SETAC PRESS

Environmental Toxicology and Chemistry, Vol. 30, No. 4, pp. 988–996, 2011

Printed in the USA
DOI: 10.1002/etc.470

EFFECTS OF AGING AND MIXED NONAQUEOUS-PHASE LIQUID SOURCES IN SOIL SYSTEMS ON EARTHWORM BIOACCUMULATION, MICROBIAL DEGRADATION, SEQUESTRATION, AND AQUEOUS DESORPTION OF PYRENE

ELIJAH J. PETERSEN, JIXIN TANG, and WALTER J. WEBER JR.*
Department of Chemical Engineering, The University of Michigan, Ann Arbor, Michigan, USA

(*Submitted* 28 *June* 2010; *Returned for Revision* 13 *September* 2010; *Accepted* 26 *November* 2010)

Abstract—The effects of loading and aging pyrene in soils in the presence of four environmentally common nonaqueous-phase liquids (NAPLs) (hexadecane, 2,2,4,4,6,8,8-heptamethylnonane [HMN], toluene, and dimethyl phthalate [DMP]) on its subsequent desorption from those soils, earthworm accumulation, biodegradation, and extractability were tested by using two dissimilar soils. The presence of each of the four NAPLs increased fractions and rates of pyrene desorption, and hexadecane slowed the effects of aging on these same parameters. Loading with hexadecane and HMN caused earthworm accumulation of pyrene to decrease. These results contrast with generally observed faster desorption rates resulting from NAPL addition, suggesting that additional factors (e.g., association of pyrene with NAPL phases and NAPL toxicities to earthworms) may impact bioaccumulation. The presence of HMN and toluene increased pyrene biodegradation, whereas hexadecane and DMP had the opposite effects. These results correlate with changes in the extractability of pyrene from the soils. After aging and biodegradation, hexadecane and DMP substantially increased pyrene residues extractable by methanol and decreased nonextractable fractions, whereas HMN and toluene had the opposite effects. Environ. Toxicol. Chem. 2011;30:988–996. © 2011 SETAC

Keywords—Bioavailability Polycyclic aromatic hydrocarbons Phenanthrene

INTRODUCTION

Widespread contamination of the environment by hydrophobic organic compounds (HOCs) has frequently resulted from spills or disposal of tar-like and oily organic compounds in surface and subsurface soil and sediment systems [1]. Polycyclic aromatic hydrocarbons (PAHs) have been identified as a dominant constituent of many nonaqueous-phase liquids (NAPLs), particularly coal tars, petroleum fuels, and organic solvents; because of their respective toxicities and carcinogenicities, they may pose significant hazards to the environment and to human health. Therefore, assessing the exposure and environmental risks of NAPL-associated PAH contaminants is important to fully understand their environmental fates, biodegradation rates, and bioavailabilities to ecological organisms in surface and subsurface systems.

Extensive evidence has been compiled to indicate that PAHs and other HOC contaminants in the environment are readily sorbed by various forms of geosorbents, particularly those containing significant levels of soil/sediment organic matter (SOM). The extent and reversibility of such sorption phenomena are highly dependent on contact times and on the physicochemical properties and macromolecular structures of the associated SOM [2–5]. Quantities of HOC contaminants sorbed by soils and the formation of nonextractable residues have been observed to increase with times of exposure and with amounts and degrees of condensation of associated SOM [6–9]. This results in smaller fractions desorbed by water (readily available fractions) or extractable by organic solvents (potentially available fractions) and thus decreased HOC availability to ecological organisms with increased aging [10,11]. Similarly, increasing binding with SOM over time decreases PAH availability to most microorganisms and thus the extent of biodegradation [3,7,12]. The effects of aging on the environmental mobility and bioavailability of HOCs in the presence of NAPLs have not been fully studied.

When present in soil systems at sufficiently high concentrations, NAPLs can, like SOMs, function as discrete sorbents for HOC contaminants [13–15]. Nonaqueous-phase liquids–associated HOC contaminants transfer into the aqueous phase, and rates and extent of mass transfer of HOC contaminants from NAPLs depend on the viscosities, densities, hydrophobicities, concentrations, and biodegradability of the NAPLs themselves [13,16,17]. Nonaqueous-phase liquids also enter and occupy pores in soil particles. Small micropore diameters and tortuous diffusion paths may in such cases constrain diffusion and in turn significantly limit mass transfer of associated HOC contaminants into soil pore water, thus diminishing contaminant availabilities to soil organisms [13,18–20] For example, uptake of benzo[*a*]pyrene by earthworms (*Eisenia fetida*) has been reported to decrease in the presence of NAPLs [18]; however, effects of aging in the presence of the NAPLs were not investigated. Nonaqueous-phase liquids also may impact degradation of associated HOC contaminants through enzyme inhibition, membrane modification, extraction of cell wall components, or coating of cells by organic phases [21]. Furthermore, NAPL may itself serve as a carbon source for microorganisms and thus be degraded in preference to HOC substrates; for example, hexadecane has been observed to delay or completely inhibit the mineralization of pristane [22] and phenanthrene [17] by microorganisms while itself being metabolized. Conversely, some microorganisms are able to adhere to interfaces between hydrophobic and hydrophilic phases to overcome mass transfer limitations [23] for HOC compounds from NAPLs and act on sorbed HOCs without requiring prior desorption [24], thus

All Supplemental Data may be found in the online version of this article.

* To whom correspondence may be addressed (wjwjr@umich.edu).

Published online 1 February 2011 in Wiley Online Library (wileyonlinelibrary.com).

accelerating degradation of NAPL-associated or sorbed contaminants. In addition to HOC mineralization, some fractions of NAPL-associated contaminants can become sequestered within organic matrices through sorption, covalent binding, or hydrophobic interaction and entrapment during biodegradation, forming nonextractable residues [25,26]. The impacts of NAPL characteristics on such processes are, however, largely unknown.

This study was designed to investigate the combined effects of NAPL characteristics and aging on desorption, biodegradation, mobility, and bioavailability to *E. fetida* of a representative PAH, pyrene. Pyrene was spiked to soils and aged in the presence of one of four different NAPLs at NAPL concentrations ranging from 1 to 10% (v/w), and its bioaccumulation by *E. fetida* was measured, as well as the extent and rates of its aqueous dissolution/desorption, microbial degradation, and distribution in soils. This is, to the authors' knowledge, the first study to investigate the effects of aging HOCs in the presence of various NAPLs on this broad range of environmentally critical behaviors tested in concert. Although numerous previous studies have focused on one or two aspects of the effects of NAPLs in soils on co-contaminants, investigation of such phenomena acting together can yield insights into the interrelationships among these various processes.

MATERIALS AND METHODS

Materials

Two sandy loam soil samples having different organic carbon (OC) contents were employed: North Campus (NC) soil (2.47% OC) and Chelsea soil (5.95% OC). Descriptions of the collection and sterile treatment of these two soils were given in a previous paper [27]. Unlabeled and radiolabeled (9-^{14}C) pyrene reagents were purchased from Sigma-Aldrich Chemical. Four commonly used NAPLs, hexadecane, 2,2,4,4,6,8,8-heptamethylnonane (HMN), toluene, and dimethyl phthalate (DMP), were chosen to provide different structures, solubilities, densities, and volatilities, properties that may affect partitioning and bioavailability of pyrene dissolved in NAPLs. These well-defined NAPL phases were selected instead of more complex, though likely more environmentally relevant, mixtures such as crude oils or coal tars [28,29], with the intent that their simpler, more homogeneous compositions could lead to insights between their respective physicochemical properties and the observed environmental behaviors of pyrene. Some of these important properties are presented in the Supplemental Data, Table S1. Table S1 also includes NAPL–water distribution coefficients measured similarly to the shake-flask method for determining octanol–water distribution coefficients [30]. Vials were filled with a water solution with dissolved pyrene (0.0135 mg/L) and NAPL at 3% v/v, capped, and shaken horizontally at 100 rpm for the time of the test. Vials were sacrificed at 7 and 14 d; after 14 d, equilibrium was reached for toluene and DMP, whereas it had not yet been reached for hexadecane and HMN. Duplicates of 2-ml aliquots from the aqueous phases were removed after centrifugation of vials at 10 *g* for 5 min. The aliquots were each mixed with 4 ml Instagel scintillation cocktail and analyzed for residual ^{14}C by liquid scintillation counting (LSC). Concentrations of pyrene in the NAPL were determined by mass balance.

Test organisms

Earthworms (*E. fetida)*, obtained from the Carolina Biological Supply, were used to assess the availability of the sorbed pyrene to biological uptake. *Eisenia fetida* were maintained in worm bedding at $21 \pm 2^{\circ}$C and kept moist with deionized water. The worms were fed twice per week with food consisting of a mixture of crude proteins and carbohydrates (Magic Worm Products).

Soil spiking and aging

The radiation-sterilized soils were spiked aseptically in sterilized 250-ml wide-mouth glass jars with pyrene in unlabeled and ^{14}C-labeled forms dissolved in hexadecane, HMN, toluene, DMP, and dichloromethane to yield a final pyrene soil concentration of 100 μg/g and a radioactivity of 1.7 kBq/g soil. The jars were agitated on an end-over-end shaker for 24 h to ensure thorough mixing of their contents. For soil samples spiked with pyrene in hexadecane, HMN, toluene, or DMP, these solvents were maintained in NC soil as NAPLs at a level of 5.0% (v/w). The concentration of HMN was varied in a separate set of experiments by adding pyrene dissolved in HMN to NC soil at HMN concentrations of 1.0, 3.0, or 10% (v/w). The experiments with different NAPLs at 5.0% (v/w) and the different concentrations of HMN were only conducted in NC soil. For soil samples spiked with pyrene dissolved in dichloromethane, the lids of the jars were removed, replaced with sterilized aluminum foil, and the solvent evaporated in a fume hood. These samples were used as reference samples containing no NAPLs. Autoclaved Milli-Q water (Millepore) was added to provide a moisture level of 22% (v/w; moisture measurements were determined by mass loss after drying at 110°C for at least 24 h), and the samples were then mixed and transferred aseptically to sterilized 125-ml glass jars. The jars were then sealed with sterilized screw caps fitted with Teflon® liners and stored in the dark at $10 \pm 1^{\circ}$C to initiate the aging process. Each jar was filled until it was almost completely full to minimize headspace and minimize NAPL and pyrene volatilization during the aging period. At the end of the aging process, freshly spiked samples were made by adding pyrene dissolved in dichloromethane to clean samples of the NC and Chelsea soils, allowing the dichloromethane to evaporate, and moistening and holding the soils for 1 d before use. At the beginning and end of the aging period and for the freshly spiked soils, duplicate or triplicate samples of aged soils were taken for measurements of moisture, high temperature combustion, and extraction by methanol.

Aqueous desorption

The mobility of NAPL-associated pyrene from aged soil samples was determined by its aqueous dissolution/desorption, using C18 membranes to create infinite-sink desorption conditions. *Dissolution* is a term typically used to describe the movement of organic chemicals from NAPLs into the water phase, whereas the term *desorption* is similarly used for contaminant releases from soils. Our experimental setup did not explicitly differentiate between dissolution and desorption phenomena, so pyrene available in the aqueous phase in these experiments may have resulted from dissolution, desorption, or both of these factors. The term *desorption* is used here to simplify ensuing discussions. Empore extraction C18 membrane disks (polytetrafluoroethylene) impregnated with a bonded octadecyl silica sorbent and demonstrated to possess strong sorption capacities for PAHs and other HOC compounds [31,32] were purchased from Alltech Associates. Each of duplicate soil samples (0.5 g dry wt) was transferred to a 50-ml glass centrifuge tube containing 45 ml of 0.01 M $CaCl_2$ aqueous solution (with NaN_3 at 200 mg/L to prevent microbial

degradation) and having a Teflon-lined cap. A C18 membrane disk (47 mm) was cut into four pieces and conditioned in methanol for 30 min and rinsed twice with 400 ml deionized water before it was placed in the centrifuge tube. The tubes were shaken in an end-over-end shaker, and the C18 membranes were replaced at predetermined intervals. On each occasion, the test tubes were placed on the laboratory bench for 30 to 60 min to allow soil particles to settle. The membranes were then gently removed from the tubes and rinsed with deionized water, dried on a paper towel, and transferred to a 7-ml glass vial. Methanol (2.0 ml) was added to elute sorbed pyrene, and 1 ml of this methanol was taken to measure ^{14}C-radioactivity by LSC, using an LS6500 liquid scintillation counter (Beckman).

Earthworm bioaccumulation

The bioavailability of pyrene in each soil sample was determined by earthworm accumulation using a modified standard method [33]. Three adult worms were transferred to moist (22% water) soil samples (75 g) in 250-ml glass jars. The jars were loosely closed with a cap to prevent worm escape and allow air exchange, then were held in the dark for 14 d at $21 \pm 1^{\circ}$C for measurements of pyrene uptake; this soil-to-earthworm ratio is the same as or higher than that used in many of our recent studies [27,34–36]. Three replicates were tested for each condition. Dimethyl phthalate and toluene caused 100% earthworm fatality at a soil concentration of 5% (v/w), and thus results for these NAPLs were not obtained. At the end of 14 d of exposure, the earthworms were taken from the soil samples and washed with Milli-Q water, transferred to wet filter paper in Petri dishes for 24 h in the dark to allow purging of gut contents, again rinsed with clean Milli-Q water, transferred to preweighed glass tubes, freeze-dried, and weighed for analyses of ^{14}C radioactivity.

Microbial degradation

A bacterial consortium was obtained by an enrichment culture technique from a PAH-contaminated sediment collected near an abandoned gasification plant using mineral medium amended with phenanthrene, anthracene, fluoranthene, and pyrene as carbon sources. The mineral medium comprised 0.8 g K_2HPO_4, 0.2 g KH_2PO_4, 0.2 g $FeCl_3 \cdot 6H_2O$, and 0.1 g each of NH_4NO_3, $MgSO_4 \cdot 7H_2O$, and $CaCl_2 \cdot 2H_2O$ per liter of water [37]. Previous studies showed that this bacterial consortium was able to degrade pyrene [37].

Microbial inocula were prepared as reported previously [37]. Briefly, the bacterial consortium was transferred into a mineral medium containing pyrene as the sole carbon source and incubated at 30°C on a rotary shaker operating at 100 rpm for 14 d. The cells were collected by centrifugation, washed with the mineral solution, and resuspended in the medium. For degradation of pyrene in soil, a 2.5-ml portion of the cell suspension of bacterial consortiums containing 10^6 to 10^7 cells/ml was inoculated into 250-ml glass flasks containing 80 ml mineral medium and 15 g (dry wt) pyrene-contaminated soil samples; the soils had been aged under sterile conditions before microorganism addition. The flasks were fitted with Teflon tape-wrapped silicon stoppers with an 18-gauge needle and a cannula inserted through each stopper. A 2.0-ml glass vial was attached to the end of the cannula that was put inside of the flask. A 1.5-ml quantity of 0.5-N NaOH was injected through the cannula into the vial, using a 5.0-ml syringe. The alkali solution was removed periodically and replaced with fresh NaOH solution. The NaOH solution was transferred into a 7-ml glass vial and mixed with 5.0 ml scintillation cocktail for determination of its radioactivity.

At the conclusion of the biodegradation experiment, 3.0 ml of 1 M HCl was added to each flask to arrest bacterial growth and remove CO_2 residues from the medium. The reactors were immediately closed with stoppers, mixed by hand, and set overnight to allow solid soil phase separation from the liquid phase. The remaining alkali solution in the 2-ml glass vials was sampled for measuring ^{14}C activity from the $^{14}CO_2$. A 2.0- or 5.0-ml aliquot of the supernatant from the flasks was taken to mix with 5 or 15 ml scintillation cocktail to measure the radioactivity remaining in the aqueous medium. The remainder of the supernatants were carefully decanted from the flasks and weighed for each flask. The soil sample remaining in each reactor was freeze dried for measurement of total ^{14}C radioactivity, aqueous desorption, methanol extraction, and high-temperature combustion of pyrene and its metabolites. These experiments were performed only with NC soil.

Extraction and chemical analysis

Residual concentrations of pyrene were determined for all soil samples before and after aging and uptake experiments. Soil samples (1.5 g) were freeze dried in 35-ml glass centrifuge tubes and extracted first with 20 ml methanol for 3 h, then with an additional 10 ml methanol for another 2 h. The methanol was carefully decanted each time, and 2 ml of the methanol was taken for measuring ^{14}C radioactivity by LSC; 1 ml methanol was used for high-performance liquid chromatograph (HPLC) analysis to determine the concentration of the pyrene parent compound. Pyrene concentrations in the methanol extracts were analyzed using an Agilent 1100 high-performance liquid chromatograph with a 125×3.20 mm Envirosep PP C18 column (Phenomenex) and an ultraviolet or fluorescence detector. An acetonitrile–water (80:20, v/v) mixture was used as the mobile phase at 0.8 to 1.0 ml/min. After methanol extraction, the soil samples were combusted in an OX-500 Biological Oxidizer (R.J. Harvey Instrument) to measure the non-extractable pyrene fractions. This process combusts material at elevated temperatures and then captures the released carbon 14 dioxide in scintillation fluid. The concentration of radioactive pyrene in earthworm samples was similarly analyzed. Mass balances were performed for the soils before and after the aging and earthworm exposures, and pyrene recovery ranged from 86 to 97%, thus indicating good recovery.

The distribution of pyrene residues and its metabolites in the NC soil samples after aging and microbial degradation was determined by aqueous desorption (readily available fractions), methanol extraction (potentially available fractions), and non-extractable fractions (nonavailable fractions) [10,32,38]. Soil samples (2–3 g) transferred into preweighed 35-ml glass centrifuge tubes were first extracted with 30 ml 0.01 M $CaCl_2$ aqueous solution (with NaN_3 of 200 mg/L to inhibit microbial growth) for 48 h, then the tubes were centrifuged at 2,200 *g* for 15 min, an aliquot of 2 ml supernatants was transferred to 7-ml plastic vials for LSC, and the remainder of the supernatants was gently decanted. The soil samples were then sequentially bath sonicated with 20 and 15 ml methanol. The tubes were centrifuged at 2,200 *g* for 15 min after each extraction, and methanol solutions were maximally decanted and pooled in 50-ml glass tubes. An aliquot of 2 ml methanol solutions was mixed with 5 ml LSC cocktail. Finally, the tubes were weighed, and the remaining soil samples were dried in a freeze-drier. The ^{14}C radioactivity remaining in each soil sample was determined by biological oxidation, and this radioactivity was referenced as

the nonextractable fraction of pyrene and its metabolites. Percentages of pyrene mineralized and extracted in different fractions were calculated.

Data analysis

Bioavailability of pyrene was determined by quantities accumulated in dry worm tissues. Statistically significant differences among values of three replicates for the measurements were determined using a one-way analysis of variance procedure in conjunction with a Duncan multiple range test procedure in SAS (SAS Institute 2002).

Rates of pyrene desorption from soil samples were characterized using a three-parameter, two-component, first-order rate model developed by Johnson and Weber [39]

$$\frac{q_t}{q_0} = \phi_r \exp(k_{-r} t) + (1-\phi_r)\exp(-k_s t) \quad (1)$$

in which q_0 is the initial concentration of a chemical in a soil after the aging period but before initiation of the desorption experiment, q_t is the concentration of that chemical in the soil at time t after the desorption experiment started, ϕ_r is the fraction of that chemical released rapidly from the soil, $(1-\phi_r)$ is the fraction of chemical released slowly, k_r and k_s are first-order rate constants for the rapidly and slowly released fractions, respectively; the determination of these two different fractions is based on a large number of studies that indicate a rapidly desorbing contaminant fraction that occurs over a few hours or days and a slower fraction that can desorb over months or years [39]. Data for the fraction of pyrene desorbed as a function of time was fitted to the model, and desorption parameters were determined using a nonlinear regression model employing the Levenberg-Marquart least squares procedure in SAS.

RESULTS AND DISCUSSION

Aqueous desorption of pyrene

As shown in Table 1 and Figure 1 (A and B), the presence of 5% hexadecane significantly slowed the effects of pyrene aging in both NC and Chelsea soils. Despite large differences in percentages of organic carbon in the two soils, statistically significant differences in desorption parameters between the soils were rarely seen. For unamended soils, a statistically significant decrease was observed in the rapidly released fraction and the rapidly and slowly released rate constants (k_r and k_s, respectively) after 30 d of aging. In the presence of 5% hexadecane, however, no significant decrease was noted during that time frame between the fresh soil and the soil spiked with hexadecane. Only after the soils with hexadecane were aged for 167 d did the desorption parameters approach or exceed those for unamended soils aged for 30 d. This result likely stems from a large fraction of pyrene remaining associated with the hexadecane, which is more energetically favorable than soil pore water, and the more rapid dissolution compared with desorption from the soil. As such, NAPL appeared to slow pyrene sorption by the SOM in general and adsorption specifically by the hard carbon fraction, which, although it is most energetically favorable, is only a very slowly adsorbing and desorbing medium [2,4,5].

Although desorption rates varied substantially for soils exposed to different types of NAPL (see Table 2 and Fig. 1C), 5% NAPL addition for each NAPL type increased the fraction that was readily desorbed and the desorption rates of pyrene compared with the aged control without NAPL addition. 2,2,4,4,6,8,8-Heptamethylnonane showed the fastest desorption rates, and hexadecane showed the smallest increase. These results do not show any pattern relative to the NAPL properties listed in the Supplemental Data, Table S1. One possibility beyond the scope of this study is that of substantial variations in mass transfer coefficients for pyrene from different NAPL sources in the soil matrices as a result of aging. Dissolution from crude oils also can slow substantially during aging processes as a result of the formation of interfacial films [28], and thus changes in the different NAPLs during aging also may partly account for the observed differences.

Increasing the fraction of HMN added to the soils up to 10% in NC soil increased rapidly released fractions and desorption rates of pyrene (Table 2 and Fig. 1 D). This is likely a result of decreasing the fraction of pyrene associated with the soil organic matter as a result of the larger volume of NAPL present, which is more energetically favorable for pyrene than for pore water. The NAPLs at sufficiently high concentrations can be present as a discrete phase in environmental systems, a phase into which pyrene can partition [13–15]. This phase also may interact with SOM, and perhaps change their respective structures with increasing regularity as the HMN concentration increases.

Bioaccumulation of pyrene by earthworms

As shown in Table 3, the availabilities of pyrene to *E. fetida* in both NC and Chelsea soils were decreased by aging and the presence of hexadecane. The substantial decrease of pyrene accumulation by earthworms in the presence of NAPL is similar to what was observed for benzo[*a*]pyrene in soils in the presence of pristane or Sentry 19 oil [18]. The body burden values provided can readily be converted to bioaccumulation factors given the high pyrene recovery or biota-soil accumulation factor values using organic carbon values provided in the Methods section and an estimate of 2.0% lipid content determined in our laboratory previously [35]. Earthworm accumulation of pyrene was substantially smaller for Chelsea soil compared with NC soil for all conditions tested (Table 4), an expected result given the higher OC percentage for Chelsea soil and previous studies that indicated similar results [27,35]. Although addition of hexadecane slowed the aging process

Table 1. Nonaqueous-phase liquid (NAPL) and aging time effects on desorption of pyrene from different soils with 95% confidence intervals

	North Campus soil			Chelsea soil		
Soil treatment	ϕ_r[a]	k_r[b] (/d)	$k_s \times 10^{-2}$ (/d)	ϕ_r	k_r (/d)	$k_s \times 10^{-2}$ (/d)
Fresh	0.74 ± 0.03	0.29 ± 0.03	2.3 ± 0.7	0.73 ± 0.04	0.27 ± 0.02	1.5 ± 0.4
30-d No hexadecane	0.71 ± 0.03	0.17 ± 0.03	0.58 ± 0.32	0.64 ± 0.04	0.12 ± 0.02	0.52 ± 0.28
30-d + Hexadecane	0.74 ± 0.06	0.27 ± 0.04	2.0 ± 0.4	0.72 ± 0.05	0.27 ± 0.03	1.3 ± 0.3
110-d + Hexadecane	0.73 ± 0.04	0.18 ± 0.01	0.77 ± 0.29	0.67 ± 0.03	0.12 ± 0.01	0.57 ± 0.37
167-d + Hexadecane	0.70 ± 0.02	0.14 ± 0.01	0.35 ± 0.21	0.62 ± 0.03	0.10 ± 0.01	0.36 ± 0.22

[a] ϕ_r, rapidly released fractions.
[b] k_r and k_s are first-order rate constants for the rapidly released fraction and the slowly released fraction, respectively.

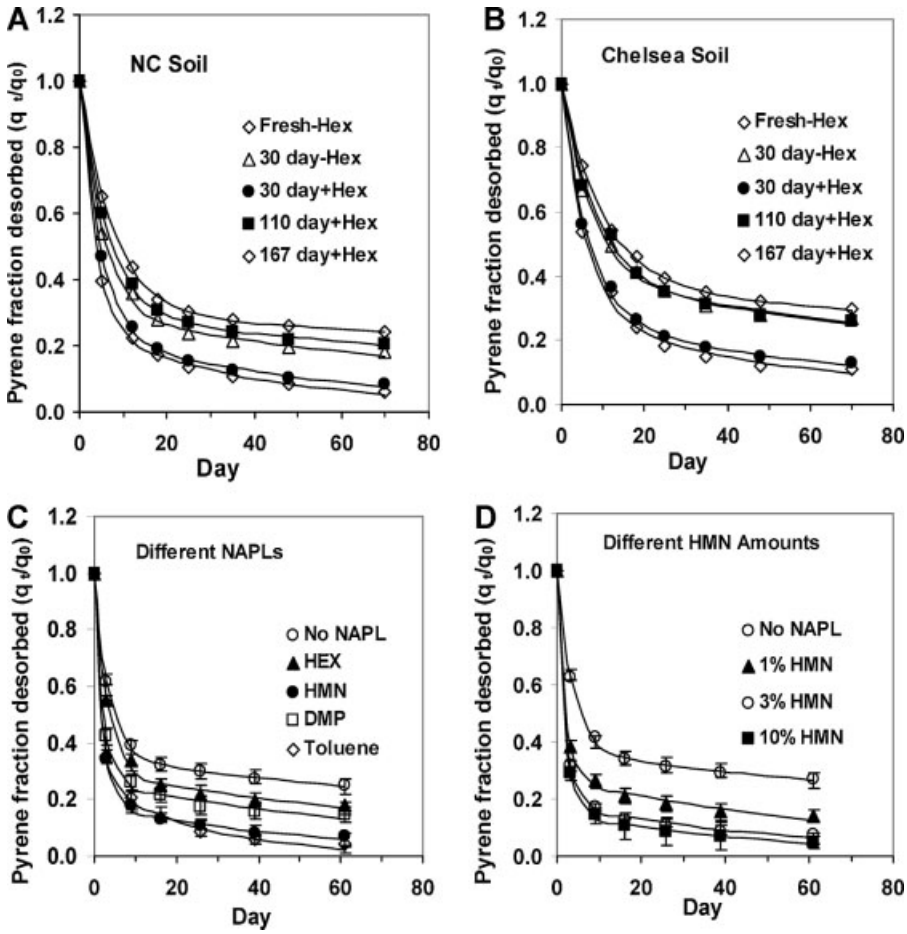

Fig. 1. Aqueous desorption of pyrene. Effects of aging for various durations in the presence of hexadecane (Hex) as nonaqueous phase liquids (NAPLs) in North Campus (NC) soil (**A**) and Chelsea Soil (**B**), the effects of aging for 145 d in Hex, 2,2,4,4,6,8,8-heptamethylnonane (HMN), toluene and dimethyl phthalate (DMP) (**C**), or in different concentrations of HMN (**D**). Each data point represents the average of duplicate data points, and error bars represent standard deviations.

of pyrene and accordant decreases in its desorption rates (Table 1), the presence of hexadecane also decreased uptake by earthworms in both soils to a much more substantial extent than aging of pyrene for 30 d without hexadecane (Table 4). This decrease in earthworm accumulation cannot as such be explained solely by relative changes in desorption rate parameters. Instead, the presence of NAPL likely affected earthworm health factors such as a smaller lipid fraction or lower feeding rates, even though the worms promptly burrowed into the soil under these exposure conditions. Pyrene aging and the addition of hexadecane in both soils caused decreases in dry earthworm masses (see Supplemental Data, Table S2a), although no substantial difference was seen in the earthworm masses for soils aged 30 days with or without hexadecane for Chelsea soil. The finding of mass decrease after pyrene aging was unexpected, but the purpose of this study was not to investigate the subacute

Table 2. Effect of different nonaqueous-phase liquids (NAPLs) and amounts on the desorption rate parameters of pyrene aged 145 d in North Campus soil with 95% confidence intervals

	Different NAPLs				
	No NAPL	Hexadecane	2,2,4,4,6,8,8-Heptamethylnonane	Dimethyl phthalate	Toluene
ϕ_r[a]	0.62 ± 0.04	0.71 ± 0.05	0.81 ± 0.06	0.74 ± 0.05	0.72 ± 0.04
k_r (/d)[b]	0.28 ± 0.02	0.32 ± 0.03	0.53 ± 0.04	0.48 ± 0.03	0.46 ± 0.03
$k_s \times 10^2$(/d)	0.52 ± 0.32	0.90 ± 0.46	2.0 ± 0.4	1.1 ± 0.3	0.63 ± 0.35
	Different 2,2,4,4,6,8,8-heptamethylnonane concentrations				
	0%	1%	3%	10%	
ϕ_r	0.62 ± 0.03	0.72 ± 0.06	0.79 ± 0.06	0.84 ± 0.06	
k_r (/d)	0.28 ± 0.02	0.58 ± 0.04	0.59 ± 0.04	0.65 ± 0.05	
$k_s \times 10^2$(/d)	0.52 ± 0.32	1.2 ± 0.3	1.8 ± 0.4	2.2 ± 0.6	

[a] ϕ_r rapidly released fractions.
[b] k_r and k_s are first-order rate constants respectively for the rapidly released fraction and the slowly released fraction.

Table 3. Effects of nonaqueous-phase liquids (NAPLs) and aging on the uptake of pyrene by earthworms in soils

	North Campus soil	Chelsea soil
Soil aging	Uptake (μg/g dry worm)	Uptake (μg/g dry worm)
Fresh	925 ± 45[a] A[b]	566 ± 34 A
30-d No hexadecane	610 ± 41 B	385 ± 27 B
30-d + Hexadecane	296 ± 22 C	212 ± 15 C
110-d + Hexadecane	220 ± 21 D	174 ± 13 D
167-d + Hexadecane	210 ± 18 D	127 ± 11 E

[a] Mean ± 1 standard deviation from triplicate samples.
[b] Values in columns followed by the same capital letter are not significantly different ($p < 0.05$).

toxicity of NAPLs to earthworms, and therefore the cause behind the finding was not further explored.

Pyrene bioaccumulation decreased in a concentration-dependent manner with increased HMN concentrations, as shown in Table 4, even though pyrene exhibited enhanced desorption with increasing concentrations of HMN. This again suggests that decreased earthworm accumulation cannot be explained solely by changes in pyrene desorption and that additional factors such as NAPL toxicity can impact bioaccumulation, a hypothesis supported by the decreasing earthworm masses with the addition of HMN (Supplemental Data, Table S2b). The presence of hexadecane decreased pyrene uptake significantly more than did HMN. These results indicate that the presence of different types and concentrations of NAPLs have a much stronger effect than that of pyrene desorption rates on earthworm bioaccumulation.

Biodegradation and distribution of pyrene in soils

Aging under sterile conditions also decreased subsequent pyrene biodegradation, whereas hexadecane substantially decreased biodegradation of pyrene in both soils (Table 5, Supplemental Data, Table S3, and Fig. 2A and B). These results were observed in both soils, and the higher percentage of organic carbon in the Chelsea soil did not have a large effect on the percent degraded in the unamended soils, although smaller percentages were degraded in Chelsea soil samples that were aged with 5% hexadecane. That desorption rates increased with addition of hexadecane suggests that some phenomenon related to addition of hexadecane, such as hexadecane potentially being preferentially metabolized by the organisms, as previously shown for pristane [22] and phenanthrene [17], was the cause of this behavior. The percentage of pyrene desorbed from the soil residues after the biodegradation experiment for soils aged with hexadecane 30 d was decreased by a factor of 1.8 (2.3 ± 0.2% compared with 4.9 ± 0.2%) compared with the aged but unamended sample, whereas the concentration that was extractable by methanol increased by a factor of 2.3 (57 ± 3% compared with 25 ± 2%). This may result from the methanol-extractable pyrene fraction being more bioavailable in the absence of hexadecane, but a large fraction of pyrene may have been associated with the methanol-extractable fraction of the NAPL phase. Hexadecane and methanol also may have acted as co-solvents, thus increasing the extractability of pyrene similarly to a previous study with phenanthrene aged in soil with diesel and extracted by an aqueous solution of hydroxypropyl-β-cyclodextrin [15]. Given the subacute toxicity of hexadecane and acute toxicity of DMP observed for earthworms in bioaccumulation experiments, likely the high NAPL concentrations used in this study were toxic to the microorganisms, which is perhaps the more likely cause for the slower mineralization in the presence of hexadecane.

The presence of 5% (v/w) of the different NAPLs had profoundly different effects on pyrene biodegradation rates after aging for 155 d (Table 5 and Fig. 2C). The presence of toluene and HMN increased pyrene degradation rates compared with the unamended control, whereas hexadecane and DMP decreased biodegradation. This result accords with previous studies that indicated faster phenanthrene degradation in the presence of HMN compared with hexadecane [17,40].

In samples spiked with hexadecane or DPM in which minimal degradation occurred, concentrations of pyrene in nonextractable residuals decreased, and its concentration in the methanol-extractable phase significantly increased (Table 5). This suggests that bioavailability was limited by the presence of the NAPL rather than by enhanced sorption to the SOM. This result agrees with a previous study of phenanthrene degradation in the presence of hexadecane, which showed that high concentrations of the latter (1:1 ratio of hexadecane and mineral salts broth) decreased phenanthrene concentrations as a result of phenanthrene partitioning into the NAPL phase [41].

For soils spiked with increasing HMN concentrations, corresponding statistically significant increases occurred in the fraction of pyrene mineralized, although the percentage of pyrene mineralized increased by only a factor of 1.13 when HMN concentration increased from 1 to 10%. The fractions of nonextractable residues and aqueous desorption were relatively similar for all samples with HMN added, although the nonextractable residue percentage for the 1% HMN condition statistically differed from that for loadings of 3 and 10%.

Table 4. Effects of different nonaqueous-phase liquids (NAPLs) and NAPL concentrations on worm uptake of pyrene aged 155 d in North Campus soil

	Different NAPLs		
	No NAPL	Hexadecane	2,2,4,4,6,8,8-Heptamethylnonane
Uptake (μg/g dry worm)	708 ± 47[a] A[b]	245 ± 24 C	486 ± 25 B
	Different 2,2,4,4,6,8,8-heptamethylnonane concentrations		
	0%	1%	3%	10%
Uptake (μg/g dry worm)	732 ± 36 A	566 ± 43 B	521 ± 40 B	378 ± 29 C

[a] Mean ± 1 standard deviation from triplicate samples.
[b] Values in rows followed by the same letter are not significantly different ($p < 0.05$).

Table 5. Effects of nonaqueous-phase liquids (NAPLs) on the environmental fate of aged pyrene in North Campus soil after microbial degradation

	Fraction mineralized (%)	Fraction in mineral medium (%)	Residues retained in soil		
			Aqueous desorption (%)	Methanol extraction (%)	Non-extractable (%)
Soil treatment	Different aging durations with or without HEX				
Fresh[a]	61 ± 4[e] A[f]	6.8 ± 0.4 A	2.5 ± 0.2 A	18 ± 1 C	12 ± 1 B
30-d No hexadecane[b]	52 ± 3 B	4.9 ± 0.2 B	2.2 ± 0.2 A	25 ± 2 B	15 ± 1 A
30-d + Hexadecane	23 ± 1 C	2.3 ± 0.2 C	1.2 ± 0.2 B	57 ± 3 A	10 ± 1 B
167-d + Hexadecane	16 ± 1 D	1.7 ± 0.2 D	1.0 ± 0.1 B	60 ± 3 A	13 ± 1 B
			Different NAPLs[c]		
Aged only		45 ± 4 B	4.4 ± 0.3 B	1.8 ± 0.2 B	26 ± 1 C	16 ± 1B
Aged + hexadecane		18 ± 2 C	1.7 ± 0.1 C	1.2 ± 0.1 C	60 ± 3 B	12 ± 1 C
Aged + 2,2,4,4,6,8,8-heptamethylnonane (HMN)		53 ± 3 A	6.3 ± 0.4 A	2.3 ± 0.1 A	13 ± 1 D	20 ± 2 A
Aged + toluene		61 ± 4 A	6.6 ± 0.4 A	2.4 ± 0.1 A	10 ± 1 D	21 ± 2 A
Aged + dimethyl phthalate (DMP)		1.4 ± 0.1 D	0.5 ± 0.1 D	0.6 ± 0.1 D	82 ± 6 A	8.5 ± 0.6 D
			Different 2,2,4,4,6,8,8-heptamethylnonane concentrations[d]		
Aged only	43 ± 4 B	4.2 ± 0.2 B	1.8 ± 0.1 B	27 ± 2 A	15 ± 1 C
1% HMN	47 ± 3 BA	6.2 ± 0.4 A	2.0 ± 0.1 A	20 ± 1 B	19 ± 1 B
3% HMN	50 ± 4 A	6.4 ± 0.2 A	2.3 ± 0.2 A	14 ± 2 C	23 ± 1 A
10% HMN	53 ± 3 A	6.8 ± 0.2 A	2.3 ± 0.2 A	11 ± 1 C	22 ± 1 A

[a] Soil was spiked for 1 d and bioremediated for 114 d.
[b] Soils were aged without (30 d) and with (30 and 167 d) hexadecane (HEX) as NAPL, and then bioremediated for 114 d.
[c] Soils were aged with/without 5% HEX, HMN, toluene, and DMP as different NAPLs for 155 d, then bioremediated for 104 d.
[d] Soils were aged with different amounts of HMN for 155 d, then bioremediated for 104 d.
[e] Mean ± 1 standard deviation from triplicate samples.
[f] Values in columns followed by the same capital letter are not significantly different ($p < 0.05$).

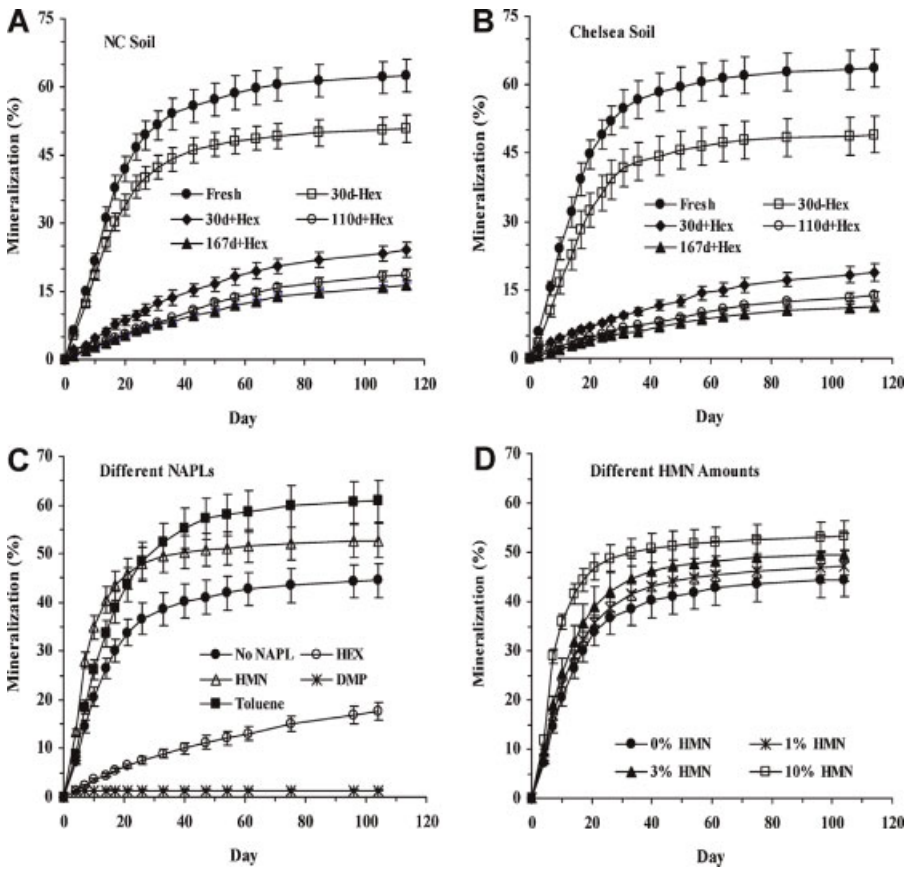

Fig. 2. Mineralization of pyrene. Effects of aging for various durations in the presence of hexadecane (Hex) as nonaqueous phase liquids (NAPLs) in North Campus (NC) (**A**) and Chelsea soils (**B**) and biodegradation for 114 d; the effects of aging in Hex, 2,2,4,4,6,8,8-heptamethylnonane (HMN), toluene, and dimethyl phthalate (DMP) for 155 d and biodegradation for 104 d (**C**); and the effect of aging for 155 d in different concentrations of HMN and biodegradation for 104 d (**D**). Each data point represents the average of triplicate data points, and error bars represent standard deviations. Percent mineralization was calculated relative to the pyrene concentration after aging but before initiation of the biodegradation experiment.

Increasing HMN concentrations caused a more substantial decrease in the methanol-extractable fraction in a concentration-dependent manner similar in proportion to enhanced mineralization. This suggests that methanol-extractable fractions were more bioavailable with increased HMN concentrations, or that increased HMN concentrations caused the fraction of pyrene to shift from methanol-extractable to a more bioavailable form.

CONCLUSIONS

This paper describes a comprehensive investigation of the effects of aging and the presence of various NAPLs on four interrelated environmental behaviors of pyrene. The presence of NAPLs consistently increased fractions of pyrene available for sorption by C-18 membranes and slowed the aging process in comparison with those of unamended soils. This result likely stems from pyrene captured by the C-18 membrane coming primarily from dissolution from the NAPL phase, a process that appeared to mitigate sorption to the hard carbon fraction of the SOM, which typically has very slow adsorption and desorption rates [2,4,5]. These increases in the aqueous phase availability of the pyrene did not, however, correspond to related increases in earthworm accumulation rates. Higher percentages of HMN in the soils actually caused decreased uptake values, even though desorption rates increased. This suggests that, in contrast with naphthalene, which showed higher uptake by *E. fetida* when readily desorbable [42], an increase in HOC availability in the presence of NAPLs may not correspond to increased bioaccumulation, likely as a result of NAPL toxicity. Additionally, the soil properties (porosity, surface area) may have been changed by aging in the presence of the NAPL amendments. Overall, the results indicate that desorption rates did not predict contaminant bioavailability with earthworms in soils containing NAPLs under these experimental conditions, despite the success with which desorption rates have been used to predict bioavailability in other studies [43]. The aging of soils spiked with hexadecane, however, did serve to decrease earthworm accumulation and pyrene biodegradation in a manner consistent with typical contaminant aging effects [11]. Decreased biodegradation after aging in the presence of hexadecane and DMP appears to stem from inhibited rates of mass transfer from the NAPL phase as previously observed with aged crude oils [28], given that methanol-extractable fractions increased and non-extractable fractions decreased. Effects arising from additions of various NAPLs on pyrene biodegradation did not follow trends similar to those observed in the earthworm accumulation experiments; HMN increased biodegradation and hexadecane decreased it, whereas both NAPLs decreased earthworm accumulation. This result highlights variations in contaminant bioavailability among different organisms even under similar environmental conditions, although toxicity of the NAPL likely influenced pyrene uptake. Overall, aging had the expected effect of decreasing bioavailability to both model organisms, whereas the presence of various NAPLs had different effects on rates of biodegradation, which were difficult to predict a priori.

SUPPLEMENTAL DATA

Table S1. Properties of nonaqueous-phase liquids (NAPLs)

Table S2. Earthworm masses after 14-d exposure for different aging times with hexadecane (a) and different NAPLs or different HMN concentrations (b)

Table S3. Effects of non-aqueous phase liquids (NAPLs) and aging on the mineralization of pyrene in soils (62 KB DOC)

Acknowledgement—The authors thank Roger Pinto for his valuable experimental assistance. This research was supported in part by the U.S. Department of Energy (DOE) through Grant DE-FG07-02ER63488, and by Grant P42ES04911-14 from the National Institutes for Environmental and Health Sciences. Conclusions set forth in the paper do not necessarily reflect the views of either agency.

REFERENCES

1. Enzminger JD, Ahlert RC. 1987. Environmental fate of polynuclear aromatic hydrocarbons in coal tar. *Environ Technol Lett* 8:269–278.
2. Weber WJ Jr, McGinley PM, Katz LE. 1992. A distributed reactivity model for sorption by soils and sediments. 1. Conceptual basis and equilibrium assessments. *Environ Sci Technol* 26:1955–1962.
3. Hatzinger PB, Alexander M. 1995. Effect of aging of chemicals in soil on their biodegradability and extractability. *Environ Sci Technol* 29:537–545.
4. Xing BS, Pignatello JJ. 1997. Dual-mode sorption of low-polarity compounds in glassy poly(vinyl chloride) and soil organic matter. *Environ Sci Technol* 31:792–799.
5. Johnson MD, Huang WH, Weber WJ Jr. 2001. A distributed reactivity model for sorption by soils and sediments. 13. Simulated diagenesis of natural sediment organic matter and its impact on sorption/desorption equilibria. *Environ Sci Technol* 35:1680–1687.
6. Golding CJ, Smernik RJ, Birch GF. 2005. Investigation of the role of structural domains identified in sedimentary organic matter in the sorption of hydrophobic organic compounds. *Environ Sci Technol* 39:3925–3932.
7. White JC, Hunter M, Nam KP, Pignatello JJ, Alexander M. 1999. Correlation between biological and physical availabilities of phenanthrene in soils and soil humin in aging experiments. *Environ Toxicol Chem* 18:1720–1727.
8. Nam K, Chung N, Alexander M. 1998. Relationship between organic matter content of soil and the sequestration of phenanthrene. *Environ Sci Technol* 32:3785–3788.
9. Lueking AD, Huang WL, Soderstrom-Schwarz S, Kim MS, Weber WJ Jr. 2000. Relationship of soil organic matter characteristics to organic contaminant sequestration and bioavailability. *J Environ Qual* 29:317–323.
10. Guthrie EA, Pfaender FK. 1998. Reduced pyrene bioavailability in microbially active soils. *Environ Sci Technol* 32:501–508.
11. Tang JX, Alexander M. 1999. Mild extractability and bioavailability of polycyclic aromatic hydrocarbons in soil. *Environ Toxicol Chem* 18:2711–2714.
12. Nam K, Kim JY. 2002. Role of loosely bound humic substances and humin in the bioavailability of phenanthrene aged in soil. *Environ Pollut* 118:427–433.
13. Heyse E, Augustijn D, Suresh P, Rao C, Delfino JJ. 2002. Nonaqueous phase liquid dissolution and soil organic matter sorption in porous media: Review of system similarites. *Crit Rev Environ Sci Technol* 32:337–397.
14. Jonker MTO, Sinke AJC, Brils JM, Koelmans AA. 2003. Sorption of polycyclic aromatic hydrocarbons to oil contaminated sediment: Unresolved complex? *Environ Sci Technol* 37:5197–5203.
15. Swindell AL, Reid BJ. 2007. The influence of a NAPL on the loss and biodegradation of ^{14}C-phenanthrene residues in two dissimilar soils. *Chemosphere* 66:332–339.
16. Labare MP, Alexander M. 1995. Enhanced mineralization of organic compounds in nonaqueous-phase liquids. *Environ Toxicol Chem* 14: 257–265.
17. Morrison DE, Alexander M. 1997. Biodegradability of nonaqueous-phase liquids affects the mineralization of phenanthrene in soil because of microbial competition. *Environ Toxicol Chem* 16:1561–1567.
18. Quiñones-Rivera A, Alexander RR, Alexander M. 2003. Effect of nonaqueous-phase liquids on the availability of polycyclic aromatic hydrocarbons in soil for worm uptake and bacterial genotoxicity. *Environ Toxicol Chem* 22:2599–2604.
19. Sun SB, Boyd SA. 1991. Sorption of polychlorobiphenyl (PCB) congeners by residual PCB-oil phases in soils. *J Environ Qual* 20:557–561.
20. Efroymson RA, Alexander M. 1995. Reduced mineralization of low concentrations of phenanthrene because of sequestering in nonaqueous-phase liquids. *Environ Sci Technol* 29:515–521.
21. Bar R. 1987. Phase toxicity in a water-solvent two-liquid phase microbial system. In Laane C, Tramper J, Lilly MD, eds, *Biocatalysis in Organic Media*. Elsevier, Amsterdam, The Netherlands, pp 147–153.
22. Pirnik MP, Atlas RM, Bartha R. 1974. Hydrocarbon metabolism by *Brevibacterium erythrogenes*: Normal and branched alkanes. *J Bacteriol* 119:868–878.
23. Ortega-Calvo JJ, Alexander M. 1994. Roles of bacterial attachment and spontaneous partitioning in the biodegradation of naphthalene initially

present in nonaqueous-phase liquids. *Appl Environ Microbiol* 60:2643–2646.
24. Calvillo YM, Alexander M. 1996. Mechanism of microbial utilization of biphenyl sorbed to polyacrylic beads. *Appl Microbiol Biotechnol* 45:383–390.
25. Steinberg SM, Pignatello JJ, Sawhney BL. 1987. Persistence of 1,2-dibromoethane in soils: Entrapment in intraparticle micropores. *Environ Sci Technol* 21:1201–1208.
26. Hartlieb N, Erturic T, Schaeffer A, Klein W. 2003. Mineralization, metabolism and formation of non-extractable residues of C-14-labelled organic contaminants during pilot-scale composting of municipal biowaste. *Environ Pollut* 126:83–91.
27. Tang JX, Petersen EJ, Huang QG, Weber WJ Jr. 2007. Development of engineered natural organic sorbents for environmental applications: 3. Reducing PAH mobility and bioavailability in contaminated soil and sediment systems. *Environ Sci Technol* 41:2901–2907.
28. Ghoshal S, Pasion C, Alshafie M. 2004. Reduction of benzene and naphthalene mass transfer from crude oils by aging-induced interfacial films. *Environ Sci Technol* 38:2102–2110.
29. Ghoshal S, Ramaswami A, Luthy RG. 1996. Biodegradation of naphthalene from coal tar and heptamethylnonane in mixed batch systems. *Environ Sci Technol* 30:1282–1291.
30. Organisation for Economic Co-operation and Development. 1981. Partition coefficients. OECD Guideline 107. Paris, France.
31. Tang JX, Liste HH, Alexander M. 2002. Chemical assays of availability to earthworms of polycyclic aromatic hydrocarbons in soil. *Chemosphere* 48:35–42.
32. Tang JX, Robertson BK, Alexander M. 1999. Chemical-extraction methods to estimate bioavailability of DDT, DDE, and DDD in soil. *Environ Sci Technol* 33:4346–4351.
33. American Society for Testing and Materials. 1998. Standard guide for conducting laboratory soil toxicity or bioaccumulation test with the lumbricid earthworm *Eisenia foetida*. E1676-97. Philadelphia, PA.
34. Petersen EJ, Huang QG, Weber WJ Jr. 2008. Bioaccumulation of radiolabeled carbon nanotubes by *Eisenia foetida*. *Environ Sci Technol* 42:3090–3095.
35. Petersen EJ, Pinto RA, Landrum PF, Weber WJ Jr. 2009. Influence of carbon nanotubes on pyrene bioaccumulation from contaminated soils by earthworms. *Environ Sci Technol* 43:4181–4187.
36. Petersen EJ, Huang Q, Weber WJ Jr. 2010. Relevance of Octanol-water distribution measurements to the potential ecological uptake of multi-walled carbon nanotubes. *Environ Toxicol Chem* 29:1106–1112.
37. Tang JX, Petersen E, Weber WJ Jr. 2008. Development of engineered natural organic sorbents for environmental applications. 4. Effects on biodegradation and distribution of pyrene in soils. *Environ Sci Technol* 42:1283–1289.
38. Kamath R, Schnoor JL, Alvarez PJJ. 2005. A model for the effect of rhizodeposition on the fate of phenanthrene in aged contaminated soil. *Environ Sci Technol* 39:9669–9675.
39. Johnson MD, Weber WJ Jr. 2001. Rapid prediction of long-term rates of contaminant desorption from soils and sediments. *Environ Sci Technol* 35:427–433.
40. Efroymson RA, Alexander M. 1994. Biodegradation in soil of hydrophobic pollutants in nonaqueous-phase liquids (NAPLs). *Environ Toxicol Chem* 13:405–411.
41. Sandrin TR, Kight WB, Maier WJ, Maier RM. 2006. Influence of nonaqueous phase liquid (NAPL) on biodegradation of phenanthrene. *Biodegradation* 17:423–435.
42. Qi YC, Chen W. 2010. Comparison of earthworm bioaccumulation between readily desorbable and desorption-resistant naphthalene: Implications for biouptake routes. *Environ Sci Technol* 44:323–328.
43. Landrum PF, Robinson SD, Gossiaux DC, You J, Lydy MJ, Mitra S, Ten Hulscher TEM. 2007. Predicting bioavailability of sediment-associated organic contaminants for Diporeia spp. and Oligochaetes. *Environ Sci Technol* 41:6442–6447.