

Assessment of phenanthrene bioavailability in aged and unaged soils by mild extraction

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Received: 20 January 2010 / Accepted: 24 February 2011 / Published online: 26 August 2011
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Abstract It has become apparent that the threat of an organic pollutant in soil is directly related to its bioavailable fraction and that the use of total contaminant concentrations as a measure of potential contaminant exposure to plants or soil organisms is inappropriate. In light of this, non-exhaustive extraction techniques are being investigated to assess their appropriateness in determining bioavailability. To find a suitable and rapid extraction method to predict phenanthrene bioavailability, multiple extraction techniques (i.e., mild hydroxypropyl- β -cyclodextrin (HPCD) and organic solvents extraction) were investigated in soil spiked to a range of phenanthrene levels (i.e., 1.12, 8.52, 73, 136, and 335 $\mu\text{g g}^{-1}$ dry soil). The bioaccumulation of phenanthrene in earthworm (*Eisenia fetida*) was used as the reference

system for bioavailability. Correlation results for phenanthrene suggested that mild HPCD extraction was a better method to predict bioavailability of phenanthrene in soil compared with organic solvents extraction. Aged (i.e., 150 days) and fresh (i.e., 0 day) soil samples were used to evaluate the extraction efficiency and the effect of soil contact time on the availability of phenanthrene. The percentage of phenanthrene accumulated by earthworms and percent recoveries by mild extractants changed significantly with aging time. Thus, aging significantly reduced the earthworm uptake and chemical extractability of phenanthrene. In general, among organic extractants, methanol showed recoveries comparable to those of mild HPCD for both aged and unaged soil matrices. Hence, this extractant can be suitable after HPCD to evaluate risk of contaminated soils.

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Keywords Accumulation · Aging ·
Bioavailability · HPCD · Mild extraction · PAHs

Introduction

Over the past decade, the relationships between contaminant desorption from soils and bioavailability has become an important research area. It has been established that a labile contaminant fraction is bioavailable, and this fraction exhibits rapid and reversible sorption to soil. A non-

labile contaminant fraction is not considered to be bioavailable and exhibits recalcitrant desorption from soil (Reid et al. 2000a). Soil properties and soil-contaminant contact time are key factors in the sorption/desorption dynamics of contaminants in soils and are important considerations for measuring bioavailability. Direct measurement of bioavailability is expensive, time-consuming, and imprecise; thus, researches have focused on the development of chemical methods to predict bioavailability (Reid et al. 2000a; Hartnik et al. 2008). This kind of assessment, if reliable and accurate, would be valuable to risk and remediation effect assessment of contaminated sites.

A range of laboratory-based approaches have been developed to predict the available fraction of chemicals present in soil. These include chemical assays such as mild organic solvent extraction (Swindell and Reid 2006; Bergknut et al. 2007; Gomez-Eyles et al. 2010; Khan et al. 2011) and mild cyclodextrin extraction (Puglisi et al. 2007; Barthe and Pelletier 2007; Barriuso et al. 2008; Wong and Bidleman 2010; Khan et al. 2011) and biological availability assays such as earthworm (EW) uptake (Parrish et al. 2006; Bergknut et al. 2007; Hartnik et al. 2008; Gomez-Eyles et al. 2010; Khan et al. 2011). All these strategies are essentially based on the principle that bioavailability is governed by contaminant mass transfer from the soil solid phase to the aqueous phase.

Mild extraction technique (nonexhaustive extraction) is a rapid and nonbiological (i.e., physical or chemical) means of quantifying or predicting bioavailability of contaminants in matrices. Aqueous solutions of cyclodextrin have been used to dissolve (form a host–guest inclusion complex) a range of low solubility contaminants to greater than their aqueous solubility limits (Hickman and Reid 2005; Allan et al. 2006). Most recently, many studies have demonstrated that nonexhaustive extraction by aqueous hydroxypropyl- β -cyclodextrin (HPCD) was able to predict the bioavailable fraction of polycyclic aromatic hydrocarbons (PAHs) in a wide range of soils (Dupuy et al. 2005; Allan et al. 2006; Barthe and Pelletier 2007; Hickman et al. 2008; Khan et al. 2011). In many cases, a 1:1 correlation between HPCD extractability and microbial degradation rate or earthworm uptake was reported, and a scaling factor

was not needed (Reid et al. 2004; Allan et al. 2006). Stokes et al. (2005) successfully correlated HPCD extractability and biodegradability of PAHs in contaminated coke-plant soils. Hartnik et al. (2008) also developed a HPCD extraction method for the currently used pesticides α -cypermethrin and chlorfenvinphos and found that HPCD extractability correlated well with those of earthworm uptake. Most recently, Khan et al. (2011) successfully correlated HPCD extractability and earthworm uptake in pyrene-amended fresh and aged soils.

PAHs are known as some of the most notorious contaminants in soil because of their extensive pollution as by-products of incomplete fossil fuel combustion and their high toxicity and carcinogenicity (Jian et al. 2004; Wang et al. 2007). Phenanthrene is a tricyclic aromatic hydrocarbon and one of the 16 PAHs on the priority list of US Environmental Protection Agency.

The present study was designed to evaluate the bioavailability of phenanthrene in aged and unaged soil as well as to compare different abiotic techniques for assessing the bioavailability of phenanthrene in soil, by using earthworms (*Eisenia fetida*) as the reference system for bioavailability. For this purpose, different organic solvents (methanol (METH), hexane (HEXA), acetonitrile, isopropyl alcohol (ISO), and a mixture of acetone and dichloromethane (A + D)) and aqueous solution of 50 mM HPCD were used to evaluate the applicability of short-term extractions in estimating the bioavailability of phenanthrene in soil.

Materials and methods

Chemicals

Phenanthrene and HPCD of 99.9% and 98% purity, respectively, were purchased from Sigma Aldrich Co. Ltd., UK. All other chemicals used in the present study were of analytical grade.

Soil

An uncontaminated soil with undetectable phenanthrene was collected from the upper 15-cm layer of an experimental rice field at Hua Jia Chi

campus of Zhejiang University, Hangzhou, China (31°16' N, 120°12' E). The soil was air-dried and passed through 2-mm sieve to remove stones and roots. The particle size distribution (50.5% sand, 37.0% silt, and 12.5% clay) identified the soil as a sandy loam soil. The organic matter content was 2.1% and the pH was 5.95. The cation exchange capacity was 7.76 cmol kg⁻¹, and electrical conductivity was 254.5 µS cm⁻¹. The nutrient levels were 1.78% of total N, 9.39 µg g⁻¹ of total P, and 0.98% of total K.

Soil spiking and storage

Soil was spiked with five different concentrations of phenanthrene (1.12, 8.52, 73, 136, and 335 µg Phe g⁻¹ dry soil) in triplicate according to Cheema et al. (2010) and Khan et al. (2011) with slight modifications. Briefly, 1-kg air-dried soil was contaminated with the stock solution of phenanthrene and placed in a jar to prepare each independent sample. Acetone was used as carrier solvent. The jars were closed for 3 h to let the solvent (i.e., acetone) disperse after the required amount of phenanthrene stock solution was added. Thereafter, the solvent was allowed to evaporate by placing the jars in a fume hood for 48 h. Mixing was performed thoroughly in each jar for 5 min with a metal spatula. Finally, double-distilled water was added to each jar to bring the soil samples to a moisture content of 60% water holding capacity and the samples stored at 20 ± 0.5°C for different aging times (0 and 150 days) before extraction and phenanthrene analysis. Each sample, including the blank soil sample for background check, was prepared in triplicate. In the present study, we used various concentrations of phenanthrene to evaluate the potential and applicability of mild extraction at varying levels of soil contamination.

Experiments were performed using both non-sterilized and sterilized soil microcosms, in order to evaluate the fate of phenanthrene in the presence and absence of microbial degradation. Abiotic sterile controls were prepared with 2,000 µg g⁻¹ of sodium azide (NaN₃). NaN₃ can effectively inhibit bacterial metabolism via electron transport disruption and hence is an ideal agent for metabolic inactivation without disrupting the

physical texture of the soil (Frederick and Babish 1982). In order to examine aging of phenanthrene, chemical and biological analyses were performed immediately after spiking (0 day) and at 150 days after aging (Puglisi et al. 2007; Khan et al. 2011). Analyses were performed in triplicate for each sample.

Accumulation of phenanthrene by earthworms

Earthworms (*E. fetida*) were raised at 22 ± 2°C in soil in the laboratory and fed on pig manure. Five adult (with clitellum) earthworms (about 0.35 g each) were placed in a 100-mL glass bottle containing 10 g (dry weight) of phenanthrene-amended soil at a moisture content of 80% of field capacity. The bottles were covered with aluminum foil with 15 small holes for aeration and then kept in the dark at 22 ± 2°C. The tests were done in triplicate. Every day, water was added to maintain the 80% moisture level. After 10 days, phenanthrene concentration in the worms reached a bioaccumulation steady state, as had been measured in a previous absorption dynamics experiment (Khan et al. 2011) and also in our preliminary earthworm accumulation experiments. Hence, at the tenth day, the worms were removed from the bottles, rinsed with water, and placed on moist filter paper in a glass Petri dish for 24 h to allow for depuration. A 24-h depuration period was chosen because, based on chemical recoveries, this was sufficient for all soil to be eliminated from the worms (Bergknut et al. 2007). The worms were sequentially washed with water, dried with paper towels, weighed, and freeze-dried. The dried worms were ground with a mortar and pestle and mixed with ten times their weight of anhydrous sodium sulfate (Na₂SO₄) prior to Soxhlet extraction. Percentage uptake by the worms was calculated by the ratio of the quantity of phenanthrene in the worm tissues and the initial phenanthrene amount in unaged soil extracted by Soxhlet extraction.

For Soxhlet extraction, 1-g ground worm tissue or 5-g freeze-dried soil was introduced into a cellulose extraction thimble and extracted for 24 h with 200 mL mixture of acetone and dichloromethane (1:1, v/v, high-performance liquid chromatography (HPLC) grade) in Soxhlet extractors at 59°C

(5 to 6 min cycle⁻¹). The extracted solutions were concentrated to about 1 mL in a rotary evaporator, dissolved in 10 mL *n*-hexane, and passed through columns packed with layers of silica gel (200–300 mesh), neutral aluminum oxide (100–200 mesh), and anhydrous Na₂SO₄ using elution with a 70-mL mixture of hexane/dichloromethane (7:3, v/v; Tang et al. 2010). The analyte fraction was reconcentrated in a rotary evaporator to 1 mL and was further carefully evaporated to dryness under a gentle stream of nitrogen. The residue was dissolved in 2 mL methanol and gently passed through a 0.22- μ m filter to remove particulate matter prior to analysis. Analysis was conducted on triplicate samples. Strict quality-control procedures were adopted during the chemical analysis. Reproducibility and recovery averages for phenanthrene in earthworms and soil by Soxhlet extraction was 89.6% ($n = 7$, RSD less than 9.66%, where RSD is relative standard deviation) and 98.9% ($n = 6$, RSD less than 8.75%), respectively. Phenanthrene, phenanthrene d₁₀, and pyrene d₁₀ surrogate standards (Accu-Standard Company, USA) were spiked into samples to measure recoveries.

Organic solvents extraction

Two-gram soil samples of different aging time (i.e., 0 and 150 days) were transferred to 50-mL glass centrifuge tubes, and 25 mL of each of the organic solvents tested (a mixture of acetone and dichloromethane, methanol, hexane, acetonitrile, and isopropyl alcohol) was added to each tube. The tubes were shaken for 20 h on a flat-bed rotary shaker (Denley) operating with a speed of 150 rpm at $25 \pm 1^\circ\text{C}$. The tubes were then centrifuged at 3,000 rpm (Sigma Laboratory Centrifuge, 4K15) for 10 min. The supernatants were evaporated to dryness under a gentle stream of nitrogen. The concentration of phenanthrene was determined by HPLC after the same pretreatment procedure as above.

Mild HPCD extraction

The HPCD extraction method was adapted from that described by Reid et al. (2000b) and Khan et al. (2011) with slight modification. An aque-

ous solution of 50 mM HPCD was prepared using deionized water. Two-gram soil samples were weighed into centrifuge tubes ($n = 3$) and 25 mL of HPCD solution added to each. The tubes were shaken on a flat-bed rotary shaker at 150 rpm for 20 h. The tubes were then centrifuged at 3,000 rpm for 10 min, and supernatants were passed through a 0.45- μ m Whatman filter.

Re-extraction of phenanthrene from the HPCD extracts was carried out as described by Hua et al. (2007). Ten milliliters of the aqueous mixture of HPCD (standard batch sample or contaminated soil extracts) was mixed with 10 mL of the immiscible solvent, *n*-hexane. The mixture was shaken in a Fast Prep (Bio101) at speed 5 for 30 s and then centrifuged at room temperature. The organic phase thereby obtained was dried by addition of anhydrous MgSO₄ and passed through a 0.45- μ m Whatman filter. The organic extraction solution was dried in a fume hood to remove the organic solvent, and the residue was re-dissolved in 1 mL of methanol and diluted further with methanol before quantitative analysis by HPLC. The average recovery of phenanthrene by HPCD extraction was 93.8%.

HPLC analysis

All methanol extracts were filtered through a 0.22- μ m filter prior to analysis. The HPLC analyses were performed with an Agilent 1100 Serials liquid chromatograph (Agilent Company, USA) by using a mobile phase composed of Milli-Q water (10%) and methanol (90%) at a flow rate of 1.00 mL min⁻¹, signal wavelength of 220 nm with 20 nm bandwidth, and a reference wavelength of 330 nm with 50 nm bandwidth. The concentrations were quantified by an external standard method. Replicate analyses gave an error in the range of ± 5 –8%. The solvents used for chromatographic analyses were of HPLC grade.

Data analysis

All values presented for the chemical and biological analyses of soil are the means of three replicates \pm standard error. The means were compared using least significant differences calculated at a significance level of $p = 0.05$, and correlations

between the parameters were analyzed using the software Statistical Package for Social Sciences (SPSS version 16.0 for Windows).

Results and discussion

Earthworm uptake of phenanthrene under different aging times

Earthworms represent particularly interesting bio-indicators and bio-monitors in soils acknowledge to their ability to ingest inorganic and organic soil particles (Edwards 2004). Direct biological measure of bioavailability of an organic contaminant is the outcome of the actual amount of chemical taken up by earthworm. It may provide the most accurate measure of bioavailability because it integrate all biotic (e.g., metabolism) and abiotic (e.g., pH, organic matter content of soil) modifying factors of chemical bioavailability (Lanno et al. 2004). Bioaccumulation is a direct measure of chemical concentrations in an earthworm resulting from the net inward flux of chemicals from the soil due to the balance between uptake and depuration processes.

In our present study, we investigated the accumulation of phenanthrene by the earthworms in aged (i.e., 150 days) and unaged soil samples at different initial (i.e., Soxhlet extracted) concentrations of phenanthrene (1.12, 8.52, 73, and

136 $\mu\text{g g}^{-1}$ dry soil). The highest level of phenanthrene used in the present study (i.e., 335 $\mu\text{g g}^{-1}$) was not included in the earthworm accumulation data because the earthworm did not survive at this level. However, no apparent toxicity sign to the earthworm was observed up to the phenanthrene concentration of 136 $\mu\text{g g}^{-1}$ in 10-day bioassay. The results of earthworm accumulation (Fig. 1) reveal that different initial concentrations greatly influenced the phenanthrene accumulation in the earthworm tissues in aged and fresh soils. Thus, the present investigation indicates that phenanthrene uptake in earthworms was concentration dependent.

For all concentrations, percentage accumulation of phenanthrene in fresh soil was significantly higher ($p < 0.05$) compared to aged soil samples (i.e., 150 days). Reduction in accumulated percentages of phenanthrene in aged soil were 40–60% at different levels of phenanthrene (1.12, 8.52, 73, and 136 $\mu\text{g g}^{-1}$) as compared to those in unaged. It is thought that during aging pollutant molecules move to sites inaccessible to soil organisms. Our results concur with the findings of Sun and Li (2005) who found that the percentage of pyrene accumulated by earthworms was significantly lower in aged pyrene spiked soil. Likewise, Gomez-Eyles et al. (2010) and Khan et al. (2011) found a significant decline in accumulation of PAHs at successive aging times. In contrast to our results, some researchers found that earth-

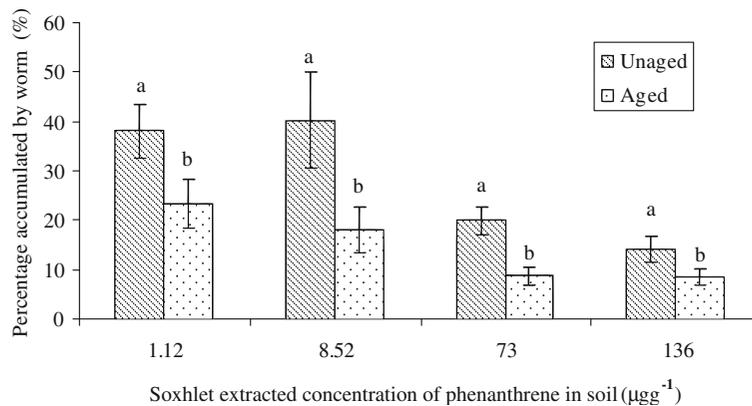


Fig. 1 Percent accumulation of aged (i.e., 150 days) and unaged (i.e., 0 day) phenanthrene in the earthworms after 10-day exposure under different initial Soxhlet extracted concentrations in soil. Columns and error bars represent-

ing mean and standard deviations ($n = 3$), respectively. Variants possessing the same letters are not significantly different at $p < 0.05$

worm accumulations were not unusually low for aged compounds (Gevao et al. 2001). Similarly, Liste and Alexander (2002) found that the amount of PAH being accumulated by earthworms was still high even in the presence of aged PAH spiked soil and a lower concentration of chrysene (i.e., $10 \mu\text{g g}^{-1}$). Moreover, a decreasing trend in accumulated percentages was found along increasing initial concentrations in both (i.e., aged and fresh) soils with the exception of $8.52 \mu\text{g g}^{-1}$ initial concentration for unaged soil.

Our results also showed that even after 150 days of aging, earthworms could accumulate more phenanthrene than those could be extracted by any of the extractants, which showed that earthworms could assimilate some of the phenanthrene that could not be extracted by any of the extraction solvents (Liste and Alexander 2002). According to equilibrium partition theory, only pollutants in soil water solution can be accumulated by terrestrial invertebrates, such as earthworms (Jager et al. 2005). As chemical in the soil water solution is gradually taken up by organisms, its concentration goes down dramatically and the chemical sorbed onto the soil solid phase is slowly desorbed into water solution and consequently utilized by organisms. As a result, the rate and extent of pollutant availability to soil biota could be limited by the rate and extent of desorption or diffusion of the pollutant into soil water solution.

Mild HPCD extraction

The cyclodextrin (HPCD) extraction could provide a reliable method to assess the bioavailability of a range of soil-associated organic contaminants as this technique is thought to mimic the mass transfer mechanisms that govern the bioavailability of nonpolar organic contaminants in soil (i.e., transfer from solid to the aqueous phase; Hua et al. 2007). HPCD extractions can predict the microbial availability of PAHs in mixtures over a range of concentrations (Doick et al. 2006).

The results of the mild HPCD extraction method are shown in Fig. 2 (unaged soil) and Fig. 3 (aged soil). Over a soil-contaminant contact time of 150 days, the HPCD extractability of phenanthrene from soil was significantly ($p < 0.05$) reduced, with respect to values at 0 day. After 150 days, 30–45% reduction in percent HPCD extractability was observed as compared with values at 0 day. This suggested a great depletion of the labile pool between fresh (i.e., 0 day) and aged (i.e., 150 days) phenanthrene. Our present results concur with the findings of Puglisi et al. (2007) and Khan et al. (2011), who found that HPCD extractability of PAHs was significantly reduced as a consequence of aging.

In addition, quite similar percent recoveries were obtained using organic solvents. We found that in unaged soil samples, HPCD percent ex-

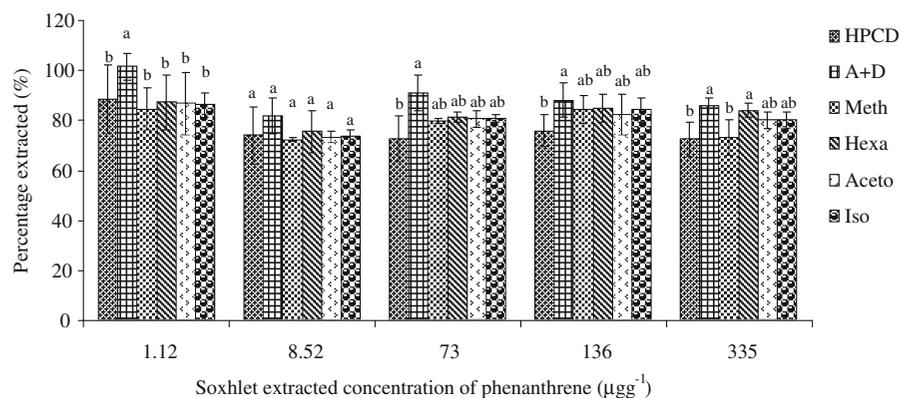


Fig. 2 Percent extraction of unaged (i.e., 0 day) phenanthrene by various solvents: hydroxypropyl- β -cyclodextrin (HPCD), acetone and dichloromethane (A + D), methanol (METH), hexane (HEXA), isopropyl alcohol (ISO), and acetonitrile (ACETO) under different

initial Soxhlet extracted concentrations in soil. Columns and error bars represent mean and standard deviations ($n = 3$), respectively. Variants possessing the same letters are not significantly different at $p < 0.05$

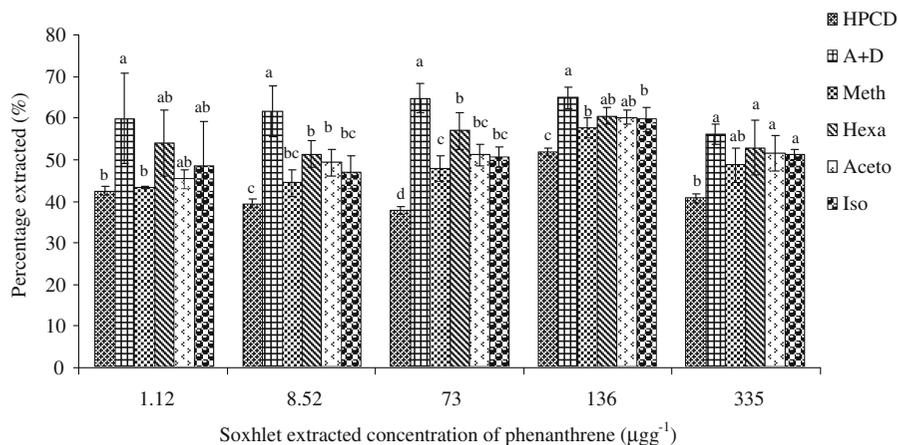


Fig. 3 Percent extraction of aged (i.e., 150 days) phenanthrene by various solvents: hydroxypropyl-β-cyclodextrin (HPCD), acetone and dichloromethane (A + D), methanol (METH), hexane (HEXA), isopropyl alcohol (ISO), and acetonitrile (ACETO) under different

initial Soxhlet extracted concentrations in soil. Columns and error bars representing mean and standard deviations (n = 3), respectively. Variants possessing the same letters are not significantly different at p < 0.05

tractability was not significantly greater (p < 0.05) as compared to the recoveries by organic solvents at 1.12 and 8.52 µg g⁻¹ initial phenanthrene concentrations in soil. These findings are in contrast to Swindell and Reid (2006), who found that HPCD and organic solvent extraction techniques had different extraction efficiencies. However, at higher phenanthrene levels, significant differences were observed between percent extraction efficiencies of HPCD and organic solvents (e.g., a mixture of acetone and dichloromethane and hexane) in unaged soil. Moreover, in aged soil (Fig. 3), we found that percent recovery by mild HPCD was significantly lower as compared to the recoveries by organic extractants with an exception of methanol, at all phenanthrene levels (i.e., 1.12, 8.52, 73, 136, and 335 µg g⁻¹). Methanol showed very similar efficiency to the HPCD in aged soil samples. However, in general, HPCD and organic solvents had different extraction efficiencies in both aged and unaged soils which concur with the findings of Swindell and Reid (2006) and Khan et al. (2011).

Organic solvents extraction

It has been established that the use of total contaminant concentrations as a measure of potential contaminant exposure to plants or soil or-

ganisms is inappropriate and that bioavailability of contaminants is a better measure of potential exposure (Alexander 2000; Swindell and Reid 2006). In light of this, non-exhaustive extraction techniques are being investigated to assess their appropriateness in determining bioavailability. The comparative assessment of phenanthrene bioavailability by mild HPCD and different organic solvents (i.e., a mixture of acetone and dichloromethane, methanol, hexane, acetonitrile, and isopropyl alcohol) extraction techniques is given in Fig. 2 (unaged soil) and Fig. 3 (aged soil). The percentage of phenanthrene recovered by all extraction methods generally decreased (p < 0.05) as a result of aging. Overall, at all initial concentrations of phenanthrene (i.e., 1.12, 8.52, 73, 136, and 335 µg g⁻¹), approximately 30% reduction in percent extractabilities of all organic extractants was observed after 150 days aging. All these results suggest considerable influence of aging on phenanthrene extraction as measured by the aforementioned nonexhaustive extraction techniques. Our present results are in conformity with the findings of Sun and Li (2005) and Gomez-Eyles et al. (2010), who concluded that aging greatly influenced the extraction percentage of pyrene when using butanol as a mild extractant. The reasons behind lower recovery of PAHs after aging could be because during aging, more

and more PAHs may have entered sites in soil particles inaccessible to organic solvent, including nanopores in clay minerals and glassy organic domains, which are difficult to expand in solvents such as butanol (Sun and Li 2005).

Furthermore, among all the organic extractants, a mixture of acetone and dichloromethane showed the best recoveries of phenanthrene in both aged and fresh soil samples as depicted in Figs. 2 and 3. In general for all initial concentrations, hexane was superior extractant next to the mixture of acetone and dichloromethane in unaged and aged soil samples. However, the percent extractabilities of methanol were inferior in both aged and unaged soils.

Like the mild extraction approaches, the percentage of total extractable phenanthrene using an exhaustive method (i.e., Soxhlet) significantly decreased after 150 days aging. About 15–30% reduction in percent recoveries of phenanthrene by Soxhlet extraction was observed after 150 days soil–chemical contact time at different initial concentrations (i.e., 1.12, 8.52, 73, 136, and 335 $\mu\text{g g}^{-1}$). Similar results have been reported by Liste and Alexander (2002) for chrysene recovery using Soxhlet plus butanol extraction methods and by Gomez-Eyles et al. (2010) and Khan et al. (2011) for PAHs recovery using organic solvents (i.e., butanol, ethyl acetate, and propanol) and Soxhlet extraction. They found significant reduction in chemical extraction along increasing soil–chemical contact time.

By comparing both mild HPCD and organic solvents extraction techniques with Soxhlet extraction, we found that percent recoveries by Soxhlet were significantly higher than those by any of the non-exhaustive extractants in aged and unaged soils. Moreover, mild HPCD showed recoveries comparable to those of methanol.

Relationship between biological and chemical availability of phenanthrene in soil

Correlations among earthworm accumulation of phenanthrene and extraction by a mixture of A + D, METH, HEXA, acetonitrile, ISO, and HPCD for both aged and unaged soil samples are shown in Table 1. The bioavailable fraction of phenanthrene in EW was significantly corre-

Table 1 Relationship between biological (earthworm uptake) and chemical availability of phenanthrene in soil

Parameter	Pearson correlation coefficients (r^2)											
	HPCD		A + D		METH		HEXA		ACETO		ISO	
	0 day of aging	150 days of aging	0 day of aging	150 days of aging	0 day of aging	150 days of aging	0 day of aging	150 days of aging	0 day of aging	150 days of aging	0 day of aging	150 days of aging
EW	0.98 ^a	0.99 ^b	0.98 ^a	0.99 ^b	0.98 ^a	0.99 ^b	0.98 ^a	0.99 ^b	0.98 ^a	0.99 ^b	0.98 ^a	0.99 ^b
HPCD												
A + D			0.99 ^b	0.99 ^b					1.00 ^b	1.00 ^b	1.00 ^b	1.00 ^b
METH					0.99 ^b	0.99 ^b			1.00 ^b	1.00 ^b	1.00 ^b	1.00 ^b
HEXA							1.00 ^b	0.99 ^b	1.00 ^b	1.00 ^b	1.00 ^b	1.00 ^b
ACETO									1.00 ^b	0.99 ^b	1.00 ^b	1.00 ^b

EW earthworm, A + D acetone + dichloromethane, METH methanol, HEXA hexane, ISO isopropyl alcohol, ACETO acetonitrile, HPCD hydroxypropyl- β -cyclodextrin

^aCorrelation is significant at the 0.05 level

^bCorrelation is significant at the 0.01 level

lated at the 1% probability level with extractable phenanthrene by all the extractants at the 150-day aging time (aged soil), while at 0 day (unaged soil) aging time, a significant relationship at 5% probability level could be seen among aforementioned parameters. The mean values of Pearson's correlation coefficient (r^2) for all the parameters were above 0.98 at all aging times (i.e., 0 and 150 days). However, in the correlation study, there was a promising relationship for all the six parameters (i.e., EW, A + D, METH, HEXA, ISO, and HPCD), which revealed the presence of a good correlation between biological and chemical bioavailability of phenanthrene for both aged and unaged soils.

Similar to the present findings regarding the correlation study, other investigators like Gomez-Eyles et al. (2010) found that the quantities of PAH extracted by mild butanol correlated well with earthworm assimilation of PAHs in soil. Likewise, Khan et al. (2011) also found a good correlation between percent recoveries of pyrene by mild organic extractant and percentage earthworm accumulation and obtained higher r^2 values (0.97–0.98), similar to those found in our present study (i.e., 0.98–0.99). Similar results have also been reported in other studies that extractability by mild organic solvents, such as methanol and butanol correlated well with bioavailability of organics in soil. A very good correlation was found in the case of our studies as well as those of others (Cuypers et al. 2002; Hickman and Reid 2005), but the organic solvent extraction approach has limitations as it often failed to predict bioavailability of a range of organic chemicals in different soils (Reid et al. 2004; Bergknut et al. 2007) and very distinct differences exist between the processes of organic solvents extraction and earthworm accumulation (Sun and Li 2005). Thus, organic solvents extraction might not be suitable approach for the evaluation of PAHs bioavailability in soils.

From our results, recoveries of phenanthrene by mild HPCD extraction were closer to earthworm uptake and substantially correlated with earthworm accumulation as compared with those obtained using organic extractants. Similarly, Hickman and Reid (2005) found no significant difference between microbially mineralized phenanthrene and extractability using HPCD in four dis-

similar soils. Overall, a single HPCD-extraction technique proved accurate and reproducible for the estimation of phenanthrene bioavailability in soil. Results of other studies also showed that HPCD could predict the fraction of PAHs bioavailable to microorganisms/earthworms in soils (Reid et al. 2000b; Allan et al. 2006; Papadopoulos et al. 2007; Hartnik et al. 2008). In addition, the HPCD extraction offered an improvement on the organic solvent extraction and provided a sound prediction of the bioavailable fraction of phenanthrene in soil. However, mild organic solvents and HPCD extraction can tell us only the feasibility of earthworm uptake, but not the actual bioavailability of phenanthrene in soil.

Conclusions

In conclusion, mild HPCD extraction may preferably be exploited to determine the bioavailable concentration of phenanthrene in aged and unaged soils as compared with organic solvents extraction. Moreover, the extraction efficiency and Pearson's correlation value (r^2) of methanol was found to be most comparable among all organic extractants used in the present study. Hence, methanol extraction might be a preferable approach after HPCD to predict the bioavailability of phenanthrene in both aged and unaged soil as compared with other organic extractants. The percentage uptake of phenanthrene by earthworms and percent extractability by each studied technique declined as the contaminant persisted in soil, indicating that aging had significant influence on earthworm uptake and chemical extractability of phenanthrene in soil. Results support previous findings that HPCD could be an appropriate extractant for distinguishing loosely and strongly bound residues and thus inferring the bioavailability of chemicals in soil. Further studies of merit would be to expand the chemical classes, to test cyclodextrin with different cavity sizes, and to investigate correlations with different bioavailability.

Acknowledgements This work was supported by Science and Technology Foundation of Zhejiang Province, China (2009C13003), National High Technology Research and

Development Program of China (2009AA063104), and program for Changjiang Scholars and Innovative Research Team in University of China (IRT0536). The authors would like to specially acknowledge all members in our group for their great assistance and cooperation during the research and manuscript writing.

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