THE BIOAVAILABILITY AND TOXICITY OF CHLOROPHENOLS TO THE EARTHWORM, *EISENIA FETIDA*

By

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS

CBR	critical body residue
ILL	incipient lethal level
k_d	desorption rate constant
k_a	sorption rate constant
k_s	dietary uptake rate constant
k_1	uptake rate constant from interstitial water
k_2	elimination rate constant
k_m	metabolic rate constant
k_g	growth rate constant
k_r	reproduction rate constant
K_{ow}	octanol-water partition coefficient
MCP	para-chlorophenol
MW	molecular weight
pK_a	- log of the acid dissociation constant
PCP	pentachlorophenol
TCP	2,4,5-trichlorophenol
$t_{1/2}$	toxicity half-life

CHAPTER ONE

INTRODUCTION

Soils are vital for sustaining terrestrial ecosystems and for agricultural production. Organic chemicals, such as pesticides, industrial chemicals, and petroleum byproducts, can be found in soils of agricultural areas, manufacturing facilities, and oil refineries. Many of these chemicals have known individual effects, but they often are present as mixtures for which effects are undetermined.

The focus of this thesis is assessment of bioavailability of chlorophenols in soil by use of earthworms (*Eisenia fetida*) as a model. Bioavailability will be assessed by examining the relationship between body residues and nominal soil concentrations of chlorophenols, both individually and as mixtures. Critical body residues (CBRs), body residues associated with a toxicological endpoint, will be used to assess and compare the toxicity of a homologous series of chlorophenols: para-chlorophenol (MCP), 2,4,5-trichlorophenol (TCP), and pentachlorophenol (PCP). The questions I will be asking are:

1. How do CBRs relate to the assessment of bioavailability of chlorophenols in soil?
2. Are CBRs an adequate tool to predict the additivity of the toxicity of chlorophenols in a mixture?
3. How do the kinetics of uptake affect the body residues of each chlorophenol in mixture assays?

These questions will be addressed by testing the null hypotheses that CBRs for chlorophenols are constant at the biological endpoint of mortality and the effects of mixtures of chlorophenols are additive.

Physical-Chemical Properties of Chlorophenols

Chlorophenols are found in the environment due to their varied industrial and agricultural applications. MCP is an intermediate and/or impurity in production of higher chlorinated phenols (Howard 1989). TCP has been used as a pesticide and pesticide intermediate in the production of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) (Howard 1991). PCP has been used primarily in the preservation of wood products, telephone poles, and fence posts (Howard 1991). This series of chlorophenols differs in chemical and physical properties due to the number of chlorine atoms substituted on the aromatic ring (Fig. 1). As the degree of chlorination increases, molecular weight and log K_{ow} increase, while water solubility, pK_a, and vapor pressure decrease (Table 1).

Table 1. Chemical and physical properties of the homologous chlorophenol series.

	MW	pK_a	log K_{ow}	Solubility in H_2O (mg L^{-1})	vapor pressure (25°C) (mm Hg)
MCP	128.56	9.41	2.39	27,000	0.087
TCP	197.46	7.43	3.72	982	0.022
PCP	266.35	4.74	5.12	14	0.00011

MCP **TCP** **PCP**

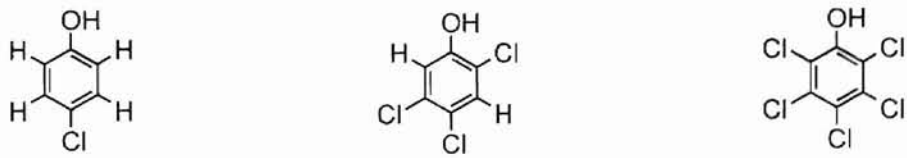

Fig. 1. Structural formulas of the homologous chlorophenol series (Howard 1989, 1991). Handbook of Environmental Fate and Exposure Data.

Also, modes of toxic action vary within this homologous series of chlorophenols. The uncoupling effect on oxidative phosphorylation decreases with decreasing chlorination (Ahlborg and Thunberg 1980), so that MCP and TCP may have other modes of toxic action such as narcosis (McCarty et al. 1992).

Bioavailability and Toxicity

One definition of bioavailability is the ability of a toxicant to move into or onto an organism (Benson et al. 1994). The total amount of chemical in soil is separated into two fractions, a bioavailable fraction and a biologically unavailable fraction. When the bioavailable fraction becomes large enough and the length of exposure to an organism is long enough, toxicity occurs. Bioavailability and toxicity cannot be determined by measuring chemical levels in soil by solvent extraction, but are determined by examining accumulation and effects of contaminants on living organisms using standardized bioassays, toxicity tests, or field exposure assessments (Fitzgerald et al. 1996, 1997; Neuhauser et al. 1985, 1986). Uptake kinetics of the bioavailable fraction by organisms are important in determining toxicity. Kinetics are controlled by the interaction of the organism (biological modifying factors) with the toxicant and the soil (physicochemical modifying factors). Biological modifying factors include test species, metabolic transformation, and excretion, whereas physicochemical modifying factors include length of exposure, pH, ionization, hydrophobicity, and sorption. These modifying factors account for much of the variation in toxicity data found in the literature (Fitzgerald et al. 1996; Sprague 1995, 1970).

Knowledge of the amount and time course of chemical exposure is critical for toxicological evaluation (McCarty and Mackay 1993). Concentration and length of exposure, along with rate of chemical uptake, determine the time to reach a toxicological endpoint or whether one will be reached at all. Regardless of the toxic potency of a chemical, without sufficient exposure duration, toxic endpoints will not be reached. Toxicity estimates based on fixed exposure-time toxicity tests neglect the importance of the time course of accumulation (toxicokinetics) as separate from that of toxic action (toxicodynamics) (McCarty et al. 1992). Uptake of a chemical by an organism is dependent on both rate of uptake and length of exposure, making time-independent toxicity assessments important for accurate toxicological assessments.

An important modifying factor affecting the toxicity of weak organic acids, such as chlorophenols, is ionization (Sprague 1995). The pH of the exposure medium causes changes in toxicity, solubility, and uptake of ionizable organics (Mayer et al. 1994; Hamelink 1977; Sprague 1970), whereas toxicity of neutral nonpolar organics is not greatly modified by pH. The pK_a of a chemical is the pH at which the chemical is 50 percent ionized. For weak organic acids, toxicity and bioaccumulation should decrease with increasing pH for $pH > pK_a$ and be roughly constant for $pH < pK_a$ as the neutral phenolic form is better able to diffuse across membranes. This trend indicates that toxicity and bioaccumulation increase as the fraction of the neutral form of the chemical increases. Modeling mixtures of chlorophenols is complicated by the impact of ionization on uptake kinetics and the effective toxic residue level, as the unionized form is thought to be substantially more toxic because it can readily diffuse across membranes (Sprague 1995; Mayer et al. 1994; McCarty et al. 1992). Effects of pH on the toxicity of

ionizable chemicals to soil organisms is dependent on the pK_a of the chemical in question and the pH of the soil.

Hydrophobic organic chemicals have the ability to bioaccumulate within organisms making it possible to compare chemicals based on their accumulation potential. Bioaccumulation is the cumulative uptake of a chemical from the test medium and ingested matter and is a measure of the ability of a chemical to accumulate in an organism. During bioaccumulation tests, organisms are exposed to a chemical at a constant concentration well below the LC_{50} to minimize toxic effects that could alter chemical uptake kinetics. Accumulation of chemicals in exposed organisms usually follows first-order kinetics, increasing quickly for a time and then reaching a plateau or steady state. As body residues reach steady state, the sorption rate (k_s) and the desorption rate (k_d) are approximately equal and the uptake from interstitial pore water (k_1) and the dietary uptake (k_s) are equal to the elimination rate (k_2) and the metabolic rate (k_m). The growth rate constant (k_g) and the reproduction rate constant (k_r) are relatively insignificant over the short duration of a bioaccumulation test (Fig. 2).

Steady state is the point in time where the chemical residue in the organism does not change over time. The bioaccumulation factor (BAF) is estimated at steady state by dividing the chemical concentration within the test organism by the chemical concentration in the exposure medium (Equation 1). Due to their toxicological significance, residue measurements are now often incorporated into toxicological assessments to provide more information about toxicokinetics and toxicodynamics. BAFs can be calculated for chemicals used in toxicity tests in which body residues are

measured. Body residues may be a better estimate of the amount of chemical at the site of toxic action than surrogate measurements based on exposure medium concentrations.

Bioaccumulation factor (BAF) = [organism] / [soil] (Equation 1)

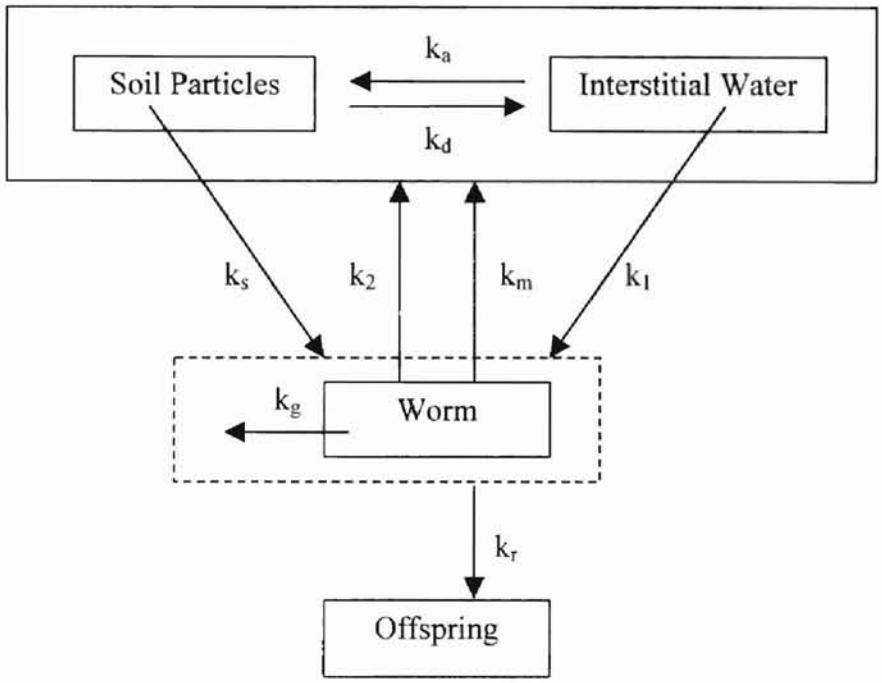

Figure 2. Processes controlling uptake kinetics and body residues within an earthworm, where k_a is the sorption rate constant, k_d is the desorption rate constant, k_s is the dietary uptake constant, k_2 is the elimination rate constant, k_m is the metabolic rate constant, k_1 is the uptake rate constant from interstitial pore water, k_g is the growth rate constant, and k_r is the reproduction rate constant (adapted from Belfroid 1994).

Temperature of the test medium potentially could be a physicochemical modifying factor (Sprague 1995). For ectothermic aquatic organisms, metabolism doubles for every 10°C change in temperature affecting respiration, metabolic rates, and diffusion across epithelial surfaces (Fitzgerald et al. 1996; Mayer et al. 1994). Increasing temperature increases respiration rates, increasing accumulation rates and leading to shorter survival times than at lower temperatures. Temperature differences should be noted when comparing differences in toxicity between species. For example, the differential between

optimal temperatures for *Lumbricus terrestris* and *Eisenia fetida* ranges from 5 to 10°C (Fitzgerald et al. 1996) and may influence LC_{50} values.

The rate of transformation of organic chemicals through photolysis or hydrolysis can be affected by the association of the chemical with certain types of organic matter (Gobas and Zhang 1994). Pentachlorophenol biodegrades quickly in the presence of UV light when dissolved in relatively clean, clear water to produce quinones and acids or tetra- and trichlorophenols (Crosby 1994). Chemicals that are sorbed to organic matter may be degraded less than freely dissolved chemicals. Sorbed chemicals are less subject to the diffusion-controlled process of volatilization and diffusion between organic matter and the aqueous phase of soil. To characterize or estimate the extent a chemical will be in association with organic matter, it is important to understand the nature of the interactions between organic chemicals and organic matter (Gobas and Zhang 1994). Chlorophenol anions are more mobile than the unionized form (You and Liu 1996). The adsorption of chlorophenols increases with increasing hydrophobicity of the adsorbent (soil organic matter) and the chlorophenol.

Earthworms compete with soil organic matter for accumulation of lipophilic organic chemicals (Fig. 2). The amount of organic matter present in the soil can affect the toxicity of chemicals (van Gestel and van Dis 1988). Sorption (k_a) and desorption (k_d), are especially important to tissue residue dynamics and toxicity as they affect the bioavailability and uptake of chemicals from the diet and interstitial water by earthworms. Soil organic matter is the primary soil property controlling the adsorption of organic chemicals in soils (Bhandari et al. 1996) with the hydrophobic nature of the chlorophenols as the driving force of the adsorption reactions (You and Liu 1996). The

amount of chlorophenol sorbed to soil organic matter decreases from PCP to TCP to dichlorophenol (DCP) (You and Liu 1996). Oxidative coupling of MCP to soil organic matter results in a non-desorbable fraction of MCP in soil (Bhandari et al. 1996). Benoit et al. (1996) found that DCP and MCP sorption to humic acid was strong. Sorption of ionizable organic compounds cannot be predicted by using the organic carbon fraction due to variation in ionization at different pH values. Processes other than partitioning can affect the behavior of organic acids. Most attention has been given to studying the partitioning or sorption of organics to suspended or solid organic matter. Natural inorganic phases such as clays, silicates, and metal oxides have the ability to sorb organics, although their ability to do so is much weaker. There is a great bulk of these inorganic phases in the environment and cumulative sorption could greatly impact the bioavailability of organic chemicals and the amount of chemical reaching the organism (Gobas and Zhang 1994).

Dose estimation is very important to the science of toxicology. Paracelsus stated in the 16th century that "All substances are poisons; there is none which is not a poison. The right dose differentiates a poison from a remedy" (Paracelsus 1567). Dose-response theory forms the basis of toxicological study and involves three assumptions. First, the amount of chemical at the site of toxic action is proportional to the concentration and nature of exposure. Second, biological responses occur when the chemical is present at the site of toxic action. Third, the biological response is proportional to the amount of chemical at the site of toxic action after the toxic threshold has been reached (McCarty and Mackay 1993). These three principles concern the uptake and depuration rates of the chemical of interest. Accumulation occurs when the uptake rate is greater than the

elimination rate. Organic chemicals accumulate within the organism at the site of toxic action until a threshold is reached and then biological response(s) begin to occur. The biological response(s) is proportional to the amount of chemical at the site of toxic action. Measuring the amount of chemical at the site of toxic action is difficult, thus the amount of chemical in the exposure medium is generally used as a surrogate (McCarty and Mackay 1993).

Toxicity Evaluation

Three factors interact to produce a toxic response: exposure, toxicokinetics, and toxicodynamics (McCarty and Mackay 1993). The toxicity information on mortalities is used to compute LC_{50} values from which comparisons of toxic potencies of chemicals are made. Kinetic behavior, bioavailability, and biotransformation can influence the results of LC_{50} tests (de Bruijn et al. 1991). Use of a simple LC_{50} at a single defined time can be a misleading indicator of toxicity due to slow uptake kinetics of a chemical. Erroneous conclusions about the toxicity and potency of chemicals may be drawn if the test has not reached a steady state with cessation of mortalities (Lanno et al. 1997; Mackay et al. 1992). Toxicity curves provide information about the toxicity half-lives ($t_{1/2}$s) and incipient lethal levels (ILLs) and as they are measurements made at a steady state with respect to time, they are less dependent on external modifying factors. The ILL is a time-independent LC_{50} value or concentration at which half of the exposed organisms will live indefinitely with the $t_{1/2}$ being the time necessary to achieve half the ILL (Fitzgerald et al. 1997, 1996; Sprague 1995).

Toxicity curves are developed using raw toxicity data to aid in evaluating of the inherent toxicity of chemicals and can provide important information about toxicant kinetics. They are based on LC_{50} values derived from mortality observations over the course of the toxicity test. A mathematical estimate of the LC_{50} is calculated for each observation time where greater than 50% mortality has occurred (Lanno et al. 1997; Hamilton et al. 1977). Confidence limits on the LC_{50} can be obtained when there is partial mortality in some of the replicates. The approach used for construction of the toxicity curves is to plot LC_{50}s (or $1/LC_{50}$s) for a geometric series of inspection times against time (Lanno et al. 1997). The ILL and $t_{1/2}$ can be estimated by nonlinear regression with the ILL as the asymptotic value of the curve and $t_{1/2}$ as the time necessary to obtain half of the ILL (Fig. 3) (Fitzgerald et al. 1997, 1996). The ILL provides information on toxicity and potency and allows toxicities of different chemicals to be compared easily and meaningfully because they are time-independent lethality values (Lanno and McCarty 1997). The $t_{1/2}$ indirectly provides information on the kinetics of toxicant uptake and can be used to compare the kinetics between different toxicants.

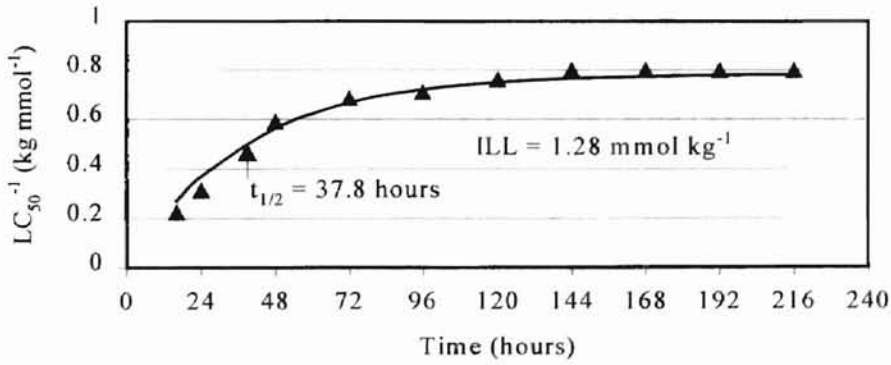

Fig. 3. Generic toxicity curve created by plotting inverse LC_{50} against time. Toxicity half-life ($t_{1/2}$) and incipient lethal level (ILL) are estimated from nonlinear regression parameters.

Short-term toxicity tests focus on acute effects of a chemical and lethality is often the desired endpoint (Abdul Rida and Bouche 1997). Standardized acute toxicity tests with *E. fetida* are usually conducted for 7-28 days (ASTM 1995). Tests should continue until mortality has ceased in order to obtain a complete toxicity curve for evaluation of ILLs and $t_{1/2}$s (Fitzgerald et al. 1996). Earthworm soil toxicity tests with some hydrophobic chemicals may not achieve an ILL in 14 days, the suggested duration for acute lethality tests with earthworms (Lanno and McCarty 1997). ILLs are usually attained after 4-5 $t_{1/2}$s (Lanno and McCarty 1997). Only toxicity data up to 14 days were used to model toxicity and estimate the ILL for *Eudrilus eugeniae* and *L. terrestris* exposed to PCP because very few mortalities occurred after 14 days and the ILL had been reached (Fitzgerald et al. 1996). Tests longer than 14 days may exhibit inconsistencies in the LC_{50} data due to mortalities other than from exposure to test chemicals, such as reduced food intake (Fitzgerald et al. 1996). Evaluation of the effect of supplemental feeding on earthworms in 21-day tests showed significant differences in growth and reproductive response (cocoon production) between fed and unfed groups (Gibbs et al. 1996).

Standardized toxicity tests are merely tools designed to compare the relative toxicity of chemicals. However, they cannot be used to predict the effects of toxicants in real-world field situations without adequate validation in the field (Abdul Rida and Bouche 1997). The contact filter paper test, a standardized protocol for earthworm toxicity testing, gives consistent and reproducible results; however, these results do not correlate well to results from other types of toxicity tests (Neuhauser et al. 1986; Callahan et al. 1985). This test system also does not describe what will happen within a soil system (Neuhauser et al. 1985). The ASTM artificial soil toxicity test better mimics what

happens in soil systems because the test medium is a representative artificial soil made up of organic matter, sand, and clay. This standard, reproducible "soil" gives consistent results among laboratories. Results of the artificial soil test should not be extrapolated to the field, but toxicity test results using field soils with similar chemical-physical characteristics to the artificial soil may be well correlated.

Critical Body Residues

Chemical residues in an organism that are linked to ecologically relevant endpoints such as survival, growth, and reproduction are termed critical body residues (CBRs) (Fitzgerald et al. 1996; Belfroid et al.1993) and are determined using bioassays and toxicity tests (McCarty and Mackay 1993). CBRs show that uptake kinetics are largely responsible for the differences in ILLs (Fitzgerald et al. 1996). Whole-body residues are better surrogates for estimating the amount of chemical at the site of toxic action than ILLs. As an example, a difference in toxicity among species of earthworms, expressed as LC_{50} values, demonstrates that smaller earthworms are more sensitive to PCP than larger earthworms (Fitzgerald et al. 1996). This may not be the case, however as the internal body concentration or residue at lethality in these different species of earthworms is similar and accounts for bioavailability since organism uptake defines the bioavailable fraction. Three earthworm species, *E. fetida, L. terrestris,* and *E. eugeniae*, varying in size by an order of magnitude, were exposed to PCP in artificial soil. ILLs for *L. terrestris* and *E. eugeniae*, 0.72 and 0.63 mmol kg^{-1} respectively, were significantly higher than ILLs for *E. fetida*, 0.14 mmol kg^{-1}, the smallest of the three species (Fitzgerald et al. 1996). However, CBRs for *E. fetida* were 0.33-2.65 mmol kg^{-1} while the combined residues of *L. terrestris* and *E. eugeniae* were 0.47-2.18 mmol kg^{1}. CBRs were

not significantly different for the PCP exposures for the three species even though there were significant differences in the ILLs. CBR assessment of toxicity and bioavailability of chemicals helps to overcome erroneous conclusions about the potency of chemicals based on exposure medium concentrations due to modifying factors of toxicity.

CBRs have several advantages over the expression of toxicity based on external ambient concentrations. Bioavailability is incorporated into CBRs and uptake kinetics can be determined by measuring residues and mortalities over time. Toxic potencies are expressed more clearly with internal concentrations, possibly allowing for determination of different modes of toxic action since chemicals with specific modes of action often have lower CBRs than chemicals with non-specific modes of action (e.g. narcosis).

Chemical residues for acute narcosis appear to be approximately constant among species and range from 2 to 8 mmol kg^{-1} (Table 2). The differences in CBRs associated with narcosis among different species may be due to differences in proximate composition or physiology rather than differences in target site concentration. Organisms with very high lipid content can store chemical in neutral lipid, minimizing the amount reaching the site of toxic action and resulting in a higher CBR for the same biological response as organisms with a low lipid content. Toxicity estimates using CBRs are less variable than LC_{50} data based on external soil concentrations (McCarty and Mackay 1993). Fitzgerald et al. (1996) observed ILLs among three earthworm species in different test soils spiked with PCP to be significantly different. The ILLs varied seven-fold whereas CBRs for the same earthworms varied less than three-fold (Fitzgerald et al. 1996). The differences in toxicity expressed as exposure concentrations and CBRs illustrate how CBRs can reduce variation in toxicity data.

CBRs can help overcome many problems associated with toxicity testing. However, there are some disadvantages to using CBRs. Metabolic breakdown or activation, internal distribution, lipid types and content, and temperature may impose problems on the toxicological evaluation (McCarty and Mackay 1993). These toxicokinetic factors also cause many problems with standard approaches to toxicity assessment (e.g. LC_{50}s). CBRs may bias the apparent toxicity of a chemical if it is metabolized and detoxified readily. In such a case, the body residues of the parent compound will be decreased and therefore the apparent toxicity may be overestimated (Mackay et al. 1992). Activation of chemicals by mixed function oxidase enzymes can result in metabolites that are more toxic than the parent chemicals and can complicate toxicity interpretation (Mackay et al. 1992). Because earthworms have a mixed function oxidase system similar to that of other invertebrates, but less active than that of vertebrates (Lee 1998), activation should pose little problem to the use of CBRs. When irreversible damage occurs to the organism from a single dose or sustained exposure, it would be incorrect to use CBRs, as the residue would not be indicative of toxicant at the site of toxic action. The damage sustained could significantly affect the sensitivity of the organism to the chemical and result in variable CBRs that are not necessarily indicative of the nature of the exposure. The chemical residue needs to act as its own marker or descriptor of exposure (McCarty and Mackay 1993).

Mode of Toxic Action

The mechanism by which a large class of organic chemicals exert their toxic action is by a nonspecific narcotic effect or anesthetic mode of action (narcosis) (Abernethy et al. 1988). The actual toxic mechanism and site of action remain unclear for narcosis. Two theories used to explain narcosis are the critical volume theory and the protein binding theory. The critical volume theory hypothesizes that narcosis results from changes in the structure of the lipid bilayer in nerve cell membranes due to an increase in volume caused by dissolved toxicant. The membrane accumulates a high volume of the chemical so that it 'swells', interfering with the structure and function of the cell membrane (Abernethy et al. 1988). A critical volume of approximately 1% was found to result in narcosis in many organisms (Mullins 1954). Further work by Miller et al. (1983) has shown the critical volume to be as low as 0.5%. Increasing atmospheric pressure has been observed to reduce or eliminate the narcotic effect and supports the critical volume theory because the reduction of volume with increasing pressure decreases the critical volume (Abernethy et al. 1988; Franks and Lieb 1982). According to the protein binding theory, narcosis occurs due to binding of the chemical to specific receptor sites located in hydrophobic regions of proteins found in nerve membranes (Abernethy et al. 1988). Franks and Lieb (1982) suggest that narcosis occurs from chemicals binding to a sensitive protein and inhibiting its normal function

Chemicals with modes of toxic action other than narcosis often are associated with differing ranges of body residues (Table 2) (McCarty and Mackay 1993). Pentachlorophenol is a metabolic inhibitor (uncoupler of oxidative phosphorylation) and not a narcotic (Ahlborg and Thunberg 1980). Because it has a specific mode of action

and a high log K_{ow}, it is more potent than other chlorophenols that act by narcosis and will subsequently have lower CBRs. When assuming that narcosis results from a minimal effect, it follows that organisms dying of exposure to chemicals with specific modes of action will have a lower CBR than those that die from narcotics. Even if lethal levels of a chemical are similar for fish and aquatic invertebrates, it must not be assumed that the mode of action is the same (Sprague 1970). Modes of action may differ between species in relation to different physiologies. Modes of action also can have negative impacts on the accurate assessment of mixtures of chemicals.

Table 2. Modes of action for organic toxicants and associated CBR estimates in fish. Adapted from McCarty and Mackay (1993).

Mode of action	Estimated residue (mmol kg^{-1})
Narcosis	
acute – summary	2 – 8
Polar narcosis	
Acute – summary	0.6 – 1.9
Acute – 2,3,4,5-tetrachloroaniline	0.7 – 1.8
Respiratory uncoupler	
acute – pentachlorophenol	0.3
acute – 2,4-dinitrophenol	0.2
acute – pentachlorophenol	0.11

Toxicity of Mixtures

Assessing the toxicity of chemical mixtures often is difficult because chemical interactions are not well understood. Chemicals in mixtures may have different modes of action, prompting many different biological responses which may result in toxicological effects that are substantially different from any of the individual components (McCarty et al. 1992). The effects of mixtures of chemicals can be described best using the terms antagonistic, additive, and synergistic (Sprague 1970). Antagonism occurs when the

effects of two chemicals combined is much less than the sum of their effects individually. Additivity of toxicity occurs when combined the effect of two or more chemicals is equal to the sum of the effects of each chemical alone. Synergism is when the combined effect is much greater than the sum of individual effects. The time course of toxicity in a mixture will be the result of the kinetics of uptake of each individual chemical. CBRs may facilitate the toxicological assessment of mixtures of chemicals since body residues can be established for each component of the mixture to determine which chemical(s) is responsible for the toxicity (McCarty and Mackay 1993). The proportion of each chemical contributing to the CBR will vary with time until all have attained a steady-state body residue or the organism dies.

The "toxic unit" model is a commonly used approach for assessing the toxicity of mixtures (Deneer et al. 1988; Sprague 1970). The toxic unit (TU) is defined as the concentration of a particular toxic chemical in a mixture divided by the ILL for the biological response endpoint in question (McCarty et al. 1992; Sprague 1970). This approach can also be expanded to include TUs expressed as CBRs (Equation 2).

$$\text{Toxic Unit} = \frac{\text{[Medium or Solution]}}{\text{[Incipient Lethal Level]}} = \frac{\text{Body residue in organism}}{\text{Critical body residue}} \quad \text{(Equation 2)}$$

Toxic units are dimensionless and can be added together for every component in a mixture allowing for a sum of all of the body residues (McCarty et al. 1992; Sprague 1970). Toxic units are based only on toxicity to one species of organism used in the toxicity tests, due to the fact that ILLs could vary between species of organisms (Sprague 1970). Organic chemicals will contribute to the narcotic effect of the mixture even at concentrations as low as 0.0025 TU. Therefore, any chemical that acts by narcosis, according to its hydrophobicity and concentration, should contribute to the toxicity of the

mixture even at extremely low concentrations (Deneer et al. 1988). For large mixtures of chemicals, Hermens et al. (1984) and has shown that the larger the number of chemicals in a mixture, the better the approximation of additivity of toxicity (McCarty and Mackay 1993). Additivity of chemicals is probably the most common occurrence for effects of mixtures of chemicals.

A molar concentration of a narcotic mixture in an organism should produce a biological response similar to that produced by the same molar concentration of a single narcotic chemical (McCarty et al. 1992). Chemicals with a specific mode of action possess some narcotic potency, depending on the hydrophobicity of the chemical. When present at concentrations well below those necessary to cause the specific mode of action, they will contribute to the total narcotic potency of the mixture (Deneer et al. 1988). One explanation for additive toxicity among chemicals with dissimilar modes of toxic action is that when chemicals with specific modes of toxic action are present at levels well below the LC_{50} for their response (0.3-0.02 of their LC_{50} or critical body residue), they do not express the toxic action and merely contribute to the narcotic effect of the mixture (McCarty and Mackay 1993). Understanding how mixtures of chemicals interact is valuable for remediating contaminated sites to safe levels.

Summary

In summary, ILLs, based on external soil concentrations, do not consider bioavailability. Using CBRs for toxicological assessment takes bioavailability into account since the amount of chemical that the organism bioaccumulates is measured and variability in toxicity data due to modifying factors is reduced. Assessment of the

toxicity of chemical mixtures may be conducted using ILLs and CBRs set to toxic unit equivalents, allowing for a determination of the constituent of the mixture that is contributing to most of the toxicity. The objectives of this thesis are to determine ILLs, $t_{1/2}$s, and CBRs for a homologous series of chlorophenols and evaluate how each chlorophenol contributes to mixture toxicity. The null hypotheses being tested are that CBRs for chlorophenols are constant at the biological endpoint of mortality and the effects of mixtures of chlorophenols are additive.

CHAPTER TWO

MATERIALS AND METHODS

This study of the toxicity and bioavailability of chlorophenols was divided into two sections. The first part was the exposure of earthworms to chlorophenols spiked in artificial soil and analysis of LC_{50} values from these tests. Secondly, earthworm tissues were extracted, chlorophenol residues quantified and comparisons of toxicity were made using both ILLs and CBRs. Tests were conducted with both individual chlorophenols and chlorophenol mixtures.

Testing protocols were modified from the ASTM (1995) methodology E 1676 – 95, "Standard Guide for Conducting a Laboratory Soil Toxicity Test With Lumbricid Earthworm *Eisenia foetida*," designed to assess lethal or sublethal toxic effects on earthworms in short-term tests (7-28 days). Spiked artificial soil is an accepted exposure medium (ASTM 1995) and was used in all toxicity tests. The results of the tests were reported as LC_{50} (median lethal concentration) values from which ILLs were estimated.

Chemicals, Organisms, and Test Medium

Para-chlorophenol (MCP, 99% pure, Fisher Scientific), 2,4,5-trichlorophenol (TCP, 99% pure, Chem Service), and pentachlorophenol (PCP, 99% pure, Chem Service) stock solutions were prepared by dissolution in reagent grade water (RGW) using volumetric glassware within 12 hours of spiking the artificial soil. Sodium hydroxide (ACS grade, Fisher) was added to the TCP and PCP stock solutions to aid dissolution (Fitzgerald et al. 1997). RGW was prepared by passing tap water through two activated carbon filters, two research-grade, mixed-bed, deionizer cartridges, one HPLC-grade resin cartridge, and

reverse osmosis treatment. The final step in RGW preparation was to pass the water through a 0.2 um filter and a UV sterilization unit to remove bacteria/viruses and to break down any chemicals that remained in the purified water.

Eisenia fetida were obtained from either Willingham Worm Farm (Butler, GA) or Granny's Hillside Farms (Gore, OK). Worms from Willingham were cultured at 25°C in peat moss and fed fermented alfalfa pellets, rolled oats, and shredded newspaper. *Eisenia fetida* obtained from Granny's Hillside Farms were cultured in fresh and composted horse manure at 25°C. Moisture of culture media was maintained by spraying the surface with reconstituted water when the media began to look and feel dry (roughly twice a week). Reconstituted water was prepared from RGW by adding salts [magnesium sulfate, calcium carbonate, and sodium bicarbonate] to adjust the alkalinity and conductivity.

Artificial soil used in toxicity tests was composed of 69% silica sand (60 mesh), 20% pulverized kaolin clay, 10% finely sieved peat (2-mm sieve), and 1% calcium carbonate. Artificial soil was prepared in 10-kg batches with the pH ranging from 5.5 to 7.0. The components of the artificial soil were mixed thoroughly and the moisture content was determined by difference.

General Toxicity Test Preparation

Test containers used for toxicity testing were 500-ml, wide-mouth glass canning jars. Canning jar lids punctured twice with an ice pick (2-3 mm holes) were placed on the jars to allow some exchange of air and to prevent escape of the earthworms during testing. Stainless steel spatulas and ceramic coated trays, 30 cm x 45 cm, were used for sorting and counting the earthworms. A constant temperature room at 25°C was used for range-

finder tests and incubators (Percival Scientific) at 25°C were used for definitive tests. Photoperiod in both the exposure room and incubators was 12 L : 12 D, which deviates slightly from ASTM (1995) which states that continuous lighting is preferred. All tests except the range-finder assays had at least three replicates.

All glassware and other items were scrubbed to remove soil and residue, soaked in hot water with Micro® detergent for 15 minutes, and rinsed with tap water (Greene et al. 1989). An acetone rinse and two distilled water rinses were followed by soaking items in a 10% nitric acid bath for 24 hours. Finally, the items were rinsed with reagent grade water and allowed to dry.

General Toxicity Test Outline

Aliquots of artificial soil (200 g, dry weight) were weighed into 500-ml canning jars and spiked with the appropriate amount of chlorophenol stock solution. Distilled water was then added to bring moisture content to 54% (dry weight) and soils were mixed with long-handled spoons until they appeared homogeneous. Lids were placed on the jars and the soils were allowed to equilibrate for 24 hours in the dark before organisms were added to the test containers. Each range-finder assay was conducted without replication. For each definitive test, four replicates per concentration were used, three earthworm exposure replicates and a fourth for monitoring physical/chemical characteristics. The only exception was the second MCP/TCP/PCP test which had four replicates of earthworms and no physical/chemical replicate. Subsamples of 40-50 g of soil were taken from the physical/chemical replicates of the definitive tests at the 24-hour equilibration time. *Eisenia fetida* were randomly removed from culture tubs, rinsed,

blotted dry, and placed in holding containers in groups of ten. Weights of each group of ten *E. fetida* were taken at this time for the second PCP and second MCP/TCP/PCP mixture assays. One group of ten *E. fetida* was placed on the surface of the artificial soil in each replicate and the lids were fastened in place. Earthworms were not acclimated to artificial soil prior to commencing the tests. Observations on mortalities were made in a geometric time series for the first 16 hours and at 24-hour intervals thereafter (e.g. 1,2,4,8,16,24,48,72...) until mortalities ceased for a period of 48-72 hours at which time the ILL was assumed to have been reached. At each observation time, each jar was individually examined by dumping contents onto a porcelain tray and sorting through the soil with a stainless steel spatula to find the earthworms. Earthworms were determined to be dead when no response to gentle mechanical stimulus to the anterior end was observed. Dead earthworms were removed from the soil, rinsed with distilled water, blotted dry, wrapped in hexane-rinsed aluminum foil, and stored at -40°C for body residue analysis. Test jars were examined in order of increasing concentration to prevent contamination of controls and lower concentrations with soils of higher chlorophenol concentrations. Once the ILL had been reached, the test was terminated. Remaining earthworms from each replicate were removed from the soil, rinsed with distilled water, blotted dry, and placed in holding containers. At this time, weights of earthworms in replicates with no mortalities were taken for the second PCP and second MCP/TCP/PCP tests. Earthworms were then wrapped in hexane-rinsed aluminum foil, and stored at -40°C for body residue analysis. As earthworms were removed, the soils were placed in pint or quart Ziploc® bags and stored at -40°C.

Range-finder Toxicity Tests

The range-finder tests for MCP and TCP were composed of five, nominal logarithmic concentrations (0.1, 1, 10, 100 and 1,000 mg/kg) with a negative control. The TCP test had an additional carrier control with sodium hydroxide. A PCP range-finder test was not conducted because LC_{50} values from the literature were available (Fitzgerald et al. 1997, 1996). There was no replication of concentrations as these tests were only range-finders for the later definitive tests.

Definitive Toxicity Tests

Four single chemical tests were conducted, one each for MCP and TCP and two for PCP. The definitive tests for MCP and TCP were designed to bracket the LC_{50}s determined by the range-finder tests. Nominal concentrations of 100, 150, 220, 320, 460, 680, and 1000 mg/kg MCP were used with a control. TCP tests comprised nominal concentrations of 10, 16, 25, 40, 63, and 100 and two controls, a negative control and a carrier control with sodium hydroxide. The first PCP test nominal concentrations were 10, 16, 25, 40, 63, and 100 with negative and sodium hydroxide controls and the second PCP test nominal concentrations were 10, 18, 32, 56, and 100 with a sodium hydroxide control. The negative water control was omitted. Three mixture tests were conducted using the chlorophenol series. Two tests were conducted using the three chlorophenol components, MCP, TCP, and PCP. A third test included only TCP and PCP. Nominal concentrations for all mixture tests were 0.25, 0.5, 1, 2, and 4 toxic units with a sodium hydroxide control. Toxic units were based on LC_{50} values at termination of the

individual chemical toxicity tests as ILLs were assumed to have been obtained. The LC_{50} values for MCP and TCP approximate the ILL whereas the LC_{50} value for PCP is 2.28 times than the ILL for PCP.

Tissue Extraction and Sample Concentration

The procedure for extracting the TCP and PCP from earthworm tissue was modified from Belfroid et al. (1993) which specified a 5-hour soxhlet extraction with hexane for removal of chlorobenzenes from *E. fetida* tissue samples. To increase the number of samples extracted at a time, the earthworms for this project were extracted in hexane (95% n-hexane, organic residue analysis grade, J.T. Baker) by a hot solvent extraction method using 100-ml digestion tubes placed in a 65°C water bath. Earthworms were removed from the freezer and weighed to five decimal places on a Mettler H20T analytical balance. Worms were homogenized using a hexane-rinsed mortar and pestle with 1 to 2g of Na_2SO_4 (ACS grade, 10-60 mesh, Fisher). Homogenate was then placed into the 100-ml digestion tube. Hexane used to rinse the mortar and pestle was added to the digestion tube bringing the total volume of hexane to 20-25 ml. The tissue samples were extracted for five hours in a 65°C water bath using the digestion tube with a small funnel on top to prevent solvent evaporation.

Contents of the digestion tube were cooled to room temperature and filtered through Whatman GF/B 25-mm filters (1um pore size) in a 15-ml microanalysis filter holder apparatus. The digestion tube and filter assembly were rinsed twice with hexane to remove any residual chlorophenol. Filtrate was collected in a 25x150-mm test tube placed inside a 500-ml Erlenmeyer vacuum flask. The test tube was removed from the

flask to concentrate the sample. Samples were placed in the 65°C water bath and N_2 (high purity, Sooner Airgas) was bubbled through the filtrate via pasteur pipettes to concentrate them to approximately 0.25 ml. Samples were transferred to 8-mm GC vials along with two hexane rinses of the test tube used to concentrate the samples. The vial was then capped and stored at 4°C for cleanup and lipid analysis.

Sample Cleanup

Cleanup of earthworm extracts was accomplished using a method modified from Mundy and Machin (1981) for PCP cleanup after extraction from animal tissues. Waters Sep Pac Vac cartridges containing florisil (3 cc / 500 mg, Phase Separations) were used in the cleanup of the samples containing TCP and PCP. Methanol (gas chromatography and residue analysis grade, EM Science), chloroform (residue analysis grade, Fisher), and ethyl ether (anhydrous, reagent grade, Fisher) were used in the preparation of the cartridges. The cartridges were prepared by washing with 10 ml methanol/chloroform (1:9 v/v), 10 ml chloroform, 10 ml of ethyl ether, and 10 ml of hexane. Following preparation of cartridges, one half of the concentrated extract was measured and removed from the GC vial using an SGE® 1.0 ml syringe. Volume was recorded for calculation of CBRs. The cartridge was eluted twice with 2 ml hexane, twice with 2 ml ethyl ether, and twice with 2 ml methanol/chloroform (1:9 v/v) using 10-ml serological pipettes. The methanol/ chloroform fraction containing the chlorophenols was placed in a graduated test tube containing 2-3 ml of 2,2,4-trimethylpentane (gas chromatography and residue analysis grade, EM Science). The test tube was placed in a heating block at 65°C with N_2 bubbled through the sample. The sample was concentrated to less than 0.5 ml, 2 more ml

of 2,2,4-trimethylpentane was added to the tube, and the sample was again concentrated to less than 0.5 ml. The volume was increased to 1.0 ml with 2,2,4-trimethylpentane after the test tube had cooled and the sample was transferred to a GC vial for analysis. Due to incompatibility of MCP with this extraction and cleanup procedure, MCP residue data are lacking from this thesis.

Spike Recoveries

Spike recoveries using six earthworms were conducted to determine percent recoveries of TCP and PCP, respectively. Earthworms were divided in half, with one half used for control and the other half as the spiked sample. The six halves for recovery analysis were spiked with 10 ug TCP and treated as the other earthworm samples from extraction through analysis. Percent recoveries were 54.7% (*SE* = 1.92). The six halves for PCP recovery analysis were spiked with 1.30 ug of PCP and treated as the other earthworm samples from extraction through analysis. Percent recoveries were 49.2% (*SE* = 4.76).

Gas Chromatography

A Tracor model 560 gas chromatograph with electron capture detector (ECD) and a megabore DB-5 MS fused-silica capillary column (J&W Scientific) were used in the analysis. Helium (high purity grade, Sooner Airgas) (flow rate = 10 ml min^{-1}) was the carrier gas with argon/methane (high purity grade, 5% methane, Sooner Airgas) (flow rate 20 ml min^{-1}) as the makeup gas resulting in a combined flow through the detector of 30 ml min^{-1}. Two temperature programs were used, 130°C (4 minute hold) with 50°C/min ramp to a final temperature of 250°C (3 minute hold) was used to analyze some

residues from the first PCP test and 175°C (1 minute hold) with 50°C ramp to a final temperature of 250°C (2 minute hold) was used for the remaining PCP residues, TCP residues, and the TCP/PCP residues.

Concentrations of TCP and PCP in purified samples were estimated by external calibration using peak area with calibration curves using Maxima® Chromatography Software (version 3.30, Dynamic Solutions Inc. 1990) or Peaknet® Chromatography Software (version 4.20, Dioniex 1995). Outputs of residue data were in pg/ul, from which body residues were calculated.

pg/ul * 1000 = pg/ml = pg in half of worm extract
pg in half of worm extract / 0.5 = pg in worm
pg in worm / worm weight (g) = pg/g worm
pg/g worm / 1000000 = ug/g worm
ug/g worm / MW (g/mole) = mmol/kg worm
mmol/kg worm / % recovery = corrected body residue

For example, the output for sample X is 2000 pg PCP/ul (MW=266.35 g $mole^{-1}$)

$$\frac{2000\,pg-PCP}{ul}\times\frac{1000ul}{ml}\div 0.5=\frac{4000000\,pg-PCP}{worm}\div 0.250g=\frac{16000000\,pg-PCP}{g}$$

$$\frac{16000000\,pg-PCP}{g}\div\frac{10000000\,pg}{ug}\div\frac{266.35g-PCP}{mole}\div 0.453=\frac{0.133mmol-PCP}{kg}$$

Data Analysis

LC_{50}s were calculated for each time interval where > 50% mortality was observed by trimmed Spearman-Karber analysis (Hamilton et al. 1977). Toxicity curves were obtained by plotting inverse LC_{50} (mmol kg^{-1}) against time (hours). From the toxicity curves, the ILLs and $t_{1/2}$s were calculated using nonlinear regression (SYSTAT® version 5.0, Systat Inc. 1990). Statistical comparisons were made of CBRs using SAS® (version

6.12, SAS Institute Inc. 1989-1996). Controls were not included in the statistical analyses because this was a non-zero analysis. Variances were homogeneous for the TCP and PCP tests and analyzed by ANOVA using SAS. Variances were heterogeneous for the TCP fraction, PCP fraction and combined TCP/PCP residues for the TCP/PCP mixture assay as determined by SAS. A mixed model for data with unequal variances (SAS) was used to determine differences in CBRs for the TCP/PCP mixture. *A posteriori* comparisons of the mean CBRs for TCP and PCP for single chemical toxicity tests and for the TCP/PCP mixture were made using SAS least square means.

CHAPTER THREE

RESULTS

Incipient Lethal Levels and Toxicity Half-Lives

ILLs for the individual chlorophenol toxicity tests decreased as the degree of chlorination, molecular weight, and hydrophobicity increased and vapor pressure decreased (Table 3). All toxicity curves showed a good fit to the data as exhibited by r^2 values of 0.993 and above. Mean ILLs for MCP, TCP, PCP #1, and PCP #2 were 1.28, 0.326, 0.050, and 0.047 mmol kg^{-1}, respectively. The mean $t_{1/2}$s for MCP, TCP, PCP #1, and PCP #2 were 39, 51, 608, and 411 hours, respectively, indicating slower kinetics of uptake with increased hydrophobicity, and decreased vapor pressure.

Table 3. ILLs and $t_{1/2}$s of the individual chemical toxicity tests and mixture toxicity tests with correlation coefficient for curve fit.

Figure	Test	ILL (mmol kg^{-1} or TU)	$t_{1/2}$ (hours)	r^2
1	MCP	1.28	39	0.998
2	TCP	0.326	51	0.999
3	PCP #1	0.050	609	0.998
4	PCP #2	0.047	417	0.993
5	MCP/TCP/PCP #1	1.32	22	1.000
6	MCP/TCP/PCP #2	0.96	52	0.999
7	TCP/PCP	0.59	166	0.999

Similar trends were observed from the mixture toxicity tests (Table 3). The MCP/TCP/PCP #1, MCP/TCP/PCP #2 and TCP/PCP tests had mean ILLs of 1.32, 0.96, and 0.59 TUs, respectively, and mean $t_{1/2}$s of 22, 52, and 166 hours, respectively. The chemical mixtures with MCP had lower $t_{1/2}$s relative to the TCP/PCP mixture and approximately the same as for the MCP and TCP tests. The $t_{1/2}$ of the TCP/PCP mixture was intermediate to $t_{1/2}$s of individual TCP and PCP tests.

All toxicity data except for PCP exhibited well-defined toxicity curves (Fig. 4-10). The PCP curves (Fig. 6-7) do not reach their asymptotic value during the toxicity test due to the slow kinetics of PCP as indicated by the large $t_{1/2}$s.

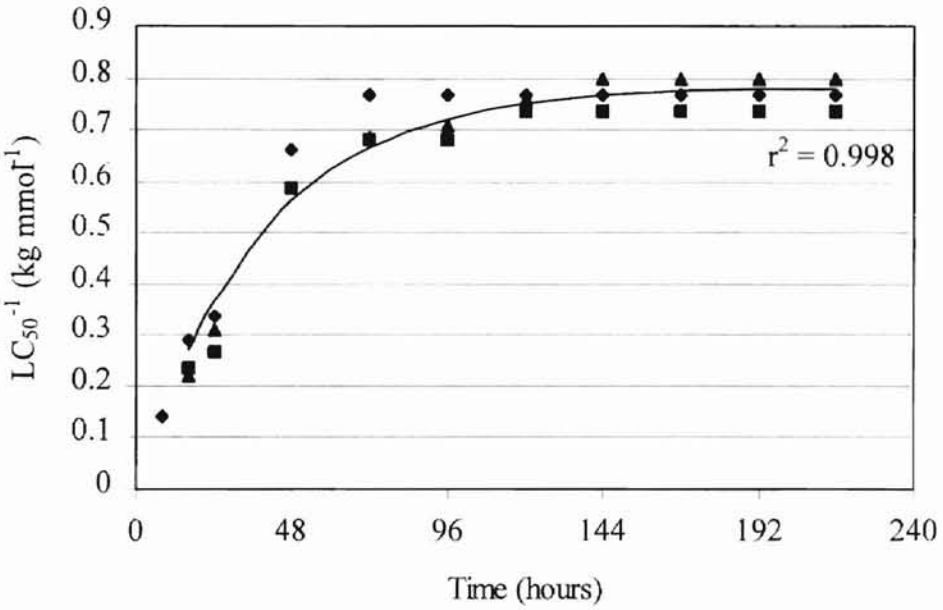

Fig. 4. Toxicity curve for *E. fetida* exposed to MCP in artificial soil. Different symbols at each time point represent replicates and the curve is fitted to the points by nonlinear regression ($r^2 = 0.998$).

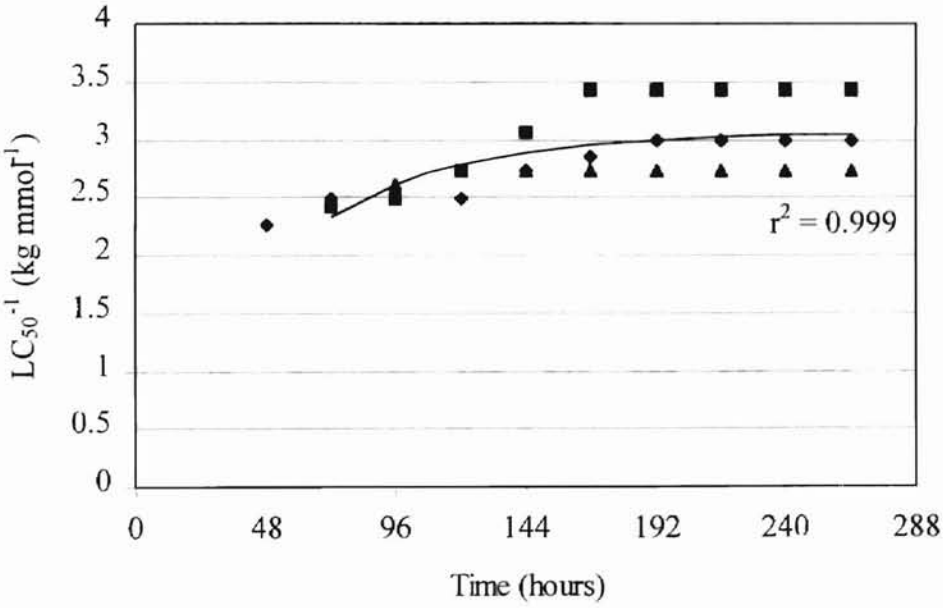

Fig. 5. Toxicity curve for *E. fetida* exposed to TCP in artificial soil. Different symbols at each time point represent replicates and the curve is fitted to the points by nonlinear regression ($r^2 = 0.999$).

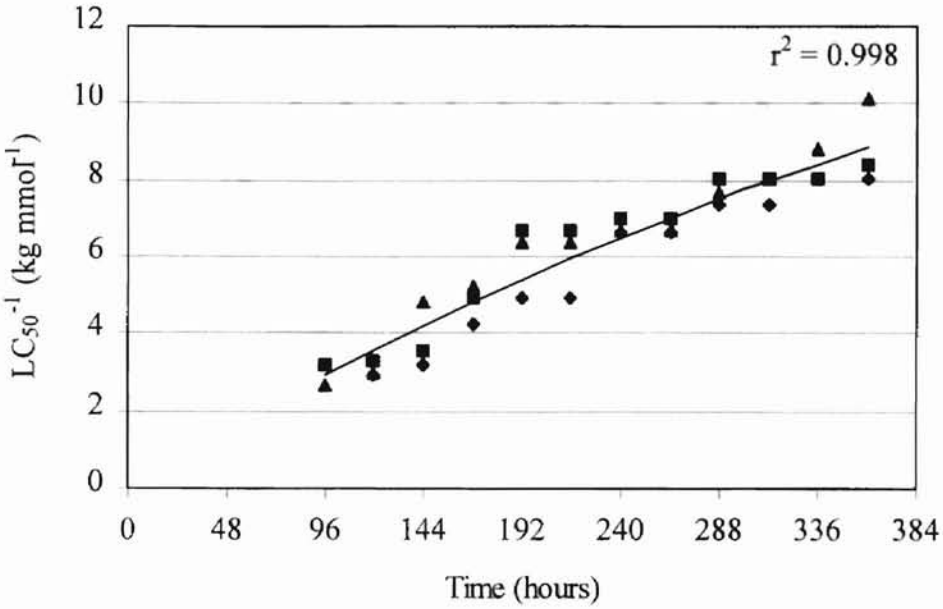

Fig. 6. Toxicity curve for *E. fetida* exposed to PCP #1 in artificial soil. Different symbols at each time point represent replicates and the curve is fitted to the points by nonlinear regression ($r^2 = 0.998$).

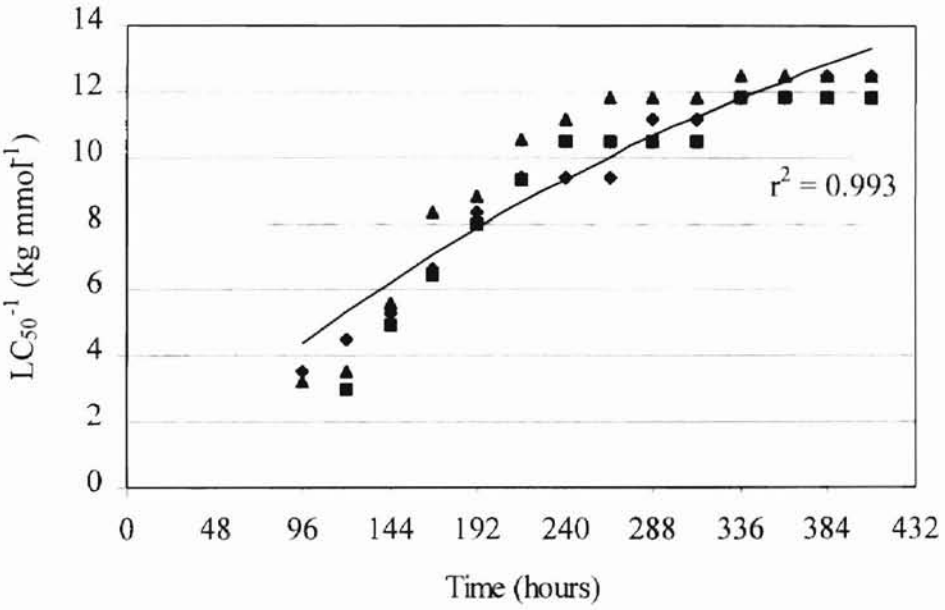

Fig. 7. Toxicity curve for *E. fetida* exposed to PCP #2 in artificial soil. Different symbols at each time point represent replicates and the curve is fitted to the points by nonlinear regression ($r^2 = 0.993$).

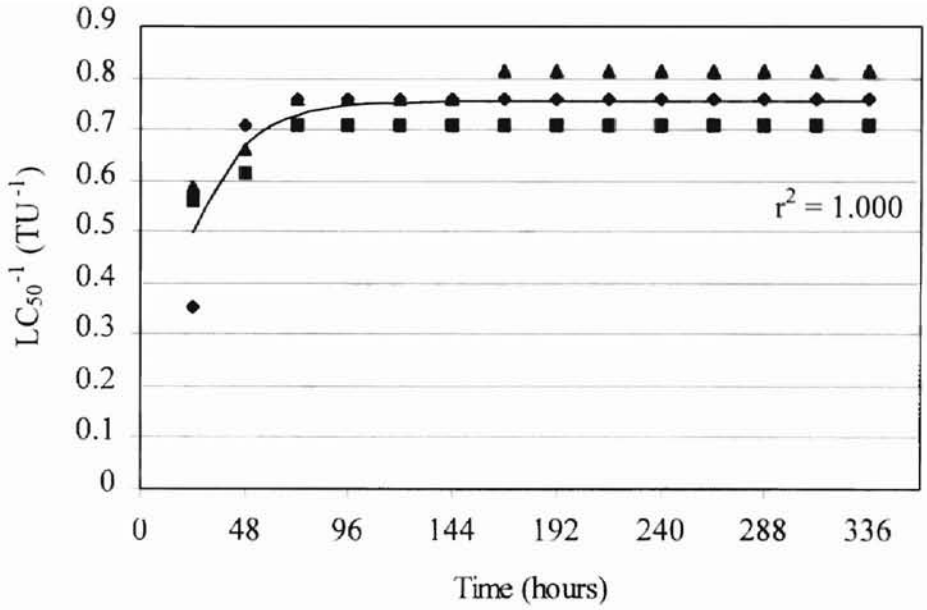

Fig. 8. Toxicity curve for *E. fetida* exposed to the MCP/TCP/PCP #1 mixture in artificial soil. Different symbols at each time point represent replicates and the curve is fitted to the points by nonlinear regression ($r^2 = 1.000$).

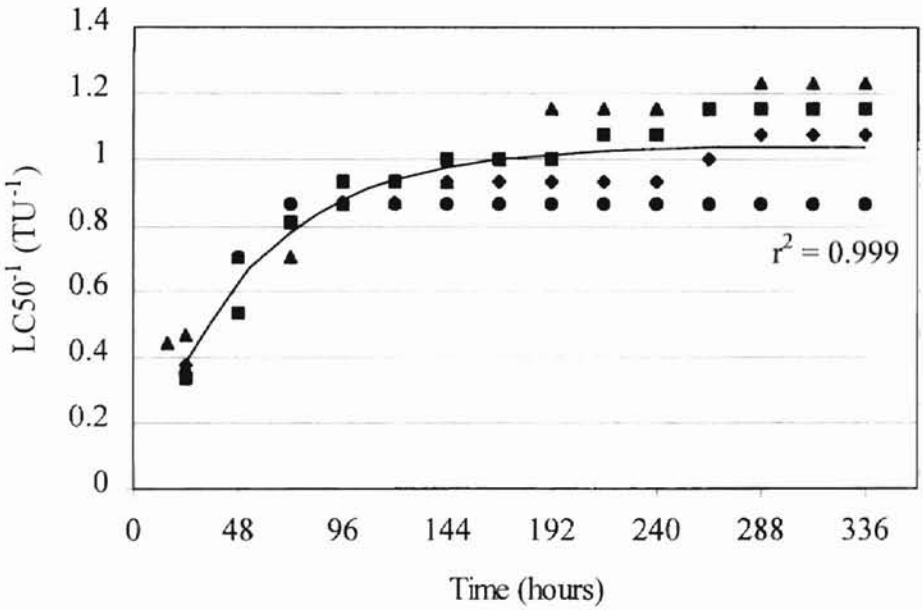

Fig. 9. Toxicity curve for *E. fetida* exposed to the MCP/TCP/PCP #2 mixture in artificial soil. Different symbols at each time point represent replicates and the curve is fitted to the points by nonlinear regression ($r^2 = 0.999$).

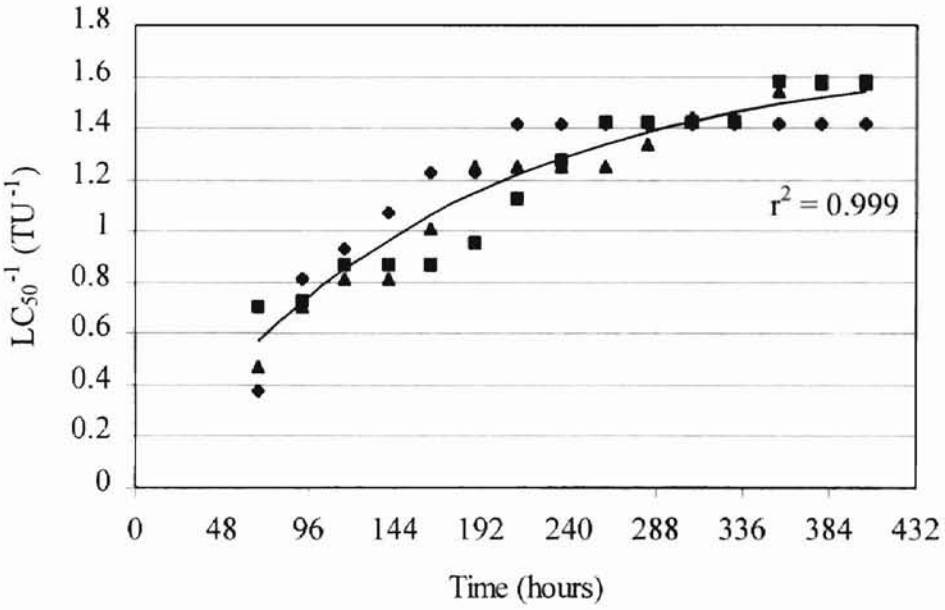

Fig. 10. Toxicity curve for *E. fetida* exposed to the TCP/PCP mixture in artificial soil. Different symbols at each time point represent replicates and the curve is fitted to the points by nonlinear regression ($r^2 = 0.999$).

Critical Body Residues

TCP and PCP body residues in control earthworms in all experiments were below detection limits (Tables 4-7). The body residues of the TCP-exposed earthworms increased with exposure concentration (Table 4, Fig. 11). Average TCP concentrations ranged from 0.0057 mmol kg^{-1} in earthworms exposed to 0.051 mmol kg^{-1} to 0.439 mmol kg^{-1} in worms exposed to 0.506 mmol kg^{-1}. Earthworms exposed to concentrations above the ILL of 0.326 mmol kg^{-1} exhibited significantly higher body residues than earthworms at or below the ILL. The CBR at mortality of TCP was estimated to be 0.347 mmol kg^{-1} from residues of 2 dead earthworms exposed at 0.319 mmol kg^{-1} and 6 dead earthworms exposed at 0.506 mmol kg^{-1}. BAFs for TCP in earthworms were calculated at 0.403 for all measured body residues in the TCP test.

The body residues of the PCP exposed earthworms also increased with exposure concentration (Tables 5 and 6, Fig. 12), but the range of concentrations was narrower. Average PCP concentrations in worms ranged from 0.042 mmol kg^{-1} at the exposure concentration of 0.038 mmol kg^{-1} to 0.083 and 0.093 mmol kg^{-1} at the exposure concentrations of 0.375 mmol kg^{-1} in PCP #1 and PCP #2, respectively. CBRs were calculated to be 0.073 mmol kg^{-1} from an average residue of all dead earthworms. BAFs for PCP were calculated to be 0.550 for all body residues determined in the PCP tests.

Body residues of both TCP and PCP in the TCP/PCP mixture test were higher for earthworms present in concentrations resulting in mortality (0.5, 1, 2, and 4 TU) (Table 7 and Fig. 13). The TCP fraction residues in earthworms exposed to 0.5, 1.0, 2.0, and 4.0 TU were not significantly different from each other, but were significantly higher than earthworms exposed to 0.25 TU. The PCP fraction residues in earthworms exposed to 0.5, 1.0, 2.0, and 4.0 TU were significantly higher than 0.25 TU exposed earthworms. The combined TCP and PCP residues for 0.5, 1.0, 2.0, and 4.0 TU exposed worms were also significantly higher than 0.25 TU exposed earthworms.

Table 4. Body residues in earthworms exposed to TCP in artificial soil. Means with common superscripts are not significantly different ($P > 0.05$, differences of least squares means); nd – not detected

Exposure concentration (mmol kg^{-1})	TCP residue in worm (mmol kg^{-1})	SE	n
Control	nd	--------	10
0.051	0.006^{A}	0.0010	6
0.081	0.010^{A}	0.0010	9
0.127	0.083^{AB}	0.0346	7
0.203	0.064^{AB}	0.0481	9
0.309	0.132^{B}	0.0233	15
0.506	0.439^{C}	0.0558	6

Table 5. Body residues in earthworms exposed to PCP in artificial soil during the first PCP toxicity test. Means with common superscripts are not significantly different (P > 0.05, differences of least squares means); nd – not detected

Exposure Concentration (mmol kg^{-1})	PCP residue in worm (mmol kg^{-1})	SE	n
0.060	0.077*	-------	1
0.094	0.022^{A}	0.011	2
0.150	0.046^{AC}	0.012	5
0.237	0.103^{B}	0.034	3
0.375	0.083^{BC}	0.008	3

* 0.060 mmol kg^{-1} residues are not significantly different due to n=1

Table 6. Body residues in earthworms exposed to PCP in artificial soil during the second PCP toxicity test. Means with common superscripts are not significantly different (P > 0.05, differences of least squares means); nd – not detected

Exposure Concentration (mmol kg^{-1})	PCP residue in worm (mmol kg^{-1})	SE	n
NaOH control	nd	--------	9
0.038	0.042^{A}	0.004	9
0.068	0.046^{A}	0.008	9
0.120	0.056^{AB}	0.010	8
0.210	0.077^{BC}	0.009	9
0.375	0.093^{C}	0.015	5

Table 7. Body residues in earthworms exposed to TCP and PCP in artificial soil during the TCP/PCP mixture toxicity test. Means with common superscripts are not significantly different ($P > 0.05$, differences of least squares means); nd – not detected

	TCP residues (mmol kg^{-1})			PCP residues (mmol kg^{-1})			Total residues TCP + PCP (mmol kg^{-1})		
Exp Conc (TU)	$\bar{X}$	SE	n	$\bar{X}$	SE	n	$\bar{X}$	SE	n
control	nd	-------	15	nd	-------	15	nd	-------	15
0.25	0.014^{A}	0.008	15	0.037^{A}	0.006	15	0.050^{A}	.014	15
0.5	0.155^{B}	0.034	25	0.217^{B}	0.035	25	0.372^{B}	.066	25
1	0.160^{B}	0.032	22	0.148^{BC}	0.041	22	0.308^{BC}	.065	22
2	0.128^{B}	0.040	15	0.107^{C}	0.023	15	0.235^{BC}	.045	15
4	0.134^{B}	0.032	15	0.060^{C}	0.012	15	0.194^{C}	.038	15

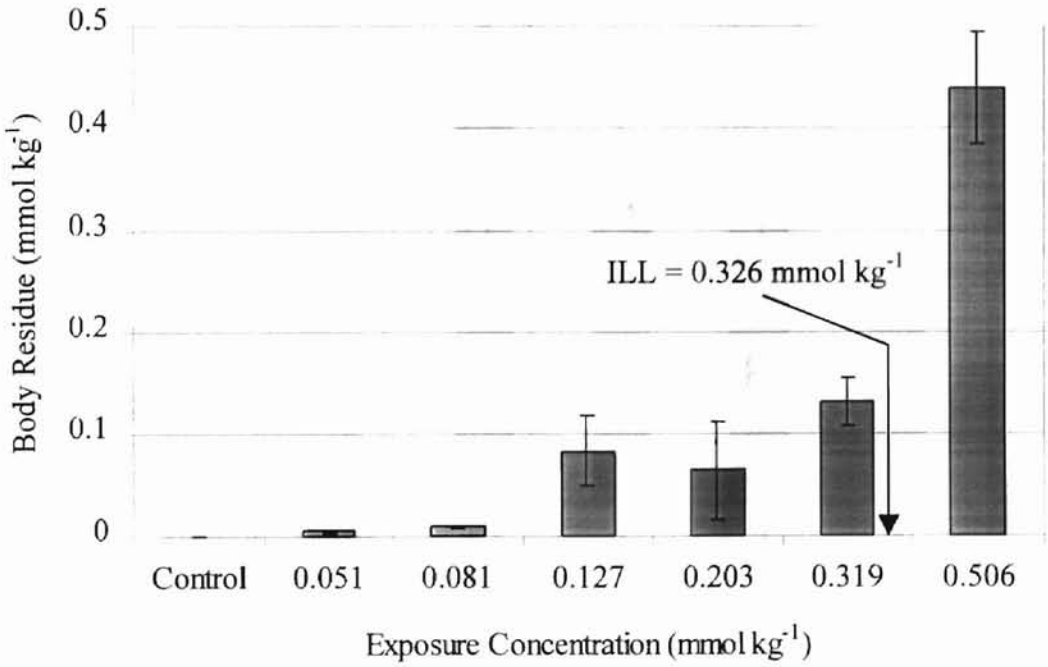

Fig. 11. Average body residues (± SE) in earthworms exposed to TCP in artificial soil. The majority of the earthworms below the ILL were alive and those above the ILL were dead.

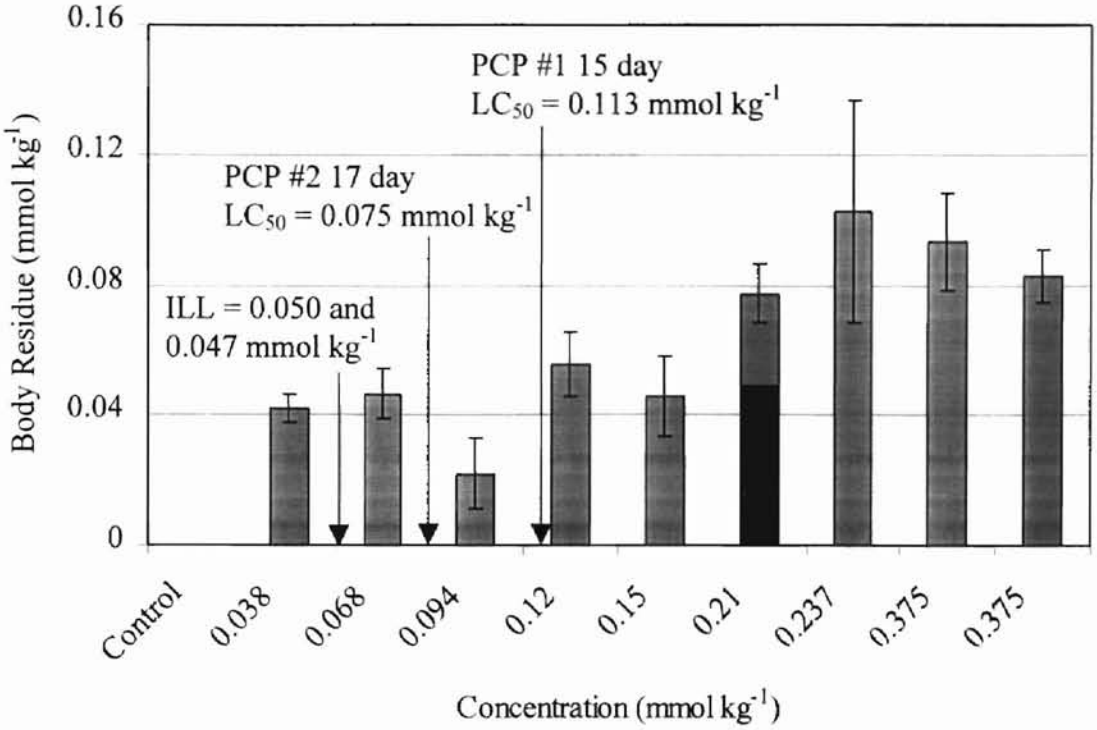

Fig. 12. Average body residues (± SE) in earthworms exposed to PCP in artificial soil with the ILL and LC_{50} values at termination of the PCP tests. The majority of the earthworms below 0.1 mmol kg^{-1} were alive and those above 0.1 mmol kg^{-1} were dead at the termination of the two PCP toxicity tests.

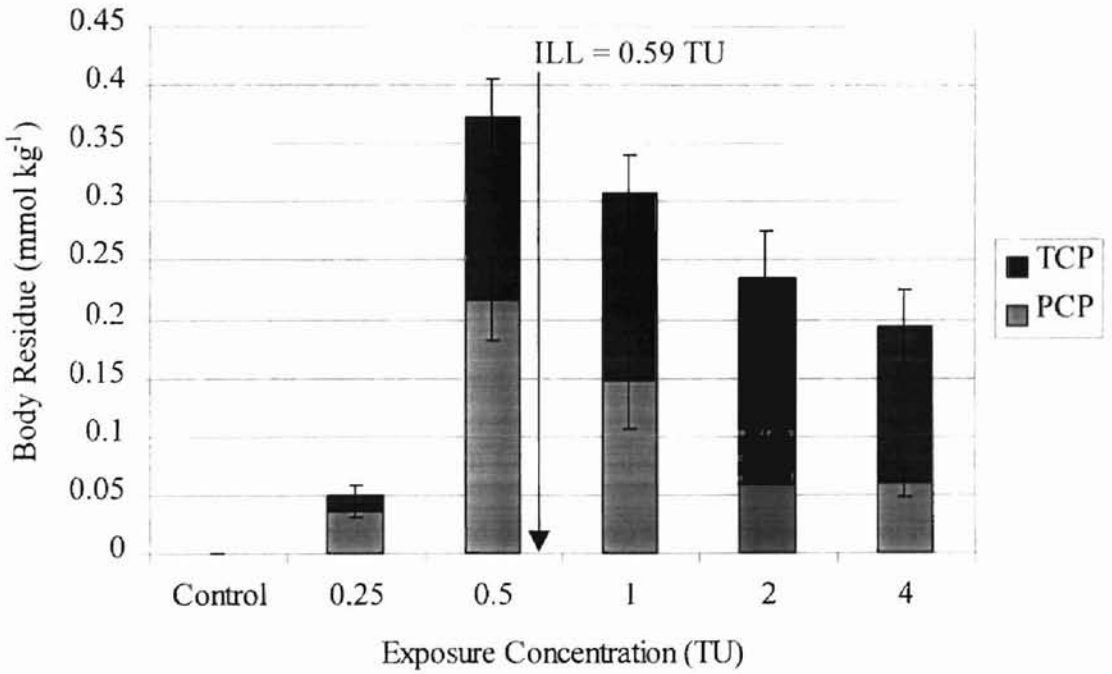

Fig. 13. Average body residues (± SE) in earthworms exposed toTCP and PCP in artificial soil with the calculated ILL. The majority of the earthworms below 0.59 TU alive and those above 0.59 TU were dead.

CHAPTER FOUR

DISCUSSION

MCP, TCP, and PCP

Toxicity Curve Analyses

ILLs determined for MCP, TCP, and PCP are similar to values observed in the literature. The 4-MCP ILL of 1.28 mmol kg^{-1} is similar to the 14-day LC_{50} of 3-MCP (1.01 mmol kg^{-1}) in artificial soil reported by van Gestel and Ma (1990) assuming no difference in toxicity between the two monochlorophenols. The ILL of 2,4,5-TCP (0.326 mmol kg^{-1}) is similar to the 14-day LC_{50} of 2,4,5-TCP in artificial soil (0.319 mmol kg^{-1}) reported by van Gestel and Ma (1990) and the 14-day LC_{50} for 2,4,6-TCP (0.294 mmol kg^{-1}) determined by Neuhauser et al. (1985) as there should be little differences in uptake or mode of action between the two trichlorophenols. PCP ILLs (0.050 and 0.047 mmol kg^{-1}) were threefold lower than those reported by Fitzgerald et al. (1997) of 0.137 mmol kg^{-1} and sixfold lower than the PCP 14-day LC_{50} of 0.311 mmol kg^{-1} (van Gestel and Ma (1990). These differences in ILLs of PCP are likely due to curve fit of the LC_{50} values. Fitzgerald et al. (1997) exposed worms to concentrations ranging from 0.038 to 6.75 mmol kg^{-1}, more than an order of magnitude higher, and obtained a better defined toxicity curve. Van Gestel and Ma (1990) didn't report exposure concentrations. The differences in $t_{1/2}$ for PCP from this thesis (417 and 609 hours) and that reported by Fitzgerald et al. (1997) of 94 hours are also explained by a better curve fit due to the wider range of concentrations.

The ILLs and $t_{1/2}$s for the individual chlorophenol tests differ due to the chemical properties and their interaction with the soil and earthworms (Fig. 14, 15). The main route of uptake for chlorophenols and chlorobenzenes is diffusion from soil pore water (Belfroid et al. 1994; van Gestel and Ma 1988). Hydrophobicity (e.g., log K_{ow}) is inversely related to solubility and has the greatest impact on pore water concentrations, but vapor pressure and pK_a may also contribute to the uptake of chlorophenols. Chlorophenol adsorption is determined by hydrophobicity. Increasing log K_{ow} increases adsorption, decreases pore water concentrations and uptake kinetics, and increases potency (Fig. 14). Similar results are also observed for earthworms exposed to a homologous series of chlorobenzenes with increasing hydrophobicity. Belfroid et al. (1994) also observed that time to steady state for uptake of chlorobenzenes by earthworms increased as log K_{ow} increased.

The pK_as of MCP, TCP, and PCP affect whether they are present in the phenolic (unionized) form or as phenolate ions. At the average artificial soil pH of 6.5, MCP and TCP are present predominantly in the phenolic form while PCP is present as the phenolate anion. Increased bioavailability of MCP and TCP would result in more rapid uptake kinetics and toxicity than for PCP. The effect of pK_a on uptake kinetics is reflected by varying $t_{1/2}$s, as is solubility and vapor pressure, but effect of pK_a on toxicity may be very low when compared to log K_{ow} and vapor pressure effects.

In summary, uptake occurs predominantly by diffusion from the pore water across the epithelium and is most affected by the K_{ow} of the chlorophenols. A decrease in uptake kinetics and an in increase potency are associated with an increase of K_{ow}.

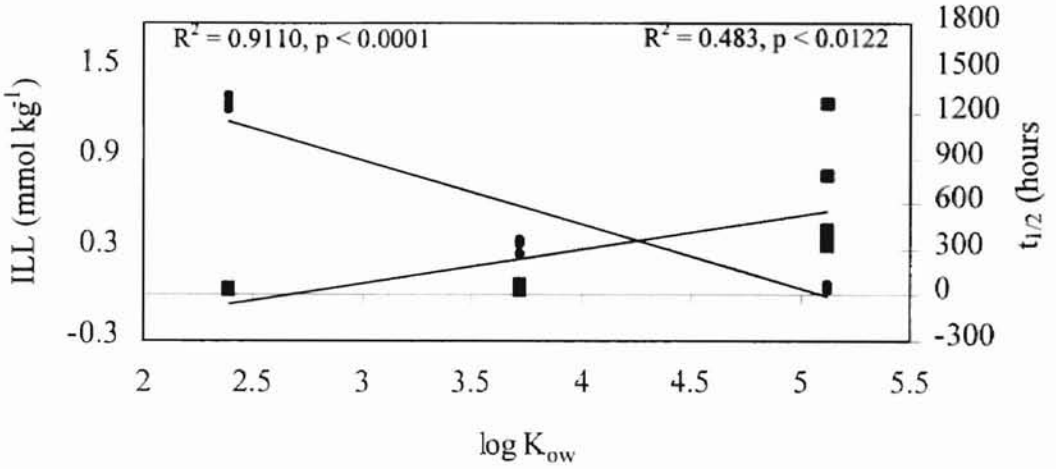

Fig. 14. Effects of K_{ow} on ILL (●) and $t_{1/2}$ (■) with linear regression analysis

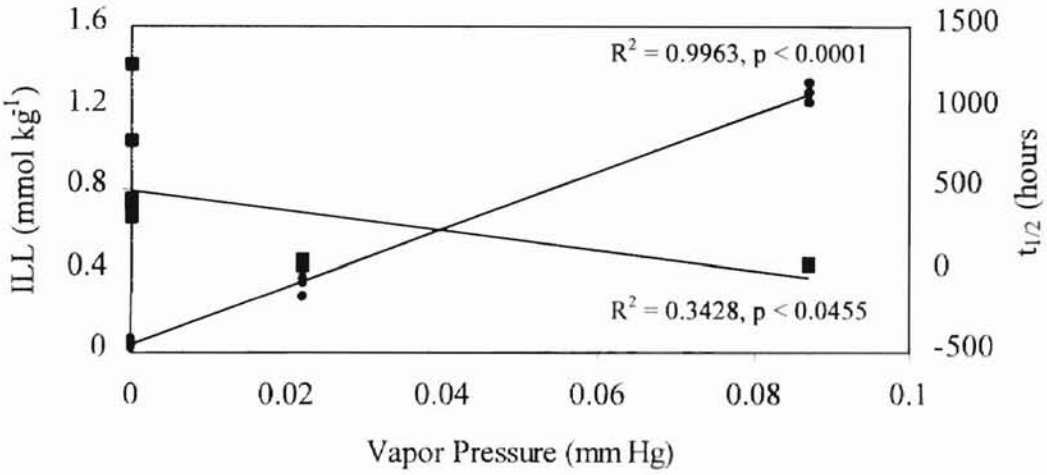

Fig. 15. Effects of vapor pressure on ILL (●) and $t_{1/2}$ (■) with linear regression analysis

Critical Body Residue Analyses

CBRs and ILLs also decrease with increasing K_{ow} for TCP and PCP. This correlation between log K_{ow} and toxicity has been observed for neutral organic chemicals in fresh-water fish (McCarty and Mackay 1993) and for tetra-, penta-, and hexachlorobenzene in earthworms (Belfroid et al. 1994). Literature values of TCP CBRs could not be found. CBRs for TCP were 0.347 mmol TCP kg^{-1} for earthworms exposed to 0.506 and 0.319

mmol kg^{-1}. This CBR may be slightly inflated due to diffusion into earthworms after mortality has occurred. Fitzgerald et al. (1996) observed a similar trend of high body residues in earthworms exposed to very high levels of PCP. Due to the small sample size for dead worms at 0.319 mmol kg^{-1}, CBRs of earthworms exposed to 0.506 mmol kg^{-1} were included in mean CBR estimates for TCP. CBRs estimated for earthworms exposed to PCP ranged from 0.011 to 0.161 mmol kg^{-1}, more than an order of magnitude lower than PCP CBRs reported by Fitzgerald et al. (1996) which ranged from 0.47-2.18 mmol kg^{-1}. Differences in CBRs may be explained partially by the amount of moisture present in the worms. Earthworms in this study were exposed to PCP spiked artificial soil at 35% moisture on a wet weight basis (54% dry weight), whereas earthworms in Fitzgerald et al. (1996) were exposed to 35% moisture on a dry weight basis. Body mass may change over the course of a toxicity test as result of loss of water from earthworm tissue while lipid mass does not change (Belfroid et al. 1994).

Mixture Toxicity Tests

Toxicity Curve Analyses

The three mixture tests exhibit well defined toxicity curves with $t_{1/2}$s similar to MCP and TCP. This likely is due to the uptake kinetics of the less hydrophobic components of the mixture. Ninety-five percent confidence intervals (CI) for the ILLs of the mixture tests did not include 1 TU suggesting that none of the combinations of chlorophenols are strictly additive. However. MCP/TCP/PCP #1 had an ILL of 1.32 TU ± 0.01 (95% CI), and the second MCP/TCP/PCP mixture toxicity test had an ILL of 0.96 TU ± 0.025 (95% CI). The fact that the ILL for one test was slightly above 1 TU and one was slightly

below 1 TU do suggest additivity of toxicity for MCP, TCP, and PCP. These tests were conducted using the same molar concentrations of MCP, TCP and PCP, although the batch of artificial soil was different. Slight differences in the makeup of each batch of artificial soil might account for the slight differences in toxicity for the three-chemical mixture tests. The TCP/PCP mixture toxicity test ILL was 0.59 TU ± 0.025 (95% CI). The lower ILL suggests that TCP and PCP act in a synergistic manner when present at 0.5 TU each in a mixture.

Critical Body Residue Analyses

CBRs for TCP and PCP in the TCP/PCP mixture test show differences in uptake kinetics (Table 7, Fig. 13). TCP residues remain constant and are not significantly different in the 0.5, 1.0, 2.0, and 4 TU exposures. PCP residues are significantly higher in 0.5 and 1 TU exposures than in 2 and 4 TU exposures (Table 7, Fig. 13). This is likely due to the decreased time to mortality with increasing exposure concentration. At 4 TU, earthworms were sampled (determined to be dead) at 68 hours. The average sampling time for worms exposed to 2 TU was 82.4 hours (68-92 hours), where the average time to mortality for worms exposed to 1 TU was 161.3 hours (68-356 hours) for 18 dead worms. Four live worms were sampled at 404 hours. Only 4 dead earthworms were sampled at 0.5 TU, with an average time to mortality of 254 hours (188-356 hours), but 21 live worms were sampled at 404 hours. There were no differences in residues between live and dead worms exposed to 0.5 TU. These differences in time to mortality with corresponding body residues show that the TCP residue fraction has achieved a steady

state by approximately 68 hours, but PCP residues continue to increase throughout the 17-day toxicity test (Figure 13).

CBRs for TCP and PCP in the TCP/PCP mixture test indicate that TCP and PCP are acting antagonistically. If TCP and PCP were acting additively, the combined residues

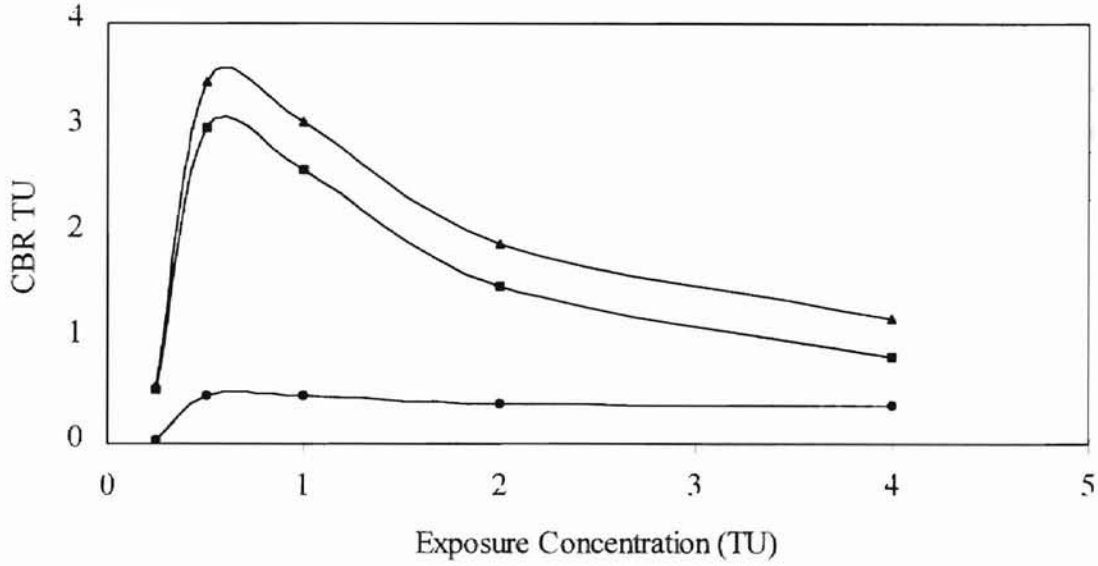

Fig. 16. CBR TUs for TCP (●), PCP(■), and TCP+PCP (▲) illustrated the antagonistic effect of TCP and PCP.

should fall somewhere in the range of the individual PCP or TCP CBRs, 0.073-0.347 mmol kg^{-1}. Combined TCP + PCP residues ranged from 1.19-3.45 CBR TUs for dead worms in the mixture test (Fig. 16). TCP and PCP appear to be acting antagonistically in this experiment.

The TCP fraction is present at 0.37, 0.39, 0.46, 0.45, and 0.04 TU when comparing CBR-based TU calculations in 4, 2, 1, 0.5, and 0.25 TU exposure concentrations, respectively, and the PCP fraction is present 0.82, 1.5, 2.6, 3.0, and 0.51 TU when comparing CBR-based TU calculations in 4, 2, 1, 0.5, and 0.25 TU exposure concentrations, respectively. The TU assessment of the TCP fraction residues may be

slightly underestimated due to calculations with inflated TCP CBR values, since diffusion after mortality can give an inflated estimate of CBRs (Fitzgerald et al. 1997).

TCP fraction residues were relatively constant at approximately 1 TU in earthworms that were exposed to concentrations resulting in morality, 0.5, 1, 2, and 4 TU, although PCP fraction residues were higher than 1 TU in earthworms in the 0.5, 1, 2, and 4 TU exposures. TCP is interfering with the mode of action of PCP and the effects of the mixture are considered antagonistic. One possible explanation for these differences is that TCP has faster uptake kinetics than PCP and that TCP anesthetizes the earthworms and slows down their physiological functions before PCP is accumulated to interfere with oxidative phosphorylation. Metabolic processes could be reduced dramatically due narcotic effects of TCP. Another explanation may be that TCP interferes with the toxicity of PCP in some other manner, allowing it to accumulate to levels much higher than the CBR for PCP.

Summary

The main route of uptake for chlorophenols is diffusion with kinetics being determined by K_{ow}. ILLs decrease with an increase in K_{ow} of chlorophenols. CBRs were calculated as the average of body residues of dead worms. The mixtures of MCP, TCP, and PCP appear additive based on ILLs, while the TCP/PCP mixture appears to act synergistically from ILL observations and antagonistically when looking at CBRs and TUs.

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APPENDIX A – BODY RESIDUE DATA FOR TCP TOXICITY TEST

Analysis conducted using Tracor 560 Gas Chromatograph

ID #	Test	Conc. (mmol kg^{-1})	Jar	Length of Exposure (hours)	Live or Dead	Body Residue (mmol kg^{-1})
226	TCP	NaOH	B	264	L	nd
227	TCP	NaOH	B	264	L	nd
228	TCP	NaOH	C	264	L	nd
229	TCP	NaOH	C	264	L	nd
250	TCP	0	A	264	L	nd
251	TCP	0	A	264	L	nd
274	TCP	0	B	264	L	nd
275	TCP	0	B	264	L	nd
276	TCP	0	C	264	L	nd
277	TCP	0	C	264	L	nd
252	TCP	0.051	B	264	L	0.008
254	TCP	0.051	B	264	L	0.004
230	TCP	0.051	C	264	L	0.005
231	TCP	0.051	C	264	L	0.007
232	TCP	0.051	C	264	L	0.009
233	TCP	0.081	A	264	L	0.011
234	TCP	0.081	A	264	L	0.014
235	TCP	0.081	A	264	L	0.011
262	TCP	0.081	B	264	L	0.012
263	TCP	0.081	B	264	L	0.007
264	TCP	0.081	B	264	L	0.007
238	TCP	0.081	C	264	L	0.007
239	TCP	0.081	C	264	L	nd
240	TCP	0.081	C	264	L	0.008
241	TCP	0.127	B	264	L	0.115
242	TCP	0.127	B	264	L	0.158
243	TCP	0.127	B	264	L	0.028
255	TCP	0.127	C	264	L	0.010
256	TCP	0.127	C	264	L	0.014
257	TCP	0.127	C	264	L	0.013
244	TCP	0.203	B	264	L	nd
245	TCP	0.203	B	264	L	0.006
246	TCP	0.203	B	264	L	0.010
265	TCP	0.203	C	264	L	0.032
266	TCP	0.203	C	264	L	0.026
267	TCP	0.203	C	264	L	0.400

ID #	Test	Conc. (mmol kg^{-1})	Jar	Length of Exposure (hours)	Live or Dead	Body Residue (mmol kg^{-1})
258	TCP	0.319	A	264	L	0.092
259	TCP	0.319	A	264	L	0.099
260	TCP	0.319	B	264	L	0.155
261	TCP	0.319	B	264	L	0.033
247	TCP	0.319	C	96	D	0.033
248	TCP	0.319	C	120	D	0.113
249	TCP	0.319	C	264	L	0.108
268	TCP	0.319	C	264	L	0.087
269	TCP	0.319	C	264	L	0.080
270	TCP	0.506	A	48	D	0.523
271	TCP	0.506	A	72	D	0.475
236	TCP	0.506	B	48	D	0.283
237	TCP	0.506	B	48	D	0.302
272	TCP	0.506	C	48	D	0.642
273	TCP	0.506	C	72	D	0.408

Analysis conducted using Hewlett Packard Gas Chromatograph

ID #	Test	Conc. (mmol kg^{-1})	Jar	Length of Exposure (hours)	Live or Dead	Body Residue (mmol kg^{-1})
82	TCP	0.051	A	264	L	0.002
79	TCP	0.127	A	264	L	0.243
58	TCP	0.203	A	264	L	0.023
59	TCP	0.203	A	264	L	0.013
60	TCP	0.203	A	264	L	0.004
7	TCP	0.319	A	144	D	0.230
8	TCP	0.319	A	144	D	0.105
9	TCP	0.319	A	168	D	0.338
10	TCP	0.319	B	144	D	0.145
11	TCP	0.319	B	168	D	0.061
12	TCP	0.319	B	168	D	0.297

APPENDIX B – BODY RESIDUE DATA FOR PCP TOXICITY TESTS

Analysis conducted using Tracor 560 Gas Chromatograph

ID #	Test	Conc. (mmol kg^{-1})	Jar	Length of Exposure (hours)	Live or Dead	Body Residue (mmol kg^{-1})
310	PCP 2	NaOH	A	408	L	nd
311	PCP 2	NaOH	A	408	L	nd
312	PCP 2	NaOH	A	408	L	nd
313	PCP 2	NaOH	B	408	L	nd
314	PCP 2	NaOH	B	408	L	nd
315	PCP 2	NaOH	B	408	L	nd
322	PCP 2	NaOH	C	408	L	nd
323	PCP 2	NaOH	C	408	L	nd
324	PCP 2	NaOH	C	408	L	nd
364	PCP 2	0.038	A	408	L	0.042
365	PCP 2	0.038	A	408	L	0.017
366	PCP 2	0.038	A	408	L	0.048
316	PCP 2	0.038	B	408	L	0.042
317	PCP 2	0.038	B	408	L	0.040
318	PCP 2	0.038	B	408	L	0.034
325	PCP 2	0.038	C	408	L	0.044
326	PCP 2	0.038	C	408	L	0.067
327	PCP 2	0.038	C	408	L	0.044
328	PCP 2	0.068	A	408	L	0.017
329	PCP 2	0.068	A	408	L	0.049
330	PCP 2	0.068	A	408	L	0.083
367	PCP 2	0.068	B	408	L	0.077
368	PCP 2	0.068	B	408	L	0.054
369	PCP 2	0.068	B	408	L	0.028
319	PCP 2	0.068	C	408	L	0.053
320	PCP 2	0.068	C	408	L	0.033
321	PCP 2	0.068	C	408	L	0.025
286	PCP 2	0.120	A	144	D	0.043
287	PCP 2	0.120	A	192	D	0.093
288	PCP 2	0.120	A	192	D	0.094
289	PCP 2	0.120	B	192	D	0.015
290	PCP 2	0.120	B	192	D	0.055
291	PCP 2	0.120	B	192	D	0.060
284	PCP 2	0.120	C	168	D	0.056
285	PCP 2	0.120	C	216	D	0.029
298	PCP 2	0.210	A	120	D	0.084

ID #	Test	Conc. (mmol kg^{-1})	Jar	Length of Exposure (hours)	Live or Dead	Body Residue (mmol kg^{-1})
299	PCP 2	0.210	A	168	D	0.077
300	PCP 2	0.210	A	168	D	0.062
281	PCP 2	0.210	B	168	D	0.129
282	PCP 2	0.210	B	168	D	0.075
283	PCP 2	0.210	B	168	D	0.027
292	PCP 2	0.210	C	144	D	0.091
293	PCP 2	0.210	C	144	D	0.076
294	PCP 2	0.210	C	168	D	0.077
280	PCP 2	0.375	A	48	D	0.050
295	PCP 2	0.375	A	96	D	0.120
296	PCP 2	0.375	B	120	D	0.084
297	PCP 2	0.375	B	120	D	0.131
303	PCP 2	0.375	C	144	D	0.080

Analysis conducted using Hewlett Packard Gas Chromatograph

ID #	Test	Conc. (mmol kg^{-1})	Jar	Length of Exposure (hours)	Live or Dead	Body Residue (mmol kg^{-1})
18	PCP 1	0.060	A	336	D	0.077
42	PCP 1	0.094	C	360	L	0.033
13	PCP 1	0.094	C	336	D	0.011
15	PCP 1	0.150	A	336	D	0.011
16	PCP 1	0.150	C	360	D	0.048
17	PCP 1	0.150	C	360	D	0.083
40	PCP 1	0.150	A	360	L	0.027
41	PCP 1	0.150	B	360	L	0.059
37	PCP 1	0.237	A	240	D	0.161
38	PCP 1	0.237	A	240	D	0.104
39	PCP 1	0.237	A	240	D	0.043
93	PCP 1	0.375	A	72	D	0.095
94	PCP 1	0.375	B	72	D	0.067
96	PCP 1	0.375	B	96	D	0.086

APPENDIX C - BODY RESIDUE DATA FOR TCP/PCP MIXTURE TOXICITY TESTS

Analysis conducted using Tracor 560 Gas Chromatograph

ID #	Test	Conc. (TU)	Jar	Length of Exposure (hours)	Live or Dead	TCP Residue (mmol kg^{-1})	PCP Residue (mmol kg^{-1})
142	TCP/PCP	NaOH	A	404	L	nd	nd
143	TCP/PCP	NaOH	A	404	L	nd	nd
144	TCP/PCP	NaOH	B	404	L	nd	nd
145	TCP/PCP	NaOH	B	404	L	nd	nd
202	TCP/PCP	NaOH	B	404	L	nd	nd
203	TCP/PCP	NaOH	B	404	L	nd	nd
204	TCP/PCP	NaOH	B	404	L	nd	nd
178	TCP/PCP	NaOH	C	404	L	nd	nd
179	TCP/PCP	NaOH	C	404	L	nd	nd
180	TCP/PCP	NaOH	C	404	L	nd	nd
214	TCP/PCP	NaOH	A	404	L	nd	nd
215	TCP/PCP	NaOH	A	404	L	nd	nd
216	TCP/PCP	NaOH	A	404	L	nd	nd
217	TCP/PCP	NaOH	C	404	L	nd	nd
218	TCP/PCP	NaOH	C	404	L	nd	nd
140	TCP/PCP	0.25	A	404	L	0.004	0.019
141	TCP/PCP	0.25	A	404	L	0.002	0.014
146	TCP/PCP	0.25	A	404	L	0.003	0.034
148	TCP/PCP	0.25	A	404	L	0.002	0.026
149	TCP/PCP	0.25	A	404	L	0.004	0.025
150	TCP/PCP	0.25	B	404	L	0.003	0.022
151	TCP/PCP	0.25	B	404	L	0.004	0.022
152	TCP/PCP	0.25	B	404	L	0.004	0.018
153	TCP/PCP	0.25	B	404	L	0.007	0.037
181	TCP/PCP	0.25	C	404	L	0.021	0.058
182	TCP/PCP	0.25	C	404	L	0.006	0.030
183	TCP/PCP	0.25	C	404	L	0.131	0.110
205	TCP/PCP	0.25	B	404	L	0.005	0.044
206	TCP/PCP	0.25	C	404	L	0.006	0.044
207	TCP/PCP	0.25	C	404	L	0.004	0.043
219	TCP/PCP	0.5	A	404	L	0.184	0.208
220	TCP/PCP	0.5	A	404	L	0.219	0.291
221	TCP/PCP	0.5	B	404	L	0.109	0.180
222	TCP/PCP	0.5	B	404	L	0.267	0.290
208	TCP/PCP	0.5	C	404	L	0.019	0.100

ID #	Test	Conc. (TU)	Jar	Length of Exposure (hours)	Live or Dead	TCP Residue (mmol kg^{-1})	PCP Residue (mmol kg^{-1})
209	TCP/PCP	0.5	C	404	L	0.020	0.251
210	TCP/PCP	0.5	C	404	L	0.008	0.165
134	TCP/PCP	0.5	A	404	L	0.008	0.053
135	TCP/PCP	0.5	A	404	L	0.007	0.041
136	TCP/PCP	0.5	B	404	L	0.008	0.004
137	TCP/PCP	0.5	B	404	L	0.023	0.081
138	TCP/PCP	0.5	C	404	L	0.007	0.017
139	TCP/PCP	0.5	C	404	L	0.135	0.169
398	TCP/PCP	0.5	A	404	L	0.048	0.205
399	TCP/PCP	0.5	A	404	L	0.261	0.386
400	TCP/PCP	0.5	A	404	L	0.086	0.306
401	TCP/PCP	0.5	B	404	L	0.132	0.211
402	TCP/PCP	0.5	B	404	L	0.725	0.712
403	TCP/PCP	0.5	B	404	L	0.230	0.330
404	TCP/PCP	0.5	C	404	L	0.447	0.626
405	TCP/PCP	0.5	C	404	L	0.137	0.272
118	TCP/PCP	0.5	A	212	D	0.227	0.020
119	TCP/PCP	0.5	B	260	D	0.025	0.008
394	TCP/PCP	0.5	B	356	D	0.295	0.295
395	TCP/PCP	0.5	C	188	D	0.248	0.209
130	TCP/PCP	1	A	404	L	0.016	0.126
131	TCP/PCP	1	B	404	L	0.018	0.038
132	TCP/PCP	1	B	404	L	0.041	0.064
133	TCP/PCP	1	C	404	L	0.027	0.020
211	TCP/PCP	1	A	140	D	0.029	0.180
212	TCP/PCP	1	A	140	D	0.375	0.282
213	TCP/PCP	1	B	116	D	0.228	0.197
223	TCP/PCP	1	C	356	D	0.152	0.159
224	TCP/PCP	1	B	116	D	0.367	0.225
225	TCP/PCP	1	B	116	D	0.210	0.315
112	TCP/PCP	1	A	140	D	0.061	0.034
113	TCP/PCP	1	C	188	D	0.013	0.017
114	TCP/PCP	1	B	188	D	0.025	0.015
115	TCP/PCP	1	C	116	D	0.036	0.010
116	TCP/PCP	1	C	116	D	0.021	0.022
117	TCP/PCP	1	B	212	D	0.197	0.114
120	TCP/PCP	1	A	92	D	0.256	0.024
121	TCP/PCP	1	A	116	D	0.397	0.071
122	TCP/PCP	1	A	212	D	0.057	0.165

ID #	Test	Conc. (TU)	Jar	Length of Exposure (hours)	Live or Dead	TCP Residue (mmol kg^{-1})	PCP Residue (mmol kg^{-1})
123	TCP/PCP	1	B	260	D	0.280	0.026
396	TCP/PCP	1	B	212	D	0.455	0.903
397	TCP/PCP	1	A	68	D	0.266	0.239
124	TCP/PCP	2	C	92	D	0.163	0.142
125	TCP/PCP	2	C	92	D	0.416	0.064
126	TCP/PCP	2	C	92	D	0.000	0.018
109	TCP/PCP	2	A	92	D	0.044	0.015
110	TCP/PCP	2	A	92	D	0.010	0.004
111	TCP/PCP	2	A	92	D	0.012	0.003
184	TCP/PCP	2	B	92	D	0.144	0.258
185	TCP/PCP	2	B	68	D	0.001	0.163
186	TCP/PCP	2	B	68	D	0.039	0.126
190	TCP/PCP	2	A	92	D	0.423	0.101
191	TCP/PCP	2	A	92	D	0.079	0.286
192	TCP/PCP	2	B	68	D	0.058	0.170
193	TCP/PCP	2	B	68	D	0.033	0.164
194	TCP/PCP	2	C	68	D	0.384	0.059
195	TCP/PCP	2	C	68	D	0.116	0.028
106	TCP/PCP	4	C	68	D	0.074	0.018
107	TCP/PCP	4	C	68	D	0.025	0.004
108	TCP/PCP	4	C	68	D	0.061	0.014
127	TCP/PCP	4	B	68	D	0.060	0.036
128	TCP/PCP	4	B	68	D	0.002	0.008
129	TCP/PCP	4	B	68	D	0.059	0.051
187	TCP/PCP	4	A	68	D	0.047	0.069
188	TCP/PCP	4	A	68	D	0.205	0.084
189	TCP/PCP	4	A	68	D	0.164	0.200
196	TCP/PCP	4	A	68	D	0.018	0.074
197	TCP/PCP	4	A	68	D	0.318	0.067
198	TCP/PCP	4	B	68	D	0.308	0.059
199	TCP/PCP	4	B	68	D	0.343	0.058
200	TCP/PCP	4	C	68	D	0.279	0.092
201	TCP/PCP	4	C	68	D	0.043	0.067

APPENDIX D - SAS CODE FOR ANALYZING BODY RESIDUES FOR THE TCP TEST

```
dm 'log; clear; output; clear; ';
options ls=72 ps=54 pageno=1;

***filename: a:\tcp p.sas***;

title;
filename one 'a:\tcp.txt';

data a;
infile one missover firstobs=5 dsd dlm='09'x ;
input ID Test $ Conc  Jar $  Time  LorD $  Wwt injvol tcp ;

*proc print data=a;

proc sort data=a; by conc;
proc mixed data=a covtest; where conc ne 0;
classes conc;
model tcp = conc / ddfm=satterth ;
lsmeans conc / pdiff;
run;
```

APPENDIX E - SAS CODE FOR ANALYZING BODY RESIDUES FOR THE PCP #1 TEST

```
dm 'log; clear; output; clear; ';
options ls=72 ps=54 pageno=1;

***filename: a:\pcp1 p.sas***;

title;
filename one 'a:\pcp1.txt';

data a;
infile one missover firstobs=5 dsd dlm='09'x ;
input ID Test $ Conc  Jar $  Time  LorD $  Wwt injvol pcp ;

*proc print data=a;

proc sort data=a; by conc;

proc mixed data=a covtest; where conc ne 0;
classes conc;
model pcp = conc / ddfm=satterth ;
repeated / group=conc;
lsmeans conc / pdiff;
run;
```

APPENDIX F - SAS CODE FOR ANALYZING BODY RESIDUES FOR THE PCP #2 TEST

```
dm 'log; clear; output; clear; ';
options ls=72 ps=54 pageno=1;

***filename: a:\pcp2 p.sas***;

title;
filename one 'a:\pcp2.txt';

data a;
infile one missover firstobs=5 dsd dlm='09'x :
input ID Test $ Conc  Jar $  Time  LorD $  Wwt injvol pcp ;

*proc print data=a;

proc sort data=a; by conc;

proc mixed data=a covtest; where conc ne 0;
classes conc;
model pcp = conc / ddfm=satterth ;
repeated / group=conc;
lsmeans conc / pdiff;
run;
```

APPENDIX G - SAS CODE FOR ANALYZING TCP AND PCP BODY RESIDUES FOR THE TCP/PCP TEST

```
dm 'log; clear; output; clear; ';
options ls=72 ps=54 pageno=1;

***filename: a:\tcppcp p.sas ***;

title;
filename one 'a:\tcppcp.txt';

data a;
infile one missover firstobs=5 dsd dlm='09'x ;
input ID Test $ Conc  Jar $  Time  LorD $  Wwt injvol tcp pcp tcppcp;

*proc print data=a;

proc sort data=a; by conc;
proc mixed data=a covtest; where conc ne 0;
classes conc;
model tcp = conc / ddfm=satterth ;
repeated / group=conc;
lsmeans conc / pdiff;

proc mixed data=a covtest; where conc ne 0;
classes conc;
model pcp = conc / ddfm=satterth ;
repeated / group=conc;
lsmeans conc / pdiff;

proc mixed data=a covtest; where conc ne 0;
classes conc;
model tcppcp = conc / ddfm=satterth ;
repeated / group=conc;
lsmeans conc / pdiff;

run;
```

VITA

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Employed by Oklahoma State University, Department of Zoology as a graduate research assistant, August, 1996 to present to evaluate the bioavailability and toxicity of chlorophenols to the earthworm, *Eisenia fetida*

Employed by Southeastern Oklahoma State University, Department of Biology, as an undergraduate research assistant, October, 1995 through July 1996 to evaluate the effects of sex hormones on the development of neuroreceptors in rats

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