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

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 pentachloropheriol (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

MONTANA STATE UNIVERSITY—BOZEMAN
Bozeman, Montana

April 1997

APPROVAL

of a thesis submitted by

Ronald Allen Doughten

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.

William P. Inskeep — (Signature) — 4/15/97 (Date)

Approved for the Department of Plant, Soil, and Environmental Science

Jeffrey S. Jacobsen — (Signature) — 4/17/97 (Date)

Approved for the College of Graduate Studies

Robert L. Brown — (Signature) — 4/26/97 (Date)

STATEMENT OF PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirement for a master's degree at Montana State University—Bozeman, I agree that the Library shall make it available to borrowers under rules of the Library.

If I have indicated my intention to copyright this thesis by including a copyright notice page, copying is allowable only for scholarly purposes, consistent with "fair use" as prescribed in the U.S. Copyright Law. Requests for permission for extended quotation from or reproduction of this thesis in whole or in parts may be granted only by the copyright holder.

Signature Ronald A Dryhter

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.

ACKNOWLEDGEMENTS

I would like to thank my major advisor Dr. William Inskeep for the ideas, support, and encouragement he has given me as an undergraduate and graduate student at Montana State University.

I would also like to thank the other members of my committee, Dr. Jon Wraith, Dr. Timothy McDermott, and Dr. Anne Camper, for their assistance and use of laboratories and equipment. I owe thanks also to Dr. Carl Johnston and Michael Beccera of Mycotech Corporation for their assistance in my work with the white-rot fungi.

I am grateful to my fellow lab workers, Richard Macur, Dr. Hesham Gaber, Dr. Robert Grosser, Dr. Shaobai Sun, Clain Jones, Heiko Langner, and countless other students who have contributed in some part to my life and research in Bozeman.

Finally, I would like to give special thanks to my parents, Mr. and Mrs. Ralph Prosser, for their unending moral and spiritual support through my education.

TABLE OF CONTENTS

LIST OF TABLES

Table

LIST OF FIGURES

Figure

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 $^{14}CO_2$ 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 1

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.

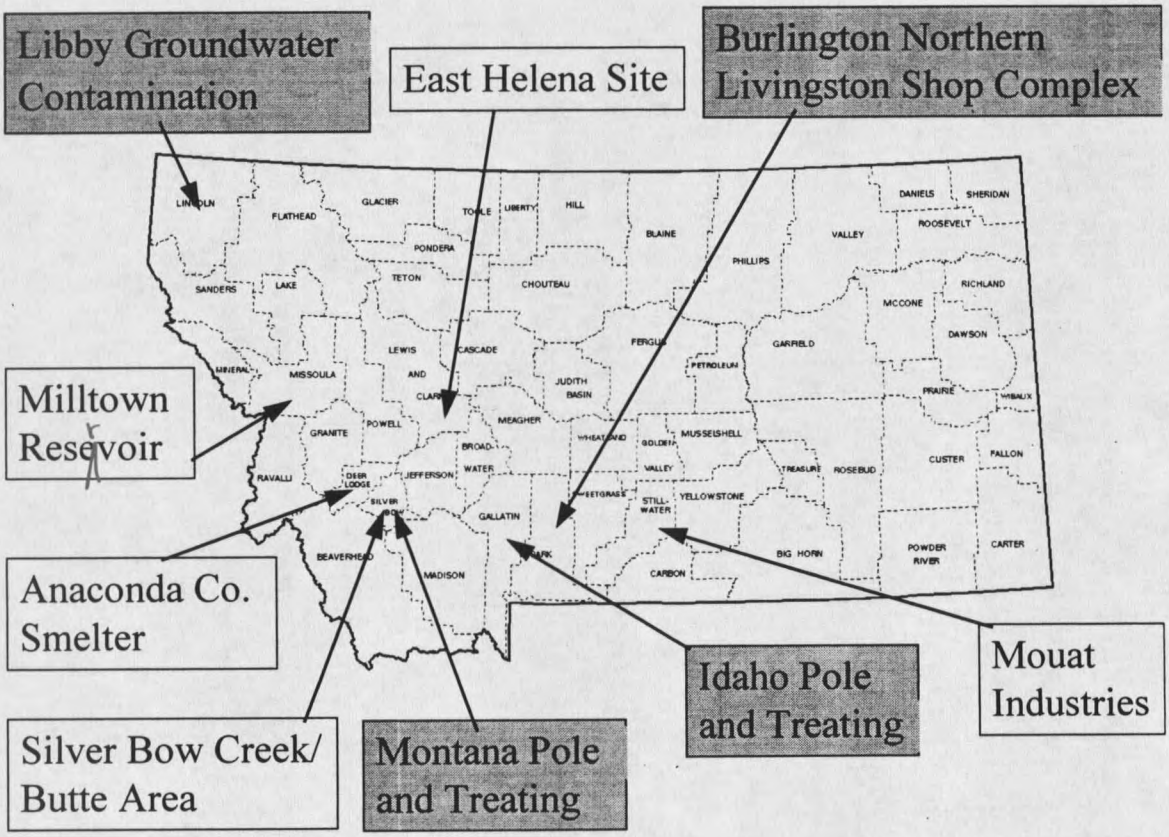

Figure 1. Locations of the nine EPA National Priority Sites in Montana. The shaded labels are those contaminated with organic chemicals; the others are contaminated with inorganics.

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,

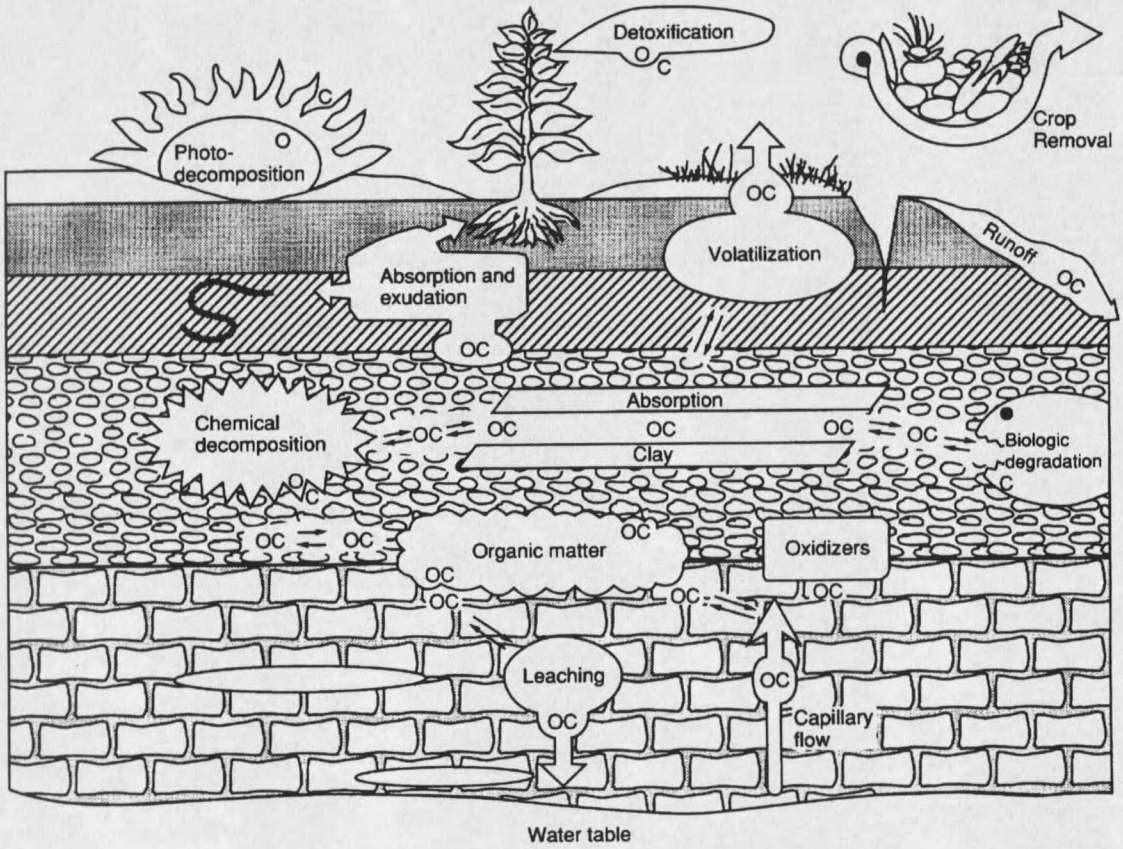

Figure 2. Processes influencing the fate of organic chemicals (OC) in the environment (Weber and Miller, 1989).

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 know 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

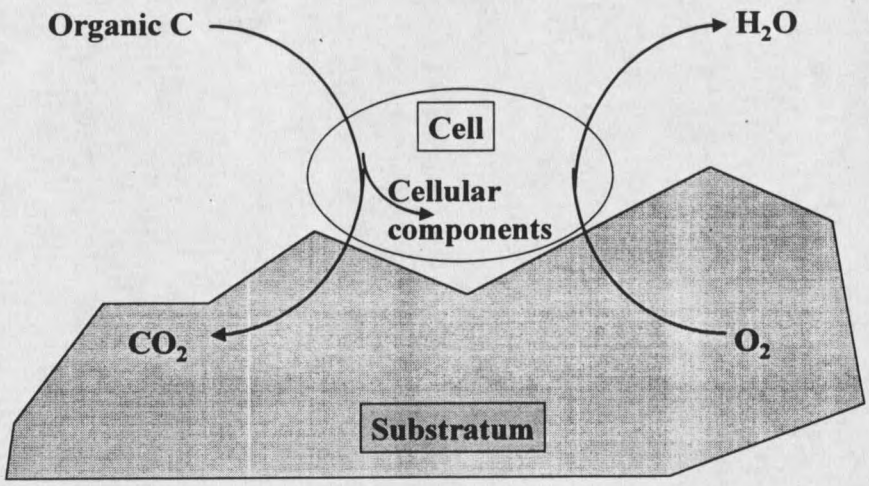

Figure 3. Aerobic metabolism of a substrate to cellular components and CO_2 by an attached bacterium.

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

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_2O_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 chyrsosporium*, 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,

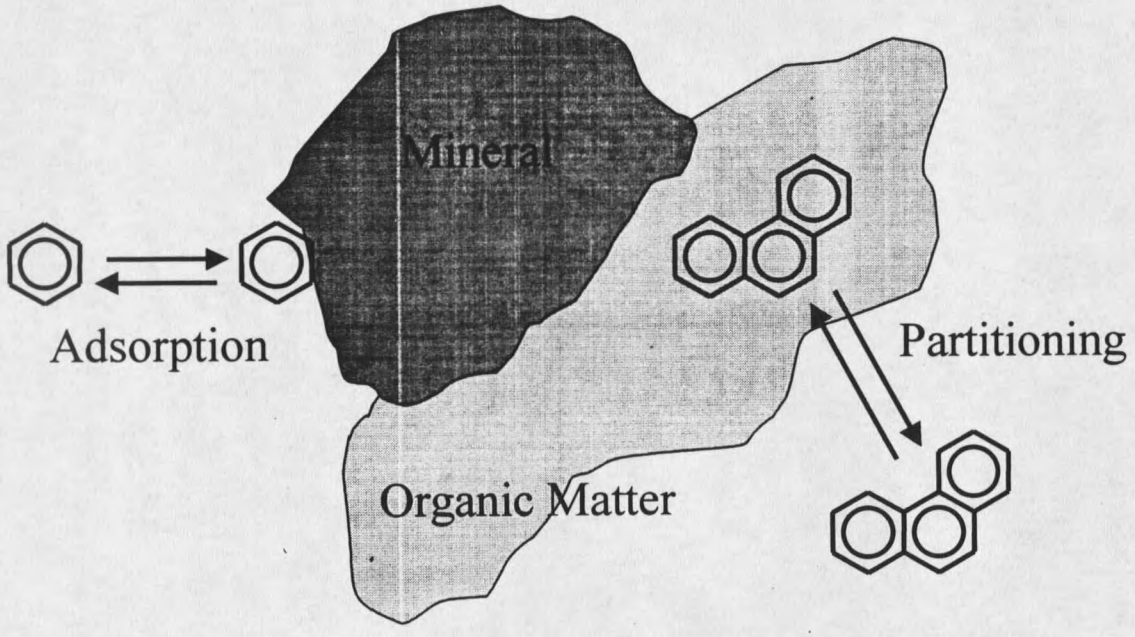

Figure 4. Sorption in soil involves both adsorption to surfaces and partitioning into the bulk solid.

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:

$$K_d = \frac{C_{sorbed}}{C_{aqueous}}$$

where C_{sorbed} is the concentration of sorbed compound (mg kg^{-1}) and $C_{aqueous}$ 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_{om}:

$$K_{om} = \frac{K_d}{X_{som}}$$

where X_{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_{oc}):

$$K_{oc} = \frac{K_d}{X_{oc}}$$

where X_{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

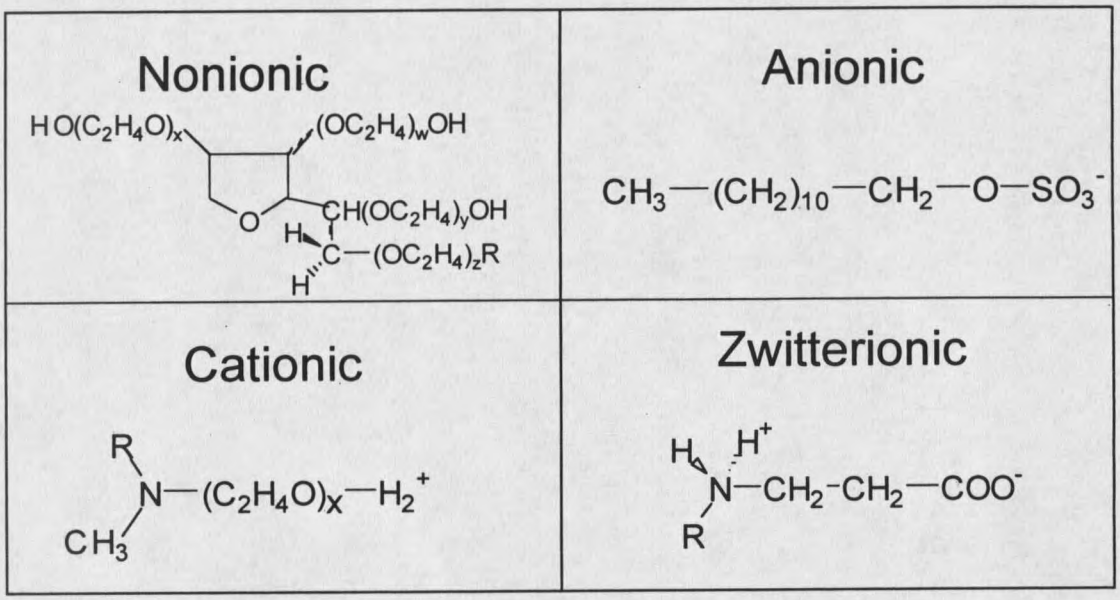

Figure 5. Surfactants can be classified into four groups based upon the charge of the polar group—nonionic, anionic, cationic, and zwitterionic—containing no charge, a negative charge, a positive charge, and both positive and negative charges, respectively.

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

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 solubilitzation. 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) micellular solubilization that is thermodynamically driven by the concentration

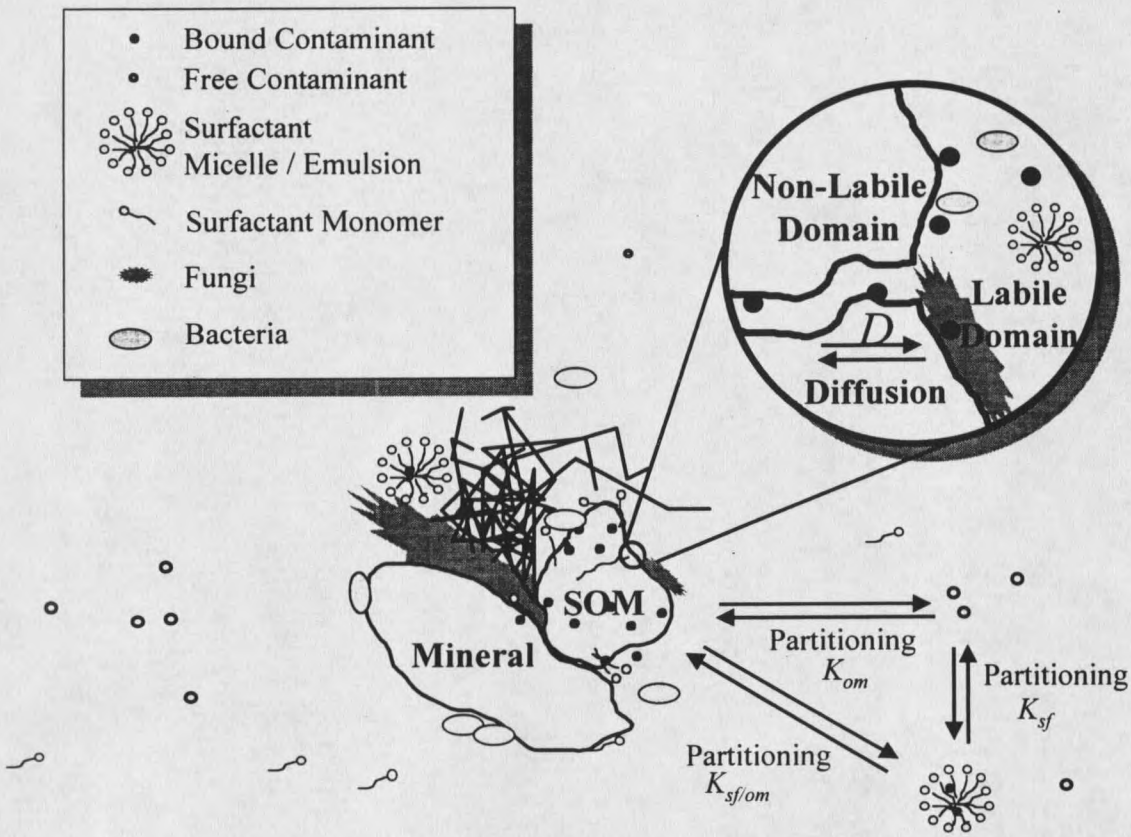

Figure 6. Interactions between sorbed contaminants, soil bacteria and fungi, and surfactants in enhancing solubility of NOCs in soil environments.

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 micellular

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 (K_d) 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 a *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 subtrates (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 Brusseu, 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 rhamnlipids 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_{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_{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_{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_{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_{oc} = 2.00 x 10^{-2} m^3kg^{-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.

Soil Code	Soil Series	Location	Land Use	--------%-------- Sand	Silt	Clay	Textural Class	2:1 pH	EC*	Organic C (%)	K_d ($L\ kg^{-1}$)
AMST	Amsterdam	Gallatin	Crop-Fallow	15	52	33	Silt Loam	6.9	0.26	0.50	0.64
BACF	Farnuf	Fergus	Crop-Fallow	28	44	28	Clay Loam	7.1	0.38	2.27	1.52
BANR	Farnuf	Fergus	Native-Range	26	46	28	Clay Loam	7.5	0.53	2.53	1.62
BECF	Farland-Cherry	Daniels	Crop-Fallow	46	32	22	Silt Loam	7.8	0.24	0.97	0.22
BENR	Farland-Cherry	Daniels	Native-Range	45	34	21	Silt Loam	7.5	0.66	1.76	0.37
BROC	Brocko	Gallatin	Crop-Fallow	24	56	20	Silt Loam	8.1	NA	0.03	0.11
CRCF	Fairfield-Danvers	Fergus	Crop-Fallow	34	30	36	Clay Loam	7.3	0.22	1.34	0.51
CRNR	Fairfield-Danvers	Fergus	Native-Range	34	32	34	Clay Loam	7.2	0.28	1.23	0.62
HICF	Danvers	Fergus	Crop-Fallow	30	36	34	Clay Loam	5.4	0.25	1.79	1.00
HINR	Danvers	Fergus	Native-Range	39	32	29	Clay Loam	5.3	0.19	1.83	0.56
INSK	Beaverell	Gallatin	Pasture	37	36	27	Loam	7.3	0.58	2.49	0.88
LACF	Turner	Daniels	Crop-Fallow	62	19	19	Sandy Loam	6.0	0.15	0.83	0.19
LANR	Turner	Daniels	Native-Range	65	20	15	Sandy Loam	6.4	0.12	0.93	0.17
LONN	Lonna	Custer	Native-Range	16	54	30	Silty Clay Loam	8.0	0.28	1.63	0.72
NECF	Winifried	Judith Basin	Crop-Fallow	30	32	38	Clay Loam	7.1	0.47	2.21	1.68
P1CF	Turner-Beaverton	Roosevelt	Crop-Fallow	81	8	11	Loamy Sand	6.4	0.07	0.64	0.19
P1NR	Turner-Beaverton	Roosevelt	Native-Range	81	8	11	Loamy Sand	6.4	0.09	1.17	0.17
P2CF	Tally	Roosevelt	Crop-Fallow	70	15	15	Sandy Loam	7.0	0.10	0.68	0.35
P2NR	Tally	Roosevelt	Native-Range	70	16	14	Sandy Loam	6.4	0.14	0.90	0.24
P3CF	Farnuf	Roosevelt	Crop-Fallow	69	17	14	Sandy Loam	5.5	0.14	0.61	0.12
P3NR	Farnuf	Roosevelt	Native-Range	67	17	16	Sandy Loam	6.1	0.12	0.74	0.17

* $dS\ m^{-1}$

Table 1. --Continued

Soil Code	Soil Series	Location	Land Use	--------%-------- Sand	Silt	Clay	Textural Class	2:1 pH	EC	Organic C	K_d ($L\ kg^{-1}$)
P4CF	Williams-Zahill	Roosevelt	Crop-Fallow	65	17	18	Sandy Loam	5.8	0.15	0.26	0.06
P4NR	Williams-Zahill	Roosevelt	Native-Range	63	20	17	Sandy Loam	6.3	0.12	0.38	0.09
POCF	Judith	Judith Basin	Crop-Fallow	29	32	39	Clay Loam	7.2	0.16	1.45	0.49
PONR	Judith	Judith Basin	Native-Range	33	29	38	Clay Loam	7.2	0.21	1.99	0.66
PUCF	Farland-Cherry	Daniels	Crop-Fallow	43	32	25	Loam	7.4	0.21	1.53	0.50
PUNR	Farland-Cherry	Daniels	Native-Range	48	34	18	Loam	7.7	0.33	2.68	1.06
SONN	Sonnet	Custer	Native-Range	6	57	37	Silty Clay Loam	7.6	0.18	2.11	0.68
SUCF	Willams-Zahill	Daniels	Crop-Fallow	25	33	42	Clay	8.0	0.24	1.21	0.34
SUNR	Willams-Zahill	Daniels	Native-Range	30	36	34	Clay Loam	7.9	0.25	1.79	0.33
WILL	Williams	Custer	Native-Range	20	50	30	Clay Loam	5.6	0.33	3.72	1.16

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-

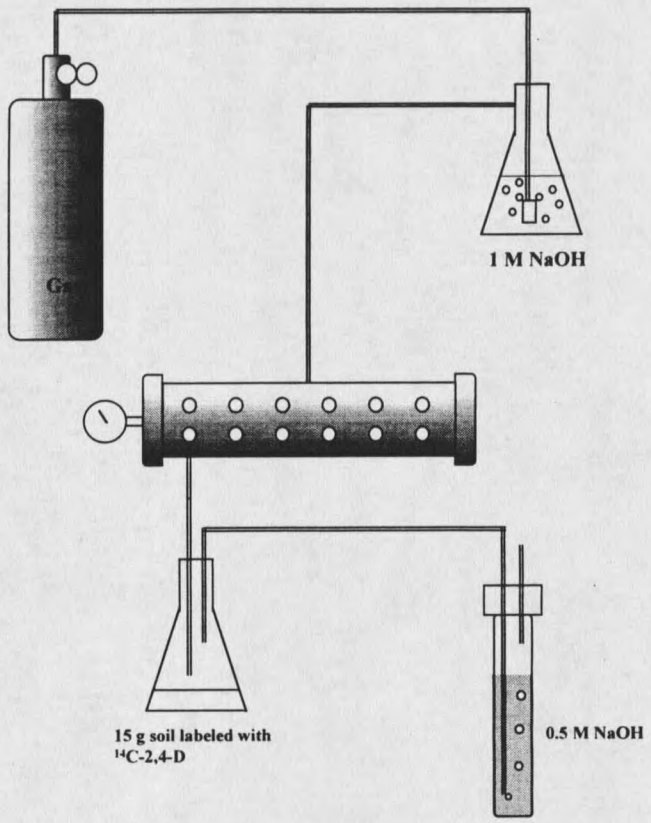

Figure 7. Experimental setup used in the batch biodegradation studies. Compressed air or nitrogen was passed through a NaOH trap into a manifold that controlled gas flow to individual flasks containing ^{14}C-2,4-D labeled soil. Degradation was measured as the accumulation of $^{14}CO_2$ in 0.5 M NaOH traps changed regularly.

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_2PO_4, 1.35 g K_2HPO_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:

$$\frac{C}{C_o} = 1 - e^{kt}$$

where C/C_o is the fraction of total ^{14}C-2,4-D evolved as $^{14}CO_2$, k is the first-order rate constant, and t is time.

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

$$P = P_{max}(1 - e^{kt})$$

where P is the fraction of total ^{14}C-2,4-D evolved as $^{14}CO_2$, P_{max} is the maximum extent of mineralization in terms of total ^{14}C added, k is the rate constant, and t is time.

Logistic Equation (Characklis, 1990):

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

where X is the fraction of ^{14}C-2,4-D evolved as $^{14}CO_2$, X_o is the initial fraction of CO_2 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

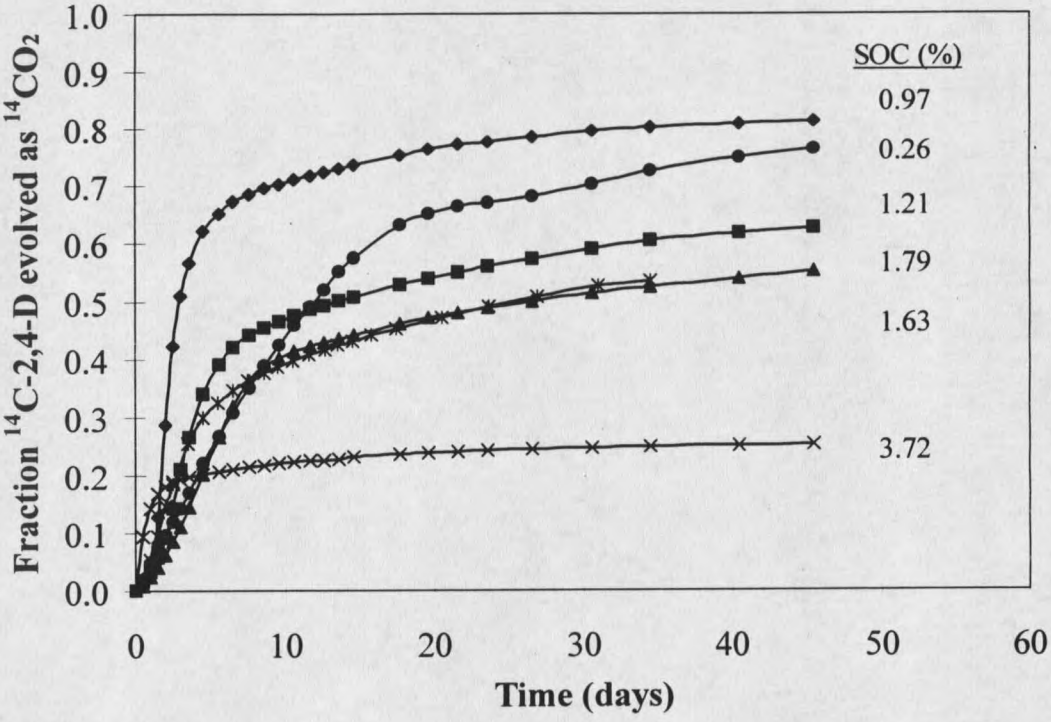

Figure 8. Degradation curves showing the fraction of ^{14}C-2,4-D evolved as $^{14}CO_2$ from six of the 31 soils. The organic carbon content (SOC, %) of the soil is shown adjacent to each curve.

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

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_{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_{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.

	Observed	First-order		Modified First-order			Logistic			
Soil	P_{max}	k (h^{-1})	r^2	k (h^{-1})	P_{max}	r^2	k (h^{-1})	X_o	X_m	r^2
AMST	0.78	0.005	0.32	0.030	0.74	0.96	0.163	0.040	0.73	0.96
BACF	0.66	0.003	0.00	0.020	0.59	0.95	0.053	0.081	0.58	0.91
BANR	0.66	0.003	0.65	0.009	0.61	0.99	0.022	0.081	0.58	0.95
BECF	0.80	0.005	0.73	0.011	0.78	0.96	0.054	0.028	0.74	0.97
BENR	0.72	0.003	0.65	0.009	0.69	0.99	0.027	0.073	0.66	0.96
BROC	0.69	0.003	0.82	0.010	0.66	0.93	0.073	0.016	0.63	0.97
CRCF	0.66	0.003	0.37	0.013	0.59	0.96	0.030	0.097	0.57	0.91
CRNR	0.75	0.005	0.27	0.020	0.68	0.96	0.070	0.058	0.66	0.93
HICF	0.59	0.002	0.00	0.021	0.52	0.91	0.027	0.138	0.52	0.86
HINR	0.62	0.003	0.00	0.021	0.55	0.94	0.049	0.089	0.54	0.89
INSK	0.65	0.003	0.10	0.018	0.60	0.97	0.059	0.056	0.58	0.94
LACF	0.79	0.006	0.56	0.017	0.74	0.96	0.079	0.024	0.72	0.97
LANR	0.62	0.002	0.61	0.009	0.58	0.98	0.029	0.051	0.64	0.97
LONN	0.54	0.002	0.64	0.007	0.50	0.98	0.019	0.064	0.46	0.94
NECF	0.62	0.003	0.36	0.013	0.58	0.99	0.032	0.077	0.56	0.95
P1CF	0.84	0.014	0.06	0.039	0.78	0.94	0.139	0.052	0.77	0.93
P1NR	0.55	0.002	0.00	0.015	0.50	0.95	0.039	0.070	0.49	0.91
P2CF	0.81	0.005	0.64	0.015	0.75	0.98	0.036	0.109	0.73	0.94
P2NR	0.69	0.003	0.41	0.013	0.62	0.97	0.036	0.078	0.59	0.94
P3CF	0.73	0.005	0.19	0.024	0.70	0.94	0.114	0.019	0.68	0.96
P3NR	0.67	0.003	0.33	0.016	0.63	0.63	0.049	0.069	0.61	0.93

Table 2. Continued

	Observed	First-order		Modified First-order			Logistic			
Soil	P_{max}	k (h^{-1})	r^2	k (h^{-1})	P_{max}	r^2	k (h^{-1})	X_o	X_m	r^2
P4CF	0.75	0.002	0.96	0.003	0.79	0.99	0.011	0.070	0.70	0.99
P4NR	0.71	0.002	0.64	0.007	0.63	0.98	0.012	0.129	0.65	0.94
POCF	0.71	0.004	0.00	0.024	0.65	0.93	0.071	0.068	0.63	0.90
PONR	0.56	0.002	0.17	0.010	0.51	0.93	0.014	0.129	0.50	0.88
PUCF	0.68	0.003	0.49	0.010	0.66	0.98	0.034	0.058	0.62	0.96
PUNR	0.51	0.001	0.49	0.007	0.50	0.98	0.017	0.071	0.47	0.94
SONN	0.40	0.001	0.22	0.010	0.35	0.96	0.024	0.057	0.34	0.90
SUCF	0.61	0.002	0.67	0.006	0.59	0.97	0.024	0.043	0.54	0.96
SUNR	0.53	0.001	0.72	0.005	0.54	0.98	0.032	0.032	0.48	0.98
WILL	0.25	0.001	0.00	0.033	0.23	0.93	0.058	0.048	0.23	0.88

not compensate for extents of degradation that are less than one. Consequently, first-order rate constants would not be accurate predicators 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_{max} from the modified first-order model (or X_m in the logistic model) were negatively correlated with SOC (r^2 =

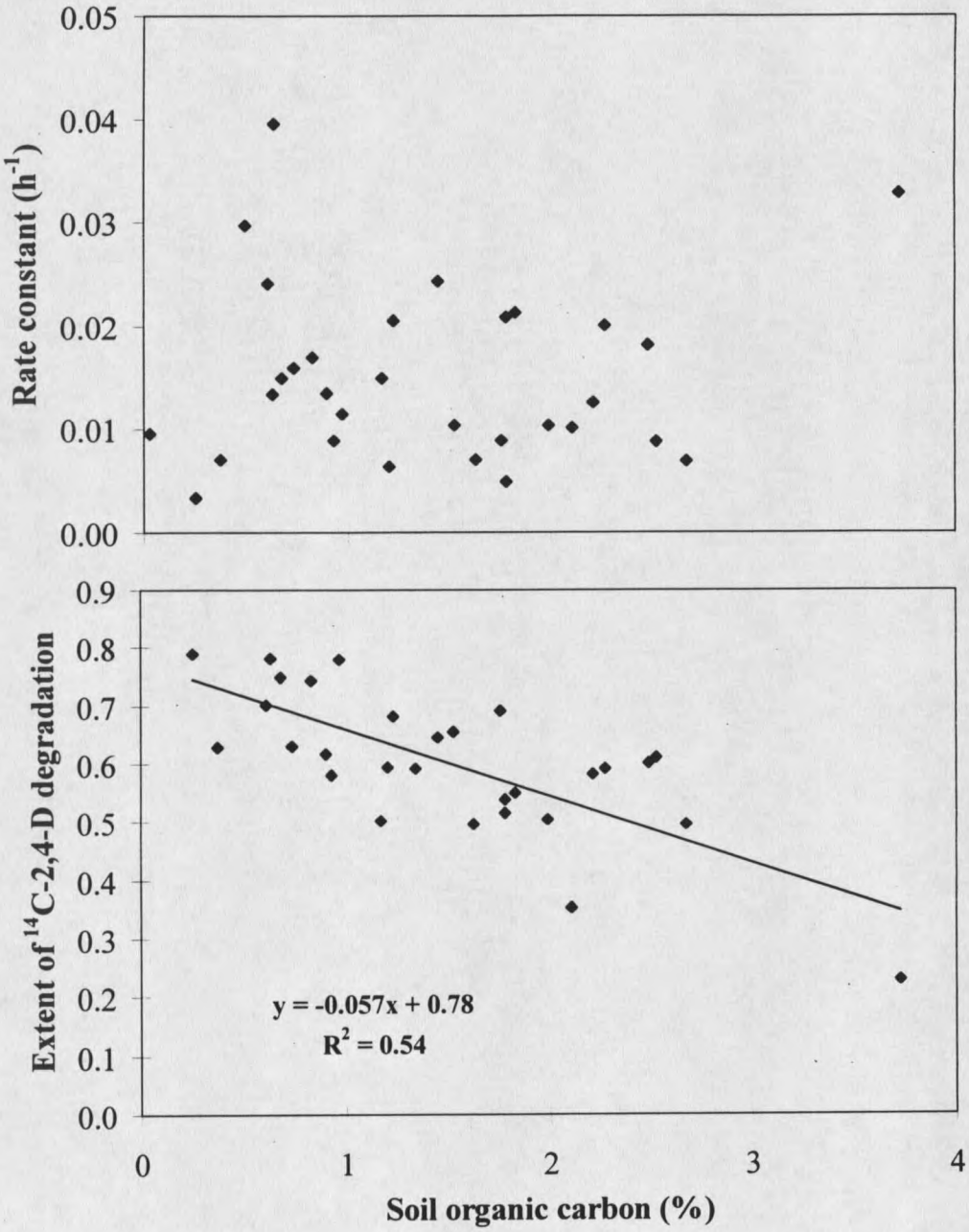

Figure 9. Top figure: Comparison of fitted 2,4-D degradation rate constant (h^{-1}) from the modified first-order model to SOC. Bottom figure: Maximum extent of degradation fitted from the modified first-order model compared to SOC.

0.54, Figure 9), 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_{max} (or X_m in the logistic model; Figure 10), indicating that the rate constant taken by itself

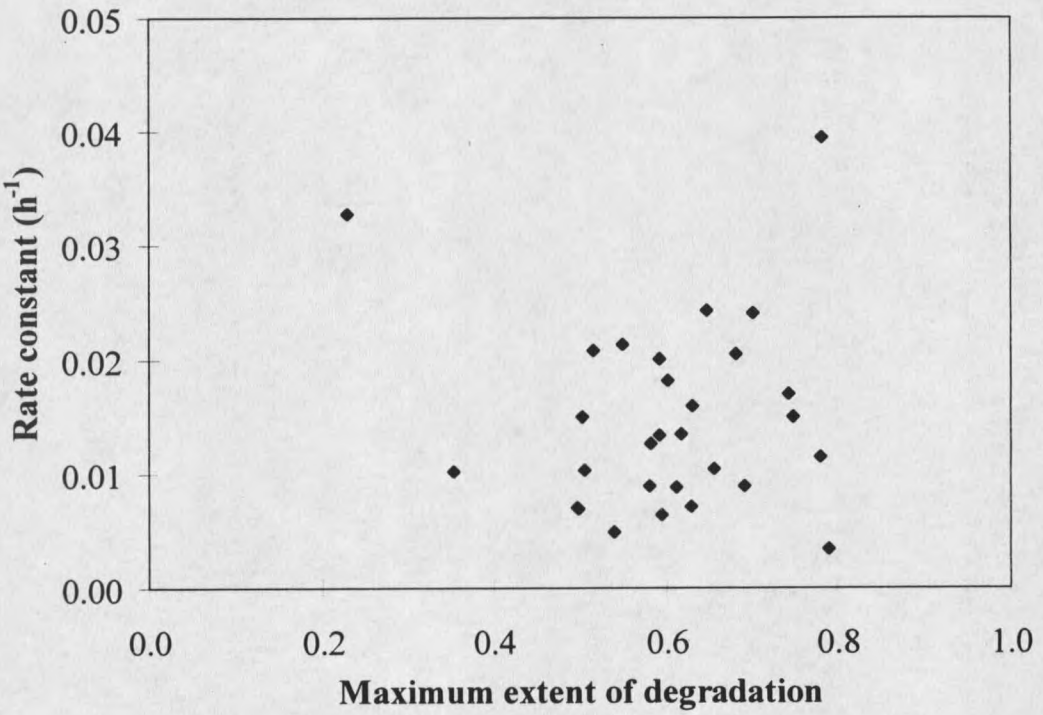

Figure 10. Comparison of the degradation rate constant (h^{-1}) and P_{max}, values obtained from modified first-order model.

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

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

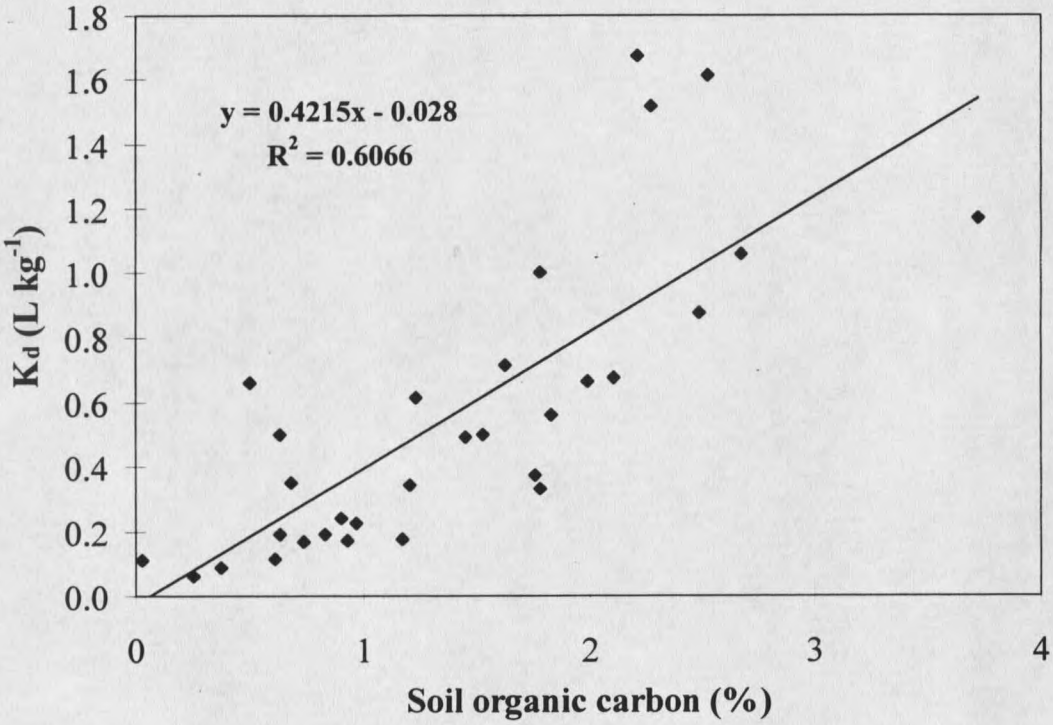

Figure 11. Relationship of 2,4-D partition coefficients (K_d) to soil organic carbon contents for all 31 soils. The slope yields an average K_{oc} value of 422 L kg^{-1}.

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-

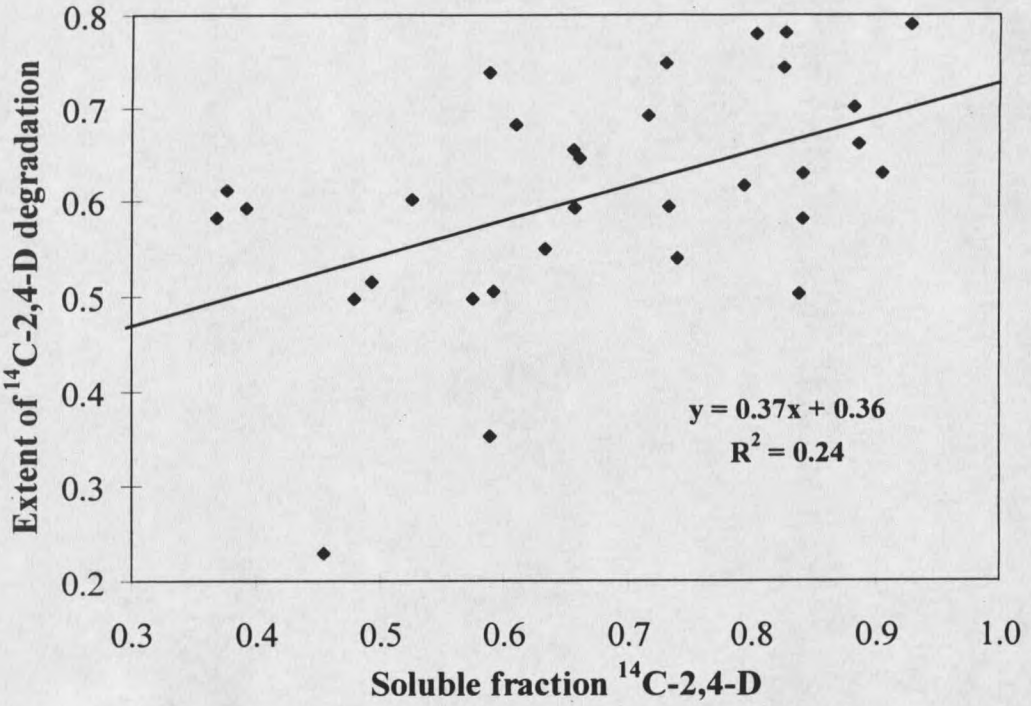

Figure 12. Maximum extent of ^{14}C-2,4-D degradation plotted against soluble 2,4-D in solution at t = 0 (calculated from K_d). Solid line is the observed relationship between available and degraded 2,4-D.

D present at time 0 (from the K_d values) and extents of degradation was considerably poorer than the relationship of P_{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_{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_{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

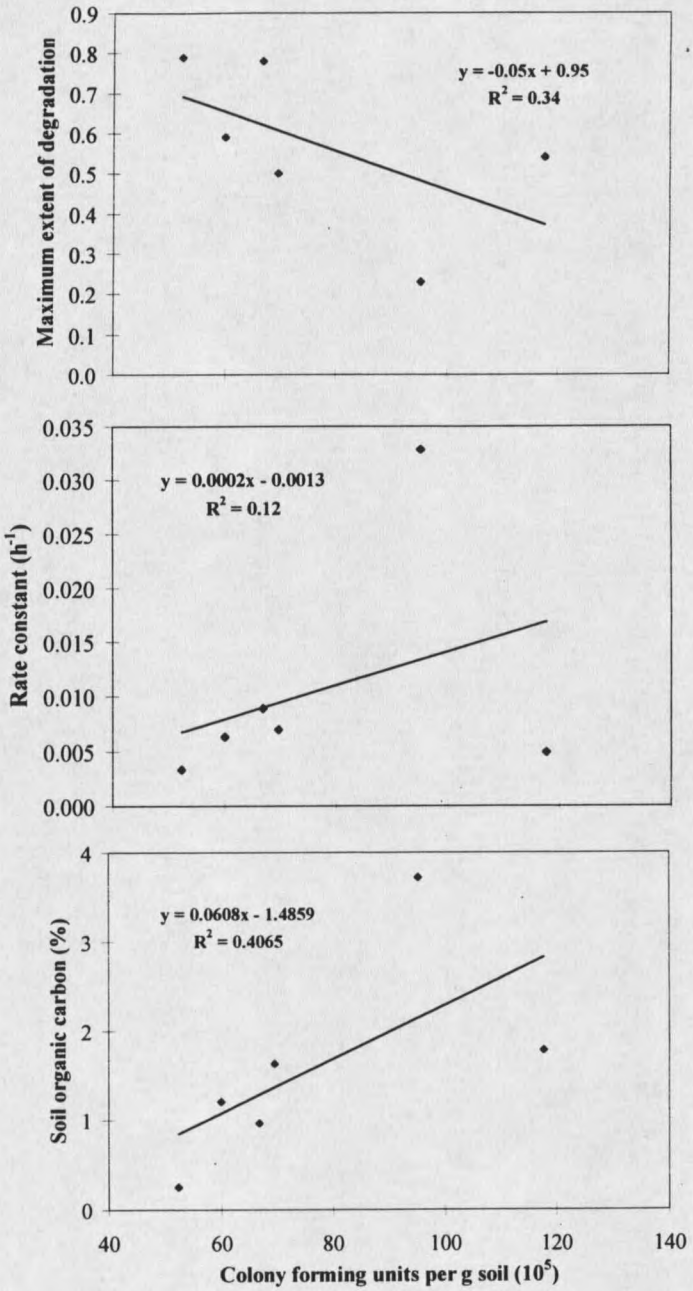

Figure 13. Relationship between fitted extent of 2,4-D degradation (P_{max}), rate constants, and SOC to the number of colony forming units of bacteria isolated on non-selective media six soils (bracketing the range in SOC contents observed for the soils studied).

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

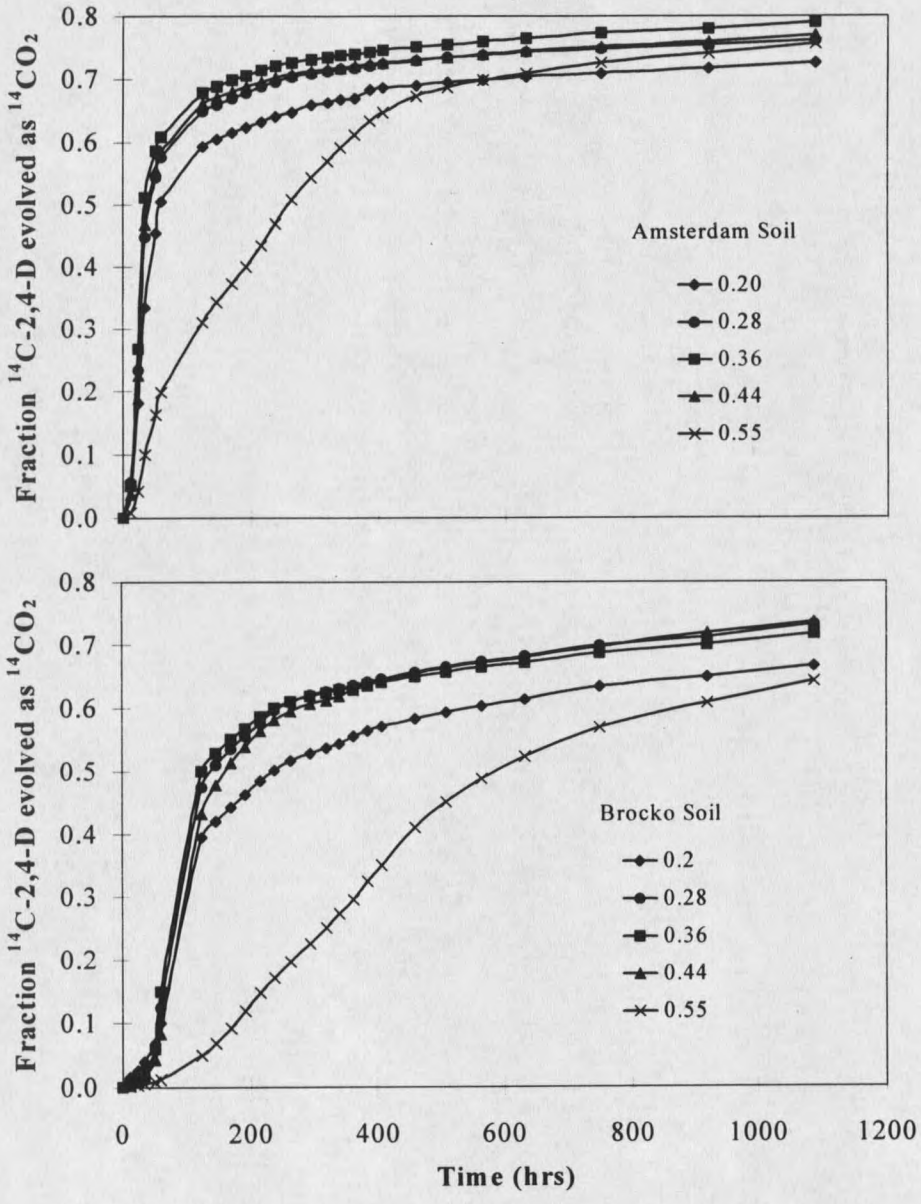

Figure 14. Effect of moisture content on the degradation of 14C-ul-2,4-D in two soils. Moisture content of 0.55 is above saturation; 0.36 is approximately field capacity.

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).

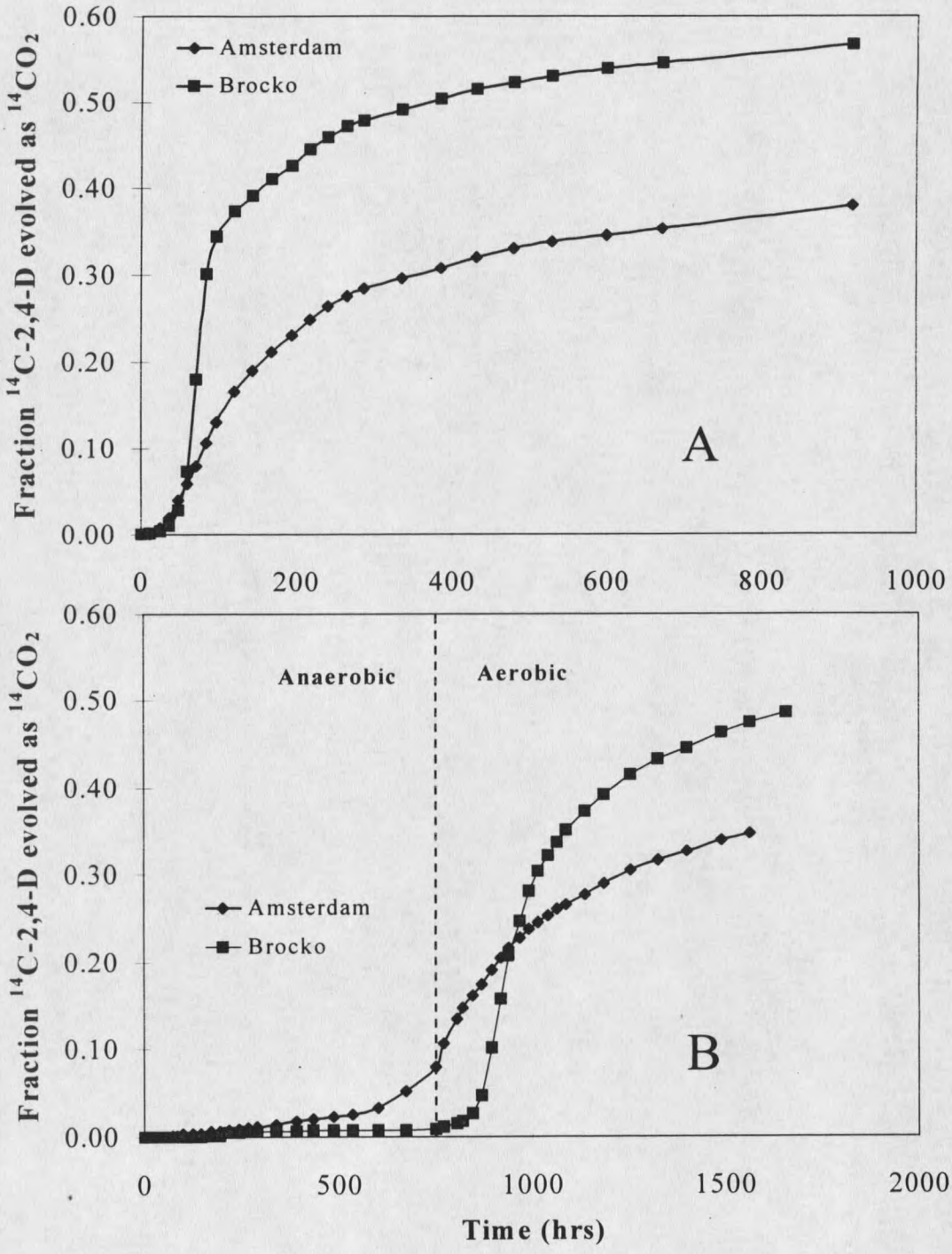

Figure 15. Effect of soil preconditioning under anaerobic conditions on the mineralization of 2,4-D in Amsterdam and Brocko soils (θ_m = 0.46). A: $^{14}CO_2$ evolution from ^{14}C-ul-ring-labeled 2,4-D under aerobic conditions; B: $^{14}CO_2$ evolution from ^{14}C-ul-ring-labeled 2,4-D preconditioned 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.

CHAPTER 3

MINERALIZATION OF PENTACHLOROPHENOL (PCP) IN SOIL BY WHITE-ROT FUNGI IN THE PRESENCE OF SURFACTANTS

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-β-cyclodextrin; Tween 80; and Triton X-100). Each surfactant was applied to the contaminated soil at 25 mg g soil^{-1}, 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 $\mu g\ day^{-1}$ (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 ($pK_a = 4.35$), 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 ($\log K_{oc} = 4.51$). 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

Table 3. Characteristics of the Amsterdam Silt Loam (Typic Cryoboroll).

% Sand	% Silt	% Clay	Textural Classification	Organic C (%)	CEC (mmol charge kg^{-1})	pH (1:1)	θ_m (1/3 bar)
15	52	33	silt loam	1.80	0.21	6.9	0.26

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 ^{14}C-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 ^{14}C concentration in the soil was verified by oxidation of triplicate soil subsamples at 800°C under an O_2 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).

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, $\theta_m = 0.26$).

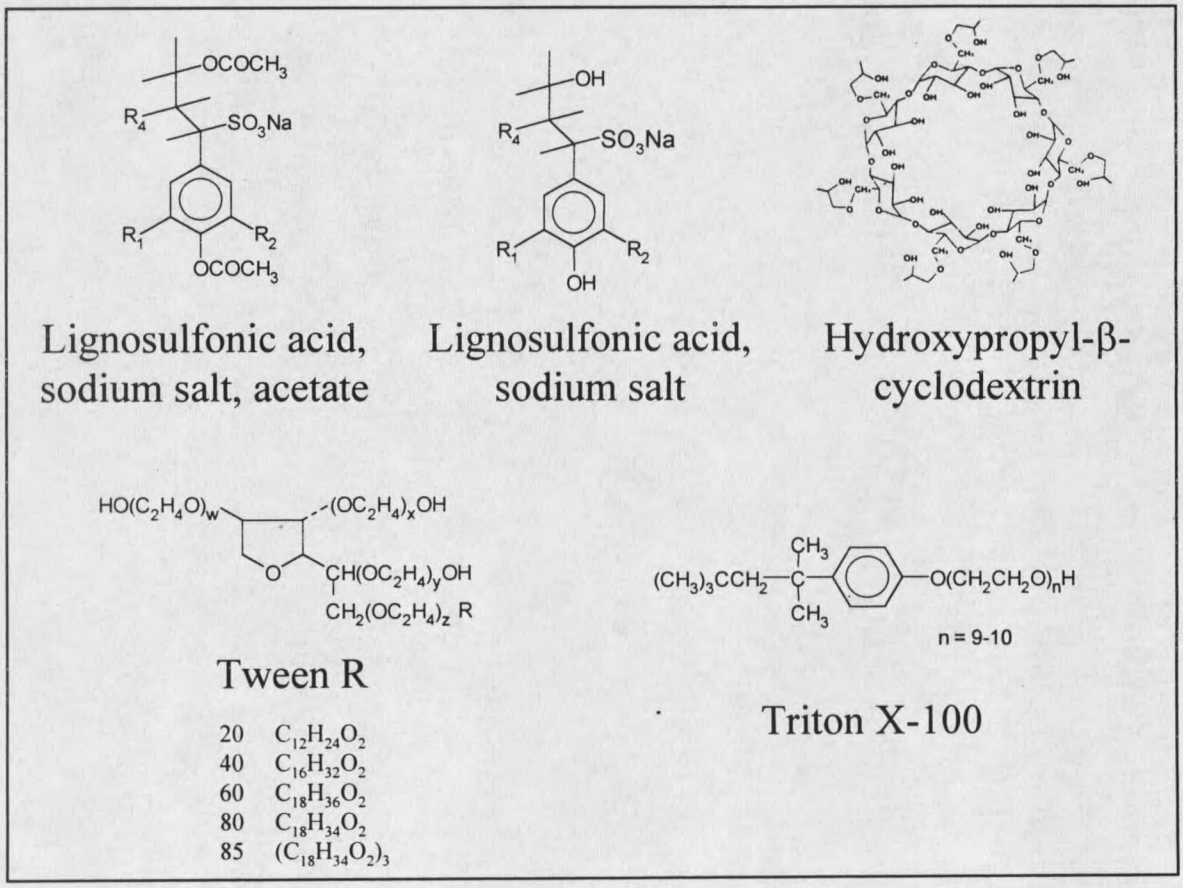

Figure 16. Structures of the nine surfactants used in the study. The chemical formulas for the Tween series are given as the R group.

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.

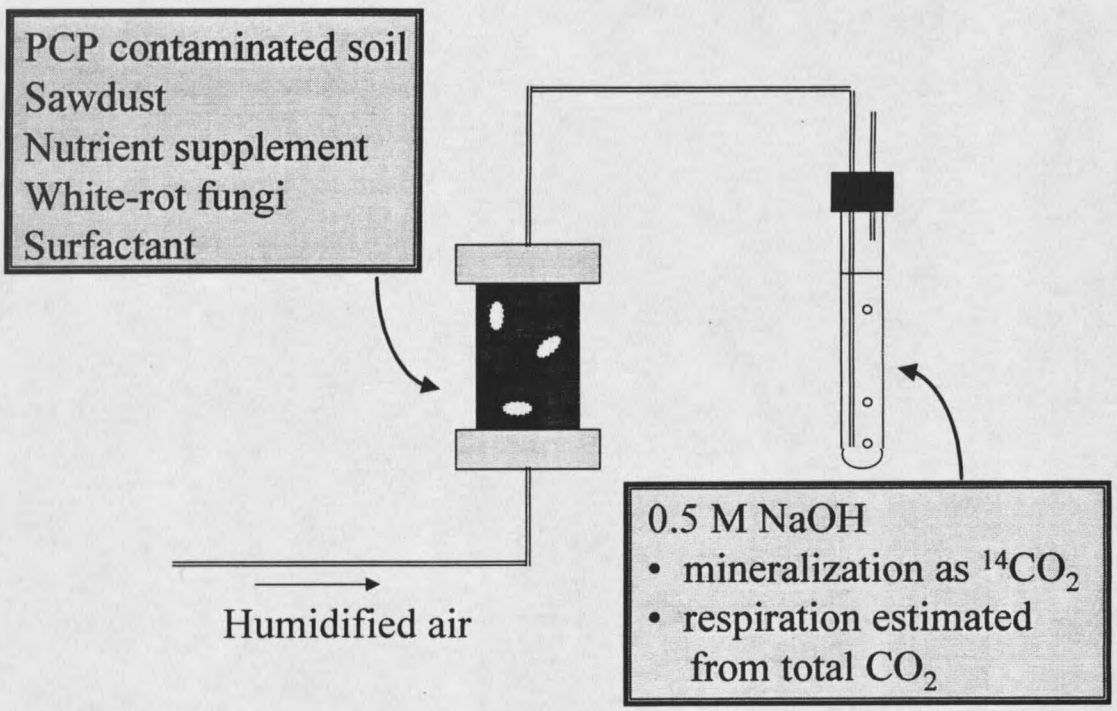

Figure 17. Experimental setup for measuring PCP mineralization in contaminated soil.

PCP Extraction and HPLC Analysis

Soils were solvent-extracted by mixing 5 g soil, 10 g anhydrous Na_2SO_4 (dried for 2 h at 105°C), one microspatula of $Na_2S_2O_4$, 9.75 mL 1:1 acetone:hexane, and 0.25 mL concentrated H_2SO_4 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 N_2, 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 $Q = K_dC$, 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 μm filter into clean tubes containing 0.3 g KOH. Tubes were capped and incubated at 75°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_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^{-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

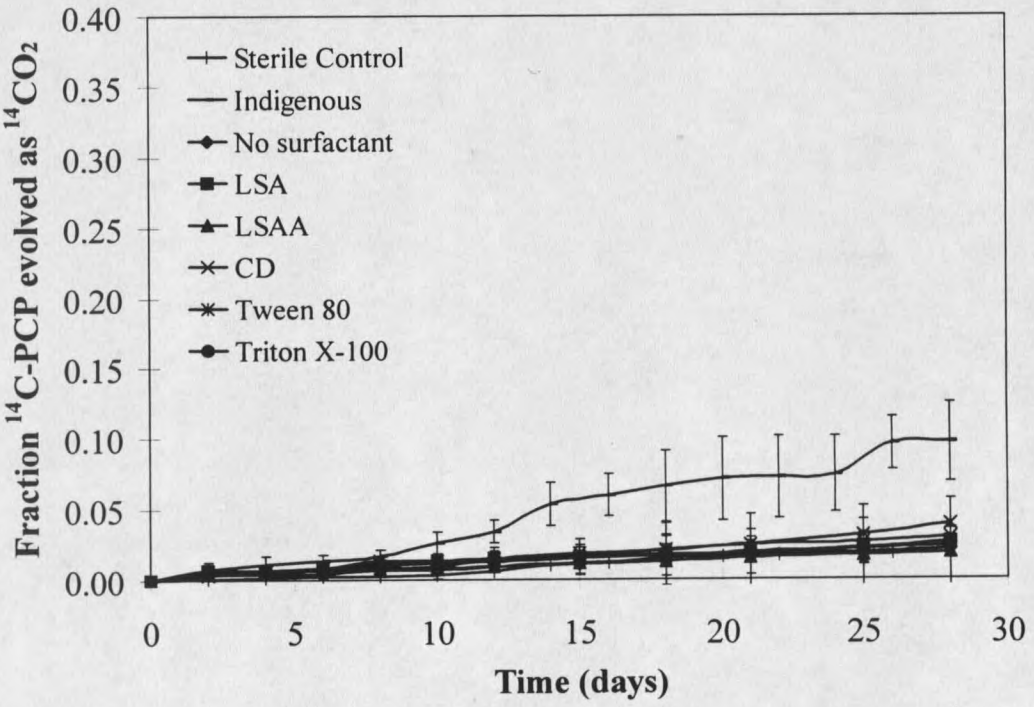

Figure 18. Mineralization of PCP in Amsterdam soil by *Phanerochaete chrysosporium* in the presence of 25 mg surfactant g $soil^{-1}$.

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

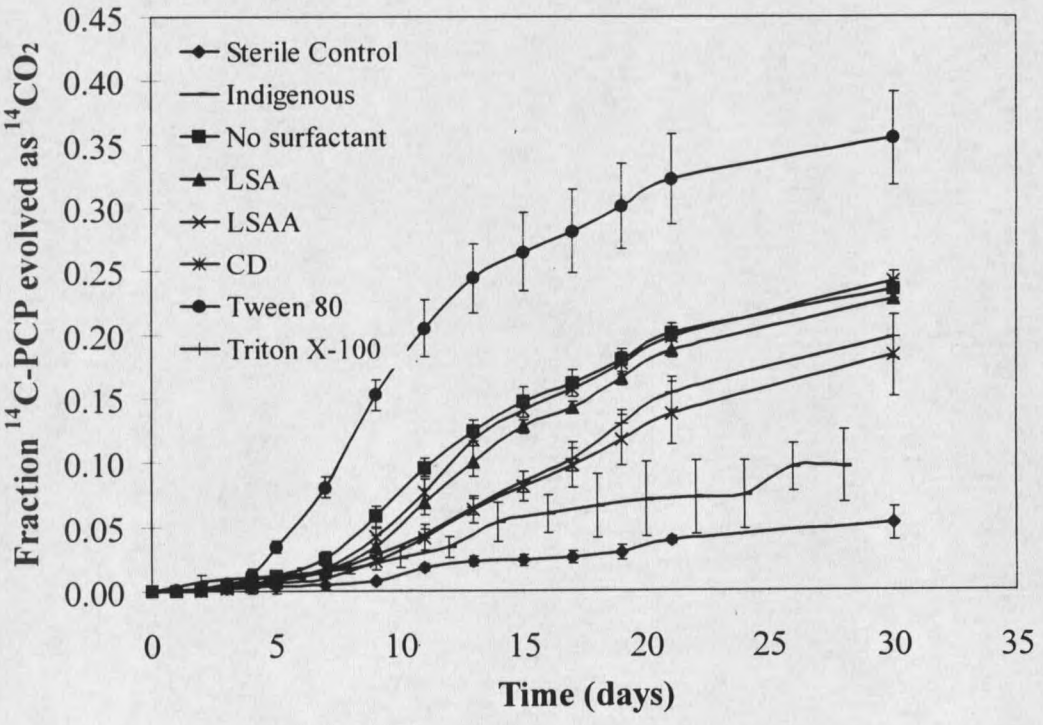

Figure 19. Mineralization of PCP in Amsterdam soil by a non-*Phanerochaete* species in the presence of 25 mg surfactant g soil^{-1}.