EFFECTS OF SUBLETHAL EXPOSURE OF *EISENIA FETIDA* TO PHENANTHRENE AND DEVELOPMENT OF A LIPID ANALYSIS TECHNIQUE

By

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Introduction

Earthworms play a significant role in the functioning of soil ecosystems. Edwards and Bater (1992) showed that the mean height of barley plants grown in soil inoculated with earthworms was significantly higher ($P < 0.05$) than intact soil, suggesting that earthworms may have some effect on soil fertility. Earthworms also play an important role in the initial breakdown of organic litter and the transportation of soil aggregates from the soil surface downward in the soil profile. It has been estimated that over 50% of the soil macro-organism biomass in most soil types consists of earthworms (Belfroid, 1994). Earthworms are also a common prey species for many birds and vermivorous creatures such as moles, shrews, and badgers. Understanding the impact of contamination in a non-target species, the earthworm, may prove useful in understanding greater ecological impact of chemicals.

The use of earthworms in regulatory practice has been well established; however, environmental risk is still largely based only on the measurement of chemical levels in soils. Chemical measures do not take into account the potential effects of all unknown contaminants and possible metabolites formed during biological processes (Hund and Traunspurger, 1994). The use of sensitive biological measures (e.g., indicator species) may also account for effects on biological processes that may not be reflected in chemical measures. Organisms partition available energy for various processes such as growth, reproduction, metabolism, and locomotion. Exposure to environmental stressors, including contaminants, results in a reallocation of available energy, often reducing energy available for growth and reproduction. A better understanding of energy

partitioning on sublethal effects in organisms, communities, and ecosystems is needed to help reduce the uncertainty associated with ecological risk assessment (Gibbs et al., 1995).

Organic contaminants are a major class of chemicals of concern today. Organic contaminants include those used as poisons (pesticides) and compounds such as solvents, industrial by-products, and human activity by-products that are not designed as poisons but still have adverse effects when released into the environment (Newman, 1998). One major sub-group of the organic contaminants of concern is the polycyclic aromatic hydrocarbons (PAHs).

Polycyclic aromatic hydrocarbons constitute an extraordinarily large class of organic molecules. Major sources of PAHs are crude oil, coal, and oil shale (Harvey, 1997). Petroleum product contamination of the environment is a concern throughout industrialized countries. Significant levels of PAHs are also found in air, food (charcoal cooked steak), and water. Of particular interest is the fact that some PAHs (e.g., 7,12-dimethylbenz[a]anthracene) are known among some of the most potent carcinogenic compounds (Harvey 1997). The U.S. Environmental Protection Agency (USEPA) and the Agency for Toxic Substances and Disease Registry (ATSDR) list PAHs as one of their top twenty hazardous substances (ATSDR HazDat database, 1999).

PAHs present in the atmosphere are derived principally from the combustion of fossil fuels in heat and power generation, refuse burning, and coke ovens. Vehicle emissions are another major source of PAHs (Harvey, 1997). Natural sources, such as forest fires and volcanic activity add to the overall burden, but anthropogenic sources are the most important concern (Harvey, 1997).

For the current research phenanthrene (PHE) was the chemical toxicant of choice due to its relevance to soil systems. PHE is a lower molecular weight PAH (178.22), consisting of three fused benzene rings and a K_{ow} of 4.16 (Landrum et al., 1994). It is also resistant to degradation, has a high affinity for organic matter, and relatively low water solubility (Van Brummelen et al., 1995).

Bioavailability as defined by Newman (1998) is the extent to which a contaminant in a source is free for uptake. In terms of applying bioavailability to environmental toxicology, it is the bioavailable concentration of a contaminant that dictates the chemical exposure that an organism will experience. This exposure then dictates the probability and intensity of effects to the organism. As stated by Wells and Lanno (2001) bioavailability is a very difficult parameter to measure. The bioavailability of a chemical can be measured in various ways, but the accumulation of residues in an organism is the most direct measure. Chemical residues that accumulate in an organism may be related to a biological response (e.g., mortality, reproduction). Such residues, termed critical body residues (CBRs) (McCarty and Mackay, 1992), may be the most accurate measure of chemical bioavailability as they integrate bioavailability over time and space. However, CBRs are not suitable measures of bioavailability for chemicals that do not bioaccumulate or are metabolized rapidly. For these types of chemicals, alternative measures of bioavailability must be used. Determining levels of chemicals other than total chemical concentration may serve such a purpose, but these chemical measurements must be related to biological responses. Solid-phase extraction techniques have been correlated with the bioavailability of organic chemicals in soils (Wells and Lanno, 2001;

Tang and Alexander, 2000) and may provide an alternative to measuring total chemical levels.

Fat or lipid stores are the major compartments for non-polar organic chemical partitioning in organism tissues. Accurate and consistent lipid determination methods are important for the following reasons: 1) lipid reserves are often correlated with behavior (i.e. growth, reproduction, and survival), and 2) whole body chemical residues are often normalized to lipid levels. Arts and Wainman (1999) state that lipids play a critical role in the health of benthic and planktonic invertebrates. This may also be the case for terrestrial invertebrates and warrants further investigation

The thesis will be divided into chapters. Chapter 1 will explore the effects of sublethal PHE exposure on the earthworm *Eisenia fetida*, and examine the bioavailability of sublethal PHE exposure measured by investigating the relationship between solid-phase microextraction fiber determinations of PHE and body residues. Chapter 2 will explore the development of the sulphophosphovanillin technique to measure total lipid levels in earthworms (Knight et al., 1972). Each chapter will contain an introduction, methods and materials, results, and discussion section. Finally a discussion section will follow the two chapters, tying the relevance of this work together and presenting its relevance to environmental toxicology.

Chapter 1

The effects of sublethal exposure of *Eisenia fetida* to the polycyclic aromatic hydrocarbon phenanthrene and the comparison of phenenathrene availability measured by body residues and solid phase microextraction fiber

Introduction

Man's activities have resulted in increasing concentrations of potentially toxic materials being introduced into soil ecosystems. Soils can no longer be regarded as a waste depository without consideration of the effects on soil ecosystems (Neuhauser and Callahan, 1990). There is a need for more information regarding potential toxicity of chemicals to non-target organisms.

Earthworms have a major impact on soil fertility, forming water-stable aggregates, increasing the availability of nutrients, and increasing soil drainage and aeration (Neuhauser et al., 1985). Due to their importance in soil ecosystems, standard methods have been developed to evaluate the toxicity of chemicals (mostly pesticides) to earthworms (ASTM 1997). However, most of these tests have involved determining the lethality (LC_{50}) of chemicals, with less emphasis placed on sublethal effects such as reproduction.

The LC_{50} of a given chemical is often not a useful indicator of potential ecological consequences, because feeding, growth, and reproduction can be disrupted at chemical levels far below the LC_{50} (Neuhauser and Callahan, 1990). It is also known that survival is a less sensitive toxicity endpoint than growth and reproduction (Van Gestel et al.,

1992). To gain further insight into the sublethal effects of organic chemicals, the effects of a polycyclic aromatic hydrocarbon, phenanthrene (PHE), on earthworm growth and reproduction will be examined.

Bioavailability is a difficult concept to define. Bioavailability, as defined by Newman (1998), is the extent to which a contaminant in a source is free for uptake. Wells and Lanno (2001) define bioavailability as the interaction between a biological receptor and a chemical that has the capacity to interact with the receptor. Classical toxicology defines bioavailability as the extent of systemic absorption of a chemical (Klaassen, 1996). Along with the difficulty in defining bioavailability it can also be problematic to measure. Bioavailability of a contaminant is specific to many factors such as the exposure matrix, type of organism, chemical concentration, and route of exposure. The bioavailability of a hydrophobic organic chemical in water is reduced when it is bound to suspended particles and the bioavailability of a metal in soils or sediment is increased when the pH is lowered (Sijm et al., 2000). It is clear that bioavailability is a complex process, which includes several relationships and interactions.

Soils can be highly variable in physical and chemical characteristics such as moisture content, organic matter, clay content, porosity, lipids, and humic substances (Sijm et al., 2000; Lanno and McCarty, 1997). All of these factors modify the complex interaction between the toxicant and the soil matrix. Total chemical measures are commonly used to help simplify chemical exposure estimates, but they often are not well correlated with biological endpoints (Wells and Lanno, 2001; Landrum et al., 1994).

Weak solvent extractions have been used with only moderate success as surrogates to assess bioavailability due to the analyte or medium specificity of solvent

extraction and relatively poor correlation with biological responses (Wells and Lanno, 2000). Passive sampling devices are another technique that has been used in an attempt to mimic bioavailability by providing a phase for chemical partitioning (Verbruggen et al., 1999; Prosen, 1998; Wells and Lanno, 2001). If properly correlated with biological responses, chemical determination using passive sampling devices may predict toxicity as a screening tool, providing an alternative for conventional chemical extraction and/or biological methods.

The following research will utilize solid-phase microextraction (SPME) fiber technology. SPME devices are constructed of an optical fiber 1 cm in length coated with a non-polar, polydimethyl siloxane phase 7 um thick which adsorbs analytes of interest by diffusion from the exposure medium. SPMEs have been used for measuring nonpolar compounds in aquatic and atmospheric media (Mayer et al., 2000; Ramos et al., 1998, Verbruggen et al., 1999). Analytes are thermally desorbed from the fiber in the injection port of the gas chromatograph.

Another approach to simplifying the complex interactions of a toxicant with the exposure matrix is the use of body residues (Lanno et al., 1998). Body residues may reduce the variability in relating bioassay endpoints to measures of chemical exposure for test results with variable exposure medium. The interaction between a chemical and the physical-chemical properties of the exposure matrix determine what fraction of the chemical is bioavailable and thus potentially toxic (Lanno and McCarty, 1997). Chemical body residues represent the portion of chemical in the environment that is directly bioavailable to an organism, integrating all facets of biotic and abiotic modifying

factors into an explicit measure of bioavailability and may provide a useful tool for examining environmental factors that affect bioavailability.

The objective of this research is to examine phenanthrene (PHE) kinetics in the soil and earthworm, examine the effects from sublethal exposure to PHE, and assess the bioavailability of PHE using body residues of earthworms and SPME fibers. The research consisted of a time-series exposure experiment (Experiment I) to monitor soil and earthworm uptake kinetics and a 28-day exposure experiment (Experiment II) to study the endpoints of growth, cocoon production, and hatchability. The hypotheses of the study are that: 1) Earthworm growth and cocoon production will decrease as the sublethal exposure concentrations increase, 2) SPME will serve as a predictive tool used to measure available contamination when correlated with earthworm body residues.

Materials and methods

Earthworms

All tests were performed with synchronized aged (12.3 weeks) earthworms of the species *Eisenia fetida*. The worms were obtained from Oklahoma State University's cultures grown on horse manure at an ambient temperature of 25 ± 5 °C. Wet weights of individual worms ranged from 304 - 439 mg. Mean (n=4) moisture content of earthworms was 83 % ± 1.1 (SD).

Artificial soil

The artificial soil (10 % organic matter) in this experiment was composed (% dry weight) of 69% silica sand (60 mesh, Blasting Specialties, Tulsa, OK), 20% pulverized

EPK Kaolin clay (Tomorrow's Treasures, Oklahoma City, OK), 10% *Sphagnum* peat moss (Wal-Mart, Stillwater, OK), and 1 % calcium carbonate ($CaCO_3$, Fisher Scientific) (ASTM, 1997). Prior to the mixing of the artificial soil, the water content of the peat moss was determined and the amount used to prepare the artificial soil was adjusted for moisture content. The pH of the artificial soil was 6.4 ± 0.02 (± SD, n=6). Each test container was prepared individually with 345 g of sand measured into each test container and then spiked with the desired solution of PHE (Sigma-Aldrich, St. Louis, MO, 98% pure) dissolved in acetone. Acetone was allowed to evaporate overnight in a dark fume hood until dry. All artificial soil components were added into test containers to yield final weight of 500 g (dry weight). Dry components were mixed until uniform texture and color were achieved (using stainless steel impeller, 5-cm dia., attached to ¾ -hp drill press at 190 RPM). The soil was moistened to a level of 45 % (w/w) using reagent grade water (pH 7.0, resistivity 18 MΩ). PHE-spiked artificial soil was mixed again until uniform texture and color were achieved. Temperature and moisture equilibration of the test containers were allowed for 24 hr in a Percival Scientific environmental chamber (24 ± 1 °C, 24 hr light) prior to the addition of earthworms.

Toxicity tests

Prior to the start of the definitive experiment, a range-finding experiment was conducted to determine sublethal exposure concentrations. In this case only three replicates were used per concentration (acetone control, 1.010, 3.142 mmol/kg nominal) and the duration of the test was two weeks. Jars were monitored for the presence of cocoons and mortality of earthworms was also noted. Results showed that 98% mortality

occurred in the 3.142 mmol/kg concentration by 96 hr. There was zero percent mortality in the acetone control and 1.010 mmol/kg nominal concentration. Seventeen, four, and zero cocoons were found in the acetone control, 1.010, and 3.142 mmol/kg nominal concentration, respectively.

Definitive Experiments Outline

Experiment I comprised two sublethal PHE concentrations (0.180, and 0.561 mmol PHE / kg dry wt) with three independent replicate test containers, containing ten worms, for each sublethal concentration per observation time (0, 6, 12, 24, 48, 96, 336 hr). Earthworms were removed from triplicate test containers at each observation time and frozen for PHE analysis. Experiment II was performed using five sublethal PHE concentrations (0.101, 0.180, 0.314, 0.561, 1.010 mmol/kg) with replicate jars containing ten earthworms for each concentration. In both experiments replicate jars with soil containing acetone at the maximum concentration used to dissolve PHE served as the solvent controls. Two acetone washed feeding chambers containing moist ground horse manure were placed in exposure soil in both experiments to ensure food was not a limiting factor during the experiment. Each jar was capped with a lid containing two small holes to allow for air exchange and minimize moisture loss. The duration of the test was four weeks with spiked-artificial soil renewal, mortality check, and cocoon count and removal at two weeks. Parameters measured in Experiment II were growth, feeding activity, number of cocoons produced, and hatchlings per cocoon. Growth was determined as the difference in fresh mass before and after chemical exposure. Mean weights (n=10) were used because it was not possible to identify and track the growth of

individual worms. Feeding activity was assessed every other day by removing feeding balls from the exposure chamber, sorting through the ground manure, and counting the number of worms found in the food ball. Earthworms found were then buried below the surface of the exposure soil. Cocoons were counted twice, at the end of the first exposure period (two weeks) and then again at the termination of the test and were removed from the soil using a spatula and breaking the soil in to pieces smaller then 4 mm. Cocoons were then placed in 3-oz solo cups with moist filter paper and checked every other day for hatchlings. Hatchlings were removed from the solo cup, counted, and placed in amber microcentrifuge tubes (Fisher Scientific) and frozen at –35°C.

Chemical analysis of test soil

Test soils in Experiment I and II were extracted using a Dionex ASE 200 Accelerated Solvent Extractor following the Dionex application note 313, which is a proposed method to meet U.S. EPA method 3545. Five g of test soil was mixed with 20 g of oven dried (400 °C for 24 hr) anhydrous sodium sulfate and the mixture was ground before filling the extraction cell. The dried sample was placed into an extraction cell and extracted with 40 mL 1:1 methylene chloride:acetone (v/v) at 2000 psi and an oven temperature of 100 °C.

The sample extract was diluted or concentrated as needed for HPLC analysis and solvent replaced into *n*-hexane using a Kuderna-Danish apparatus. HPLC analysis was performed using a SUPLELCOSIL LC-PAH (Supelco), 5 cm x 4.6 mm ID x 3 μm reversed-phase analytical column at ambient temperature (20-25 °C). The gradient elution initially consisted of 60% reagent grade water and 40% acetonitrile (Optima

grade, Fisher Scientific), 0.3 min hold, and then ramped to 100% acetonitrile at 4.0 min with a flow rate of 3.0 mL/min. Injection volumes were 25 µL using a Dionex LC-10 autoinjector from a Dionex AS-40 autosampler and detection of PHE using a Waters 484 deuterium arc lamp UV/VIS detector. Peak integration was conducted at 254 nm with an optical band of 8 nm. Peaks from samples were identified by comparison of retention times with a certified reference standard (Chem Service, F81MS, 100 µg/mL) and concentrations were determined by peak area from standard curves. All data collection and chromatogram analysis was done using Dionex Peaknet software version 5.1 (Dionex, 1999).

Earthworm Body Residue Analysis

Earthworms were removed from storage, thawed to room temperature, and wet mass was recorded. Individual earthworms were transferred to 10-mL Teflon Oakridge centrifuge tubes and 3 mL of reagent grade water was added. Tissue homogenization was conducted for two minutes using a TH115 tissue homogenizer (Omni International, Warrenton, VA). Three mL of *n*-hexane (95%, Fisher Scientific) was added to the homogenate, ultra sonification of the homogenate occurred for two minutes at 40% duty cycle, and then the homogenate was extracted for 1 hour in a rotary evaporator in complete darkness at 40 rpm (Rotamix, ATR Inc. Laurel, MD). The homogenate was then centrifuged at 5000 rpm (5-10 °C) to separate reagent grade water from the hexane. The hexane layer was removed using *n*-hexane-rinsed Pasteur pipettes, and concentrated to 1.5 mL under a N_2 stream. The concentrated extract was cleaned by passing extract through conditioned solid-phase microextraction columns (500 mg silica gel, 3 mL

volume, BakerBond J.T. Baker, Phillipsburg, NJ) topped with 1 cm of anhydrous sodium sulfate (Fisher Scientific). Three column volumes of *n*-hexane were used to elute the sample from the solid-phase extraction column. The eluate was solvent replaced in acetonitrile and concentrated to 1.5 mL using a gentle stream of N_2.

Adding 6.25 or 0.7815 ng/uL of PHE to reference earthworm tissue and subjecting to above methods and chemical analysis conducted spike recovery. Blank analysis was conducted to correct for any earthworm impurities not removed by the solid-phase microextraction column. Earthworm body residues reported in the results have not been corrected for spike recovery of PHE (89.1 % ± 1.9, SD, n=3).

Solid-Phase Microextraction Fiber Exposure (Experiment I)

Solid-phase microextraction fibers were used to assess PHE availability directly from the aqueous phase of the soil at the earthworm exposure temperature (24 ± 1 °C) and followed methods described in Wells and Lanno (2001). Each determination was made using 0.500 g artificial soil (dry weight) placed in a screw-top amber vial with Teflon septum (Supelco) with 15 mL reagent grade water and a Teflon coated magnetic stir bar. Each vial was aligned on a magnetic stir plate, the SPME needle was inserted through the septum, and fiber was deployed. Partioning of PHE between the fiber and soil reached steady state in approximately five hours. Residue analysis was completed by using gas chromatography (Tracor 565 GC-FID, megabore fused silica capillary column). Helium was used as the carrier and makeup gas. The flow rate was set to 35 cm/sec linear velocity and make-up flow rate was set to 45 mL/min. Hydrogen flow rate was 35 mL/min and breathing airflow rate was 350 mL/min. The temperature program for direct

injection GC analysis was: injection port temerature-290 °C, detector temperature-300 °C, initial oven temperature-160 °C (5 minute hold) with 35 °C/min ramp to 210 °C (7 minute hold) (Wells and Lanno, 2001).

SPME fiber performance was determined before and after each soil determination by measuring a reference standard solution (1 mg PHE/ L RGW). Integration of peaks was done by external calibration using injections from PHE standards with a certified PHE check standard (Chem service, F81MS). Chromatography data was collected and analyzed using Peaknet chromatography software (Dionex, 1999).

Statistical analysis

Standard descriptive statistics, ANOVA, and linear regression techniques were used to analyze the data. Data interpretation was completed using SAS (Statistical Analysis System version 8.00, SAS Institute, Inc 1989-2000), TOXSTAT (version 3.4 Western EcoSystems Technology Inc. 1994), and Microsoft Excel 2000 (version 9.0, Microsoft 1983-1999). SYSSTAT (version 7.0, Chicago, IL., 1997) was used to integrate the area underneath the exposure curves.

Results

Cocoon and hatchling production in Experiment II was reduced as the concentration of PHE increased. Regression analysis results of the cocoon data and hatchling data indicate significant regression (α=0.05, $F_{1,28}$=60.2, $p< 0.001$; α=0.05, $F_{1,28}$=73.7, $p< 0.001$, respectively). Based on the slopes of the regression lines (R^2 =

0.9424 and 0.9494, respectively) from cocoon and hatchling data, the number of viable hatchlings per cocoon decreased as the concentration of PHE increased (Figure 1.1).

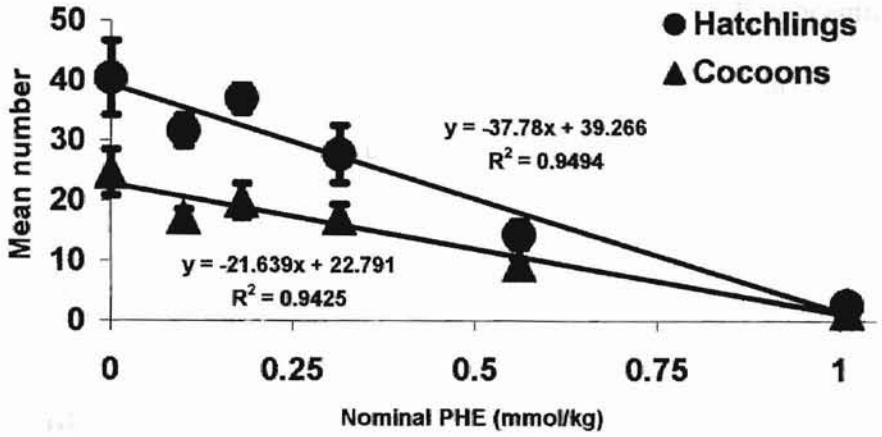


Figure 1.1: Effects of PHE on cocoon ($p<0.001$) and hatchling ($p<0.001$) production of *Eisenia fetida*. Values are means (n=5) ± SEM.

Chemical concentrations having some biologically significant effect, such as 10% reduction in fecundity, can be interpolated using a linear model. The interpolation method used will provide a point-estimate, called the inhibition concentration (IC) of a single toxicant that causes a percent reduction, in this case a reduction in number of cocoons produced that may have a significant impact on the future of the population. Use of the ICp method will generate concentration data that represents the effect level for the above test method using a continuous model. For the ICp method the responses are assumed 1) to be monotonically non-increasing, where the mean response for each higher concentration is less than or equal to the mean response for each previous concentration, 2) to follow a linear response function, and 3) the data are from a random, independent, and representative sample of test data (or population) (Norberg-King, 1993). The following data are point estimates, giving the concentration of PHE that would inhibit the

production of cocoons by p percentage, all values are based on mean value of n=5, ± SD: IC_{50} = 0.492 ± 0.08 mmol/kg, IC_{25} = 0.213 ± 0.12 mmol/kg , and IC_{10} = 0.079 ± 0.08 mmol/kg.

Earthworm weight in Experiment II was not affected by PHE concentrations. The mean % range of wet-weight loss during the four weeks of exposure, across all treatments, was 51-57 % (Table 1.1). Feeding activity in Experiment II was significantly different from control groups in exposure concentrations of 0.180 and 1.010 mmol PHE/kg dry soil (nominal) (Dunnett value $_{24,5}$ = 2.36, $P < 0.05$) (Table 1.1).

Table 1.1: Effect of PHE on the growth and feeding activity of *Eisenia fetida* in artificial soil.

Concentration (mmol PHE kg^{-1})	% Wet-weight*	Feeding activity*
0.0	-52±2.7	1.2±0.6
0.101	-51±5.0	0.8±0.4
0.180	-57±3.7	6.6±1.6**
0.314	-53±4.8	4.4±2.0
0.561	-57±2.7	3.6±0.8
1.010	-51±4.7	7.2+1.6**

*Mean of five replicates ± standard error of mean
**$P < 0.05$

Experiment I and II soil results

The nominal PHE concentrations tested in Experiment I were 0.180 and 0.561 mmol PHE / kg dry soil. However, when PHE was measured, a declining PHE concentration was observed over the two-week exposure period with the rate of change (slope = -0.0003 mmol PHE/kg soil/hr and –0.001 mmol PHE/kg soil/hr, respectively) between the two concentrations differing by an order of magnitude (Figure 1.2)

Regression analysis results indicate a significant regression in both concentrations (α=0.05, $F_{1,10}$=73.4, p< 0.001; α=0.05, $F_{1,10}$=295.9, p< 0.001, respectively).

However, at the end of the two-week exposure period no statistical differences were observed in mean (± SEM, n=5) PHE concentrations in Experiment II (Tukey value $_{6,12}$ = 4.748, p>0.05) (Figure 1.3). Upon analysis of the soil concentration at the end of a two week exposure period, the corresponding accelerated solvent extractable concentrations appeared to be 5.45E-04, 1.41E-02, 2.28E-02, 1.46E-01, 8.36E-02, 2.95E-02 mmol PHE/ kg dry soil, respectively. The mean (n=8,± SD) % water content (w/w) of the artificial soil at the end of a two week exposure was 31% ± 3.0.

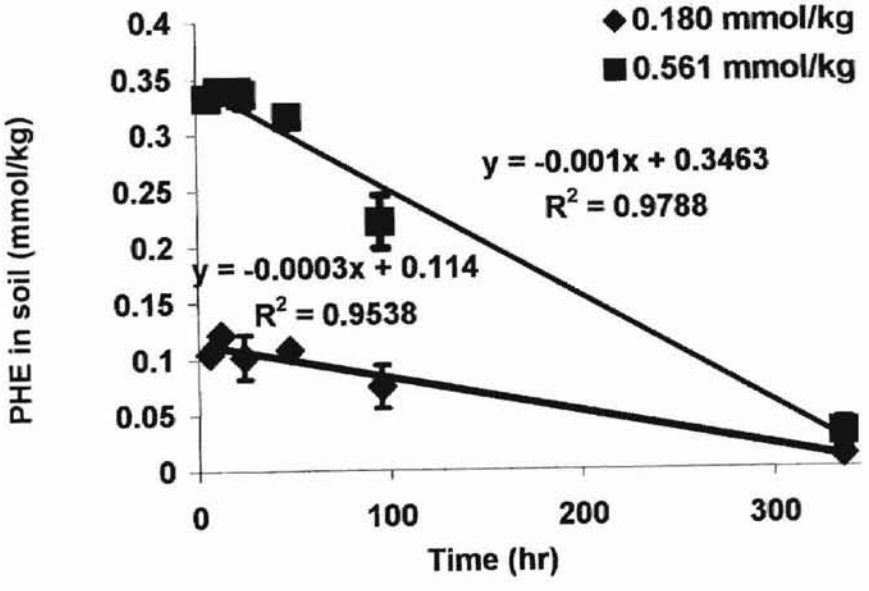


Figure 1.2: Experiment I total PHE soil concentrations (mean ± SEM, n=3) of 0.180 mmol/kg (p<0.001) and 0.561 mmol/kg (p<0.001) at various time periods.

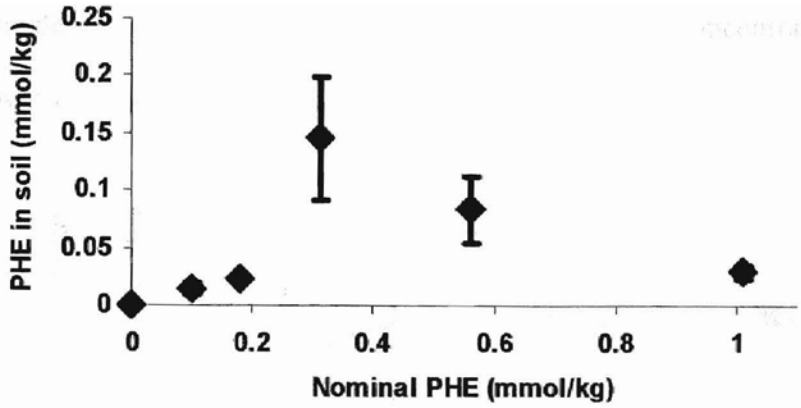


Figure 1.3: Experiment II total PHE soil concentrations (mean ± SEM, n=5) at day 28.

For nonpersistent chemicals, constant exposure will occur only when there is a constant infusion of chemical into the exposure soil. As seen above (Figure 1.2), when the concentration declines a time-weighted average can be taken as a substitute for constant exposure. To obtain exposure estimates for earthworms in the declining concentration of PHE, the area under the soil exposure curve over time was calculated using SYSTAT (ver 7.0, Chicago, IL., 1997). Estimated values were 19.2 mmol/kg/336 hr in the 0.180 mmol PHE/kg dry soil and 57.5 mmol/kg/336 hr in the 0.561 mmol PHE/kg dry soil or 0.057 mmol/kg/hr and 0.171 mmol/kg/hr.

Experiment I and II earthworm body residues

No mortalities were observed in either Experiment I or II. In Experiment I, earthworm body residues reached a maximum level of PHE at approximately 48 h (Figure 1.4). Regression analysis results indicate significant regression in both earthworm body residue concentrations (α=0.05, $F_{1,16}$=9.0, p=0.008; α=0.05, $F_{1,16}$=5.8, p< 0.029, respectively). By the end of 2 weeks (336 hr), PHE levels had decreased to

below the detection limit of 0.02 mmol/kg in both exposure concentrations. Experiment II earthworm body residues are depicted in Figure 1.5.

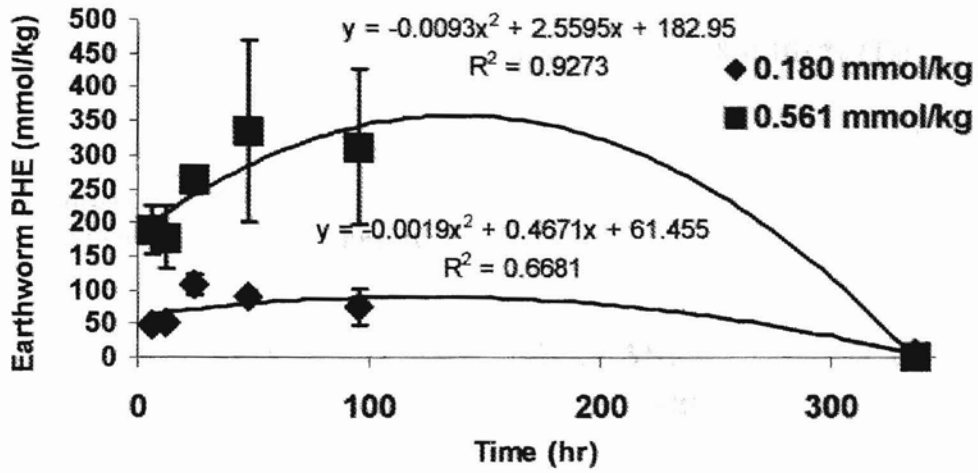


Figure 1.4: Experiment I PHE earthworm body residue concentrations (mean ± SEM, n=3) of 0.180 mmol/kg (p=0.008) and 0.561 mmol/kg (p=0.029) exposure at various time periods.

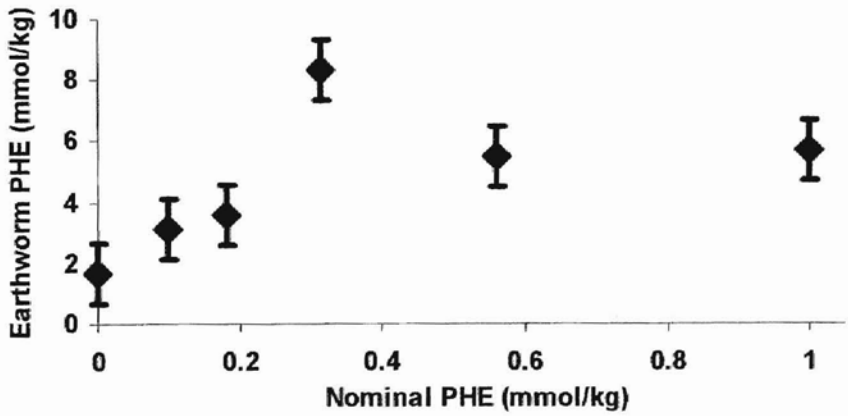


Figure 1.5: Experiment II PHE earthworm body residue concentrations (mean ± SEM, n=5) at day 28.

***Solid-phase microextraction** (SPME) analysis of soils*

PHE was detected in Experiment I at all time points in both concentrations (0.180 and 0.562 mmol PHE / kg dry soil) using SPME fiber analysis. The percent extactable PHE (SPME) compared to that extractable ASE ranged from 0.048-0.163 % (Table 1.2-1.3).

Table 1.2: Comparison of PHE levels (0.180 mmol PHE/kg, dry soil, nominal) determined at various sampling times by SPME or ASE.

Time	*Mean SPME (mmol/kg)	*Mean ASE (mmol/kg)	% ± SD (SPME/ASE)
6	5.07E-05	1.05E-01	0.048 ± 0.005
24	8.53E-05	1.02E-01	0.091 ± 0.052
96	4.54E-05	7.50E-02	0.163 ± 0.011
336	1.95E-05	6.00E-03	**0.163

* N=2
** Sample 2 of 2 was too small to calculate standard deviation.

Table 1.3: Comparison of PHE levels (0.560 mmol PHE/kg, dry soil, nominal) determined at various sampling times by SPME or ASE.

Time	*Mean SPME (mmol/kg)	*Mean ASE (mmol/kg)	% ± SD (SPME/ASE)
6	2.26E-04	3.33E-01	0.068 ± 0.007
24	2.10E-04	3.37E-01	0.063 ± 0.005
96	1.86E-04	2.23E-01	0.087 ± 0.049
336	1.85E-05	3.10E-01	0.055 ± 0.018

* N=2

PHE concentrations in the test soil of Experiment I appear to decrease with time in a linear manner (Figure 1.6). Regression analysis indicates significant regression in both SPME extractable concentrations ((α=0.05, $F_{1,6}$=7.1, p= 0.038; α=0.05, $F_{1,6}$=42.1, p< 0.001, respectively).

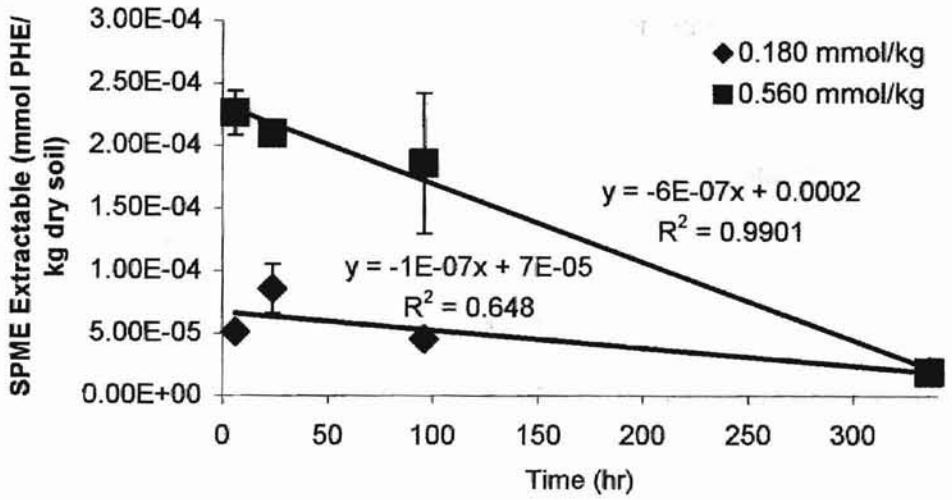


Figure 1.6: Change in SPME extractable PHE concentrations of 0.180 mmol/kg (p=0.038) and 0.560 mmol/kg (p=0.0006) over time in Experiment I

Discussion

Lowered fitness of individuals due to reproductive impairment is arguably the most useful sublethal effect measured (Newman, 1995). It is clear that based on the results of this study that exposure of *E. fetida* to sublethal levels of PHE will have a negative effect on cocoon and hatchling production. In a study of reproduction of *E. fetida* sublethally exposed to nine different organic chemicals by Neuhauser and Callahan

(1990), all tests showed decline in reproduction as the concentration of the chemical increases. Van Gestel et al. (1992) showed significant reduction in the cocoons produced and the number of hatchlings produced to a variety of chemicals and pesticides. However, in the absence of understanding the reproductive biology and ecology of the above species, understanding the effectiveness of the consequences of the sublethal effect is uncertain. As stated by Bolen and Robinson (1995), stochastic events – chance or random occurrences – are threats to the existence of organisms. These events do not immediately destroy populations instead, they reduce numbers of organisms in populations to levels at which chances of survival are greatly diminished, especially if another unfavorable random event should occur.

In many cases growth is often chosen as the response variable when measuring sublethal effects. Not only is it easy to quantify, growth incorporates a suite of biochemical and physiological effects that is often linked to individual fitness. However, when working with organisms where it is difficult to track individuals, growth may not be the best sublethal effect to measure. In the current study, the highest sublethal concentration of PHE tested showed no significant effect on weight reduction when compared to the controls. The high percent loss of weight may have been caused from an effect of the artificial soil. Hartenstein (1984) showed that *E. fetida* raised on moist-ashed loam at 25 °C lost approximately 58 % percent of their fresh weight. The lost of water in the soil by the end of a two week exposure may have masked the ability to detect any changes in tissue weight (Gibbs et al., 1996). The large change in earthworm mass may have simply resulted from the loss of water in the soil and partial desiccation of the earthworms. Measuring changes in dry mass may prove to be a better method of

monitoring change in growth and designing a method to track individuals will lead to a better understanding of effects on growth.

Behavioral effects are underutilized in environmental toxicology for three reasons: 1) it is difficult to objectively score behavioral parameters, 2) considerable variability can exist, and, 3) it is difficult to extrapolate to the field. The testing method mentioned above, monitoring feeding activity using moist-ground horse manure in the feeding chambers, seemed to be an objective score. However, no pattern of decreased feeding activity as concentration of PHE increased was found. A statistically significant increase in feeding activity was observed in two PHE concentrations tested when compared to the control. The higher feeding activity in the 0.180 mmol PHE / kg (dry soil) concentration may be an initial positive effect. It is not uncommon for some toxicants when found at high levels produce a negative effect but when found at low levels to produce a positive effect (Reinecke et al., 1997; Van Gestel et al., 1991). This is commonly referred to as hormesis. The increased feeding activity in the highest concentration (1.010 mmol PHE / kg dry soil) simply may be an avoidance behavior to PHE.

This research suggests that more consideration should be given to evaluating sublethal effects. It is clear that reproduction and possibly growth are more sensitive and relevant to measuring environmental toxicity then the use of an LC_{50} value. LC_{50} value estimates should be used as a first approximation and that more sensitive assays should be used to evaluate toxic substances and set environmental standards.

In static toxicity tests, organisms are placed into a series of exposure concentrations. The advantage of this design is that it is easy to perform and inexpensive.

However, toxicant concentrations can change during exposures due to sorption to the container walls, volatilization, bacterial transformations, photolysis and other processes. Soil toxicity tests are typically conducted in static systems where it is difficult to achieve a constant exposure level if the chemical is not highly persistent. Exposure is usually expressed as a nominal or total concentration at a specific point in time and does not take into account the declining concentration over the course of the experiment (Widianarko and Straalen, 1996). Often effects are attributed to the nominal initial concentration, without taking the decline of the concentration in the course of the experiment into account.

The loss of PHE from the soil can possibly be attributed to degradation by several factors such as microbes, uv-light, and/or the earthworms. Ma et al. (1995) suggests that the presence of earthworms increases the disappearance rate of PHE. Based on the above results, the initial spiking concentration must be adjusted to achieve the testing concentration desired or a more persistent chemical used. This will be experiment specific (matrix, moisture, organic matter, experiment duration) and require further research to fully understand and possibly develop a method to alleviate the problem of a declining dose in a static soil test system. In this experiment, to estimate exposure, a time-weighted average was taken. The observed difference in the rates of change between the two concentrations in Experiment I suggests that the level of contamination may influence the rate of decline (Figure 1.2).

Body residues allow for a meaningful comparison to be made of the toxicity of various contaminants between soil types, various worm species, and differing environmental conditions since body residues explicitly account for the bioavailability of

a chemical. This expression of the internal dose of a contaminant received by an organism has distinct advantage over the traditional expression of dose as a concentration of a contaminant in a test medium. The earthworm body residues reached a maximum value at approximately 48 h corresponding to the highest concentration on the soil concentration curves (Figure 1.2). The earthworms for approximately another 48 h maintained this level before a decline in body concentration was observed. This also suggest that it may take 72 h after spiking, in 10% organic matter and an initial moisture concentration 45 % w/w for PHE to come to equilibrium with the soil. The earthworm body residues decline after 96 h is likely due to metabolism of the sublethal PHE concentrations in the worm, the declining PHE concentration in the soil, and the loss of water.

Based upon the preceding discussion of availability and the preceding data it becomes apparent that total chemical, as a measure of exposure in environmental toxicology should be questioned. PHE can be detected and quantified at sublethal levels by SPME fibers. Based on results from Experiment I, the SPME fibers can discriminate between the declining concentrations (Figure 1.6). It is also noted that the SPME fiber concentration is less than 1% of the ASE extractable (Table 1.2-1.3) and possibly relates to the potentially PHE available fraction. This suggests that total chemical measures may be an inappropriate way to assess exposure in soil matrices. SPMEs have the benefit of no liquid chemical extraction or concentration procedures. Based on the comparison of current chemical extraction methods and SPME methods, SPMEs are a rapid method to detect PAHs. The above data (Figure 1.6) also supports the sensitivity by discriminating between the sublethal levels. SPMEs appear to be a promising screening tool the may be

used to predict potential toxicity associated with the bioavailable fraction of organic chemicals in soil systems. Their application in estimating the bioavailable fraction of PAHs in soil requires further validation in soils differing in physical/chemical characteristics. In the current study, it is only possible to suggest that SPME analyses represents a bioavailable fraction of PHE since SPME measures are proportional to total PHE measures.

Chapter 2

The development and evaluation of the sulphophosphovanillin-lipid analysis technique used in earthworm (*Eisenia fetida*) toxicology

Introduction

The purpose of the research in this chapter is to develop a simple and reliable technique to measure total lipids in earthworms. Accurate and consistent lipid determination methods are important for the following reasons: 1) lipid reserves are often correlated with behavior, growth, reproduction and survival, and 2) whole body chemical residues are often normalized to lipid levels. Arts and Wainman (1999) state that lipids play a critical role in the health of benthic and planktonic invertebrates. This may also be the case for terrestrial invertebrates and warrants the development of a simple and reliable technique to measure total lipids in soil-dwelling invertebrates.

Many methods of lipid analysis such as high pressure liquid chromatography (HPLC), gas chromatography (GC), and chromatography rods (Parrish 1999) are being used to measure, separate, and classify different fractions and total lipids. There is a demand in ecological work for a simple, reliable estimate for total lipids (Barnes and Blackstock 1973), especially for small tissue samples. Two common, frequently used, 'simple' methods exist for determining total lipids.

The rapid method of total lipid extraction and purification developed by Bligh and Dyer (1959) is a gravimetric method that isolates lipids by a solvent extraction, in one step, and works well for large samples (>5 g). An alternative method that is effective

with small samples (0.003 g – 0.5g) is the colorimetric analysis of the total lipids (Knight et al, 1972). The gravimetric method is more time consuming due to the evaporation process and requires the use of more solvents than the micro-colorimetric method. Barnes and Blastock (1973) also showed that the coefficient of variation was less in the colorimetric method (± 2.8%) compared to the gravimetric method (±5%). An in-house earthworm (n=500) study showed that a sample size of less than 1.5 mL of pooled extract was difficult to determine gravimetrically. This was reflected in the variation of the data after normalizing the weight of lipid in the sample size back to a known volume. A study by Huckaby et al. (1985) validated the micro-colorimetric by comparing it to the macro-gravimetric method (Table 2.1). The results compared well in medaka *Oryzias latipes*, but not in the amphipod *Leptocherius plumulosus*, possibly due to the small sample size.

Table 2.1: Results of sulphophosphovanillin validation study by Huckaby et al (1985).

	Medaka % lipid	**Leptocheirus % lipid**
Van Handel (micro-colorimetric)	3.92±0.59	5.4±0.47
Bligh-Dyer (macro-gravimetric)	4.15±0.33	7.25*

* only 2 samples run (range: 6.94-7.55)

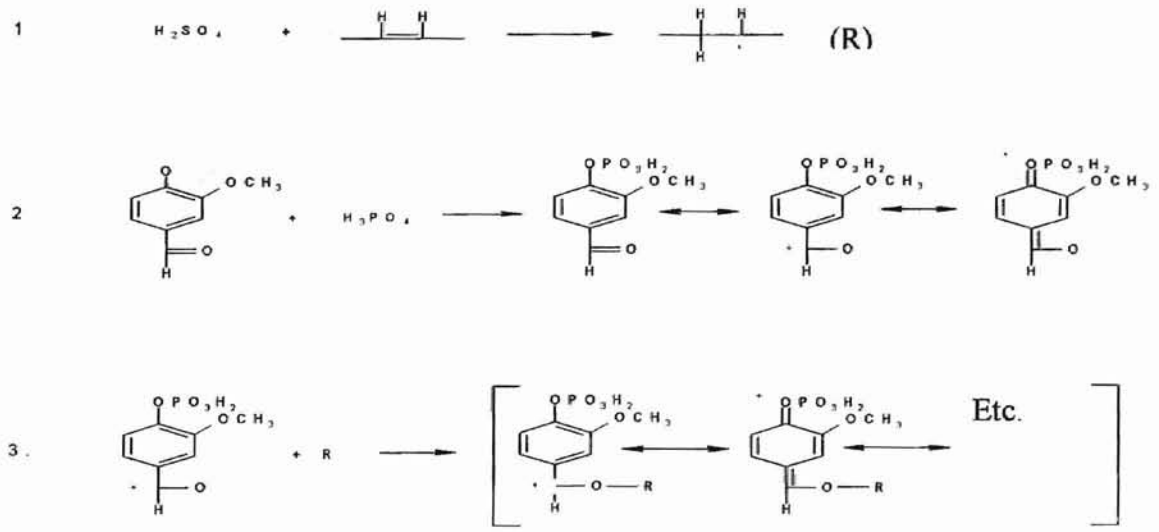
The sulphophosphovanillin (vanillin) method takes advantage of the reaction of double carbon bonds in fatty acids [(-C=C-)] with sulphuric acid, phosphoric acid, and vanillin producing a pink complex which can be measured spectrophotometrically (Van Handel 1985, Barnes and Blackstock 1973). The many advantages of the sulphophosphovanillin method over the gravimetric method including sample size, amount of solvent required, and analysis time, are summarized in Table 2.2.

Table 2.2: Comparison of the phosphovanillin method versus the gravimetric for total lipid determination.

	Vanillin Method	Gravimetric
Sample size	0.003-0.5 g	> 5 g
Solvent	chloroform/methanol	chloroform/methanol
Volume of Solvent	3 mL	15 mL
Time	2 h / 28 samples	8 h / 14 samples

The sulphophosphovanillin method has been used to measure lipids in many tissues such as blood, muscle, and whole organisms (Meyer and Walther, 1988; Van Handel, 1985; Barnes and Blackstock, 1973; Knight et al., 1972) Figure 2.1 depicts the sulphophosphovanillin reaction with unsaturated compounds. In the depiction, the

Fig. 2.1. The sulphophosphovanillin reaction with unsaturated compounds.



following reactions occur: (1) Unsaturated lipids react with sulfuric acid to produce a carbonium ion, (2) vanillin reacts with phosphoric acid to produce an aromatic ester, and (3) then the carbonium ion reacts with the activated carbonyl group of phosphovanillin to produce a charged color complex (Knight et al. 1972). Each method of analysis varies slightly depending on the tissue analyzed. The following procedure outlines the method to be used for analysis of total lipids in *E. fetida.*

Methods

Simple lipids are often found in large aggregates in storage tissues and are easy to extract. Complex lipids are often associated with proteins, polysaccharides, and found in membranes (Hamilton et al. 1992). The following procedure, adopted from protocol 8 (Bligh and Dyer method) found in Lipid Analysis A Practical Approach (Hamilton and Hamilton, 1992) was used for the extraction of simple and complex lipid tissues. A modification to protocol 8 was made. After step 5, the tissue was placed on a rotating bar at 40 rpm for 1 h. Then the samples were centrifuged at 5000 rpm for ten minutes ensuring the rapid separation of the polar and non-polar phases.

After the extraction process, the volume of the chloroform layer was recorded. A portion of the chloroform layer was removed and placed in a clean, dry test tube and incubated at 100 °C, evaporating the chloroform and methanol solvent to dryness.

Concentrated sulfuric acid (0.3 mL) was added to the dry tubes and capped with a marble to prevent evaporation. The sample was placed in a dry-bath test tube incubator at 100 °C for exactly ten minutes. After incubation the sample was placed in a cold-water bath to prevent the reaction from over developing and then 1.0 ml of the

sulphophosphovanllin reagent (600 mg vanillin, 100 mL reagent grade water, 400 ml 85% O-phosphoric acid) was added. The tube was vortexed to ensure mixing and reaction allowed to develop in the dark for five minutes. Aliquots of 250 uL were taken from the tubes, placed in a microplate, and absorbance measured at 520 nm using a microplate reader. Absorbance measurements must be made between 5 and 30 minutes after the addition and mixing of the sulphophosphovanillin reagents since the reaction has been shown to degrade with time (Barnes and Blackstock 1973).

The sulphophosphovanillin method was calibrated to a standard curve of earthworm extract. Five hundred earthworms were pooled, homogenized, and extracted using chloroform and methanol. One hundred mg of butylated hydroxytoluene (BHT) was added to the extract to prevent oxidation of lipids during storage (Gunstone, 1992). Various aliquots of the sample were taken and the amount of total lipid was determined gravimetrically. Mean spike recovery using tissue extract was 80.3 % ± 17.9 and 103.7 % ± 16.6 (± SD, n=4). Mean blank spike recovery using tissue extract was 84.5 % ± 6.4 and 81.8 % ± 22.8 (± SD, n=2).

The vanillin method was also calibrated for the amount of lipid in corn oil that could be reacted with 0.3 mL sulfuric acid by dissolving increasing amounts of corn oil in chloroform and methanol and then reacting with the sulfophosphovanillin. The concentration of corn oil was plotted versus the absorbance (520 nm). As the concentration of corn oil exceeded the volume of sulfuric acid, the plotted curve reached an asymptotic value. The results showed that 0.3 mg of corn oil was the maximum amount of corn oil that could used before the amount of lipid in the corn oil exceeded the digestion capacity of 0.3 mL of sulfuric acid.

After determining the calibration of corn oil mass to volume of sulfuric acid, the method was optimized for determining total lipids in earthworm extracts. Nine earthworms (>250 mg, fresh weight) were pooled, and as with the corn oil, increasing amounts of earthworm extract were added to the sulfuric acid. Absorbance was measured at 520 nm and plotted against the volume of earthworm extract to determine the range of the curve.

It was found that no more than 0.07 ml of earthworm (>250 mg, fresh weight) extract could be digested before the capacity of the sulfuric acid was exceeded. However, an aliquot that fell approximately in the linear portion of the standard curve was used to allow for differences in total lipid content during the experiments.

A performance control experiment was conducted to determine if the vanillin method was capable of detecting changes in the total lipid content of earthworms cultured in various media. Juvenile *E. fetida* (n=195) were obtained from the culturing facilities at Oklahoma State University. Forty-five worms were assigned randomly to each of four treatments (Table 2.3) and 15 worms were allowed to depurate gastrointestinal contents for 48 hours to obtain baseline data. The control treatment consisted of horse manure, soil, and reagent grade water and was used in the normal practice of culturing earthworms. A coconut bedding treatment was ground coconut shell with reagent grade water. The ground coconut shell was void of nutrients, acting as a negative control. Two additional treatments containing nutrient supplements were prepared. Both of the supplement treatments were similar to the control treatment except for the addition of the supplement. Two rates of nutrient supplementation were selected to determine if increased nutrient availability would increase total lipid content of earthworms. The

hypothesis of this research is as follows: as the supplement treatment increases or decreases (coconut bedding) the sulphophosphovanillin technique would detect a change in the normalized total extractable lipids.

Table 2.3: Summary of the four treatments used in the total lipid technique validation study.

	Manure	Soil	Water	Oats	Milk powder
Control	150g	50g	300ml		
Coconut bedding			30ml		
Supplement 2g	150g	50g	300ml	1g	1g
Supplement 4g	150g	50g	300ml	2g	2g

The experiment was three weeks in duration, with sub-sampling of 15 worms at one-week intervals. The sub-sampling schedule was designed to allow the worms to depurate for 48 hr, avoiding any possible interference from lipid containing compounds in the digestive system. Tissues from this experiment were analyzed fresh due to the uncertainty of lipid-oxidation during storage. A change in the percent total lipids would be necessary to validate the sulphophosphovanillin technique.

As a validation technique, three starved fathead minnows and three fed fathead minnows were obtained from a culture. These fish were sacrificed, by freezing, and homogenized in 30 mL of reagent grade water. Ten mL of the homogenate was transferred to 50 ml Teflon test tubes. Thirty mL of chloroform and methanol (2:1 v/v) was added and the test tubes placed on the rotor bar at 40 rpm for one hour. After the extraction the samples were spun in a cold centrifuge (5° C) for 30 minutes. An aliquot

(0.3-1.0 mL) was removed from the non-polar phase and analyzed using the sulphophosphovanillin method.

To also validate and test the sulphophosphovanillin lipid analysis method, varying aged earthworm and "native" adult worms were also analyzed using the above method. A synchronous culture of *Eisenia fetida* was optimally grown in moist horse manure. At various time points (Figure 2.5) earthworms were removed and analyzed for lipid content.

An unknown species of earthworm was collected from about 15 cm below the surface of the leaf litter, in the flood plain of a creek running through a scrub forest area in Stillwater, Oklahoma. The earthworms appeared a pale grayish-pink in color, has blunt terminal ends, and the gastrointestinal tract was readily visible. The earthworms have not been taxonomically identified. The purpose of collecting the earthworms was to analyze a different species of earthworm hypothesizing that the less rich diet of the "native" species would produce a lower mean percent lipid content then that of the cultured earthworm raised in highly optimal conditions.

Results and Discussion

Mean wet-weight and mean total lipid levels increased (Figure 2.2, 2.3) over the three-week experiment.($F_{7,30}$= 35.850, $p<0.005$) However, when the mass of total extractable lipid was normalized to wet body mass (Figure 2.4), the percent total lipid remained constant ($F_{7,30}$= 2.356, $0.025<p<0.05$). The working hypothesis that the percent lipid would change as the amount of supplement increased, or in the case of the coconut bedding, that the percent lipid would decrease to lack of nutrition, was not supported by

our results. Two possible reasons for the lack of change exist, 1) the sulphophosphovanillin technique is not sensitive enough to detect change or, 2) there was no physiological response by the earthworms.

Results indicated that the starved fathead minnows had 0.36 % total extractable lipid on a wet weight basis. The fed fathead minnows had 1.35 % total extractable lipids on a wet weight basis. This difference was not statistically significant ($F_{1,4}$= 4.129, $p>0.10$). This may have been due to the overlap of one the data points in the two treatments and the small sample size. The change between the two values was significant

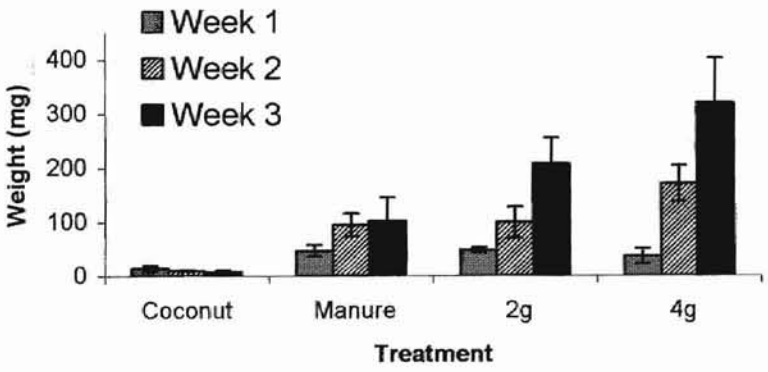


Figure 2.2: Mean wet-weights (mg) of SP-vanillin performance control experiment.

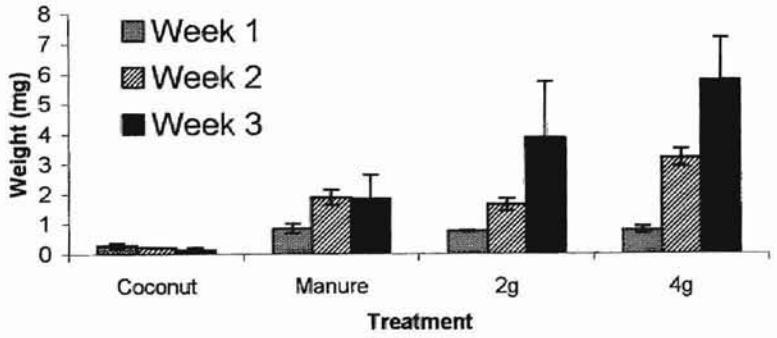


Figure 2.3: Mean total lipid of SP-Vanillin performance control experiment.

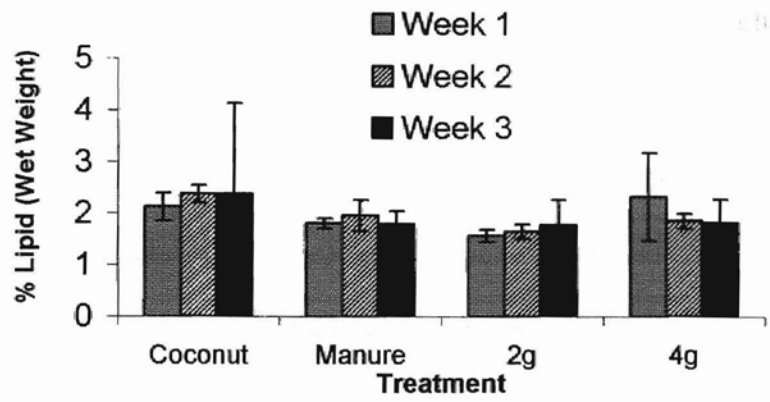


Figure 2.4: Normalized lipid values of SP-Vanillin performance control experiment.

enough to confirm that the vanillin technique was capable of detecting a change in the total extractable lipids.

Results from an independent analysis show that *E.fetida* of different ages have a different percent lipid content (Figure 2.5). As the young hatch from the cocoon it is believed that they have a high store of endogenous lipids from the parents. As the hatchlings begin to grow they use up the lipid store until they begin exogenous feeding. When they reach maturity the average lipid content of *E. fetida* grown in Oklahoma State University's cultures is about 2.3 % (fresh weight).

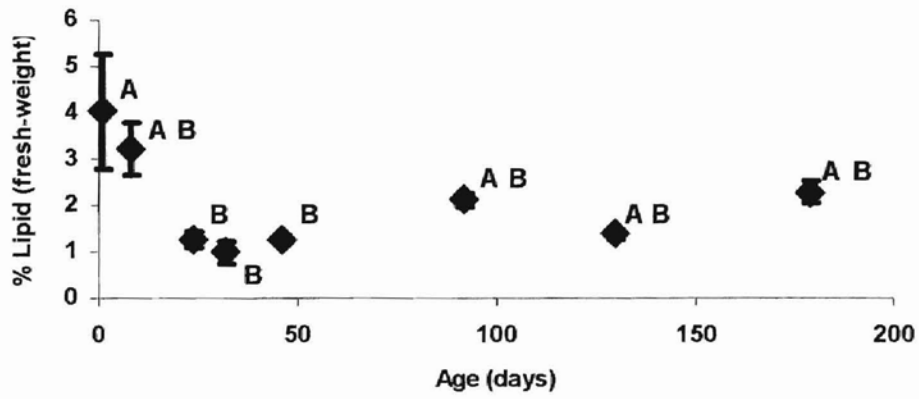


Figure 2.5: Total lipid levels (% fresh weight) in *E. fetida* of various ages as determined using the sulphophosphovanillin method.

Results showed that the mean value (n=5, ± SD) of the wet weights, mass of total extractable lipid, and normalized lipid value (wet weight) was 607.2 ± 283.5 mg, 6.7 ± 1.9 mg, and 1.1 ± 0.2 %, respectively. The normalized value (%lipid) of the "native" species (1.1 %) is less then the optimally grown species *E. fetida* (2.3 %).

Based on the results of this study the sulphophosphovanillin appears to be an adequate method for measuring total extractable lipids in earthworms. Results indicate that the vanillin technique is capable of detecting change in the percent total extractable lipids based on the comparison among treatments and compared to the controls. The use of a smaller tissue sample size alleviates the need for pooling samples and in turn allows for greater statistical power. Less solvent and the ability to analyze increased amount of samples in a shorter period of time permits the sulphophosphovanillin to become an acceptable method for analyzing total extractable lipids in earthworms.

Conclusion

Earthworms are important in the soils of many ecosystems in terms of biomass, as food for many animals and in soil formation. For these reasons, earthworms have gained acceptance for use is soil tests to assess the effects of chemicals on soil organisms. Several tests have been devised to assess the effects of chemicals on earthworms (*E. fetida*). In a 14-day artificial soil test, the toxicity of a chemical is estimated by its effect on survival. An LC_{50} is computed from the dose-response curve by counting the mortalities in a soil sample containing known concentrations of the test chemical. This method may be useful in regulating chemicals before its use (Gibbs et al., 1996). Evidence is mounting that organisms may suffer from the effects of environmental contaminants not only through death but also through direct and indirect effects on their reproduction, growth, and behavior. Organisms that must allocate energy from other sources (i.e. reproduction, growth, competition) to survive a stress, such as the metabolism of a contaminant, will experience reduced individual fitness which may lead to detrimental population effects. Thus, a better understanding of sublethal effects of contaminants on organisms, communities, and ecosystems is needed to help improve the accuracy of ecological risk assessment.

The need for more sensitive endpoints, (i.e. reproduction, growth, and behavior) and relevant than mortality, and a better understanding of the sublethal effects to *E.* fetida drove my efforts in conducting this research. It is clear that the results of this study show negative effects of increasing PHE concentrations on the number cocoons produced and

the number of viable hatchlings produced from each cocoon. What is not clear is the effect that this may have at the population, community, and ecosystem level.

In previous studies, changes in adult dry mass (post contaminant exposure) have revealed the effects of growth that could otherwise be masked by fresh weight (Gibbs et al., 1996). It has also been suggested that nontoxicological soil factors (moisture, lack of food, pH) may also influence growth rates of earthworms. The use of food supplementation and periodic checks of soil moisture content throughout an experiment may reduce variability in growth measurements.

The design of toxicity experiments, the interpretation of the results obtained from them, and the consequent derivation of environmental standards usually start from the premise that the chemical exposure level is constant (Widianarko and van Straalen, 1996). However, this only appears to be true in the case of highly persistent chemicals. In the current study, PHE is a less persistent chemical and constant exposure will only occur if there is a constant source. In the field, constant exposure will only occur in well buffered environment and in many cases erratic fluctuations occur which cause a peak of contamination followed by a rate of decline. In the above experiments a simple two-episodic event occurred followed by a period of decline. Negative biological effects were observed, soil and earthworm kinetics were monitored. However, few sublethal-toxicological studies have been conducted taking into account nonconstant exposure conditions and how to estimate criteria (i.e. body residue, availability) used from these studies.

Conclusion

In conclusion, this research demonstrates that several basic factors in environmental toxicology such as aging of soil prior to the addition of organisms to achieve constant exposure, moisture content, lipid content of various aged organisms, and more sensitive endpoints (reproduction and growth) need to be more fully understood before relevant and applicable environmental standards and guidelines can be derived from ecological risk assessment.

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