil organisms (11%); the *Penicillium* fungus, however, resulted in greater than 40% degradation of the PCP after four weeks. More research into PCP degradation by this organism is warranted.
Figure 36. PCP degradation in Libby Pole soil inoculated with a non-Phanerochaete white rot fungus and a Penicillium fungus isolated from soil. Neither fungus enhanced degradation above that of the indigenous soil microorganisms in this particular soil.
Figure 37. Surface tensions measurements of Tween 80 solutions at concentrations of 0, 10, 50, 200, 500, 1000, 2000, 4000, 6000, 8000, 10000, and 20,000 mg Tween 80 L\(^{-1}\). The diamonds are surface tension measurements made in the absence of soil; the CMC is estimated at 7.5 mg L\(^{-1}\). The squares are surface tension measurements made after 24 h equilibration of 15 mL of surfactant solution with 1 g of Amsterdam soil. The estimated concentration required to achieve CMC in a soil containing system is 2500 mg L\(^{-1}\) or 2.5 orders of magnitude higher. The impact of surfactant sorption to soil is well illustrated by this comparison.