Do Single and Sequential Solvent Extractions Determine Similar Total-Extractable Benzo[a]pyrene Fractions in Contrasting Soils?

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ABSTRACT

The fate and behaviour of polycyclic aromatic hydrocarbons (PAHs) in soil is of interest in the risk assessment of contaminated lands and are usually based on determinations of fractions extracted from soil. For decades, either single or sequential solvent extractions have been used to determine PAH extractability in soils; however, there is a lack of certainty as to which fractions are being extracted by these techniques. This study is the first report of changes and similarities in extractability of benzo[a]pyrene (B[a]P) in four contrasting soils (sandy loam, loamy sand, clayey loam, and sandy) when determined, simultaneously using both single (dichloromethane-DCM/acetone-Ace mixture) and sequential solvent (butanol followed by DCM/Ace) extractions. Residues after extraction were subjected to methanolic saponification (MeKOH). Butanol (BuOH)- and total-extractability of B[a]P, following sequential solvent extraction, decreased significantly ($p < 0.05$) with time after addition of B[a]P. The decrease in BuOH extractability was particularly marked in the organic matter-rich clayey loam soil which also had the largest (> 40%) amounts of non-extractable residues. The cumulative amounts of B[a]P extracted in each soil by single and sequential solvent extractions were similar ($p > 0.05$) at each aging period, which indicate access to similar B[a]P fractions in soil by both solvent extractions. The similarity in the amounts of B[a]P non-extractable residues recovered by MeKOH of pre-extracted soils, through either of the extraction methods, confirms that the total-extractable B[a]P fractions from both methods are similar.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous in the environment with soil serving as a major sink for these hydrophobic organic contaminants, reducing environmental mobility and leading to long-term persistence in soil [1]. Of the 16 USEPA PAHs, benzo[a]pyrene (B[a]P) is one of the most concerning due to its carcinogenicity and toxicity [2]. B[a]P is often the risk driver at most PAH-contaminated sites. In soil, PAHs display biphasic or triphasic release (desorption) behaviour [3]. The first phase of release is rapid corresponding with the extraction of readily-available fractions by chemical solvents or to soil organisms [4,5]. The second and/or third desorption phases are slow or very slow due to PAHs partitioning or their diffusion into soil organic matter (SOM) and other sorption sites within the soil matrix where they become sequestered or strongly sorbed [6,7]. The reliable extraction and quantification of PAHs in soil may, therefore, be difficult and may impact understanding of PAH fate and behaviour in soil, especially in soils with highly sequestered fractions.

A range of extraction techniques, mostly using single solvents or solvent mixtures, in a single step have been used to study PAH extractability in soil [8-10]. A single-step exhaustive solvent extraction is used to determine total-extractable PAHs in soil; in terms of risk assessment, this is considered to be overly conservative or protective as it is assumed that 100% of the extracted PAHs are available [11,12]. Sequential solvent extraction involves the use of a mild solvent (e.g. butanol or methanol/water) and an exhaustive solvent (e.g. dichloromethane, acetone and/or hexane) extraction. The soil-PAH fractions that are recovered using mild solvent extraction are often considered to be readily-available to earthworms or microbes [13,14], whereas the fractions further extracted by exhaustive solvents are not readily-available [4,15]. Sequential solvent extractions provide information on PAH

fractions in soil relevant to where they may partition, as well as their mobility and availability, and their likelihood to cause significant harm to human and/or environmental health. However, only a few studies have investigated hydrophobic organic contaminants extractability using sequential solvent extractions [15-18].

Soil-PAH extractions are well developed and described, but nothing has been published to determine if single and sequential solvent extractions measure similar PAH fractions in soil. Such studies would be valuable to determine whether similar PAH fractions are extracted, as well as allowing valid comparisons of PAH extractions in studies where only one of the two methods has been used. In addition, time-dependent changes in the extractability of PAHs in contrasting soils determined by simultaneous single and sequential solvent extractions have not been previously investigated. The objectives of this study are; (i) to assess the effects of temporal changes in B[a]P extractability in four contrasting soils using both single and sequential solvent extractions; and (ii) to compare total extractability of B[a]P in soils by the extraction methods.

EXPERIMENTAL METHODS

Chemicals. Analytical grade B[a]P (> 96% purity), analytical grade acetone (Ace), acetonitrile (ACN), 1-butanol (density = 0.81 g/ml, ≥ 99.4%), dichloromethane (DCM), methanol (MeOH, HPLC Grade), toluene (Tol, 99.8%), potassium hydroxide (KOH) and silica sand were obtained from Sigma Aldrich Pty Ltd., Sydney, Australia. Hexane (Hex, HPLC grade) was purchased from Fisher Scientific, Loughborough, UK.

Soils. Four soils, labelled I (Kurosol), M (Ferrosol), B (Black Vertosol), and N (Tenosol) were collected from a depth of 5 – 20 cm from the Adelaide Hills, South Australia, Mount Tamborine and Beaudesert, Queensland, and Dublin, South Australia, respectively. The soils were described previously [19] and their properties are summarised in Table 1. Soils I, M, B, and N were sandy loam, loamy sand, clayey loam and sandy, respectively, based on USDA textural classification. Soil organic matter in oven-dried soil was determined by loss on ignition in an automatic temperature-controlled muffle furnace at 375 ^{o}C for 2 h.

Table 1. Soil properties (Modified after [9])

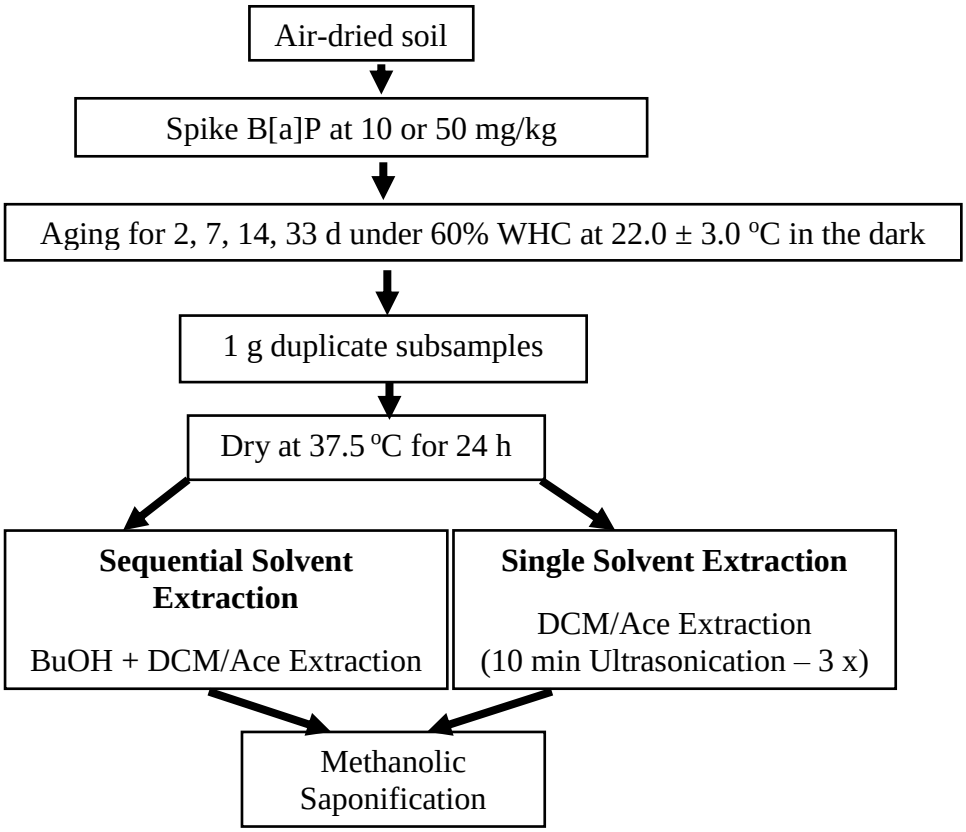
soil		I	M	B	N
particle size fraction	sand (%)	68.1	61.9	53	87.6
	silt (%)	21.2	16.8	16.1	6.7
	clay (%)	10.7	21.2	30.9	5.7
clay mineralogy		illite, kaolinite, quartz, montmorillonite	kaolinite, gibbsite, quartz	kaolinite, feldspar, montmorillonite-kaolinite interstratifications	quartz, illite, kaolinite, feldspar
pH	H_2O	5.3	5.8	6.7	7.1
SOM [a] (%)		13.3	21.4	11.0	4.8
TOC (%)		4.3	7.4	3.5	1.2
DOC (mg/L)		108.0	103.0	95.5	47.7
CEC_b[c] (cmolc/kg)		7.9	6.4	38.7	9.4
surface area (m^2/g)		6.0	51.7	4.0	7.9

[a] soil organic matter.

Experimental Design. Air-dried subsamples of soils were passed through a 2 mm sieve and spiked with 10 mg/kg or 50 mg/kg B[a]P [20] and moistened to 60% of water holding capacity (WHC). Spiked soils were incubated in the dark for 2, 7, 14, and 33 d. After each aging period, four subsamples from each treatment were dried at 37.5 ºC. Of the four dried samples, duplicate subsamples (1 g each) were subjected to either sequential or single solvent extraction [15,21,22] (Figure 1). The sequential extraction was designed to include BuOH extraction for the removal of weakly-sequestered B[a]P fractions in soils [21], followed by a DCM/Ace extraction to remove more strongly-sequestered fractions [21,22]. The remaining duplicate subsamples were extracted with DCM/Ace only (single solvent). The extracted soils, from both extraction methods, were allowed to dry and then subjected to methanolic saponification (MeKOH) [23] for mass balance purposes. The extracts were vacuum-concentrated, redissolved in ACN and filtered using 0.45 µm PTFE syringe filters prior to

96 HPLC analysis. All percentage extractability calculations were based on the initial amounts
97 of B[a]P spiked in the soil, unless where otherwise specified:

98 $$\text{Extractability (\%)} = \left(\frac{\text{Amount of B[a]P Extracted by Solvent (mg)}}{\text{Amount of B[a]P Spiked into Soil (mg)}}\right) \text{X } 100\% \; ----(1)$$



99

100 Figure 1. Experimental design

101 **Spiking and Aging of Soils.** A previously described spiking method known to result in
102 homogeneous hydrophobic organic contaminant distribution in soil was followed [20]. Briefly,
103 250 g air-dried soil was placed in a wide-mouth 2 L glass container in a fume hood. Stock
104 B[a]P solution (5 ml of 10000 mg/L B[a]P in Ace:Tol = 2:1, *v/v* for 50 mg/kg, or 5 ml of
105 2000 mg/L B[a]P in Ace:Tol = 2:1, *v/v* for 10 mg/kg) was added drop-wise using an air-tight
106 glass pipette. The pipette was rinsed with additional Ace and the content transferred into the
107 glass container. The glass container was covered to allow solvent dispersal, and then
108 uncovered overnight to allow solvent to volatilise. Soil was homogenised manually using a
109 stainless steel spoon. Another 750 g of clean soil was added batch-wise and mixed with the

spiked 250 g. Soil moisture content was then adjusted to 60% of its WHC. Blank soils without B[a]P spikes were similarly treated. In a similar manner, 50 g silica sand was concurrently spiked and used as a reference material to monitor and test spike recovery. Soils were kept in sealed amber glass jars and aged in the dark at 22 ± 3 ºC for 2, 7, 14, and 33 d.

Determination of Butanol-Extractable B[a]P. Soils were extracted with BuOH following a previously described procedure with some modifications [21,22]. Briefly, approximately 1 g (duplicate) soil was weighed into a clean pre-weighed 22 ml glass centrifuge tube with a PTFE-lined silicone cap and mixed with 3 ml BuOH. The soil-BuOH mixture was vortexed for 50 s and centrifuged at 1800 *g* for 10 min. The supernatant was decanted, vacuum-concentrated, and redissolved in ACN. An aliquot was then filtered through a 0.45 µm polytetrafluoroethylene (PTFE) syringe filter and transferred into 2 ml amber HPLC vials ready for analysis. Butanol extractability (%) was then determined. BuOH is viscous and can be retained in the soil residue after decantation of the supernatant solvent. Correction for the retained volume was made gravimetrically:

$$\text{Retained Volume (ml)} = \frac{W_1 - W_2}{D}$$

Where: W_1 (g) = Weight of glass centrifuge bottle + air-dried soil + BuOH before extraction, W_2 (g) = Weight of glass centrifuge bottle + extracted soil + BuOH after decantation, and D = density of BuOH (0.81 g/ml). The retained concentration of BuOH-extractable B[a]P was estimated relative to the supernatant concentration determined after HPLC analysis, and then subtracted from the subsequent DCM/Ace extractability results.

Determination of Total-Extractable B[a]P. For the single solvent extraction, 1 g of dried soil subsamples (duplicate) were extracted with 3 ml DCM/Ace (1:1, *v/v*) in an ultrasonication bath following previously described procedures [21,22]. For the sequential

133 solvent extraction, the resulting soil residue after BuOH extraction was extracted with
134 DCM/Ace in a similar manner. In a preliminary test (data not shown), good recovery (≥ 90%)
135 was obtained from extraction of fresh B[a]P-spiked soil in an ultrasonication bath (40 KHz,
136 10 min per cycle, and extraction frequency of 3 times). The 90% recovery was comparable to
137 those from other studies where more sophisticated extraction techniques such as accelerated
138 solvent (ASE), Soxhlet, and DCM-Soxtec extraction were used [10,15]. After extraction, the
139 combined supernatant was vacuum-concentrated, re-dissolved in ACN and filtered through a
140 0.45 µm PTFE syringe filter prior to HPLC analysis. The DCM/Ace extractability (%) was
141 determined, and pre-extracted soil residue was left in the fume hood overnight. The residue
142 after the exhaustive DCM/Ace extraction was referred to as B[a]P non-extractable residues [24].

143 **Methanolic Saponification of Soils.** Methanolic saponification can cleave ester bonds of
144 SOM, thereby releasing mostly non-covalently bound non-extractable residues (Type 1) that
145 are entrapped and adsorbed to soil matrices without substantially affecting covalently-bound
146 non-extractable residues (Type II) [10,24]. Soil residue after DCM/Ace extraction, following
147 both extraction methods, was hydrolysed with 10 ml MeOH/2 M KOH (14:1, *v/v*). The
148 mixture was vortexed for 10 s and then heated in a water bath at 80.0 ± 5.0 ºC for 5 h.
149 Release of B[a]P non-extractable residues in soils by methanolic saponification tends to
150 stabilise after 5 h [25]. Thereafter, the sample was allowed to cool, sonicated for 60 s, vortex-
151 extracted with 5 ml Hex and centrifuged. The topmost non-polar layer was transferred to a
152 clean glass bottle using a pipette tip, after which the extraction was repeated 2 more times.
153 The pipette tip was finally rinsed with 5 ml Ace into the clean glass bottle to ensure
154 maximum transfer of any trace of adsorbed B[a]P on the pipette walls. We used a pipetting
155 method for liquid-liquid extraction (LLE) instead of a separation funnel because
156 approximately 25% B[a]P was lost from silica sand freshly spiked at 50 mg/kg B[a]P
157 throughout the LLE process using a separation funnel, compared to the pipette method from

158 which no detectable loss was observed. The combined Hex extracts were vacuum-
159 concentrated, redissolved in ACN and mixed with anhydrous Na_2SO_4 (pre-baked at 450 ºC
160 for 12 h). The extracts were then filtered through 0.45 µm PTFE syringe filters prior to HPLC
161 analysis.

162 **HPLC Analysis of B[a]P.** The concentrations of B[a]P in the extracts were analysed with
163 an Agilent 1100 Serials HPLC equipped with a fluorescence detector (excitation wavelength
164 = 230 nm and emission wavelength = 460 nm). A reverse-phase C_{18} column (Agilent Eclipse
165 PAH, 4.6 x 50 mm, 1.8 µm particle size) equipped with a Kinetex security guard cartridge
166 (with a Krudkatcher in-line filter, 0.5 µm depth x 0.004 inch from Phenomenex, Lane Cove,
167 NSW, Australia) that was thermostated on both sides at 37 ºC was used. A sample volume of
168 10 µl was injected into the HPLC by an autosampler and isocratically-eluted with ACN:H_2O
169 mobile phase (85:15, v/v) at 1.0 ml/min. The total run time was 5 min including a post-run of
170 30 s prior to subsequent injection, with needle rinses between successive injections.

171 **Summary of Operational Definitions.** The following definitions were adopted in this
172 study:

173 i. BuOH extractability (%): estimates readily-available B[a]P fractions in soils;
174 ii. Total extractability (%): i + DCM/Ace extractability for sequential extractions, or
175 only DCM/Ace extractability for single solvent extractions;
176 iii. B[a]P non-extractable residues recovered by alkaline treatment (%): MeKOH
177 extractability;
178 iv. Non-extractable residue (%): 100 – ii;
179 v. Mass balance: ii + iii.

QA/QC. Solvent-rinsed clean glass bottles with PTFE-lined caps were used throughout, and were tightly-capped throughout the experiment. A standard calibration curve using 10 calibration levels (0.1 ng/ml to 1 μg/ml) was used to determine extracted B[a]P concentrations and consistently gave R^2 greater than 0.999. The detection limit using linear regression was calculated from the lowest detectable concentrations (0.1, 0.5, 1, 5, and 10 ng/ml) using 8 replicates for each concentration. The limit of detection and limit of quantitation were estimated as 0.07 ng/ml and 0.21 ng/ml respectively. Background concentrations of B[a]P in the four soils and silica sand used were below the detection limit.

Data Analysis. Extractability data were analysed statistically with SPSS (IBM Corp, Version 24), and graphing was done by both Origin (Microcal Software Inc. USA, version 6) and SPSS. No data transformation was applied. There were 2 independent and 2 or more outcome variables. The independent variables included: 4 soil types and 4 aging periods. The outcome variables comprise BuOH, DCM/Ace, total, and MeKOH extractability (mg/kg or %). The levels of significance was taken to be $p < 0.05$. A Student T-test was used to compare the means of B[a]P extractabilities between the sequential- and single-solvent extractions, and the differences between percentage extractabilities at the 2 concentrations of B[a]P spiked into soil. One-way ANOVA was used to test between-group differences, such as effects of soil properties or aging on extractability, with Games Howell's test for Post Hoc analysis [26]. Where data were not normally-distributed (Shapiro-Wilk Normality Test, $p < 0.05$), the Student's t-test and one-way ANOVA were replaced by Mann-Whitney U and Kruskal Wallis tests, respectively.

RESULTS AND DISCUSSION

Variability of Extractability Data. The average standard deviations (SDs) in total extractability of B[a]P, determined with single solvent extraction of soils spiked at 10 mg/kg and 50 mg/kg, ranged from 2.6 – 5.2% and 1.6 – 5.9%, respectively, after 33 d of aging (Figures 2 and 3). For the sequential solvent extraction of soils spiked at 10 mg/kg and 50 mg/kg B[a]P, the average SD ranged from 1.3 – 7.8% and 2.5 – 6.6% (BuOH extractability), 0.9 – 7.7% and 0.8 – 2.1% (Total extractability), and 1.9 – 10.8% and 3.1 – 8.4% (MeKOH extractability), respectively (Figures 4 and 5). Hence, the single solvent extractions showed better precision (average SD = 11% or smaller) for total extractability than the sequential solvent extractions. The BuOH extraction in the sequential solvent extraction may have contributed to the larger variability observed in total extractability data, because it is a non-exhaustive extraction that does not reach equilibrium [10]. Soils are highly heterogeneous matrices, and variability in extractability data can be expected to some extent. Although the variations in extractability data were small, they reflect the difficulty of homogeneously spiking high molecular weight PAHs, such as B[a]P, directly into soils [10].

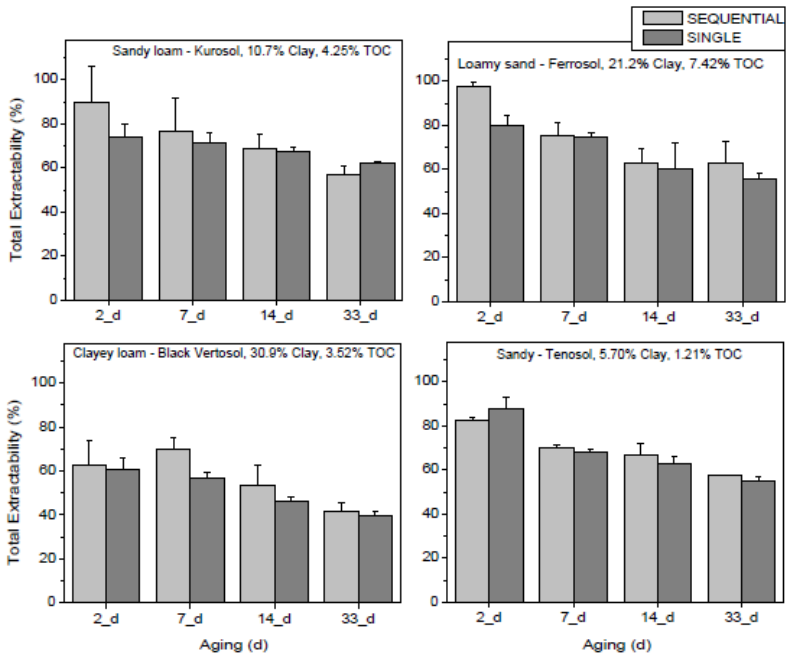


Figure 2. Temporal changes in total B[a]P extractability in soils following sequential and single solvent extractions (10 mg/kg B[a]P only). For single solvent extraction, 1 g soil was extracted with 3 ml DCM/Ace in an ultrasonication bath (40 KHz, 10 min per cycle, and extraction frequency of 3 times). After extraction, combined supernatant was vacuum concentrated and redissolved in acetonitrile, and then filtered through a 0.45 µm polytetrafluoroethylene syringe filter prior to HPLC analysis. For single solvent extraction, DCM/Ace extractability equals total extractability. Sequential extraction involves extraction of soil with butanol (BuOH) and dichloromethane/acetone (DCM/Ace) mixture. For BuOH Extraction, 1 g soil was extracted with 3 ml BuOH. The soil-BuOH mixture was vortexed for 50 s, centrifuged at 1800 g for 10 min. Resulting supernatant was prepared for HPLC analysis as in DCM/Ace. The residue after BuOH extraction was then extracted with DCM/Ace as described previously. Total extractability is the sum of BuOH- and DCM/Ace-extractable B[a]P. Values are mean of duplicates ± standard deviation.

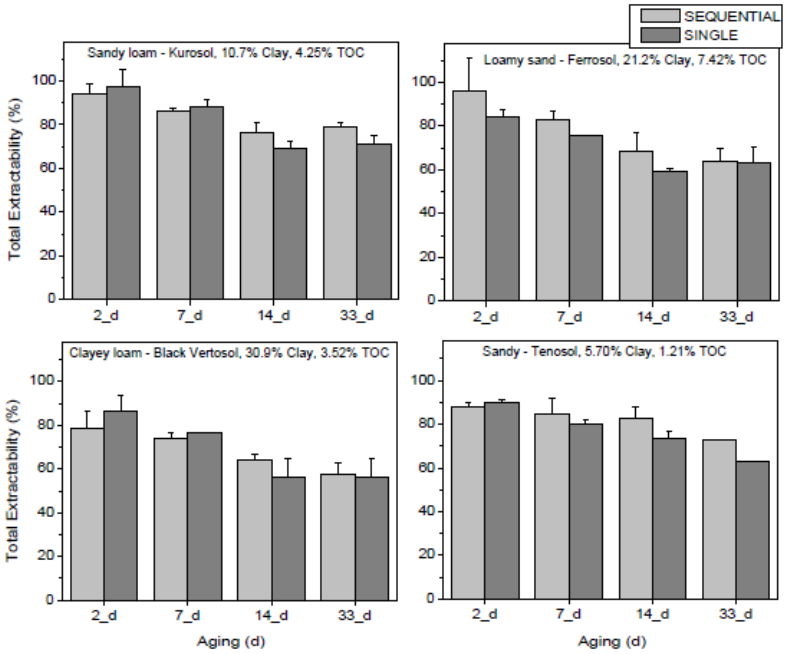
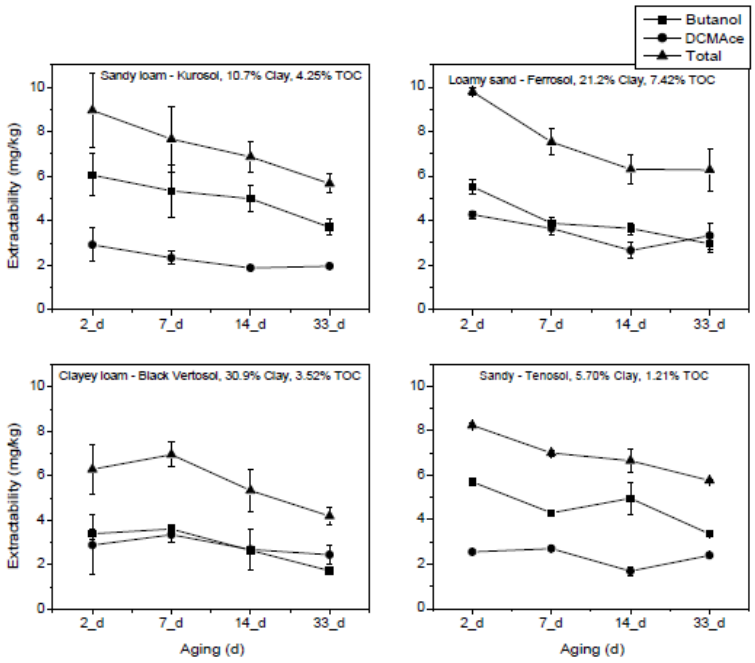


Figure 3. Temporal changes in total B[a]P extractability in soils following sequential and single solvent extractions (50 mg/kg B[a]P only). For single solvent extraction, 1 g soil was extracted with 3 ml DCM/Ace in an ultrasonication bath (40 KHz, 10 min per cycle, and extraction frequency of 3 times). After extraction, combined supernatant was vacuum concentrated and redissolved in acetonitrile, and then filtered through a 0.45 µm polytetrafluoroethylene syringe filter prior to HPLC analysis. For single solvent extraction, DCM/Ace extractability equals total extractability. Sequential extraction involves extraction of soil with butanol (BuOH) and dichloromethane/acetone (DCM/Ace) mixture. For BuOH Extraction, 1 g soil was extracted with 3 ml BuOH. The soil-BuOH mixture was vortexed for 50 s, centrifuged at 1800 g for 10 min. Resulting supernatant was prepared for HPLC analysis as in DCM/Ace. The residue after BuOH extraction was then extracted with DCM/Ace as described previously. Total extractability is the sum of BuOH- and DCM/Ace-extractable B[a]P. Values are mean of duplicates ± standard deviation.

Temporal Changes in B[a]P Extractability in Contrasting Soils.

*Butanol Extraction***.** BuOH-extractable PAH has sometimes been reported to correlate with PAH fractions that are readily-available to sentinel soil organisms, such as earthworms and microbes [13,14], as well as oral bioavailability in surrogates that model human gastrointestinal ingestion pathways [21,27]. Butanol extractability (mg/kg) generally decreased from 2 d to 33 d of aging especially in soil M at 33 d ($p < 0.05$) and soil N after 14 d ($p < 0.05$) (Figures 4 and 5). Previous aging studies also indicated that BuOH extractability tend to plateau approximately after 30 d [8]. B[a]P extractability (mg/kg) by BuOH was generally greater in the sandier soils I or N compared to soils M and B, and the percentage decreases in

252 extractability from 2 d to 33 d of aging was relatively steeper for soils B (48.9%) and M
253 (46.4%), compared to soil I (40.9%) or N (38.6%) (Figure 4). This indicates that B[a]P was
254 more strongly retained in soils B and M than in soils I and N. A similar trend was observed in
255 soils spiked with 50 mg/kg B[a]P (Figure 5). The spiked concentration (10 mg/kg or 50
256 mg/kg) did not significantly influence ($p > 0.05$) percentage BuOH extractability in each soil.
257 Overall, BuOH extractability was greater in the sandy organic matter-poor soils compared to
258 the clayey loam organic matter-rich soils that had comparatively larger capacity for B[a]P
259 sequestration.



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261 Figure 4. Temporal changes in extractable-B[a]P in soils following sequential extraction (10
262 mg/kg B[a]P only). For BuOH Extraction, 1 g soil was extracted with 3 ml BuOH. The soil-
263 BuOH mixture was vortexed for 50 s, centrifuged at 1800 g for 10 min. Resulting supernatant
264 was vacuum concentrated and redissolved in acetonitrile, and then filtered through a 0.45 µm
265 polytetrafluoroethylene syringe filter prior to HPLC analysis. For DCM/Ace extraction, residue
266 after BuOH extraction was extracted with 3 ml DCM/Ace in an ultrasonication bath (40 KHz,
267 10 min per cycle, and extraction frequency of 3 times). After extraction, combined supernatant
268 was prepared for HPLC analysis as in BuOH. Total extractability is the sum of BuOH- and
269 DCM/Ace-extractable B[a]P. Values are mean of duplicates ± standard deviation.

*Dichloromethane/Acetone Extraction following BuOH Extraction***.** Dichloromethane/Acetone was used to extract B[a]P that was not extracted by BuOH. Extractability by DCM/Ace (mg/kg) also decreased with aging at both B[a]P concentrations; although, decreases were not observed at all aging times (Figures 4 and 5). The extractability between successive aging times, especially after 2 d of aging, suggest that DCM/Ace extractability (%) increased when BuOH extractability was smaller, or alternatively DCM/Ace extractability became comparatively smaller when BuOH extractability was larger. Both solvents used in this study showed different extraction capacities (Figures 4 and 5); thereby, indicating that BuOH and DCM/Ace access different B[a]P fractions in soil [9]. Weakly-associated B[a]P fractions in soil were released into BuOH, while strongly-associated fractions were released into DCM/Ace. However, BuOH may show similar extraction efficiency as DCM/Ace, if the extraction time with BuOH is extended [23]. Both solvents may be used to access similar B[a]P fractions in soil, depending on extraction conditions.

Total Extractability following Single- and Sequential-Solvent Extractions. Spike recoveries were greater than 95% from silica sand at each aging time, indicating only minimal B[a]P losses during laboratory procedures (data not shown). Total B[a]P extractability, following single solvent extraction, in soils after 2 d of aging ranged from 60.6% (soil B) to 87.9% (soil N) at 10 mg/kg (Figure 2), and from 84.2% (soil M) to 97.3% (soil I) at 50 mg/kg (Figure 3). For the sequential solvent extraction, total B[a]P extractability ranged from 62.9% (soil B) to 97.9% (soil M) at 10 mg/kg (Figure 2), and from 78.5% (soil B) to 95.9% (soil M) at 50 mg/kg (Figure 3). At 2 days of aging, complete mass balance of B[a]P (≥ 100%) were generally obtained following methanolic saponification of soils which had been previously exhaustively extracted by both single and sequential solvent extractions (Figure S1 in Supplementary Information), except in soil B. The mass balances of B[a]P in soil B were

78.1 ± 10.4% (10 mg/kg) and 88.9 ± 8.5% (50 mg/kg) in the sequential treatments (Figure S1). Benzo[a]pyrene is highly hydrophobic (Log octanol-water partition coefficient = 6.3) [28] and its sequestration can be rapid [15], particularly in soils with large contents of clay and organic matter [29]. The subsequent decreases in B[a]P extractability following aging were attributed to sequestration in soil, although losses due to biodegradation cannot be completely disregarded.

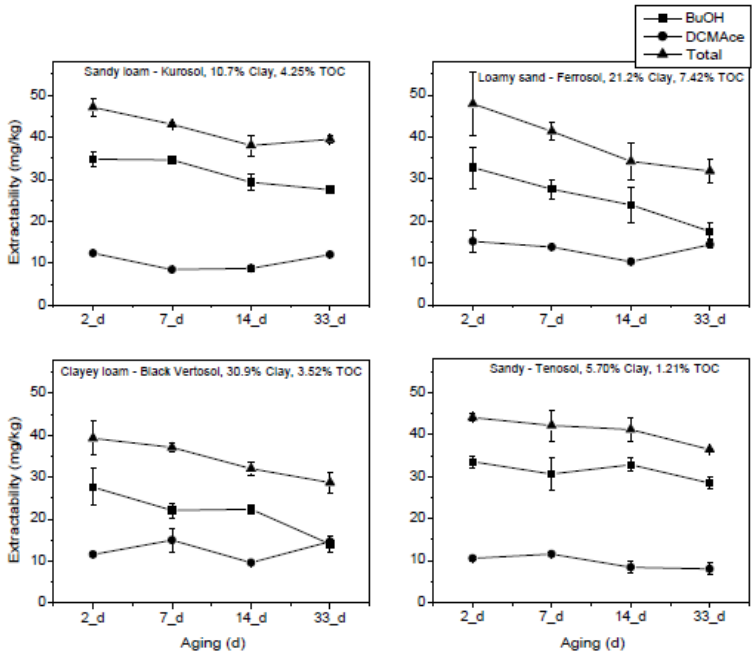

Figure 5. Temporal changes in extractable-B[a]P in soils following sequential extraction (50 mg/kg B[a]P only). For BuOH Extraction, 1 g soil was extracted with 3 ml BuOH. The soil-BuOH mixture was vortexed for 50 s, centrifuged at 1800 g for 10 min. Resulting supernatant was vacuum concentrated and redissolved in acetonitrile, and then filtered through a 0.45 µm polytetrafluoroethylene syringe filter prior to HPLC analysis. For DCM/Ace extraction, residue after BuOH extraction was extracted with 3 ml DCM/Ace in an ultrasonication bath (40 KHz, 10 min per cycle, and extraction frequency of 3 times). After extraction, combined supernatant was prepared for HPLC analysis as in BuOH. Total extractability is the sum of BuOH- and DCM/Ace-extractable B[a]P. Values are mean of duplicates ± standard deviation.

Changes in total B[a]P extractability data at both spiked concentrations in soils show similar trends with time ($p > 0.05$) (Figures 2 and 3). Also, a comparison of total extractability as a percentage of the amount of B[a]P spiked (Figures 2 and 3), mass balances (Figure S1), as well as non-extractable residues and MeKOH-extractable B[a]P (data not shown) did not show significant differences ($p > 0.05$) between the sequential and single solvent extractions

316 in any soil over the aging period. The similarity in total extractability results indicated that
317 similar B[a]P fractions in soil were accessed by both extraction methods. Therefore, either
318 extraction methods may be used to determine total-extractable B[a]P in soil. This result
319 further implies that extractability data obtained from studies using single or sequential solvent
320 extraction schemes as in this study may be compared. The total extractabilities for the two
321 methods were thereafter aggregated and averaged (Table 2), as well as the estimated B[a]P
322 non-extractable residues (%) (Figure S2), MeKOH-extractable B[a]P (%) (Table 2), and mass
323 balance (Figure S3). Total-extractable B[a]P (%) in soils decreased over time but slightly
324 (Table 2). This indicates the time-dependent effects of sequestration on B[a]P extractability
325 in soil were more realistically reflected by the mild (BuOH) extraction, than the harsh
326 (DCM/Ace) extraction.

Table 2. Total- and MeKOH-extractable B[a]P in Soils

soil	aging (d)	10 mg/kg total (%)	SD [a]	MeKOH (%)	SD	50 mg/kg total (%)	SD	MeKOH (%)	SD
I	2	81.7^{aA}	13.8	27.6	19.2	95.8^{A}	5.5	11.1	1.3
	7	74.0^{AB}	9.5	3.8	0.2	87.3^{AB}	2.2	2.3	0.6
	14	68.1^{ABC}	4.3	4.1	1.1	72.5^{C}	5.5	2.5	0.7
	33	59.5^{BCD}	4.2	4.8	0.8	75.0^{CD}	5.4	4.9	0.3
M	2	67.5^{A}	14.6	37.6	6.0	74.9^{A}	12.4	17.0	15.7
	7	75.0^{AB}	3.6	4.4	0.6	79.2^{AB}	4.8	2.6	0.3
	14	61.7^{C}	7.8	5.5	1.1	63.9^{C}	7.2	2.6	0.3
	33	59.2^{D}	7.1	6.3	0.6	63.5^{D}	5.2	3.6	0.3
B	2	61.7^{A}	7.3	36.9	25.2	82.6^{A}	7.8	10.8	0.9
	7	63.1^{AB}	8.4	13.4	1.2	75.3^{AB}	1.8	7.9	0.3
	14	49.7^{ABC}	6.9	9.8	1.9	60.2^{C}	6.6	6.3	1.1
	33	40.7^{BCD}	2.8	10.8	0.6	57.0^{CD}	5.6	7.3	0.7
N	2	85.1^{A}	4.4	41.7	28.2	89.1^{A}	1.6	13.0	0.7
	7	69.2^{B}	1.3	16.1	0.2	82.0^{AB}	5.1	11.3	1.1
	14	64.8^{BC}	3.9	12.0	1.9	77.9^{C}	6.5	5.1	2.4
	33	56.3^{D}	1.8	16.9	0.5	67.9^{D}	5.8	7.4	3.8

Total extractability is the average of extractable B[a]P in soils following single solvent extraction with DCM/Ace and sequential extraction with BuOH and DCM/Ace. Residues after DCM/Ace extractions were mixed with 10 ml methanol/2 M potassium hydroxide (MeKOH, 14:1, v/v). Soil I is sandy loam, soil M is loamy sand, soil B is clayey loam, and soil N is sandy. [a] In the column for each soil, the cells for aging time (d) of 2, 7, 14, and 33 were initially assigned upper case letters A, B, C, and D respectively. Against each column categorised per soil type, when any cell contains same letter which has been coded for a different cell, then values within those cells were statistically similar ($p > 0.05$), else they are significantly different ($p < 0.05$). For example, values in cells 1 and 2 within column 3 (Total), row 1 (soil I) were not different ($p > 0.05$). Values throughout a column per soil without any letters show no significant difference ($p > 0.05$), except MeKOH column. [b] Standard deviations.

B[a]P Non-Extractable Residues Released by Methanolic Saponification. With increasing soil-B[a]P contact time, the amounts of B[a]P non-extractable residues (%) increased (Figure S2). All four soils, and especially in soil B, at 10 mg/kg B[a]P showed substantial B[a]P sequestration after 33 d of aging, as the non-extractable residues generally became greater than 40% of the initially spiked concentration (Figure S2). At 50 mg/kg, B[a]P non-extractable residues constituted up to 25%, 32%, 36%, and 43% of the initially spiked concentrations in soils I, N, M, and B, respectively (Figure S2). The larger amounts of B[a]P non-extractable residues at 10 mg/kg compared to 50 mg/kg illustrate concentration influences of PAH sequestration in soils with similar contamination history [6,15]. The similarity ($p > 0.05$) in the amounts (%) of B[a]P non-extractable residues in soils recovered by MeKOH, following single and sequential solvent extractions allowed the aggregation and averaging of MeKOH extractability data (Table 2). This observation is further confirmation that both extraction methods accessed similar total-extractable B[a]P fractions in the soils, as well as resulted in similar amounts of non-extractable residues (data not shown). Given that B[a]P is persistent in soil and resistant to biodegradation, the estimated non-extractable residues (%) in this study refer to Types I and/or II [24]; segregating both NER types was not the focus of this study.

Recoveries (%) of B[a]P non-extractable residues by MeKOH were generally greater in soils spiked with 10 mg/kg B[a]P compared to soils spiked with 50 mg/kg B[a]P (Table 2). Large amounts of MeKOH-extractable B[a]P were observed in the pre-extracted (after DCM/Ace) soils after 2 d of aging (Table 2), indicating that B[a]P sequestration occurred rapidly [15]. The more sandy soil N generally showed the largest MeKOH-extractable B[a]P (%) at 2 d to 33 d of aging (Table 2). This observation suggested that B[a]P non-extractable residues were loosely sequestered in soil N compared to the other soils during the aging periods investigated, and were therefore more susceptible to methanolic saponification. Our

recent investigations also showed that the amounts of B[a]P non-extractable residues re-extracted or remobilised by additional solvent extractions, after re-equilibration periods of 30 d or 60 d, were greater in soil N than the other soils (unpublished), further suggesting strong B[a]P sequestration in organic matter-rich and clayey loam soils. In this study, B[a]P non-extractable residues recovered by MeKOH treatment at 33 d of aging are likely Type I non-extractable residues [24].

CONCLUSIONS

This study is distinctive in that it monitored the time-dependent changes and similarities in the extractability and sequestration of B[a]P in four contrasting soils through a combination of sequential or single solvent extractions, and methanolic saponification. Benzo[a]pyrene extractability decreased over time, especially in organic-matter rich clayey loam soils rather than in sandier soils. The amounts of total-extractable B[a]P in soils were similar following sequential or single solvent extractions, indicating that both extraction methods access similar total-extractable B[a]P fractions in soils and are comparable. This is the first report of such observations. Similar amounts of sequestered B[a]P fractions were recovered by methanolic saponification of soils, following either single or sequential extractions further confirming similarities of both extractions. Sequential solvent extractions, first involving a mild solvent (e.g. BuOH) extraction, have an advantage over single solvent extractions in that B[a]P fractions loosely sequestered (or readily-available) in soil can be monitored along with total-extractable B[a]P fractions. However, where total-extractable fractions are suitable for their intended use, e.g. in risk assessments, or for estimating exposure to target organisms, the simpler and less expensive single extraction may be preferable.

ASSOCIATED CONTENT

Supporting Information Available. Figure S1. Comparing mass balance of B[a]P in soil between sequential (SEQ) and single solvent (SIN) extractions; Figure S2. Temporal changes in the amounts (%) of B[a]P non-extractable residues in soils at two spiked concentrations; and Figure S3. Average mass balance of B[a]P in soils. This information is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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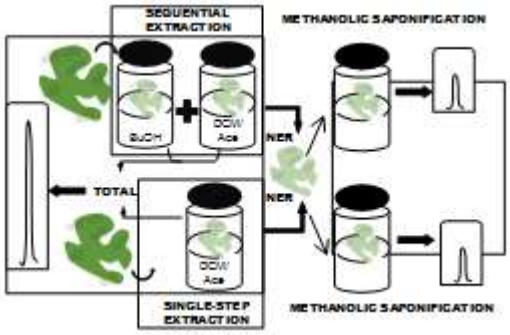

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SUPPORTING INFORMATION

Do Single and Sequential Solvent Extractions Determine Similar Total-Extractable Benzo[a]pyrene Fractions in Contrasting Soils?

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This supporting information is 4 pages including: Figure S1. Comparing mass balance of B[a]P in soil between sequential (SEQ) and single solvent (SIN) extractions; Figure S2. Temporal changes in the amounts (%) of B[a]P NERs in soils at two spiked concentrations; and Figure S3. Average mass balance of B[a]P in soils.

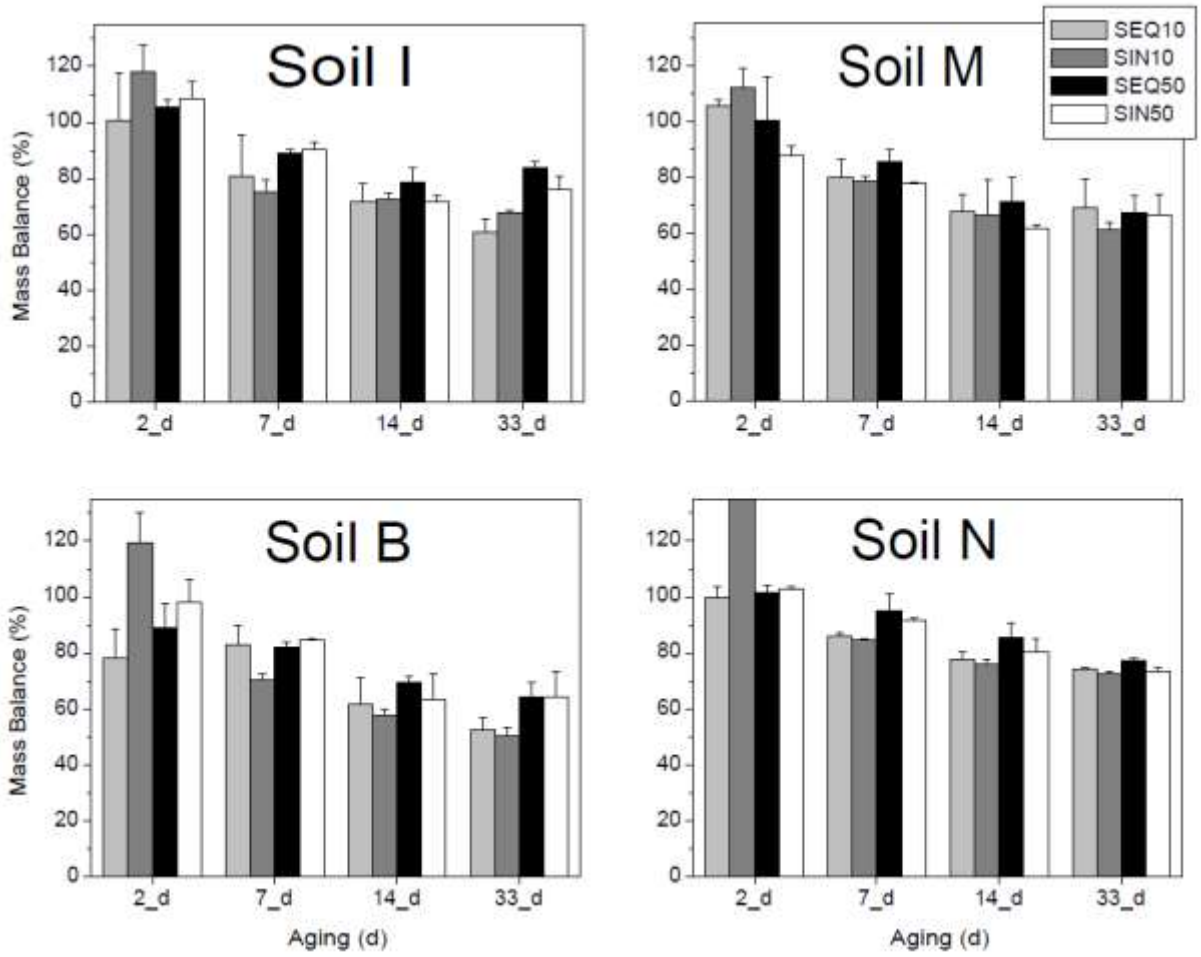

Figure S1. Comparing mass balance of B[a]P in soil between sequential (SEQ) and single solvent (SIN) extractions. 10 and 50 = 10 mg/kg and 50 mg/kg B[a]P spiked amounts in soil. Soil I is sandy loam, soil M is loamy sand, soil B is clayey loam, and soil N is sandy. Values are mean of duplicates ± standard deviation.

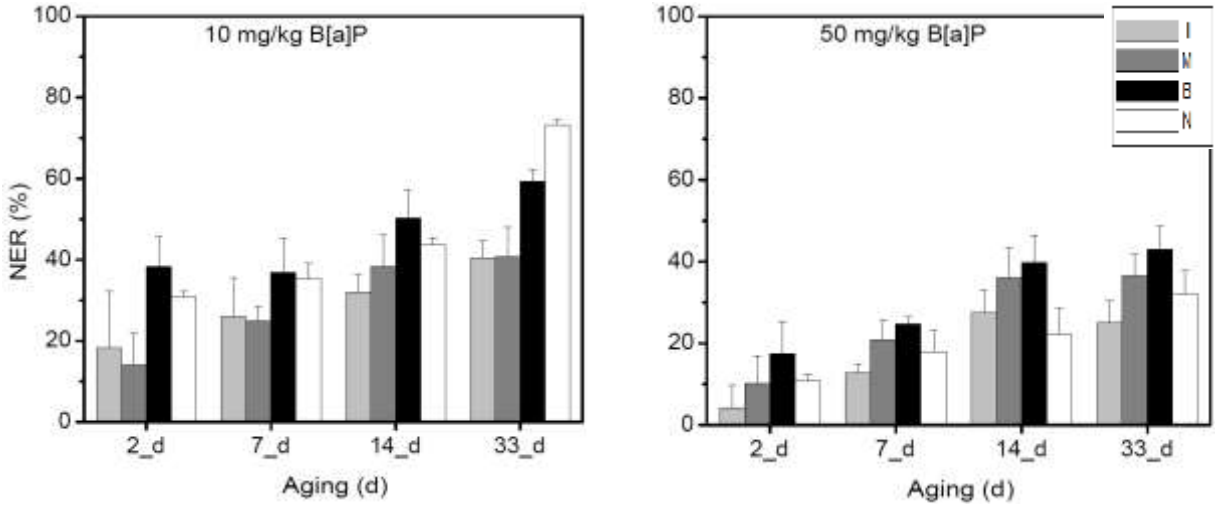

Figure S2. Temporal changes in the amounts (%) of B[a]P NERs in soils (I, M, B, N) at two spiked concentrations. Soil I is sandy loam, soil M is loamy sand, soil B is clayey loam, and soil N is sandy. Each data is an average between sequential (n = 2) and single solvent (n = 2) extractions ± standard deviation.

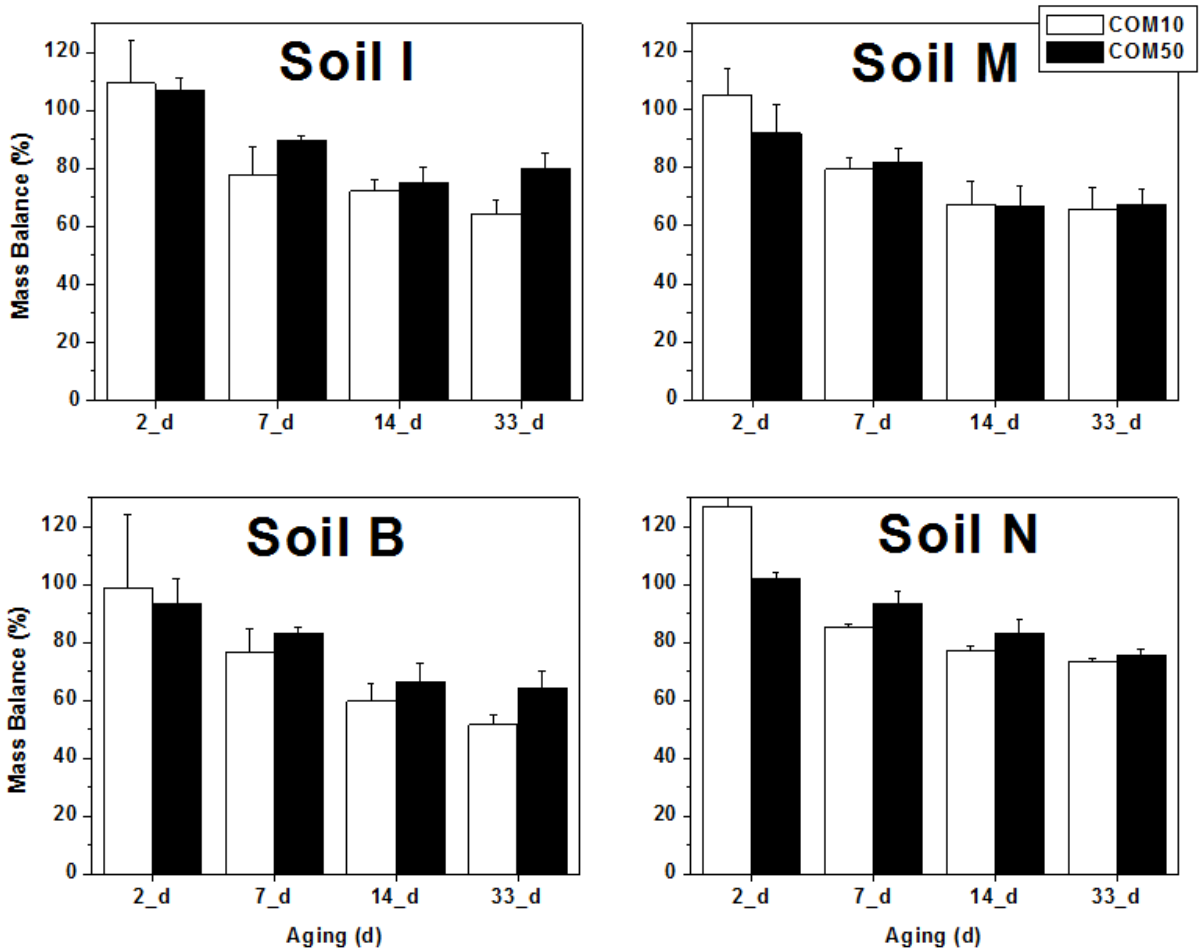

Figure S3. Mass balance of B[a]P in soils. COM = average extractability between sequential and single solvent extractions of soils spiked at 10 mg/kg (10) and 50 mg/kg (50) B[a]P. Soil I is sandy loam, soil M is loamy sand, soil B is clayey loam, and soil N is sandy. Values are mean (n = 4) ± standard deviation.