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Evaluation of Mechanisms of Alteration and Humification of PAHs for Water Quality Management

Ronald C. Sims
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Evaluation of Mechanisms of Alteration and Humification of PAHs for Water Quality Management

Utah Center for Water Resources Research
Utah Water Research Laboratory
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FOREWORD

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INTRODUCTION

Creosote-pentachlorophenol (PCP) is a mixture commonly used as a wood preservative in the U.S. (1). A 1988 survey (2) indicated that 1,397 wood preserving waste contaminated sites exist in the United States consisting of 555 active wood treatment plants and 842 inactive plants. Stinson (3) identified 58 wood preserving sites on the National Priorities List, of which 51 have PCP and/or creosote or polycyclic aromatic hydrocarbon (PAH) contamination.

Principal classes of organic constituents present in creosote waste are PAHs (~85% by weight) and phenolics. PAHs with less than three fused benzene rings comprise 69% (i.e., naphthalene, anthracene and phenanthrene); PAHs with more than three rings, such as pyrene, benzo(a)pyrene, benz(a)anthracene, dibenz(a,h)anthracene, and indeno(1,2,3-c,d)pyrene comprise 16% by weight of creosote. Phenolics comprise 2% to 17% of creosote. Nitrogen- and sulfur-containing heterocyclic compounds may comprise up to 13% of creosote by weight. Creosote and creosote components including phenol and several PAHs have been reported to be mutagenic, teratogenic, fetotoxic and/or toxic (4, 5) and have been designated as hazardous wastes under the Resource Conservation and Recovery Act of 1976 (6) and as hazardous substances under the Comprehensive Environmental Response, Compensation, and Liability Act of 1980.

PCP is often added to creosote to enhance the wood preservation potential due to its bactericidal and fungicidal properties. PCP is also toxic to lower and higher plants (algicide, herbicide), to invertebrate and vertebrate animals (insecticide, molluscicide), and to man. Toxicity of PCP and potential for uptake by organisms are pH-dependent, since PCP is a weak acid with a Ka of about 10^-5. Both bioaccumulation and toxicity increase as pH decreases due to the greater penetration of cell membranes by non-ionized PCP molecules than by pentachlorophenate ions (1). Therefore PCP may inhibit microbial degradation of other compounds in creosote-PCP waste, including oil and grease.

Contaminated vadose zone soil systems generally consist of four phases: 1) aqueous; 2) gas; 3) oil (commonly referred to as non-aqueous phase liquid, or NAPL); and 4) solid, which has two components, an inorganic mineral compartment and an organic matter compartment (organic carbon-humic substances). Interphase transfer potential for waste constituents among oil (waste or NAPL), water, air, and solid (organic and inorganic) phases of a soil system is affected by the relative affinity of the waste constituents for each phase. Measurement of waste constituents in all four phases is generally not done in treatability studies, especially in complex environmental samples (7).

High molecular weight (greater than 3 rings) PAHs are hydrophobic and essentially not mobile due to their low volatilities and water solubilities. Bulman et al. (8) and Keck et al. (9) observed that sorption of B(a)P to soil was the dominant mechanism of loss. Studies have shown that immobilization of some xenobiotics can be accomplished by incorporation into soil humus, or sorption into the clay lattice of soil (10). Humification and sorption have not been extensively evaluated for PAHs in creosote contaminated soil.

PCP is, in general, more mobile in high pH soils than in acidic soils. At low pH, PCP exists as a free acid (non-ionized) and readily adsorbs to soil particles. At high pH, PCP exists in the ionized form (pKa = 4.7) as the negatively charged pentachlorophenate anion, and is more mobile. In a study by Choi and Aomine (11), "apparent adsorption" of PCP was greatest in strongly acid soil and in soils with high organic matter content. "Apparent adsorption" was shown to include both the mechanisms of adsorption on soil colloids and precipitation in the soil micelle and in the external liquid phase, depending on the soil pH. Pionteck (12) also observed that although soil organic matter is important in determining the extent of adsorption of PCP, an even more important soil property is pH. Adsorption of PCP was shown to be reversible. Therefore, PCP may not be
permanently immobilized in the soil phase, but may be slowly released into and move through the soil (1).

A wide range of soil organisms, including bacteria, fungi, cyanobacteria and eukaryotic algae, have been shown to have the enzymatic capacity to oxidize PAHs. Metabolites from the degradation of large PAHs identified in these studies are responsible for toxic, mutagenic, and/or carcinogenic responses in animal species and many indicate epoxide intermediates (13-20). Presence of PCP may inhibit microbial degradation of other organics, including PAHs, oil and grease, etc.

Despite a high degree of chlorination, PCP has been shown to be degraded in soil. Microbial decomposition appears to be the primary detoxification mechanism. Success was highest in those studies that used acclimated or inoculated (with acclimated species) systems. The ability to degrade PCP may not be uniform among microorganisms, and adaptation of microbial populations to PCP and control of pH may play important roles in its degradation (1).

Laboratory studies (7, 21) of the biodegradation potential of creosote wood preservative waste have shown that hazardous parent components were degraded, transformed, or immobilized in certain soil systems. In a study by Aprill et al. (21) on the biodegradation potential of creosote, the apparent degradation of four non-carcinogenic PAHs and four carcinogenic PAHs ranged from 54% to 90% and 24% to 53% of mass added, respectively. Aprill et al. (21) defined apparent degradation as the measurement of changes in concentrations of specific constituents in solvent extracts of soil samples with time of incubation. The reduction in concentration of the higher molecular weight PAHs was correlated with oil and grease content of the waste.

Degradation of a chemical in soil may not result in the complete mineralization of a hazardous waste, but may render waste constituents less hazardous or nonhazardous through transformations (1,7,21). However in some cases detoxification does not occur (22).

Studies (23-30) conducted with 14C labeled compounds often report collection of the radiolabelled carbon in carbon dioxide trapping solutions to indicate degradation or mineralization. However, collection of the radiolabelled carbon in a carbon dioxide trapping solution may be misleading in two ways, i.e., 1) liberation of CO2 may not be concurrent with complete degradation of the total mass present because of accumulation of metabolites in the soil (31), or 2) measurement of radiolabelled carbon may not indicate mineralization if volatilized parent compound or labeled metabolites are collected in the trapping solution in addition to 14CO2 (32,33). Torstensson and Stenstrom (31) recommend that the rate of decomposition of a substance should be defined by direct measurement of its disappearance. However, direct measurement of the disappearance of hydrophobic organics from soil systems cannot be defined as degradation because of other loss mechanisms including volatilization or sorption to soil solids. Sorbed organics that cannot be removed from soil by organic solvents cannot be easily identified or analyzed.

There is a current lack of knowledge concerning the behavior of PAHs in complex environmental vadose zone soil samples. This study was undertaken, using a chemical mass balance approach, to determine the distribution of radiolabelled carbon, parent compounds, and transformation products of the radiolabelled PAH compounds, benzo(a)pyrene (B(a)P) and pyrene, among aqueous, gas, and solid phases of a non-contaminated and contaminated (creosote-PCP) vadose zone soil over time of incubation. The apparent degradation of unlabeled PAHs and changes in the toxicity of the water-soluble (aqueous) fraction were also measured.
OBJECTIVES

The goal of this research project was to evaluate mechanisms of alteration and humification of PAHs in non-contaminated and contaminated (creosote and PCP) vadose zone soil. Specific objectives are listed below:

1. Determine the distribution of spiked radiolabelled carbon, added as $^{14}$C-benzo(a)pyrene [B(a)P] and $^{14}$C-pyrene, among soil phases including aqueous, gas, and solid (humic plus fulvic acids and humin plus inorganic mineral components) phases as a function of incubation time in soil.

2. Determine the extent of humification of spiked PAHs

3. Determine the extent of mineralization of B(a)P (spiked) and pyrene (spiked), and provide for separation of $^{14}$CO$_2$ from volatile intermediates and parent compound in soil.

4. Determine the apparent degradation of unlabeled PAHs and PCP occurring in the creosote-PCP contaminated vadose zone soil.

5. Determine the initial toxicity and changes in toxicity of the aqueous phase of soil using the Microtox™ assay for contaminated (non-poisoned and poisoned) and non-contaminated (non-poisoned and poisoned) soil samples spiked with B(a)P and Pyrene.

APPROACH

The approach to meeting the objectives of the research was to conduct a chemical mass balance of radiolabelled carbon through time of soil incubation. Both non-contaminated and contaminated samples of the same vadose zone soil were spiked with either $^{14}$C-B(a)P or $^{14}$C-pyrene and incubated in enclosed vessels. Soil poisoned with propylene oxide served as controls for each of four combinations evaluated: (1) contaminated soil with B(a)P spike, (2) contaminated soil with pyrene spike, (3) non-contaminated soil with B(a)P spike, or (4) non-contaminated soil with pyrene spike. While the gas phase was monitored for mineralization ($^{14}$CO$_2$) and volatile chemicals, aqueous and solid phases were evaluated through sequential extraction using: (1) water and (2) solvent (dichloromethane), to determine parent compound, intermediates, and radiolabeled carbon, (3) base extract (humic plus fulvic acids), to determine $^{14}$C associated with extractable soil organic matter and (4) combustion of soil to determine non-extractable $^{14}$C. Using a chemical mass balance approach resulted in determination of the distribution as well as changes in distribution of spiked $^{14}$C among gas, aqueous, and solid phases of a soil system through time of incubation, as well as mineralization of applied $^{14}$C and detoxification of the aqueous phase.

Humification was measured as the incorporation of $^{14}$C into the solid phase of the soil and not extractable with water or dichloromethane, but associated with the solid phase characterized as humic plus fulvic acids (base extractable) and humin (non-extractable organic matter plus mineral components) fractions of soil.

The Microtox™ assay was used to measure changes in toxicity of the water phase (water extracts of soil) through incubation time. The Microtox™ assay is an aqueous general assay that measures the reduction in light output produced by a suspension of marine luminescent bacteria in
response to an environmental sample (21,34). The assay measures the physiological effect of a toxicant and not just mortality. Symons and Sims (35) utilized the assay to assess the detoxification of a complex petroleum waste in a soil environment. The assay was included as a recommended bioassay in the USEPA Permit Guidance Manual on Hazardous Waste Land Treatment Demonstrations (36).

CONCLUSIONS

Conclusions based on observations and results obtained in this research are identified below:

1. A chemical mass balance approach was useful for determining the distribution (fate and behavior) of spiked PAHs among aqueous, gas, and solid phases in both contaminated and non-contaminated soils as a function of incubation time. Mass balances of greater than 95% of spiked radiolabelled carbon were achieved throughout the 285 day incubation for both B(a)P spiked soil and for pyrene spiked soil.

2. Humification of spiked PAHs represented the most significant fate mechanism in non-contaminated soil, including non-poisoned and poisoned soils, and consistently increased with increased time of incubation. Results of mass balance analyses through time of incubation indicated a large fraction (70% to 80%) of 14C became associated with the soil solid phase while the solvent extract fraction decreased throughout the 285 day incubation.

3. Humification of spiked PAHs also represented the most significant fate mechanism in contaminated soils in both non-poisoned and poisoned soils. However, humification as measured, was initially rapid and did not increase with increased time of incubation, with the exception of pyrene in non-poisoned soil. Results for spiked PAHs in contaminated soil may be due to interference from the soil-associated creosote-PCP mixture (non aqueous phase liquid) with regard to extraction of B(a)P with dichloromethane.

4. Mineralization and production of polar intermediates of spiked 14C were very low for both PAHs (<5%). However, mineralization and production of polar intermediates were significantly greater in non-poisoned soil than in poisoned soil. Low rates and extent of these processes were attributed to the recalcitrance of the spiked PAHs. Mineralization can be successfully determined experimentally by separating 14CO2 from volatile 14C-intermediates through the use of different trapping solutions, i.e., monoethanolamine for CO2 and ethylene glycol monomethyl ether for volatile organic carbon.

5. Apparent degradation of PCP and PAHs including naphthalene, acenaphthylene, acenaphthene, and fluorene was obtained in non-poisoned contaminated soil, but not in poisoned contaminated soil.

6. Microtox™ toxicity was very different between non-contaminated and contaminated soils, with contaminated soil toxicity much higher than non-contaminated soil toxicity.

7. Changes in toxicity of the water phase (water extracts) were related to the biological status of soil samples (non-poisoned versus poisoned) and to the contamination status (contaminated versus non-contaminated) of soil samples. Changes in toxicity of the water phase (water extracts) through incubation time occurred only in unpoisoned (biologically active) soil samples. Changes in toxicity were more pronounced in uncontaminated soil, with both decreases and increases in toxicity observed. Changes in toxicity of water extracts were less
pronounced in contaminated soil; however, consistent detoxification trends were observed. Poisoned control samples exhibited no statistical changes in toxicity over incubation time.

RECOMMENDATIONS

1. A chemical mass balance approach that includes measuring degradation, transformation, and mineralization in non-poisoned and poisoned (control) soil samples is required to evaluate the behavior and fate of hazardous constituents in complex environmental samples, including the role as well as the rate and extent of biological reactions versus abiotic reactions.

2. A chemical mass balance should also include an evaluation of the partitioning and distribution of parent compound and transformation products among gas, aqueous, and solid phases as a function of time in contaminated soil.

3. Evaluations of fate and behavior of organic chemicals in soil systems requires that methods be used to separate CO$_2$ from volatilized intermediates and parent compounds.

4. Detoxification, as well as intoxification, of the aqueous phase should be evaluated because toxicity changes are not predictable based upon chemical mass balance.

METHODS AND PROCEDURES

Vadose Zone Soil. PCP-creosote contaminated vadose zone soil was supplied by Dr. Gary McGinnis, Mississippi State University, Forest Products Utilization Laboratory, Mississippi State, Mississippi. The soil is a McLaurin sandy loam classified as a coarse-loamy, siliceous, thermic Typic Paleudult, having a particle-size distribution of 56%, 28% and 16% by weight for sand, silt, and clay, respectively. Uncontaminated sandy loam vadose zone soil from the same site was characterized as having a pH of 6.3, 3.7 cmol (+) kg$^{-1}$ CEC and 0.41% organic carbon content. The contaminated soil used in this study had a pH of 5.0, and 2.88% organic carbon content, with oil and grease and PCP contents of 2 to 3% and 421 mg kg$^{-1}$, respectively. Bacterial enumeration indicated 4.5 X 10$^5$ bacteria /g soil dry-weight, determined by agar plate counting, for the contaminated soil and 4.5 x 10$^6$ for non-contaminated soil. Soil samples were characterized by the Soil Testing Laboratory, Utah State University, Logan, Utah. Waste characterization and soil-waste concentrations were determined by the Utah Water Research Laboratory, Logan, Utah (Table I). Waste concentration in the soil was 8% waste wet weight (creosote-PCP) to soil (dry weight).

Chemicals. Radiolabelled [7-14C] benzo(a)pyrene (B(a)P) was obtained from Chemsyn Laboratories, Lenexa, Kansas through the Cancer Research Program of the National Cancer Institute (NCI), Division of Cancer Cause and Prevention, Bethesda, MD. Radiolabelled [4,5,9,10-14C] pyrene was obtained from Chemsyn Laboratories, Lenexa, Kansas through the generosity of Drs. J.T. Dibble and I. Bossert, Texaco, Deacon, NY. Unlabeled benzo(a)pyrene and pyrene were purchased from Sigma, St. Louis, MO. Microtox™ reagents were obtained from Microbics Corp., Carlsbad, CA. Sep Pak™ C-18 preparatory columns were obtained from Waters, Milford, MA. All solvents and chemicals used were of the highest purity available.

Analyses. Radiolabelled carbon was measured in a Beckman LS1701 liquid scintillation counter, Beckman Instruments, Inc., Fullerton, CA. Microtox™ assays were performed on a Microtox™ Analyzer Model 2055, Beckman Instruments, Inc., Fullerton, CA. Soil and extracted soil samples were combusted on a Harvey Biological Oxidizer Model OX-400, R. J. Harvey Instrument Corporation, Hillsdale, NJ. Gas chromatography analysis of headspace was
performed on a Perkin Elmer Sigma 4 Isothermal Gas Chromatograph, with a Thermal Conductivity Detector, and an Alltech (Deerfield, IL) CTR1 Specialty column. HPLC analysis was performed on a Perkin Elmer Model Series 4 LC pump system with a LC90 UV Spectrometrometric Detector and a Supelco LC-PAH 25 cm x 4.6 mm 5 µm column.

Table I. Concentration (mg/kg) of PCP¹ and PAH Compounds² in Creosote Waste (wet weight) on McLaurin Sandy Loam Soil (dry weight).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Soil Concentration (mg/kg)</th>
<th>Waste Concentration (mg/kg)³</th>
<th>(CV)⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pentachlorophenol</td>
<td>421</td>
<td>3826 ± 1500</td>
<td>(4%)</td>
</tr>
<tr>
<td>Naphthalene</td>
<td>885</td>
<td>80447 ± 20100</td>
<td>(25%)</td>
</tr>
<tr>
<td>Acenaphthylene</td>
<td>275</td>
<td>24998 ± 6500</td>
<td>(26%)</td>
</tr>
<tr>
<td>Acenaphthene</td>
<td>411</td>
<td>37360 ± 11000</td>
<td>(29%)</td>
</tr>
<tr>
<td>Fluorene</td>
<td>244</td>
<td>22180 ± 5500</td>
<td>(25%)</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>696</td>
<td>63267 ± 13000</td>
<td>(21%)</td>
</tr>
<tr>
<td>Anthracene</td>
<td>605</td>
<td>54995 ± 22000</td>
<td>(40%)</td>
</tr>
<tr>
<td>Fluoranthene</td>
<td>806</td>
<td>73266 ± 16000</td>
<td>(22%)</td>
</tr>
<tr>
<td>Pyrene</td>
<td>868</td>
<td>78902 ± 15000</td>
<td>(19%)</td>
</tr>
<tr>
<td>Benz(a)anthracene</td>
<td>539</td>
<td>48996 ± 12000</td>
<td>(24%)</td>
</tr>
<tr>
<td>Chrysene</td>
<td>612</td>
<td>55631 ± 12239</td>
<td>(22%)</td>
</tr>
<tr>
<td>Benzo(b)fluoranthene</td>
<td>121</td>
<td>10999 ± 2860</td>
<td>(26%)</td>
</tr>
<tr>
<td>Benzo(k)fluoranthene</td>
<td>148</td>
<td>13453 ± 3363</td>
<td>(25%)</td>
</tr>
<tr>
<td>Benzo(a)pyrene</td>
<td>126</td>
<td>11454 ± 1718</td>
<td>(15%)</td>
</tr>
<tr>
<td>Benzo(g,h,i)perylene</td>
<td>100</td>
<td>9090 ± 2363</td>
<td>(26%)</td>
</tr>
<tr>
<td>Dibenz(a,h)anthracene</td>
<td>100</td>
<td>9090 ± 2000</td>
<td>(22%)</td>
</tr>
<tr>
<td>Indeno(1,2,3-c,d)pyrene</td>
<td>29</td>
<td>2636 ± 290</td>
<td>(11%)</td>
</tr>
</tbody>
</table>

¹Determined by gas chromatography, U.S. EPA Method 8040
²Determined by high performance liquid chromatography, U.S. EPA Method 8310
³Concentration is the average concentration of three replicate analyses ± one standard deviation
⁴CV (coefficient of variation = %).

Experimental vessels. Wide mouth Erlenmeyer flasks (1 L), sealed with rubber stoppers, were used as experimental vessels (Figure 1). Three replicates of each treatment were analyzed at each sampling event. Treatments evaluated included B(a)P in non-poisoned contaminated soil, B(a)P in poisoned contaminated soil, pyrene in non-poisoned contaminated soil, and pyrene in poisoned contaminated soil. Additional treatment included B(a)P and pyrene spiked into poisoned and non-poisoned uncontaminated soil. Two hypodermic needles (18 gauge, 1 1/2") with two-way Luer-Lok stop-cocks attached were pushed through rubber stoppers for inlet and outlet valves. The inlet needle projected into a 3 inch piece of glass tubing that was sealed to the bottom of the rubber stopper with silicone sealant and allowed to cure for 72 hours prior to use. Creosote contaminated soil (700 grams air dried) was placed in each flask. The flasks were wrapped in black plastic and secured with rubber bands around the neck of the flask. Poisoned control flasks were prepared using propylene oxide. The propylene oxide was allowed to evaporate and the soil was mixed thoroughly three times and exposed to the air for three hours.
Figure 1. Experimental flask (Microcosm) set up with impingers attached. Impingers contained ethylene glycol monomethyl ether (EGME) or scintillation cocktail:methanol:monoethanolamine solution (MEA).

Seven hundred milligrams of unlabeled B(a)P and seven hundred milligrams of unlabeled pyrene were dissolved separately in 5 mL of dichloromethane, and brought to 100 mL volume each with ethylene glycol monomethyl ether (EGME). EGME was used to simulate the waste oil and grease matrix without introducing additional toxicity. EGME adsorbs to surfaces of soil clay and can penetrate into interlayer spaces. These two stock solutions were then spiked with 1400 μCi of [7-14C] benzo(a)pyrene or [4,5,9,10-14C] pyrene. Ten milliliters of the spiked stock solution were added in small increments, mixing the soil after each addition, to 700 grams of creosote contaminated vadose zone soil in each experimental flask, resulting in the addition of 100 mg kg\(^{-1}\) B(a)P or pyrene, and a total of 14 μCi of radioactivity per flask. It was assumed in this study that the fate of the two 14C labeled compounds, the freshly added non-labeled B(a)P or pyrene, and the previously added B(a)P and pyrene from the original contamination was the same in all cases.

**Flask maintenance and incubation.** It was determined in our laboratory that the contaminated soil after air drying would absorb approximately 50% water by weight and therefore was not hydrophobic. After addition of the spiked B(a)P or pyrene solutions, the first subsamples were taken and then double deionized water (DDW) was added to each flask to bring the moisture content to 40% by weight. Moisture content was maintained throughout the study by weighing each flask weekly and before and after sub-sampling, with addition of water when necessary to maintain post sampling weights.

To avoid photooxidation/photodegradation, all procedures involving PAHs were performed under GE-40 gold fluorescent lights, and each soil microcosm was wrapped with black plastic (33).

All flasks were incubated in a constant temperature room at 20 to 22° C and removed only for head space analyses, aeration, and sub-sampling. Addition of the spiked B(a)P or pyrene stock solutions, sterile DDW, sampling, and any other manipulations involving opening the poisoned
flasks were carried out inside a sterile chamber. At time increments of 14, 30, 45, 60, 100, 130, 160, 200, and 285 days, flasks were flushed with air with impingers (traps) attached, and trapping solutions were analyzed for radiolabelled carbon using scintillation counting.

**Sampling.** A summary of the sampling and analyses conducted is shown in Figure 2. Prior to aerating the flasks, head space air was removed through one of the stop cocks with a 5 mL syringe and directly injected into the gas chromatograph for analysis of head space gases, carbon dioxide, oxygen, and nitrogen. Scott Specialty Gases (Plumsteadville, PA) oxygen, nitrogen and carbon dioxide standards were used.

![Diagram](image)

**Figure 2.** Sampling and Analyses for Soil Fractions through Incubation Time.
Flasks were aerated according to the method of Abbott et al. (32), except that three impingers were used. The first two impingers were filled with 20 mL of ethylene glycol monomethyl ether (EGME) for collection of volatile parent compound and volatile intermediates, and the third impinger with 20 mL of a (50:40:10) scintillation cocktail:methanol:monoethanolamine solution (MEA) for collection of carbon dioxide (Figure 1). Air supply, flasks and impingers were connected in series with Tygon tubing. Air flow was 50 mL/minute and was maintained for 30 minutes per flask. Each ethylene glycol monomethyl ether (EGME) trapping solution was transferred to two scintillation vials (approximately 10 mL each). Ten milliliters of Ready Gel™ scintillation cocktail was added to each vial and counted. The monoethanolamine (MEA) solution was transferred to a scintillation vial and counted in an unchanged form. Poisoned control flasks were aerated after sterile conditions were confirmed by head space analyses. All flasks were evacuated using compressed laboratory air. The air was introduced into the flasks via the stop cock with the glass tubing attached. For the poisoned control flasks, sterilized bacteria-vents were placed in line between the air source and the flasks.

For B(a)P spiked flasks, soil sub-samples were taken at selected intervals during incubation (30, 60, 130, and 200 days) after aeration. At 285 days, the remaining soil was divided into three parts, sub-samples for plating and combustion were taken, and the remaining soil was used for the extraction series. For pyrene spiked flasks, soil sub-samples were taken at 30, 60 and 100 days after aeration. Sub-samples were handled as follows: 2-1 g samples from poisoned flasks were suspended in sterile DDW, and diluted 1:10 five times. One milliliter and 0.5 mL of the three most dilute suspensions were plated on dilute nutrient agar and on dillute tryptic soy agar for microbial enumeration; 1 g samples from all flasks were used for combustion to determine non-extractable $^{14}$C, and 30 to 50 g samples were used for the extraction series to determine solvent extractable $^{14}$C.

**Extraction series.** Extraction of soil sub-samples follows the outline presented in Figure 2. Soil sub-samples from all flasks were suspended in DDW at a 1:6 soil to water ratio by weight, in glass mason jars, sealed with teflon lined lids, and tumbled for 24 hours. At the end of 24 hours, the soil and water were allowed to settle. Supernatant was decanted and measured in a graduated cylinder. Two milliliters of the supernatant were pipetted into a 7 mL scintillation vial, approximately 5 mL of Ready Safe™ scintillation cocktail were added, and then radiolabelled carbon was counted as a part of the chemical analysis procedure for the aqueous fraction shown in Figure 2.

Samples for Microtox™ assay analyses were further manipulated as follows. Supernatant pH was measured and if necessary adjusted to between 6 and 8 using phosphate buffer. Ten mL of the supernatant were prepared and used in the Microtox™ bioassay according to the method described in Beckman (34) and Symons and Sims (35).

Organic chemicals present in the water phase were extracted using C-18 Sep Pak cartridges according to the following procedure. Remaining supernatant was extracted using C-18 Sep Pak cartridges (that had been washed with 5% methanol, then flushed with 5 mL DDW). Water samples were passed through the cartridges at a flow rate of approximately 10 mL/min to allow organic chemicals to sorb to the cartridges. Organic chemicals were desorbed from the cartridges sequentially using 5 mL methanol for the first fraction, followed by 5 mL of methylene chloride for the second fraction. Ready Gel™ scintillation cocktail was added to 200 μL of each eluate in scintillation vials for counting $^{14}$C. The methanol and methylene chloride eluates were also analyzed by HPLC.

Water extracted soil was allowed to air dry. The soil was then ground with mortar and pestle and weighed into a 250 mL beaker. One gram sub-samples were taken for combustion to determine the non-water extractable fraction of $^{14}$C, as part of the chemical analysis procedure for
the solid fraction shown in Figure 2, and the remainder was solvent extracted with methylene chloride according to the tissumizer homogenization procedure of Coover et al (37). A 200 µL portion of each solvent extract was placed in scintillation vials with Ready Gel™ scintillation cocktail and counted. Organic extracts of soil and methanol and methylene chloride eluates of Sep Pak extractions were analyzed twice by reverse phase HPLC. The first HPLC analysis was for PAH parent compounds, and used a gradient mobile phase program consisting of 2 minute isocratic elution with 40% acetonitrile in water followed by 15 minute linear gradient to 100% acetonitrile at a flow rate of 1 mL/min. Analytes were detected at a wavelength of 254 nm.

The second HPLC analysis was for PCP and polar PAH metabolites. Void volume plus the first five minutes of HPLC elution volume were collected and analyzed using a gradient mobile phase program consisting of 5 minutes of 100% acidified (< 3 pH) water followed by a 5 minute linear gradient to 100% methanol at a flow rate of 1 mL/min. Analytes were detected at a wavelength of 250 nm.

14C associated with the solid phase that was not extracted using water or organic solvent (methylene chloride) was determined in the next step of the procedure as part of the chemical analysis of the solid phase. Solvent extracted soils were allowed to air dry under a laminar flow hood. When dry, solvent extracted soil was ground up with mortar and pestle and weighed. One gram subsamples were taken for combustion. The remaining dried solvent extracted soil was weighed and placed in a 500 mL Erlenmeyer flask for humic material extraction.

14C associated with the humic material part of the solid phase was determined next. Humic material extraction was carried out by the method described by Skujins and Richardson (38), with the following modifications: 1) Dowex was not used, 2) division of fulvic and humic acids into separate fractions was not attempted as the organic matter content of the soil was low, and 3) the combined humic-fulvic supernatant was not taken to dryness. Previous attempts to separate the humic and fulvic acids from the McLaurin soils were unsuccessful due to the low organic matter content of the soil. The humic-fulvic supernatant (0.5 mL) was added to scintillation cocktail and radiolabelled carbon was counted. The insoluble portion was allowed to air dry. When dry, this portion was ground up and 1 g samples were taken for combustion.

All combustion samples were oxidized in a Harvey Biological Oxidizer using the method described by Bumpus and Aust (24), with the trapping solution counted for radiolabelled carbon.

Results of 14C analysis from combusted samples were added to 14C results from aqueous and solvent extractions as well as extracted humic material to establish a complete mass balance measurement for 14C in PAH spiked contaminated and non-contaminated soil.

RESULTS AND DISCUSSION

Microbial activity and enumeration. Microbial activity of indigenous soil microorganisms was monitored through measurement of O2 utilization and CO2 production. Headspace analyses showed that carbon dioxide levels in poisoned (control) flasks remained consistently equal to ambient air concentration, and oxygen content did not change throughout the study. Enumeration by serial dilution and plating of soil subsamples confirmed microbial activity was depressed in poisoned control flasks. In non-poisoned flasks, constant production of unlabeled carbon dioxide, utilization of oxygen, and decrease in oil and grease content of the soil (50%) indicated that the indigenous microbial population was active and degraded unlabeled organic carbon to CO2 in creosote contaminated soil.
Mass Balance. With regard to results for creosote-PCP contaminated soil, radiolabelled carbon ($^{14}$C) mass balances over all soil phases indicated that greater than 95% of the radiolabelled carbon remained associated with soil solids as either solvent extractable (dichloromethane) or non-solvent extractable (humic plus fulvic acids and insoluble humin plus mineral components) fractions after 285 days in B(a)P amended soil (non-poisoned and poisoned) (Figures 3 and 4), and after 100 days in pyrene amended soil (non-poisoned and poisoned) (Figure 5). Partitioning of the radiolabelled carbon between solvent extractable and non-solvent extractable (humic plus fulvic acids, and humin plus mineral components) soil solids fractions did not change significantly through time for either B(a)P spiked non-poisoned or poisoned soils or for pyrene spiked poisoned soil. The average percent of $^{14}$C of three replicates associated with $^{14}$C-B(a)P amended soil solids were a) base extractable (humic and fulvic acid fractions) <12% and b) insoluble soil solids (humin) for poisoned (24 to 52%) and non-poisoned flasks (40 to 56%). This trend occurred initially and was independent of incubation time for spiked B(a)P. For spiked pyrene, while the average percent of $^{14}$C associated with non-solvent extractable solids was 32% for poisoned soil at 100 days, the percentage increased to 62% in non-poisoned soil at 100 days incubation, which was significantly different from the poisoned soil samples (Figure 5). Thus microbial activity is likely the agent of incorporation of $^{14}$C derived from spiked pyrene into soil humic material. Humification, therefore, as defined in this study was a dominant mechanism of behavior for spiked B(a)P and pyrene in contaminated soil, and occurred in non-poisoned as well as poisoned soil samples; in addition, microbial activity was observed to increase humification of pyrene in contaminated soil.

As part of the chemical mass balance evaluation for contaminated soil, radiolabelled carbon associated with the gas phase plus aqueous phase of the soil was measured and added to that associated with the solid phase as described above. Collection of radiolabelled carbon in the gas phase from the volatile organic and carbon dioxide trapping solutions accounted for less than 1% for both B(a)P and pyrene amended soils over the 285 day study for all flasks, non-poisoned and poisoned. The total radiolabelled B(a)P carbon associated with the aqueous fraction averaged less than 0.5% at each sampling for all B(a)P and pyrene flasks.

With regard to results for non-contaminated soil, soil samples were spiked with 100 mg/kg pyrene and $^{14}$C-pyrene or with 100 mg/kg B(a)P and $^{14}$C-B(a)P. $^{14}$C mass balances over all soil phases indicated that, as with contaminated soil results, greater than 95% of the radiolabelled carbon remained associated with soil solids as either solvent extractable (dichloromethane) or non-solvent extractable (humic plus fulvic acids and insoluble humin plus mineral components) fractions after 285 days in B(a)P amended soil (non-poisoned and poisoned) (Figures 6 and 7), and after 100 days in pyrene amended soil (non-poisoned and poisoned) (Figures 8 and 9). However, unlike results with contaminated soil, partitioning of spiked PAHs between solvent extractable and non-solvent extractable fractions changed significantly through incubation time for both spiked PAHs. For B(a)P, a decrease in the solvent extractable fraction was associated with an increase in the fraction associated with humic and fulvic acids for both non-poisoned and poisoned soil samples, thus indicating that abiotic reactions may play a dominant role in the humification process for B(a)P. For pyrene, a decrease in the solvent extractable fraction was primarily associated with an increase in the humin plus mineral component fraction for both non-poisoned and poisoned soil samples, thus indicating that abiotic reactions may play a dominant role in the humification process for pyrene. Humification, therefore, as described for results for contaminated soils, appeared to be the dominant mechanism of behavior for spiked B(a)P and pyrene in non-contaminated soil, and occurred in non-poisoned as well as poisoned soil samples.
As part of the chemical mass balance evaluation for non-contaminated soil, radiolabelled carbon associated with the gas phase plus aqueous phase of the soil was measured and added to that associated with the solid phase as described above. Collection of radiolabelled carbon in the gas phase from the volatile organic and carbon dioxide trapping solutions accounted for less than 1% for both B(a)P and pyrene amended soils over the 285 day study for all flasks, non-poisoned and poisoned. The total radiolabelled B(a)P carbon associated with the aqueous fraction averaged less than 0.5% at each sampling for all B(a)P and pyrene flasks.

**Degradation/Mineralization.** With regard to mineralization for creosote-PCP contaminated soil, there were no significant differences in the amount of radiolabelled carbon dioxide collected over incubation time for poisoned flasks for either radiolabelled B(a)P or pyrene. However, statistically significant differences in the amount of radiolabelled carbon collected in the carbon dioxide traps due to treatment (non-poisoned versus poisoned) through incubation time indicated that some mineralization/ degradation of at least the radiolabelled carbon (position 7) of B(a)P had taken place, although it was small (<1%). Also, with regard to pyrene, non-poisoned flasks produced almost twice the $^{14}$CO$_2$ compared with poisoned flasks over the 285 days incubation period, indicating mineralization of pyrene although it was less that 1%.
Radiolabelled carbon was found associated with volatile organic traps for both pyrene and B(a)P incubated flasks. Statistically significant differences in radiolabelled carbon associated with volatile organic traps from the B(a)P flasks were found due to incubation time in the non-poisoned flasks, and due to treatment (non-poisoned vs poisoned) and time, at 130 and 285 days incubation. The total amount of radioactive carbon recovered in volatile organic traps from poisoned and non-poisoned B(a)P flasks over the 285 day incubation period was 0.0052% and 0.0089%, respectively. Total percentages of radiolabelled carbon associated with the volatile organic traps from the pyrene flasks (0.014% and 0.0091% for the non-poisoned and poisoned flasks respectively) were not significantly different.

Figure 4. Mass Balance of Radiolabelled Carbon in Poisoned $^{14}$C-B(a)P Spiked Contaminated Soil. Vertical error bars show standard deviation of the data.
Radiolabelled carbon was present in water extracts of both pyrene and B(a)P incubated flasks. Radiolabelled carbon associated with water extracts did not change over time for B(a)P non-poisoned soil or poisoned soil flasks. However, there were increases in the radiolabelled carbon content in the methanol eluates of Sep Pak™ extracts of the water fractions from B(a)P non-poisoned flasks (0.12% to 0.41% of total recovered DPM, 0 and 285 day respectively), but not poisoned flasks. This observation suggests transformation of 14C-B(a)P resulting in the generation of polar intermediate compounds, which were recovered in the methanol solvent. These metabolites were not detected using reverse phase HPLC. Radiolabelled carbon associated with the water extracts from the pyrene flasks were very small and similar to results obtained for B(a)P water extracts. The percentage of radiolabelled carbon in the methanol plus methylene chloride eluates of the Sep Pak extracts of the water fractions from B(a)P flasks totalled less than 1.5% for non-poisoned flasks and less than 1% for poisoned soil flasks. Percent of radiolabelled carbon in Sep Pak™ eluates from pyrene flasks averaged a total of 1% for both poisoned and non-poisoned flasks and was significantly different for the methanol eluate due to treatment. Fractions from HPLC analyses of the Sep Pak™ methanol eluate from day 30, 60 and 100 samples from the non-poisoned pyrene flasks had 80, 80 and 100% of the label present in the HPLC void volume, respectively, indicating the presence of polar metabolites.
Results concerning mineralization/degradation for non-contaminated soil samples were essentially identical to those for creosote-contaminated soil, described above. The observations were consistent for non-poisoned and poisoned soil samples.
Apparent Degradation of PCP and PAHs. With regard to creosote-PCP contaminated soil, HPLC analysis of the methanol and dichloromethane Sep Pak™ eluates of water extracts indicated differences between non-poisoned and poisoned flasks for PCP and for PAHs including naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene and fluoranthene in both B(a)P and pyrene amended flasks. Water extracts of soil from the B(a)P amended and poisoned flasks showed greater concentrations of $^{14}$C-B(a)P, determined by HPLC and scintillation counting, than water extracts of B(a)P amended non-poisoned flasks, a trend that remained consistent throughout incubation time. Radiolabelled carbon content of water extracts of the pyrene amended and poisoned soil was significantly greater than non-poisoned soil water extracts for the 0 and 30 day samples and significantly less for the 100 day sample. Concentrations of PCP, naphthalene, acenaphthylene, acenaphthene, and fluorene from the non-poisoned soil from both B(a)P and pyrene amended flasks showed decreases (Table II), while phenanthrene, anthracene and fluoranthene concentrations remained constant in the extracts of the non-poisoned flasks. Pyrene, benz(a)anthracene, chrysene, benzo(k)fluoranthene, benzo(f)fluoranthene, benzo(a)pyrene, dibenz(a,h)anthracene, benzo(g,h,i)perylene, and indeno(1,2,3-c,d)pyrene were not present in detectable amounts in the water extracts of either the non-poisoned or poisoned soils. Decreases in PCP and in the PAHs listed in Table II were not observed in poisoned flasks.
Figure 8. Mass Balance of $^{14}$C in Pyrene Non-Poisoned Uncontaminated Vadose Zone Soil. Each bar is the average of sub-samples from three replicate flasks with vertical error bars that show standard deviation of the data.
Figure 9. Mass Balance of $^{14}$C in Pyrene Poisoned Uncontaminated Vadose Zone Soil. Each bar is the average of sub-samples from three replicate flasks with vertical error bars that show standard deviation of the data.
Table II. PCP and PAH concentrations in water fractions from non-poisoned flasks in µg/mL.

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>0</th>
<th>30</th>
<th>60</th>
<th>100</th>
<th>130</th>
<th>160</th>
<th>200</th>
<th>285</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCP</td>
<td>7.53</td>
<td>2.63</td>
<td>2.67</td>
<td>2.28</td>
<td>1.38</td>
<td>1.35</td>
<td>0.50</td>
<td>1.13</td>
</tr>
<tr>
<td>Naphthalene</td>
<td>6.90</td>
<td>5.17</td>
<td>5.02</td>
<td>4.98</td>
<td>4.59</td>
<td>3.24</td>
<td>2.93</td>
<td>2.82</td>
</tr>
<tr>
<td>Acenaphthylene</td>
<td>5.70</td>
<td>5.21</td>
<td>4.90</td>
<td>4.44</td>
<td>4.91</td>
<td>4.68</td>
<td>4.00</td>
<td>4.19</td>
</tr>
<tr>
<td>Acenaphthene</td>
<td>2.86</td>
<td>2.34</td>
<td>2.17</td>
<td>2.00</td>
<td>2.05</td>
<td>2.06</td>
<td>1.33</td>
<td>1.42</td>
</tr>
<tr>
<td>Fluorene</td>
<td>0.39</td>
<td>0.22</td>
<td>0.20</td>
<td>0.16</td>
<td>0.09</td>
<td>0.02</td>
<td>0.04</td>
<td>0.04</td>
</tr>
</tbody>
</table>

The differences in the water extractability of phenols including PCP may be attributed to the changes in pH of the soil and water extracts. Equilibrium of neutral and ionized PCP in solution can be described with a knowledge of pH and the acid dissociation constant for PCP (pKa = 4.75) using the following equation as shown in Lee et al (38).

\[
\Phi_n \text{ (neutral fraction)} = (1-10^{\text{pH}-\text{pKa}})^{-1}
\]

With regard to poisoned contaminated soil, the pH of water extracts decreased from 5 to 4.2, due to the use of propylene oxide as the sterilant. This decrease in pH was accompanied by a decrease in PCP concentration in the water fractions (from 7.43 µg/mL to 3.6 µg/mL) and a corresponding increase in solvent extractable fraction of PCP (203.29 µg/g to 405.07 µg/g). Total PCP concentration in the poisoned soil (water and solvent extract) remained constant.

With regard to non-poisoned contaminated soil, the pH of water extracts increased from 5 to 6 over the 285 day incubation period possibly due to degradation of acidic creosote constituents. Water extractable PCP decreased through time of incubation (7.1 µg/mL to 1.3 µg/mL) while there was no change in solvent extractable PCP concentration. This indicated that the difference in PCP concentration between poisoned and non-poisoned soil was due to loss by degradation of PCP in the non-poisoned flasks and not due to adsorption caused by a change in pH.

**Toxicity of Water Extracts.** The Microtox™ assay was used to evaluate changes in toxicity of soil water extracts through incubation time for contaminated and non-contaminated soil. The EC50 value, defined as the effective concentration (EC) [by percent soil water extract in buffered water] that causes a 50% reduction in light output by the test microorganisms, increases as the water extract toxicity decreases since a greater concentration (%) of extract is required to effect a 50% reduction in light output. For example, an EC50 value of 1% indicates that a water extract to buffer ratio of 1mg:99ml will decrease light output by 50%, where an EC50 value of greater than 100% indicates that the water extract, at 100%, does not decrease light output by 50%. Therefore, as the EC50 values increases, the toxicity of the soil water extract to the exposed microorganism decreases.

With regard to contaminated soil, all poisoned control samples were consistently toxic through incubation time using the Microtox™ assay with no significant changes (Figures 10 and 11). However, a decrease in toxicity of water extracts was observed for non-poisoned soil through time.
of incubation. Water extracts from B(a)P spiked non-poisoned flasks decreased in toxicity as indicated by a change in EC50 from 5% to 22% over the 285 day incubation (Figure 10) and from an EC50 of 4.3% to 9.4% over 100 days of incubation for the pyrene non-poisoned flasks (Figure 11). The amount of radiolabelled carbon in the water fractions from the B(a)P non-poisoned soil flasks as well as the concentrations of naphthalene, acenaphthylene, acenaphthene, and fluorene also decreased with time (Table II). The amount of radiolabelled carbon in the water fractions from the pyrene non-poisoned soil flasks increased over 100 days, with decreases of naphthalene, acenaphthylene, acenaphthene, and fluorene. Water extracts from B(a)P poisoned controls showed constant radiolabelled carbon concentrations and from pyrene poisoned controls showed decreased radiolabelled carbon concentrations. Therefore, detoxification was observed to occur throughout incubation time for non-poisoned contaminated soil, with no detoxification trend was apparent for poisoned soil.

The trend indicating decrease in toxicity shown in the Microtox™ assay in non-poisoned soil is similar to the trend reported by Aprill et al (21) for a creosote-PCP contaminated soil. Aprill et al (21) used 0.3% creosote-PCP to soil ratio (weight). In this study, the concentration of contamination was higher (8% creosote-PCP to soil) and the decrease in toxicity was not as dramatic.

In contrast to the detoxification trend observed with creosote-PCP contaminated soil, non-contaminated soil, from the same site, incubated with 100 mg/kg pyrene or with 100 mg/kg B(a)P showed consistent trends of increasing toxicity in non-poisoned samples with increase in incubation time for pyrene (Figure 12) and for B(a)P (Figure 13). The increase in toxicity in water extracts of pyrene and B(a)P spiked soils may indicate the production of polar metabolites, which would also be more water soluble than the parent compounds. This is indicated by the increase in the percent of 14C identified in the water extract phase of the soil sample (Figure 12). Poisoned samples, however, showed no increase in toxicity with increase in incubation time for either pyrene or B(a)P spiked samples.

**SUMMARY**

The distribution of spiked radiolabelled carbon, added as 14C-B(a)P and as 14C-pyrene to non-contaminated and creosote-PCP contaminated vadose zone soil (McLaurin Sandy Loam), was determined among aqueous, gas, and solid (humic plus fulvic acids and humin plus inorganic mineral components) phases as a function of incubation time. Four soil/PAH combinations were evaluated: (1) contaminated soil with B(a)P spike, (2) contaminated soil with pyrene spike, (3) non-contaminated soil with B(a)P spike, and (4) non-contaminated soil with pyrene spike.

Mass balance results of greater than 95% indicated that the major pathway for radiolabelled carbon for both PAH compounds was incorporation into the non-solvent extractable soil solid phase in a process defined for this study as humification. Specific soil solid phase fractions where spiked 14C-PAHs became associated in the process of humification can be characterized as humic plus fulvic acids and humin plus inorganic mineral components. Incorporation of spiked 14C in poisoned as well as non-poisoned soil samples indicated that abiotic reactions may be predominant in humification processes that occurred in the test soils with the test chemicals.

Mineralization and production of polar intermediates of spiked 14C accounted for less than 5% of added 14C for both PAHs in non-contaminated and in creosote-PCP contaminated soil. However, mineralization and production of polar intermediates were significantly greater in non-poisoned soil than in poisoned soil. Also, apparent degradation of PCP and PAHs naturally present in creosote-PCP contaminated soil was obtained in non-poisoned soil but not in poisoned soil.
Figure 10. Toxicity of Soil Water Extract Measured with the Microtox™ Assay with Incubation Time for B(a)P Spiked Creosote Contaminated McLaurin Sandy Loam Soil. EC50(5,15°C) denotes the effective concentration (vol/vol) of soil water extract that reduced light emission of the Microtox™ organisms by 50% five minutes after exposure to the test solution at 15°C.

Toxicity of the soil water extract fraction, as measured by the Microtox™, were very different between non-contaminated and creosote-PCP contaminated soil, with contaminated soil water toxicity much higher than non-contaminated soil water toxicity.

Changes in toxicity of the water phase were also related to the biological status of soil samples (non-poisoned versus poisoned) and to the contamination status (contaminated versus non-contaminated soil). Changes in toxicity of the soil water extracts through time occurred only in non-poisoned (biologically active) soil samples. Also, changes in toxicity were more pronounced in non-contaminated soil, with both decreases and increases in toxicity observed. Changes in toxicity of water extracts were less pronounced in contaminated soil; however, consistent detoxification trends were observed. Water extracts from poisoned control soil samples exhibited no statistical change in toxicity over incubation time. Changes in toxicity of the soil water extract were also correlated with the amount of 14C associated with the water extract. This was attributed to the production of polar metabolites in biological active samples that would be more water soluble than the parent PAHs that were spiked.
Figure 11. Toxicity of Soil Water Extract Measured with the Microtox™ Assay with Incubation Time for Pyrene Spiked Creosote Contaminated McLaurin Sandy Loam Soil. EC50(5,15°C) denotes the effective concentration (vol/vol) of soil water extract that reduced light emission of the Microtox™ organisms by 50% five minutes after exposure to the test solution at 15°C.

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Radiolabelled [4,5,9,10-14C] pyrene was obtained from Chemsyn Laboratories, Lenexa, Kansas through the generosity of Drs. J. T. Dibble and I. Bossert, Texaco, Deacon, NY.
Figure 12. Radiolabelled Carbon and Toxicity Associated with the Soil Water Extract with Incubation Time for Pyrene Spiked Non-Contaminated and Non-Poisoned McLaurin Sandy Loam Soil. Water Extract Toxicity Measured with the Microtox™ Assay where EC50(5,15°C) denotes the effective concentration (vol/vol) of soil water extract that reduced light emission of the Microtox™ organisms by 50% five minutes after exposure to the test solution at 15°C.
Figure 13. Toxicity of Soil Water Extract Measured with the Microtox™ Assay with Incubation Time for B(a)P Spiked Non-Contaminated and Non-Poisoned McLaurin Sandy Loam Soil. EC50(5,15°) denotes the effective concentration (vol/vol) of soil water extract that reduced light emission of the Microtox™ organisms by 50% five minutes after exposure to the test solution at 15° C. A hormetic response in the Microtox™ assay indicates an increase in light output compared to non-toxic controls.
REFERENCES


