ACUTE ECOTOXICITY OF CREOSOTE-CONTAMINATED SOILS TO EISENIA FETIDA: A SURVIVAL-BASED APPROACH

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Abstract—Quantification of risks to the ecosystem is necessary for cost-effective remediation strategies. Contaminant endpoints need to be established that consider the bioavailability of toxicants in soil. The challenge is to develop methods that assign risk to the bioavailable toxic contaminants, thereby protecting ecosystems, while balancing remediation costs. Our objective was to evaluate changes in bioavailability of creosote constituents in soils to earthworms. An acute ecotoxicological investigation of three weathered creosote-contaminated and two slurry-phase-biotreated soils was conducted using a 14-d earthworm (Eisenia fetida) survival bioassay. Soil characterization (physical and chemical) and contaminant concentration data (polycyclic aromatic hydrocarbons [PAH] and total dichloromethane extractable organics [DEO]) were also determined. The toxicity of the soils could not always be predicted based on chemical concentrations alone. Soils having a low PAH:DEO ratio had higher cumulative earthworm survival times as measured by earthworm-days. We propose that the DEO fraction may regulate toxicity by altering bioavailability of toxicants.

Keywords—Bioavailability Creosote Earthworms Ecotoxicology Soil

INTRODUCTION

Our aim was to examine the hypothesis that biotreatment changes bioavailability of contaminants from creosote to earthworms in soils aged for decades in the field. Bioavailability, as used here, refers to the internal dose of a xenobiotic chemical, which exists in the soil, enters the organism, and initiates a measurable biological response. Chemicals that are not bioavailable do not present a risk of toxicity to organisms, but they may make the soil nonhabitable. For example, they might cause soil to become hydrophobic and remain too dry for survival of soil organisms.

The primary goal of soil remediation is cost-effective reduction of risk to humans and ecosystems to an accepted level. Risk refers to the probability of an adverse outcome within a specified time period [1]. Risk from soil contamination is usually related to the concentration in soil of specified constituents by measuring responses of test organisms at known contaminant concentrations. Dose–response data are typically obtained from bioassays immediately after adding known concentrations of a specified contaminant to pristine soil. Most contaminated sites differ from these conditions in at least two important ways: Toxic constituents seldom exist alone; they are often present in a matrix of organic constituents, which may be toxic or may alter the behavior of the constituent of interest [2–5]; and toxic constituents are often present in soils for years or decades. Over time, soil processes alter the matrix and the contaminant. Contaminants may become inaccessible either by sequestration in soil micropores, by partitioning slowly into native soil organic matter, or by incorporation into soil humic substances [4,6–8]. The matrix may become more or less of a barrier to mass transfer [9]. Consequently, it is unlikely that the availability to soil organisms of a contaminant present in an organic matrix and aged in the field is represented by a contaminant alone in soil and assayed immediately. If bioavailability of a contaminant changes, then the magnitude of the toxic response is likely to change, but extractability by solvents may not necessarily change.

Regulations limited to solvent-extractable concentrations may be assigning inappropriate levels of risk to organic contaminants in the soil [10]. Several criticisms exist regarding the strict reliance on solvent-extraction data to predict environmental risks. Lambolez et al. [11] cite the difficulties in identifying certain micropollutants within a complex waste mixture and predicting synergistic or antagonistic interactions between pollutants as reasons why toxicological studies need to complement chemical analysis. Site history can dictate which classes of compounds will be screened in a site assessment. Failure to quantify all initial compounds and degradation metabolites will limit the usefulness of regulations based on chemical extractability [10].

Hazardous constituent classes found in creosote include the polycyclic aromatic hydrocarbons (PAHs), phenolic compounds, and N-, O-, and S-heterocyclics [12–15]. Acute creosote toxicity is well documented, and the ecotoxicological ramifications of environmental exposures to PAHs are detailed in the literature [16–18].

Indicators such as plants, microorganisms, nematodes, and earthworms have been used to assess the toxicity of various PAHs in soil or sediment [10,19–21]. Earthworms have been used to study the environmental impacts of pesticides, herbicides, metals, and PAHs in the soil [22,23]. Earthworms are large, numerous, and easily sampled; widely distributed geographically; in full contact with their substrate and ingest large
volumes of it; and respond to chemicals not only through mortality but also through behavioral, reproductive, and growth rate changes [22]. Thus, earthworms possess key characteristics necessary for environmental soil monitoring. We chose the earthworm Eisenia fetida as our receptor organism.

Bioassays in soil must provide suitable conditions of light, temperature, and soil moisture for the test organisms. Simple fixed ratios of water to soil may be used [24], but they make it difficult to compare soils of varying texture. A proportion of soil water-holding capacity might be used [25,26], but contaminated soils pose special problems in determining soil water-holding capacity. Linn and Doran [27] showed that a soil water-holding capacity equal to 60% of the pore volume was optimum for soil biological activity. Soil porosity varies with texture and is easy to determine. Thus, maintaining water content at a fixed fraction of the pore space should facilitate bioassays on contaminated soils even when texture varies.

We show here that maintaining 60% water-filled pore space is a practical way to achieve optimum soil moisture for bioassays in diverse contaminated soils; that, where biotreatment reduced total dichloromethane extractable organics (DEO) and PAH concentrations, it also eliminated acute toxicity of creosote-contaminated soil, even though the DEO and PAH concentrations remained as high as in acutely toxic dilutions of creosote-contaminated soil, even though the DEO and PAH concentrations remained as high as in acutely toxic dilutions of creosote-contaminated soil, even though the DEO and PAH concentrations remained as high as in acutely toxic dilutions of creosote-contaminated soil, even though the DEO and PAH concentrations remained as high as in acutely toxic dilutions of creosote-contaminated soil, even though the DEO and PAH concentrations remained as high as in acutely toxic dilutions of creosote-contaminated soil, even though the DEO and PAH concentrations remained as high as in 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MATERIALS AND METHODS

Soils

Three weathered creosote-contaminated soils were collected from two industrial sites. The first soil was collected from a wood treatment facility in Edmonton, Alberta, Canada (EDM) (53°34’N, 113°23’W; Universal Transverse Mercator [UTM] North American Datum [NAD] 83 Zone 12, Easting 341420, Northing 5939720). The EDM soil had a clay texture, contained admixed surface and subsurface material and was contaminated with creosote and related compounds. The second soil had a sandy texture and was sampled from the surface at a creosote wood treatment facility in Prince Albert, Saskatchewan, Canada (PAC) (~53°11’N, 105°48’W; UTM NAD 83 Zone 13, Easting 446300, Northing 5893930). The final soil (PAL) was from the site at Prince Albert, but it was sampled from a part of the site with lower concentrations of total DEO and total PAHs compared to the PAC soil. Contaminated soils were processed at the University of Alberta’s Research Station at Ellerslie (AB, Canada) as described by Rutherford et al. [5]. Briefly, they were passed through a 4-mm sieve, individually homogenized in a clean cement mixer for 30 min, and stored at 4°C until use in the acute earthworm toxicity testing. Prior to bioassays or chemical analyses, all air-dried contaminated and pristine soils were passed through a 2-mm sieve. All coarse fragments (>2 mm) were discarded.

Appropriate control soils were not available at or near the contaminated sites, so two pristine soils were selected as controls. Properties of the master soil variables [28] in the pristine soils were matched as closely as possible to those in the contaminated soils. The master variables used were pH, electrical conductivity, total organic carbon, total nitrogen, texture, and bulk density (Table 1). The control for the EDM soil was from the C horizon of the Malmo soil series (MAL) at the University of Alberta Research Station (Ellerslie, AB, Canada: 53°25’N, 113°32’W; UTM NAD 83 Zone 12, Easting 330680, Northing 5922400). The Malmo series is an Eluviated Black Chernozem, developed on sandy aeolian parent material [29]. The control for the PAC and PAL soils was from the C horizon of the Dune Sand series near Bruderheim, (BRU) Alberta, Canada (53°51’N, 112°55’W; UTM NAD 83 Zone 12, Easting 370630, Northing 5968630). The Dune Sand series is an Orthic Regosol, developed on sandy aeolian parent material [29].

DEO and PAH determinations

Total dichloromethane extractable organics were determined gravimetrically following extraction in a Soxhlet apparatus [30] as modified here. About 25 g of air-dried soil were...
weighed into a single-thickness cellulose thimble (30 × 80 mm), mixed with 10 g of Na₂SO₄, and extracted using 200 ml of dichloromethane. The dichloromethane was heated in round-bottom flasks containing two non-PTFE boiling chips (Henar, Philadelphia, PA, USA) for 16 h at 10 to 12 cycles/h. The extract was evaporated to 10 ml, transferred to a 50-ml volumetric flask, made to volume, and split into two. One portion was placed in a preweighed Al dish (Fischer Scientific, Nepean, ON, Canada) set in a fume hood at room temperature for 24 h to evaporate the solvent and then reweighed to determine mass of DEO. The second portion was stored at 4°C in 15-ml amber screw-top vials with 18-mm solid-cap PTFE liners (Supelco, Oakville, ON, Canada) prior to analysis for PAH content. The physical handling of the soils may have resulted in the loss of some volatile components from the creosote; however, all soils were handled in the same manner.

Sample cleanup for creosote constituents used a solid-phase separation. A 1-g/ml LC-Florisil (magnesium silicate 100/120 mesh) cartridge (Supelco) was conditioned with two column volumes of dichloromethane and air dried for 10 min. A 1-ml aliquot of the concentrated Soxhlet extract was applied to the column surface and eluted with two to three column volumes of dichloromethane, keeping the Florisil surface moist. The eluate was concentrated to 0.5 ml using N₂ in a glass volumetric centrifuge tube at 40 to 45°C. A 1-ml volume of toluene was then added for the PAHs to partition into and the volume again reduced to 0.5 ml. The final solution was transferred into a 2-ml screw-top vial with PTFE lid and stored at 4°C prior to gas chromatograph/mass selective detector analysis.

Creosote constituents were separated on a Hewlett-Packard 5890 gas chromatograph and quantified using selected ion monitoring with a Hewlett-Packard 5970 mass selective detector (Avondale, PA, USA). The column was an HP-5 (30.0 m × 0.25 mm; 0.25-μm film thickness). A method based on Zemanek [31] was used to elucidate the creosote constituents of interest. The carrier gas was helium, with column head pressure of 21 kPa. Samples (2 μl) were injected using an autosampler (Hewlett-Packard 7673). Each sample run started at an oven temperature of 70°C, which was held for 4 min, followed by a thermal ramp of 10°C/min to a temperature of 280°C, which was held for 13 min. The final temperature ramp was 4°C/min to a temperature of 300°C, which was held for 5 min. The total run time for each sample was 48 min. During selected ion monitoring, a major ion was selected for quantification along with a qualifier ion, which was used as confirmation for each compound of interest. Selected ion monitoring provided a method of reducing the interference from compounds that eluted at the same time as the compounds of interest.

A mixture of 16 PAHs and dibenzothiophene was used to construct a calibration curve for each compound of interest. Marine sediment from the National Research Council of Canada (NRC, Halifax, NS, Canada) with a certified analysis for PAHs was used as a primary standard. One soil sample was homogenized and included in each batch of analyses for quality control purposes.

**Slurry-phase biotreatment**

Two of the contaminated soils, EDM and PAC, received slurry-phase biotreatment for 52 d using the protocol described by Rutherford et al. [5]. Reactors consisted of 2-L glass mason jars (annealed overnight at 560°C to remove strain in the glass and prevent cracking or breaking) with perforated lids covered with filter paper to permit gas exchange. Each reactor was inoculated with a subsample of soil slurry that was previously biotreated for one month (13 g of slurry for EDM and 10 g of slurry for PAC). The inoculation provided a soil microbial population that was adapted to the substrates and environmental conditions present in the biotreatment of weathered creosote-contaminated soils.

A nutrient stock solution was added to the contaminated soil. It was prepared by adding to each liter of deionized water 28.6 g of NH₄NO₃, 1.46 g of KH₂PO₄, 1.87 g of K₂HPO₄, and 0.54 g of K₂SO₄, yielding a N:P:S ratio of 10:0.7:0.1 (mass basis). The pH of the solution was adjusted to 7.2 with 10 M KOH. Nutrient additions were based on the contaminant concentrations with a designed contaminant (C):nutrient (N) ratio of 15:1. Rutherford et al. [5] indicated that optimal soil mixing within the reactors occurred at soil:solution ratios of 1:1 for EDM and 3:1 for PAC. The actual soil:total solution ratios achieved were 0.88:1 for EDM and 2.3:1 for PAC, based on clean oven-dried soil (Table 2).

Biotreatment reactors were placed horizontally on a tissue culture roller bottle apparatus, kept in the dark at 22°C, and rotated at 3.7 rpm for 52 d. Water was added to the reactors once, approximately halfway through the experiment, to maintain constant moisture.

**Moistening soils: Water-filled pore space**

All soil water contents were gravimetrically determined, in triplicate, at 105°C [32] and expressed as a percentage of oven dry soil mass. Earthworm acute toxicity assays were conducted in soils in which the volume of water equaled 0.6 of total pore volume after Linn and Doran[27].

\[
TP = 1 - \frac{\rho_b}{\rho_p} \quad (1)
\]

\[
\theta_v = \left(\theta_g\right) \times \left(\rho_p\right) \quad (2)
\]

\[
WFP = \frac{\theta_v}{TP} \quad (3)
\]

where \(TP\) = total porosity (cm³ of pore volume/cm³ soil); \(\rho_p\) = dry soil bulk density (g/cm³); \(\rho_b\) = soil particle density, taken to be 2.65 g/cm³ (density of water is omitted from Eqn. 2 because it was taken as 1 g/cm³); \(\theta_v\) = volumetric moisture content (cm³ of water/cm³ soil); \(\theta_g\) = gravimetric moisture content (g of water/g dry soil); and WFP = water-filled pore space. Given \(\theta_v\) and \(\rho_p\), these equations can be rearranged to yield the quantity of water to add to dry soil (AW; g of water/g soil):

<table>
<thead>
<tr>
<th>Soil</th>
<th>Mass of moist soila (g)</th>
<th>Nutrient solution added (ml)</th>
<th>H₂O added (ml)</th>
<th>Total liquidb (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDM</td>
<td>140</td>
<td>12</td>
<td>121</td>
<td>148.9</td>
</tr>
<tr>
<td>PAC</td>
<td>140</td>
<td>10</td>
<td>44</td>
<td>58.2</td>
</tr>
</tbody>
</table>

- a See Table 1 for definitions of acronyms.
- b Soil moisture contents at the time of the experiment were 12 g water (per 100 g dry soil) in EDM and 3 g water (per 100 g dry soil) in PAC.
- c Includes the water in the moist soil.
Acute ecotoxicity of creosote-contaminated soils

\[ AW = \frac{0.6}{P_0} - \frac{1}{P_e} \]  

The calculated volume of double deionized water was sprayed onto the homogenized soil surface in the assay vessels. Normally, clumping occurs when adding water to soils with a high clay content (EDM and MAL). Applying water as a fine mist prevented such clumping. The jars were sealed and left undisturbed overnight to allow infiltration of water into soil pores. The next day the soils were mixed with a spatula to distribute evenly any remaining dry soil into the moist soil. The soil was then ready for the addition of the earthworms.

Earthworm acute toxicity testing (14-d survival)

Earthworm incubation vessels were 1-L glass wide-mouth mason jars with lids. The lids for all the jars had two 6.4-mm holes drilled into the top, allowing for air exchange. Filter paper was placed under each lid to reduce water evaporation during the toxicity tests. As with the slurry-phase treatment, the jars were annealed prior to homogenizing the contaminated and pristine soil treatments on the roller apparatus.

A total of eight soil treatments were used to test for the acute toxicity of EDM and PAC soil samples. Six treatments were twofold serial dilutions of the 100% contaminated soils: 100, 50, 25, 12.5, 6.25, and 3.13% weight per weight (w/w) with pristine soil; the seventh treatment was 100% pristine soil, which was the control. The eighth treatment was either the EDM or the PAC soil after 52 d of slurry-phase biotreatment (EDM-Bio or PAC-Bio). The same serial dilutions were used in the acute toxicity testing of PAL soil samples; however, the PAL soil was not biotreated. The MAL soil was the control used in the EDM toxicity tests, and BRU was the control for the PAC and PAL soil treatments. All acute toxicity treatments were replicated three times except for the biotreated soils, which had no replication because of limited quantities of soil.

Bioassays were initiated by adding 300 g (oven dry basis) of each air-dried soil or combination of contaminated pristine control soils to the earthworm incubation vessels. The contaminated and control pristine soils were homogenized by placing each vessel horizontally on a roller apparatus and rolling them at 3.7 rpm in the dark for 20 h at 22°C. After homogenization, each jar had 15 to 25 g of soil removed to determine the moisture content of the mix. The new homogenized soil moisture content was used to calculate the required mass of water necessary to bring each soil to WFP = 0.6.

Earthworm survival was the endpoint used for the acute toxicity assays described here. Our bioassay incorporated many of the principles described in [24–26] and is outlined in the following. Earthworms (E. fetida; Carolina Biological Supply, Burlington, NC, USA) were used for all acute toxicity tests except for the PAL acute toxicity assay, which used E. fetida from a local supplier (Dirt Willy Farm, Ardrassan, AB, Canada). The earthworms used for acute toxicity testing were mature, with clitellum. Overall, the mean individual earthworm mass was 390 ± 97 mg and ranged between 160 and 710 mg. When not in toxicity assays, the worms were kept in 15.5-L (40 × 32 × 12 cm) translucent, covered, plastic containers with 6.4-mm holes drilled into the lid allowing for gas exchange. The worms were maintained in Magic Worm Bedding (Carolina Biological Supply). Bedding was replaced with fresh material after three months. Several hundred worms were kept in each container. The containers were stored in a growth chamber at 22 ± 2°C under constant light to encourage burrowing. The average illuminance using a 1-s integration time was 4,000 ± 200 lux in the growth chamber; on the surface of the soil with the container lid closed, the illuminance was 3,200 ± 190 lux.

The earthworms were individually weighed and counted, then placed within the incubation vessels containing the various soil treatments. Each vessel received 10 worms placed on the surface of the soil. The jars were arranged randomly and kept in a fume hood under constant temperature (22 ± 1°C) and lighted conditions (800 ± 120 lux) during bioassays. The worms were observed a few hours after their introduction to the soil treatments to count the number of worms that had not burrowed into the substrate. No effort was made to force the worms to burrow into the soil.

To monitor survival, the jars were emptied and the worms counted daily. Soil was replaced in the corresponding jar, and the live worms were returned to the surface. Dead worms were removed from the test jars. A worm was considered dead if it did not move when touched. The soil + jar + worm weight was monitored and moisture kept constant at WFP = 0.6 by misting additional water to the surface of the soil as required. No food was provided during the toxicity tests.

Statistical analysis

Statistical analyses of earthworm data were performed using the SAS® System (Cary, NC, USA) [33]. Parameters for the rectangular hyperbolic model were derived using a non-linear least squares procedure.

RESULTS

Contaminant characterization and biotreatment

Analytical reproducibility was high, but recovery of PAHs appeared to be low. The average DEO concentrations had a mean of 1.67 ± 0.17% (n = 7) for the primary standard (HS-3, NRC) and 0.98 ± 0.04% (n = 9) for the secondary standard. Quality control data for standards and blanks from the individual PAH runs were consistent, and we found no appreciable quantities of analytes in the blank runs. We ran additional elutions through the Florisil cartridge and found no more recovery of PAHs than from one elution. We originally attempted to use an internal standard of BaP-d12 to quantify PAHs. Problems with inconsistent recoveries of BaP-d12 forced us to use an external calibration curve using a mixture of PAHs and dibenzothiophene. The Soxhlet extraction and quantification efficiency using the marine sediment (HS-3, NRC) and its published analysis as standard ranged from 17% foracenaphthene to 126% for benzo[a]pyrene. The overall recovery for total PAHs was 51%.

The highest DEO content was in the EDM soil followed by the EDM-Bio, PAC, and PAC-Bio soils at about 54% as much and finally the PAL soil at about 5.2% of that (Table 3). The EDM and PAC soils had similar total PAH contents, but the PAL soil had about 1.5% as much.

After 52 d of slurry-phase biotreatment, the DEO content of the EDM soil was reduced to about 56% of the original nonbiotreated soil (Table 3). The DEO content of the PAC remained unchanged. The DEO contents in both biotreated soils were still nearly 10-fold greater than the Alberta Tier 1 criterion, for total mineral oil and grease concentration of 1,000 mg/kg.

Biotreatment resulted in a decrease of PAHs from both soils (Table 3), with the greatest decrease in the EDM soil. All
Earthworm activity decreased with increasing concentration of dichloromethane extractable organics compared to the control soils, with earthworm-days dropping sharply to 30% of maximum followed by a slower progression to zero as DEO content increased. Similarly, at the lowest content of DEO in the EDM soil (0.003%), earthworm-days dropped sharply to 30% of maximum, lower than the value for the PAL soil with even more DEO. Little difference was observed between the EDM and PAC soils in the survival of earthworms exposed to 3.13% or more of PAC soil. When exposed to 100% contaminated PAL soil, all earthworms died within 2 d. In contrast, all earthworms survived for 14 d and Prince Albert, Saskatchewan, Canada (PAL) (contains lower concentrations of dichloromethane extractable organics compared to PAC), for 41 d at 22 ± 1°C, continuous light, 10 worms per vessel; and three replicates except for the biotreated soil, which was not replicated because of limited soil quantities. The 6.25 and 12.5% treatments follow a similar trend to the other soil dilutions (data not shown). (◇) 100%, (○) 50%, (▲) 25%, (△) 3.1%, (X) Biotreated, (○) Control.

Acute toxicity testing

In the control soils, *E. fetida* survival was 97 to 100% (Fig. 1). The 97% survival in the PAL soil resulted from one worm death on day 3. As the proportion of contaminated soil increased, survival times of the earthworms decreased (Fig. 1). All worms exposed to any amount (3.13% or more) of contaminated EDM soil were dead by day 7 and by day 4 when exposed to 3.13% or more of PAC soil. When exposed to 100% contaminated EDM or PAC soils, all earthworms died within 1 to 2 d. In contrast, all earthworms survived for 14 d, and 50% survived for 41 d in 100% contaminated PAL soil (Fig. 1). The EDM and PAC soils appeared to be acutely toxic, but the PAL soil was not.

Biotreatment eliminated acute toxicity in the EDM soil but not in the PAC soil (Fig. 1). All earthworms survived for 14 d in the EDM biotreated soil, but all died within 2 d in the PAC biotreated soil. Worms in the EDM biotreated soil were still alive at day 51 when the experiment was terminated.

Earthworm activity decreased with increasing concentration and time of exposure to the contaminated soils. Worms burrowed into only the 3.13% contaminated, 100% pristine, and the biotreated EDM soil. Subsurface worms were active and responsive to touch during the daily inspection of each soil. Worms that did not burrow displayed limited to no response when probed. In the PAC 100% treatment, little to no earthworm movement occurred, even after a few hours of exposure to the creosote-contaminated soil.

Earthworm-days and DEO

We define 1 earthworm-day as one worm surviving for 1 d. Earthworm-days were calculated as the cumulative survival days for each worm summed over all worms. For example, 30 worms all surviving for 14 d would correspond to 420 earthworm-days. An earthworm-day is a survival–time variable. Earthworm-days allow comparisons among survival rates, dilution factors, contaminant concentrations, and time.

Earthworm-days were calculated for all dilutions in all soils and are compared to DEO and total measured PAHs. Earthworm-days in the PAL soil, with a DEO content up to 0.043 ± 0.004%, were 97% of the maximum possible of 420 (Fig. 2). In contrast, at the lowest content of DEO in the EDM soil (0.050 ± 0.003%), earthworm-days dropped sharply to 30% of maximum followed by a slower progression to zero as DEO increased. Similarly, at DEO = 0.029 ± 0.002% in the PAC soil, earthworm-days dropped to 13% of maximum, lower than the value for the PAL soil with even more DEO. Little difference was observed between the EDM and PAC soils in the
Earthworm-days were still at 100% of the maximum possible. Canada (EDM); (% decrease in total PAH concentration, relative to the EDM soil, earthworm-days tended to be slightly higher in EDM than PAC for similar PAH concentrations (Fig. 2). EDM-Bio and PAL soils stood out with regard to earthworm-days and total PAHs. The EDM-Bio soil had a significantly lower DEO (0.87±0.01%) than the PAC-Bio soil (0.97±0.01%), which suggests different ratios of PAH to DEO in the two soils.

Earthworm-days and total PAHs

As observed with the DEO data, increasing quantities of PAHs were associated with reduced earthworm-days in the toxicity tests. Only a slight separation occurred between the EDM and PAC soils for the range of total PAHs analyzed, but earthworm-days tended to be slightly higher in EDM than PAC for similar PAH concentrations (Fig. 2).

As with relations between earthworm-days and DEO, the EDM-Bio and PAL soils stood out with regard to earthworm-days and total PAHs. The EDM-Bio soil had a significantly lower DEO concentration than the PAC-Bio soil. In contrast to the DEO data, where the content of DEO in EDM-Bio was similar to that in PAC-Bio, the PAH content of EDM-Bio was substantially lower than for the PAC-Bio soil (Fig. 2). This suggests different ratios of PAH to DEO in the two soils.

DISCUSSION

Recovery of PAH

Lower-than-expected recovery of PAHs from the standard sediment sample might be due to analyte loss through the sample cleanup procedure, possible losses to the boiling chips, or the higher-molecular-weight PAHs being sorbed to the Florisil packing. After Soxhlet extraction, our cleanup procedure differed from that used by the NRC. In the NRC procedure, the extract was added to a column of silica and copper powder and eluted with 20 and 40% solutions of dichloromethane (DCM) in diethyl ether. The eluate was transferred to a column of Sephadex LH-20 gel and eluted with a 6:4:3 mixture of cyclohexane, methanol, and DCM. In our cleanup procedure, only a LC-Florisil cartridge was used. We consider these data appropriate for comparing among treatments, but caution against their use as absolute values.

60% water-filled pore space and worm behavior

Some standard bioassays use a water content based on adding water as a percentage of the mass of soil [24]. Other bioassays use a percentage of water-holding capacity as the desired level of hydration [25,26]. Soils that differ in texture have a different balance between air- and water-filled pores if water is added based on a percentage of water-holding capacity. Soils that differ in texture have a different balance between air- and water-filled pores if water is added based on a percentage of the mass of soil. Water is added based on a percentage of the mass of soil [24].
sorb to some pore surfaces in soil. This hydrophobic character results in underestimation of water-holding capacity and hence low water supply to organisms during bioassays. Water-filled pore volume was chosen as the criterion for soil moisture because it would not be influenced by oily contaminants, and it would provide a constant soil aeration and water balance in soils that differed in texture.

Worms were often found actively entangled in earthworm balls and were mobile under the surface. Some worms were also engaged in reproductive behavior as described by Sims and Gerard [34]. Earthworm behavior was interpreted to be consistent with healthy worms engaging in regular behavior patterns.

Earthworms react to lighted conditions [22] and, when exposed to strong light, respond by the withdrawal reflex [35]. Worms burrowed into the control soils under lighted conditions. A preference for the worms to remain exposed to lighted conditions rather than burrowing into contaminated soil implies sensitivity to the chemicals within the soil. Acute toxicity may have resulted from toxic lower-molecular-weight volatile and/or water-soluble compounds such as naphthalene or phenolic compounds. Other lower-molecular-weight compounds may have been dermally irritating to earthworms, resulting in a lack of burrowing activity after exposure to contaminated soil. Edwards and Bohlen [22] comment that chemicals such as acids, pesticides, and detergents are irritants that will cause earthworms to surface.

Based on earthworm behavior and survival in the two very different control soils, we conclude that a WFP = 0.6 was an appropriate and preferable way to maintain a balanced supply of air and water.

**PAH:DEO relationship**

Total extractable organic values yield data on the total quantities of extractable organic material, but they do not provide the identities of compounds within a mixture. As a class, PAHs represent 85% (w/w) of the components that can make up a creosote mixture, excluding the carrier [12]. It was hypothesized that some PAHs may have correlated with the constituents that contributed to the observed acute toxicity found in the EDM, PAC, and PAC-Bio soils. The potential for PAHs to act as carcinogenic, mutagenic, and genotoxic agents also prompted further investigation into the relationship between 16 individual PAHs and earthworm toxicity.

We found the ratio of PAH to DEO to vary between soils and with biotreatment of the soils. The lowest ratio was found in the EDM-Bio soil, which was the least toxic. We found the highest PAH:DEO ratio in the PAC and PAC-Bio soils, which were the most toxic.

Partitioning of contaminants into soil organic matter is often thought of as a mediating parameter of toxicity in soils [36]. More recent experimental results suggest that the presence of a nonaqueous-phase liquid may be even more important in controlling sorption and desorption mechanisms of PAHs than soil organic matter [5]. The nonaqueous-phase liquid present in soil could be functioning as a sink for toxic contaminants and thereby decreasing the bioavailability of toxincants. As persistent organic chemicals age in the soil, their potential to be biodegraded as well as their bioavailability to microorganisms [7] and to earthworms [20] can decline.

Roos et al. [37] examined the bioavailability of PAHs in contaminated soils to rats as well as the bacterium *Vibrio fischeri*. However, total PAHs did not correlate well with the measured toxicity to *V. fischeri*. Using the ratio of PAH to total organic carbon (TOC), Roos et al. [37] observed that toxicity, as measured by bioluminescence inhibition, increased linearly as the PAH:TOC ratio increased. This trend suggests that TOC may retard the release of the PAHs and thereby decrease bioavailability and the measured toxicity in bacteria. Caution must be exercised when making cross-species extrapolations, as mammalian bioavailability of soil-bound PAHs may be only marginally influenced by soil properties [37].

We believe that, at least in part, the toxicity of constituents in these soils was related to PAH:DEO ratios. The discussion to follow provides examples related to worm mortality and biotreatment.

**Mortality and PAH:DEO**

Contaminant concentration is often related to mortality. We calculated cumulative worm mortality over 14 d using Equation 5 and related it to contaminant concentration using a rectangular hyperbolic function (Eqn. 6):

\[
WM = 420 - WD
\]

\[
WM = \frac{WM_{\text{max}} \times C}{K_c + C}
\]

where WM = worm mortality (cumulative days), WD = earthworm-days, \(WM_{\text{max}}\) = maximum possible worm mortality, C = DEO (%) or PAH (mg/kg), and \(K_c\) = a half-saturation constant.

The 14-d time period yields 420 for \(WM_{\text{max}}\) when 30 worms are used, as here. The constant, \(K_c\), is the concentration of contaminant when WM = 50% of \(WM_{\text{max}}\). Consequently, \(K_c\) is conceptually similar to a 50% median lethal concentration (LC50) but is not identical to LC50 determined using a dose response at a fixed time. The difference arises from the inclusion of intermediate times between \(t = 0\) and \(t = 14\) in the cumulative survival–time data used here, in contrast to the single point survival at \(t = 14\) used in time endpoint approaches. Therefore, we call the \(K_c\) parameter LC50_{cum}. Survival–time variables fall in the category of time–response approaches to toxicity in contrast to dose–response approaches that use a one-time endpoint [38]. Many time–response approaches seek to derive a time to death value. We did not do so. Instead, we chose to estimate the LC50_{cum} as a way to compare among soils and contaminants.

Parameter values for Equation 6 were calculated from both the DEO and the PAH data using a nonlinear least squares analysis [33] and are reported in Table 4. The rectangular hyperbola model conformed to the data, and correlations were high (\(R^2 \geq 0.97\) for DEO and 0.999 for PAH). Estimated values of \(WM_{\text{max}}\) approximated the actual values more closely for PAH than for DEO, suggesting a better fit for the PAH data. The parameter estimates for the DEO data differed little if any between the EDM and PAC soils. In contrast, \(K_c\) for PAH (\(K_{c_{\text{PAH}}}\)) was larger in the EDM soil than in the PAC soil. A larger value of \(K_{c_{\text{PAH}}}\) means that more PAH is needed to cause a similar cumulative mortality. The higher \(K_{c_{\text{PAH}}}\) value provides a quantitative measure for the observation of apparently greater cumulative survival of worms in the EDM than PAC soil at similar PAH concentrations (Fig. 2). A high \(K_{c_{\text{PAH}}}\) value and hence higher survival of worms may be due to lower bioavailability or a lower inherent toxicity suite of PAHs present. Although Sved et al. [14] observed that a high-molecular-weight fraction obtained by distillation from creosote was more
toxic to fish than was the low-molecular-weight fraction, lower-molecular-weight PAHs or water-soluble constituents are expected to be more toxic [17,39]. The PAC soil has a greater proportion of low-molecular-weight PAHs, especially naphthalene, than does EDM soil (Fig. 3). Sorption of contaminants onto soil constituents or sequestration within micropores is believed to reduce bioavailability [3–6]. The EDM soil has a higher clay content and organic C content, both of which may sorb toxic organics and reduce their bioavailability. The DEO component may be a competing sink for toxicants and thereby reduce their bioavailability. For example, Boyd et al. [2] reported that the partition coefficient of pentachlorophenol into residual oil in soil is about 10 times greater than for soil organic carbon. Reported that the partition coefficient of pentachlorophenol into residual oil in soil is about 10 times greater than for soil organic carbon.

### Bioremediation

Biotreatment reduced PAHs and DEO in EDM, and all the worms survived for 52 d, but in PAC soil biotreatment failed to reduce DEO or PAH, and all the worms died. Rutherford et al. [5] concluded that low microbial competence limited biodegradation in the sandy PAC soil. The reduction in toxicity in the EDM-Bio soil was greater, however, than would be predicted from the reduction of DEO or PAH (Figs. 1 and 2). Why might this greater reduction in toxicity occur, and what does it say about concentration-based remediation endpoints? First, the biotreatment may have preferentially removed the most toxic constituents. If so, the low-molecular-weight PAH constituents would be expected to form a higher mole fraction in the EDM than in the EDM-Bio soil. The EDM-Bio soil tended to be enriched in the higher-molecular-weight PAHs (Fig. 4). With the exception of naphthalene, the low-molecular-weight PAHs were proportionally more abundant before than after biotreatment. Thus, it is possible that if molecular weight is a satisfactory surrogate for relative toxicity, biotreatment may have preferentially removed the more toxic constituents. Second, biotreatment may have favored sequestration of PAHs by sorption into DEO. A lower PAH:DEO ratio after biotreatment. Thus, it is possible that if molecular weight is a satisfactory surrogate for relative toxicity, biotreatment may have preferentially removed the more toxic constituents. Second, biotreatment may have favored sequestration of PAHs by sorption into DEO. A lower PAH:DEO ratio after biotreatment confirms that PAHs were degraded faster than the residual DEO. A lower PAH:DEO ratio appears to be associated with reduced toxicity of PAHs in these soils, as seen when comparing EDM and PAC soils. These data are consistent with conclusions of reports in the literature [17,40] and demonstrate that concentration data alone without consideration of soil conditions, history, or other constituents may lead to erroneous conclusions about toxicity.

### CONCLUSIONS

Total PAHs decreased significantly in the EDM soil after 52 d of slurry-phase biotreatment. The PAC-Bio soil did not have the same reduction of creosote constituents. Unfavorable soil habitat for microorganisms as well as surface area for sorption of contaminants may be influencing degradation and availability of contaminants to microorganisms. Biotreatment was able to reduce the observed acute toxicity in EDM-Bio

### Table 4. Parameters ± 95% confidence limits for predicting worm mortality in EDM and PAC soils. WM<sub>max</sub> and K<sub>c</sub> values were determined using the NLIN procedure in SAS<sup>b</sup> [33]<sup>a</sup>

<table>
<thead>
<tr>
<th>Soil</th>
<th>WM&lt;sub&gt;max&lt;/sub&gt; (earthworm-days)</th>
<th>K&lt;sub&gt;c&lt;/sub&gt; (g/100 g or mg/kg)</th>
<th>R&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDM</td>
<td>436 ± 50</td>
<td>0.042 ± 0.022%</td>
<td>0.973</td>
</tr>
<tr>
<td>PAC</td>
<td>468 ± 53</td>
<td>0.015 ± 0.009%</td>
<td>0.976</td>
</tr>
<tr>
<td>EDM</td>
<td>401 ± 7.1</td>
<td>10.4 ± 1.7 mg/kg</td>
<td>0.999</td>
</tr>
<tr>
<td>PAC</td>
<td>425 ± 1.8</td>
<td>6.92 ± 0.46 mg/kg</td>
<td>0.999</td>
</tr>
</tbody>
</table>

<sup>a</sup> See Table 1 for definitions of acronyms.

<sup>b</sup> Parameters for the equation: WM = WM<sub>max</sub> × C/(K<sub>c</sub> + C) where WM = worm mortality (earthworm-days); WM<sub>max</sub> = maximum WM (earthworm-days); C = concentration of total dichloromethane extractable organics (DEO) (g/100 g) or polycyclic aromatic hydrocarbons (PAH) (mg/kg); and K<sub>c</sub> = value of C when WM = WM<sub>max</sub>/2. In a 14-d assay with 30 worms, the maximum possible value of WM would be 30 × 14 = 420 earthworm-days if all worms died on the first day.

See Table 1 for definitions of acronyms.
but not in PAC-Bio. Therefore, soils that have gone through a biotreatment process may remain acutely toxic to *E. fetida*. Concentrations of total PAHs were reduced to a greater extent compared to the DEO concentration for the EDM-Bio soil.

The sole use of DEO or total PAH concentration data did not precisely predict the eventual toxic consequences of creosote contamination to soil organisms such as *E. fetida*. Toxicity predictions based solely on analytical chemical data may poorly correlate to measured toxicity based on biological determinations, as was also evident with the EDM-Bio soil. The PAH data were more closely related to toxicity than the DEO for the soils used here. However, DEO seems to have an essential role in the regulation of toxicity, as expressed in the PAH:DEO ratio. Soils with low PAH:DEO (EDM-Bio and PAL) were less toxic, as measured by earthworm-days, compared to soils with higher ratios (EDM, PAC, and PAC-Bio). Bioavailability of organic toxics, such as PAHs, appears to be reduced in the presence of a residual nonaqueous-phase liquid, at least for the soils studied here. Potential exists for using the PAH:DEO ratio for *E. fetida* as a better predictor of toxicity compared to total PAH or DEO concentrations alone; however, specific experiments need to be conducted to test this conjecture.

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**REFERENCES**


