

1 Enhanced Recovery of Non-extractable
2 Benzo[a]pyrene Residues in Contrasting Soils
3 using Exhaustive Methanolic and Non-methanolic
4 Alkaline Treatments

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12 ABSTRACT

13 The fate, impacts and significance of polycyclic aromatic hydrocarbon (PAH) non-extractable
14 residues (NERs) in soils remain largely unexplored in risk-based contaminated land
15 management. In this study, 7 different methanolic and non-methanolic alkaline treatments, and
16 the conventional methanolic saponification, were used to extract benzo[a]pyrene (B[a]P) NERs
17 that had been aged for 180 d from four contrasting soils. Up to 16% and 55% of the amount of
18 B[a]P spiked (50 mg/kg) into soils was non-extractable after 2 d and 180 d of aging, respectively;
19 indicating rapid and progressive B[a]P sequestration in soils over time. The recovery of B[a]P
20 from soils after 180 d of aging was increased by up to 48% by the 7 different alkaline
21 extractions, although the extraction efficiencies of the different alkaline treatments did not
22 differ significantly ($p > 0.05$). Approximately 40% of B[a]P NERs in the sandy-clay-loam
23 organic matter-rich soil was recovered by the exhaustive alkaline extractions after 180 d of
24 aging, compared to only 10% using conventional methanolic saponification. However, the
25 amounts of B[a]P NERs recovered depend on soil properties and the amounts of NERs in soils.
26 A significant correlation ($R^2 = 0.69$, $p < 0.001$) was also observed between the amounts of
27 B[a]P recovered by each of the 7 alkaline extractions in the contrasting soils, and corresponding
28 NERs at 180 d of aging, indicating a potential association warranting further investigations.
29 Extraction techniques that estimate the amounts of PAH NERs recoverable in soil can help
30 give a better understanding of the fate of NERs in soil.

31 INTRODUCTION

32 The fate and behaviour of hydrophobic organic contaminants (HOCs), such as polycyclic
33 aromatic hydrocarbons (PAHs), in soil have been investigated for decades and are still being
34 researched. A range of solvent extractions schemes have been used to investigate PAH
35 extractability to elucidate their fate in soil. Non-exhaustive extractants including,
36 hydroxylpropyl- β -cyclodextrin (HPCD), TENAX, XAD, and *n*-butanol, are used to extract
37 weakly sequestered PAHs in soil, whereas, exhaustive solvents or solvent mixtures (e.g.
38 dichloromethane, acetone, hexane) are used to extract PAHs that are more strongly
39 sequestered in soils. These conventional solvent extractions are limited by their inability to
40 extract 100% of PAHs from soil, as there are strong interactions between PAHs and soil
41 organic matter (SOM), especially in long-term contaminated soils ¹⁻⁴. After successive
42 exhaustive solvent extractions, residual PAHs, termed ‘non-extractable residues (NERs)’,
43 may remain in soils as has been shown in studies using radio- or stable-isotope tracers ⁵⁻⁷.
44 Different terms have been used interchangeably in the literature to describe NERs, including:
45 non-bioavailable, non-labile, residual, resistant, highly sequestered, slowly-desorbing, non-
46 desorbing, desorption-resistant, recalcitrant, (ad)-sorbed, strongly sorbed, and bound among
47 others ⁸.

48 The formation of NERs results from progressive contaminant sequestration in soil ^{9,10}.
49 Sequestration as used describes the tortuous diffusion of HOCs into micro- and/or mesopores,
50 and/or physical and chemical sorption of HOCs to soil matrices ^{1,11}. The interactions between
51 PAHs with SOM, clay minerals, and carbon-rich materials such as black carbon also promote
52 PAH sequestration ^{3,10,12-14}. According to Kastner et al. ^{2,7}, non-covalently bonded NERs of
53 HOCs which are entrapped and strongly adsorbed in soils are referred to as Type I NERs,
54 whereas covalently-bonded NERs are Type II NERs. Microbial mineralisation of

55 biodegradable parent compounds into non-toxic natural compounds may result in the
56 formation of biogenic or Type III NERs which are incorporated into the SOM and undergo
57 natural turnover processes ^{2,15,16}. Total NERs in soil is therefore a summation of the three
58 types. While Type I to II have low to high stability in soil, Type III NER bears no
59 environmental risks or relevance ². Because of their stability, the amounts of NERs in soils
60 are difficult to measure, thereby, impacting thorough understanding of their fate in soils. In
61 soils where there are stronger soil-PAH interactions, there is likely to be larger amounts of
62 PAH NERs. Associated sequestration mechanisms include partitioning of PAHs into the
63 complex macromolecular structures of humic materials in soil, such as humic and fulvic acid,
64 humin, and SOM-mineral complexes within different particle size fractions or aggregates,
65 particularly silt and clay ^{2,3,17,18}. Of the humic materials in soil, humin is reported to exhibit
66 the greatest PAH sequestration ability due to its larger organic carbon content and embedded
67 micropores ^{3,6,19}. When labile ester or amide or ether bonds of SOM are disrupted, SOM may
68 be partially dissolved resulting in the release of humic materials, as well as PAHs
69 incorporated within the humic matter matrix ^{2,20}. As a result, PAH recoveries from soils can
70 be substantially increased.

71 Techniques to measure the amounts of NERs in soil would benefit risk-based approaches to
72 contaminated land risk assessment. This is because, changes in the amount of NERs in soil
73 due to the effects of dynamic processes, such as biodegradation, bioaccumulation, or
74 remobilisation, could be monitored reliably. This should result in better decision making
75 regarding NER stability in soil during contaminated land assessment and management. In
76 addition, efficient extraction of the so called ‘NERs’ would result in a better understanding of
77 total contaminant loads in soil which is useful for mass balance calculation purposes.

78 Based on the understanding of soil humic matter-HOCs interactions, methanolic
79 saponification of soils employing a 2 h or 5 h sample heating time has been used to increase
80 recovery of PAHs in soil ^{6,20,21}. Methyl isobutyl ketone extraction has also been used to
81 disaggregate humin to release substantial amounts of associated PAHs, as well as PAHs
82 associated with mineral fractions in soils ³. Other techniques involving ¹⁴C-sample
83 combustion and scintillation counting have been used for complete or near-complete mass
84 balancing of PAHs spiked in soil ^{6,19}; particularly, when complemented by chromatography
85 techniques ^{5,18}. The costs associated with ¹⁴C-sample combustion and scintillation counting
86 techniques mean that these techniques are not easily accessible. Various exhaustive alkaline
87 treatments have been used for routine soil humic matter extraction, including sodium
88 hydroxide, sodium pyrophosphate, sodium fluoride, potassium hydroxide, and a combination
89 of these chemicals ²²⁻²⁴. Specifically, modifying these exhaustive alkaline extractions for the
90 purpose of improving recovery of PAH NERs in soil may also allow better understanding of
91 PAH partitioning, mobility, and availability in soils.

92 The impact and significance of NERs in soils are uncertain ^{8,25}. The 256th American
93 Chemical Society (ACS) national meeting in Boston (August 19-23, 2018) with one of the
94 themes: “Non-Extractable Residue (NER) Bio-accessibility and Potential Risks” further
95 illustrates the paucity of information on the fate of NERs in soil ²⁶. Considering that the fate
96 of PAH NERs in soil has been largely unexplored, this study evaluates whether 7 different
97 exhaustive alkaline extractions, with or without methanol, access similar B[a]P NER
98 fractions in soils, and whether they increase recovery of B[a]P NERs from soils compared to
99 conventional methanolic saponification.

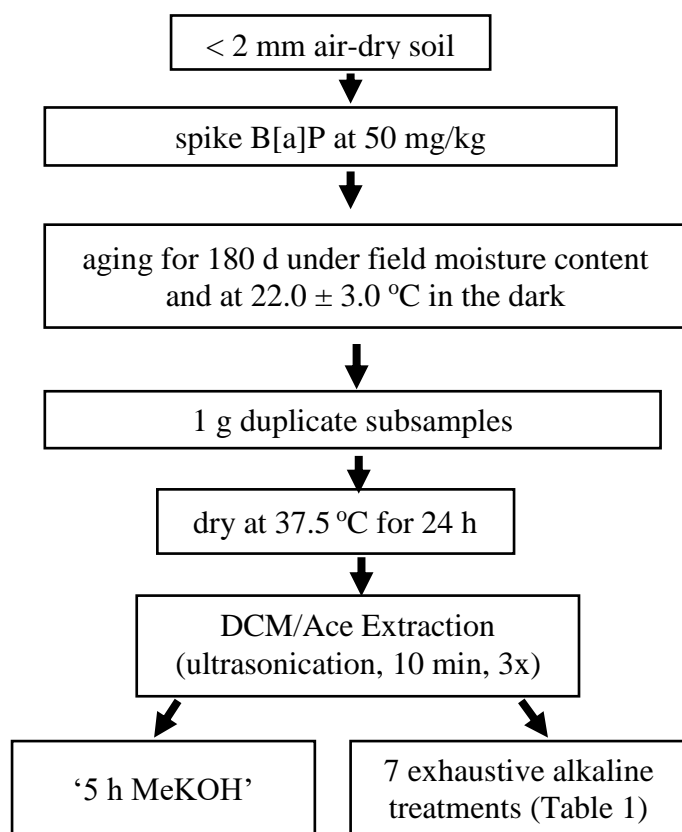
100 **EXPERIMENTAL METHODS**

101 **Chemicals.** Analytical grade B[a]P (> 96% purity), analytical grade acetone (Ace),
102 acetonitrile (ACN), dichloromethane (DCM), ethanol (Analytical Grade), methanol (MeOH,
103 HPLC Grade), toluene (Tol, 99.8%), potassium hydroxide (KOH) and silica sand were
104 obtained from Sigma Aldrich Pty Ltd., Sydney, Australia. Sodium hydroxide (NaOH) and
105 sodium fluoride (NaF) were sourced from the same suppliers. Hexane (Hex, HPLC grade)
106 was purchased from Fisher Scientific, Loughborough, UK.

107 **Soils.** Four soils (I, M, B, and N) were utilised in this study and their properties were
108 reported previously²⁷. Soils I, M, B, and N were sandy-loam, sandy-clay-loam, sandy-clay-
109 loam and loamy-sand, respectively, based on USDA textural classification. Soil organic
110 matter contents of soils I (13.3), M (21.4%), B (11.0%), and N (4.8%) were estimated by loss
111 on ignition²⁷. The total organic carbon content (TOC) of acid-hydrolysed soils I, M, B and N
112 were 4.3, 7.4, 3.5 and 1.2%, respectively. Prior to combustion of acid-hydrolysed soils at
113 1350 °C using LECO CNS analyser to determine the contents of hard organic carbon (hard
114 OC), soft OC fractions were removed by wet oxidation with persulphate according to a
115 previously described method²⁸. The difference between TOC and hard OC contents
116 estimated soft OC contents in the soils²⁸. A thermogravimetric analysis (TGA) was also used
117 to determine the contents of nonpyrogenic OC (weight losses at 200–470 °C) and black
118 carbon (BC)-rich fractions (weight losses at 470–600 °C) in soils based on the thermal
119 stability of these fractions²⁹.

120 **Experimental Design.** Previously documented quality assurance and quality control
 121 procedures were followed throughout the experiment ²⁷. Air-dried soils were sieved to less
 122 than 2 mm , spiked with 50 mg/kg B[a]P, rehydrated to field moisture contents (25% to 40%
 123 on dry weight basis), and kept for 180 d in the dark as described previously ²⁷. Dried soils
 124 were treated according to the design in Figure 1. Briefly, duplicates (1 g) of 7 subsamples
 125 were each extracted exhaustively with DCM/Ace in an ultrasonication bath and prepared for
 126 HPLC analysis as described previously ²⁷. The amount ($\mu\text{g/g}$) of B[a]P in soils that were
 127 extracted by DCM/Ace was referred to as total-extractable B[a]P, whereas the NER was
 128 estimated as the difference between the spiked concentration (50 $\mu\text{g/g}$) and the total-
 129 extractable concentration. Percentage extractability calculations were generally based on the
 130 amounts of B[a]P spiked (50 mg/kg) into the soil as described below:

131
$$\text{Extractability (\%)} = \left(\frac{\text{Amount of B[a]P Extracted by Solvent (\mu\text{g})}}{\text{Amount of B[a]P Spiked into Soil (\mu\text{g})}} \right) \times 100\%$$



132
 133 **Figure 1.** Experimental design.

134 Each of the extracted soils (n = 2) were then subjected to one of each of 7 exhaustive
 135 alkaline treatments (Table 1). A fresh subsample (1 g, n = 2) of each soil was then
 136 exhaustively extracted again and the extracted soil was subjected to methanolic
 137 saponification for 5 h. The resulting extracts were liquid-liquid extracted using Hex and
 138 prepared for HPLC analysis ²⁷. Extractability was then determined as previously described.

139 Table 1. Exhaustive methanolic and non-methanolic alkaline treatments used

ID	alkaline treatment	ratio (v/v)
1	2 M sodium hydroxide (NaOH) solution, 2 M NaOH	n/a
2	'1' + methanol (MeOH), '1' + MeOH	1:14
3	'1' + 0.4 M sodium fluoride (NaF) mixture, '1' + 0.4 M NaF	1:1
4	'3' + MeOH	1:14
5	0.1 M NaOH + 0.4 M NaF	1:1
6	'5' + MeOH	1:14
7	2 M potassium hydroxide (KOH) + MeOH, MeKOH	1:14

140 n/a: not applicable.

141 **Methanolic Saponification and Exhaustive Alkaline Treatment of Soils.** Soil containing
 142 B[a]P NERs (i.e. pre-extracted soil) was hydrolysed using different methanolic and non-
 143 methanolic alkaline treatments. The conventional methanolic saponification procedure
 144 (mixture of pre-extracted soil with 10 mL MeOH/2 M KOH (14:1, v/v)), thereafter referred to
 145 as '5 h MeKOH', has been described previously ²⁷. For the more exhaustive alkaline
 146 treatments, soils were first mixed with 10 ml of one of the alkaline solutions (Table 1). The
 147 mixtures were vortexed for 10 s and end-over-end rotated (100 rpm) for 24 h. The soil
 148 mixtures were then heat-treated in a temperature-controlled oven at 100 °C for 5 h and
 149 allowed to cool. The heat-treated samples were first liquid-liquid extracted with 5 ml
 150 ethanol:Hex (1:1, v/v) and vortexed briefly. Ethanol was added in all sample bottles to
 151 minimise the impacts of lipid emulsions especially observed in the sole alkaline treatments

152 (1, 3, and 5 in Table 1). Soil mixtures were then ultrasonicated for 10 min. The mixture was
153 centrifuged, and the hexane layer collected as described previously. The liquid-liquid
154 extraction was conducted 2 more times with only 5 ml Hex. The combined extracts from each
155 of the '5 h MeKOH' and exhaustive alkaline treatments were then prepared for HPLC
156 analysis²⁷. The fractions of B[a]P NERs recovered by the '5 h MeKOH' and each of the
157 exhaustive alkaline treatments were calculated relative to the estimated amounts of B[a]P
158 NERs in the soils.

159 **HPLC Analysis of B[a]P.** The concentrations of B[a]P in the extracts were analysed with
160 an Agilent 1100 Series HPLC equipped with a fluorescence detector (excitation wavelength =
161 230 nm and emission wavelength = 460 nm), as in our previous study²⁷.

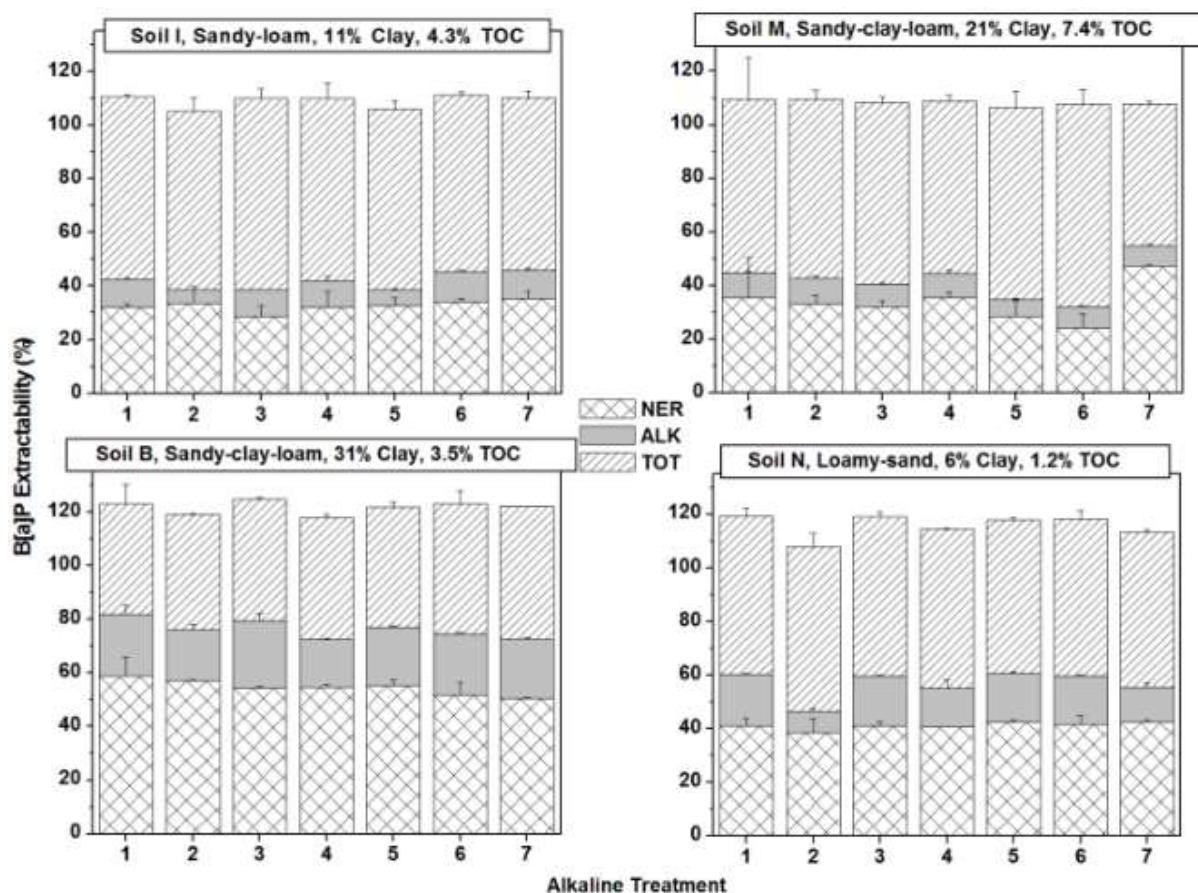
162 **Data Analysis.** Extractability data were analysed statistically with SPSS (IBM Corp,
163 Version 24), and graphing was by both Origin (Microcal Software Inc. USA, version 6) and
164 SPSS, without data transformation. There were 2 independent, and 2 or more outcome
165 variables. The independent variables included 4 soil types and up to 8 different methanolic
166 and non-methanolic alkaline treatments. The outcome variables were total extractability and
167 extractabilities by the different alkaline treatments ($\mu\text{g/g}$ or %). The levels of significance
168 adopted was $p < 0.05$. A Student's t test was used to compare B[a]P extractabilities between
169 each of the 7 exhaustive alkaline treatments and '5 h MeKOH'. One-way ANOVA was used
170 to test between-group differences, such as effects of soil types on B[a]P extractability, using
171 Games Howell's test for post hoc analysis³⁰. Where data were not normally-distributed
172 (Shapiro-Wilk Normality Test, $p < 0.05$), the Student's t test and one-way ANOVA were
173 replaced by Mann-Whitney U and Kruskal Wallis tests, respectively.

174 RESULTS AND DISCUSSION

175 **Total Extractability and Mass Balance of B[a]P in Soils.** Total extractability 2 d after
176 spiking in all four soils ranged from 84% ($42.1 \pm 1.7 \mu\text{g/g}$, soil M) to 97% ($48.7 \pm 4.0 \mu\text{g/g}$,
177 soil N), indicating good recovery. That 16% or less B[a]P (i.e. NER) could not be recovered
178 by the DCM/Ace extraction at the initial soil-B[a]P contact time (2 d) indicates that B[a]P
179 sequestration occurred rapidly. Considering B[a]P's hydrophobicity ($\text{Log } K_{ow} = 6.3$)³¹ and
180 the soils' physico-chemical properties, the amounts of B[a]P NERs formed at the initial soil-
181 contact time could be expected. Rapid sequestration of PAHs have been noted in other
182 studies^{16,19}. An additional 4% to 13% of the $50 \mu\text{g/g}$ B[a]P spiked, corresponding to $1.9 \pm$
183 $0.02 \mu\text{g/g}$ to $6.3 \pm 0.04 \mu\text{g/g}$ B[a]P, were recovered after methanolic saponification ('5 h
184 MeKOH') of pre-extracted soils 2 d after spiking and this confirmed the rapid B[a]P
185 sequestration in the soils only 2 d after spiking. Hence, mass balance (sum of total-extractable
186 B[a]P and B[a]P extracted by alkaline treatments) achieved after 2 d of spiking ranged from
187 $87.9 \pm 3.4\%$ to $108.3\% \pm 6.3\%$, indicating near-complete or complete recovery of B[a]P
188 spiked in soils.

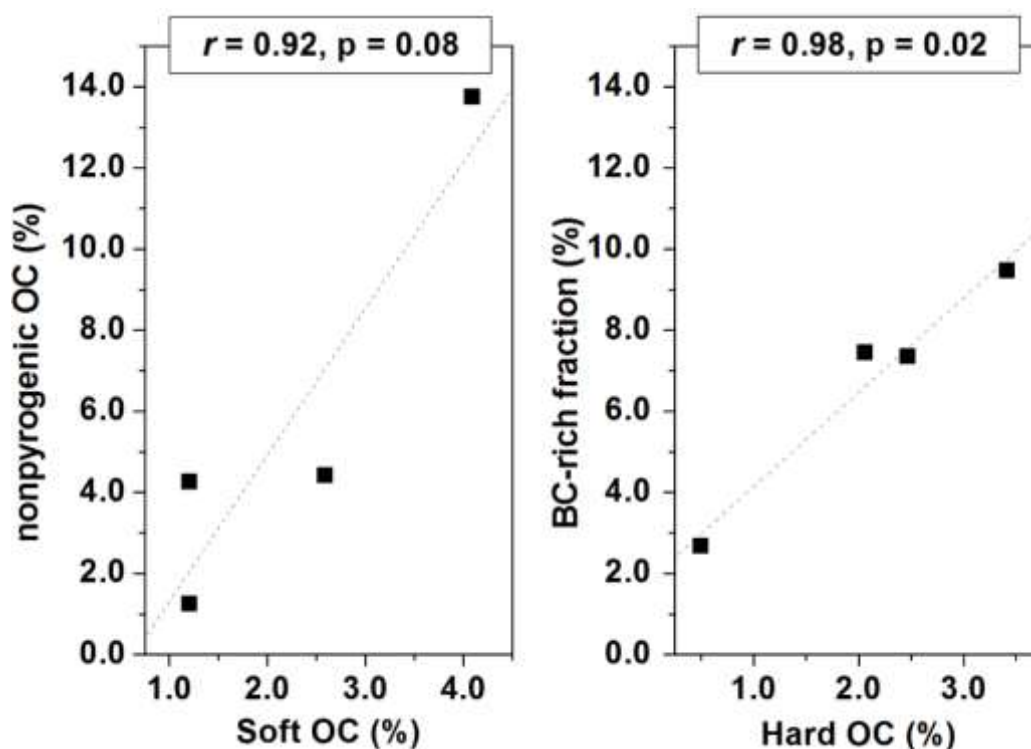
189 Spike recovery was $> 95\%$ from silica sand after 180 d of aging indicating only minimal
190 procedural losses in the laboratory. However, total B[a]P extractability in soils after 180 d of
191 aging ranged from 45% ($22.7 \pm 1.5 \mu\text{g/g}$, soil B) to 67% ($33.7 \pm 1.1 \mu\text{g/g}$, soil I) (Figure 2).
192 This suggests that approximately 33% to 55% of the amounts of B[a]P ($50 \mu\text{g/g}$) spiked in
193 soils were non-extractable after 180 d of aging. Sequestration of PAHs is known to increase
194 with aging time as a result of increased soil-PAH interactions^{1,19}. Therefore, the fractions of
195 PAHs which are extractable will be expected to decrease with increasing aging time, whereas
196 fractions of PAH NERs will be expected to increase, particularly for hydrophobic HMW
197 PAHs^{32,33}. The NERs may include fractions which are occluded in meso- or micro-pores

198 ^{18,28}, or adsorbed to surfaces of soil matrices by physical (Van der Waals) or weak non-
 199 covalent interactions (π - π) ^{2,28}, as well as fractions strongly sequestered to hard OC or BC-
 200 rich fractions in soils ^{11,13,14,28,34}.



201
 202 **Figure 2.** Extractability of B[a]P in soils subjected to 7 different alkaline treatments after 180
 203 d of aging. NER is non-extractable residue; ALK is amounts recovered by the 7 different
 204 alkaline treatments ranging from 8% ($4.2 \pm 0.6 \mu\text{g/g}$, soil M) to 22% ($10.8 \pm 1.2 \mu\text{g/g}$, soil I;
 205 and TOT is total extractability ranging from 45% ($22.7 \pm 1.5 \mu\text{g/g}$, soil B) to 67% (33.7 ± 1.1
 206 $\mu\text{g/g}$, soil I). 1 g soil was extracted with 3 mL DCM/Ace by ultrasonication (40 KHz, 10 min,
 207 3x). After extraction, combined supernatant was prepared for HPLC analysis. Pre-extracted
 208 soils containing B[a]P NERs were extracted with 10 mL alkaline solutions (Table 1). Values
 209 are means of duplicates \pm standard deviations.

210 In this study, a positive relationship was observed between hard OC and BC-rich fractions
 211 ($r = 0.98, p = 0.02$), as well as between soft OC and nonpyrogenic OC fractions ($r = 0.92, p =$
 212 0.08) (Figure 3). As a result of the recalcitrant nature of hard OC, PAHs in soils with large
 213 amounts of hard OC or BC-rich fractions can be strongly sequestered. Desorption of PAHs
 214 from these sites are likely to be slow^{13,14,28}. Extensive descriptions of the mechanisms of
 215 PAH sequestration in soils have been documented elsewhere^{1,11}.

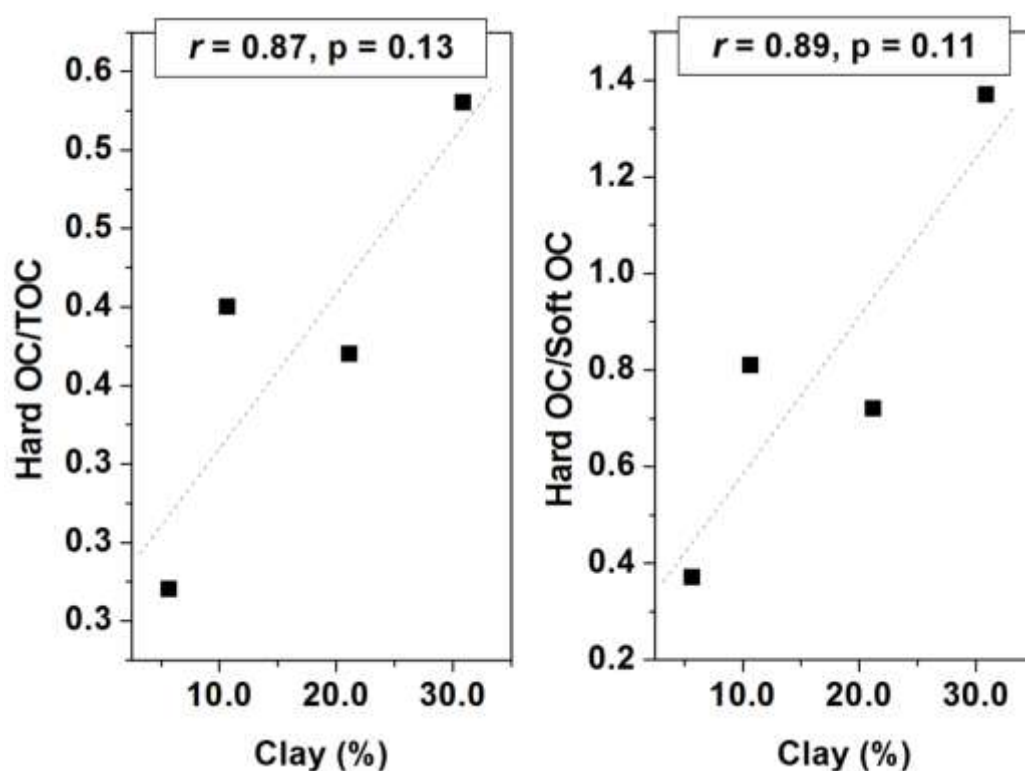


216

217 **Figure 3.** Relationship between organic carbon fractions in soils. The contents of total organic carbon (TOC) of acid-hydrolysed soils were determined by combustion at 1350 °C using LECO
 218 CNS analyser. Prior to combustion of acid-hydrolysed soils to determine the contents of hard
 219 organic carbon (hard OC), soft OC fractions were removed by wet oxidation with persulphate.
 220 The difference between TOC and hard OC contents estimated soft OC contents in the soils. A
 221 thermogravimetric analysis was used to determine the contents of nonpyrogenic OC (weight
 222 losses at 200–470 °C) and black carbon (BC)-rich fractions (weight losses at 470–600 °C) in
 224 soils based on the thermal stability of these fractions.

225 The capacity for PAH sequestration also differs with the quantity and quality of hard OC;
 226 hence, the amounts of extractable and sequestered PAHs in soils with contrasting physico-
 227 chemical characteristics will be expected to vary^{11,12}. The fraction of hard OC to TOC was

228 largest in the sandy-clay-loam soil B, which may explain the decreased extractability and
 229 stronger sequestration of B[a]P in soil B than in the other soils. It has also been reported that
 230 ^{14}C -phenanthrene extractability by HPCD and the total extents of ^{14}C -phenanthrene
 231 mineralisation decreased significantly ($p < 0.001$) by up to 50% with the increasing addition
 232 of 0.1 to 5% of activated carbon to 4 soils aged for 100 d, especially in clayey-loam soils ¹³.
 233 In addition, soils with larger amounts of fine-sized particles associated with OC fractions in
 234 soil have been reported to possess substantial sequestration capacity for B[a]P compared to
 235 soils with smaller amounts of these fine-sized fractions ¹⁸. In this study, a positive
 236 relationship was also observed between the amounts of clay and the fractions of hard OC
 237 relative to TOC content ($r = 0.87$, $p = 0.13$), and relative to soft OC fractions in soils ($r =$
 238 0.89 , $p = 0.11$) (Figure 4).

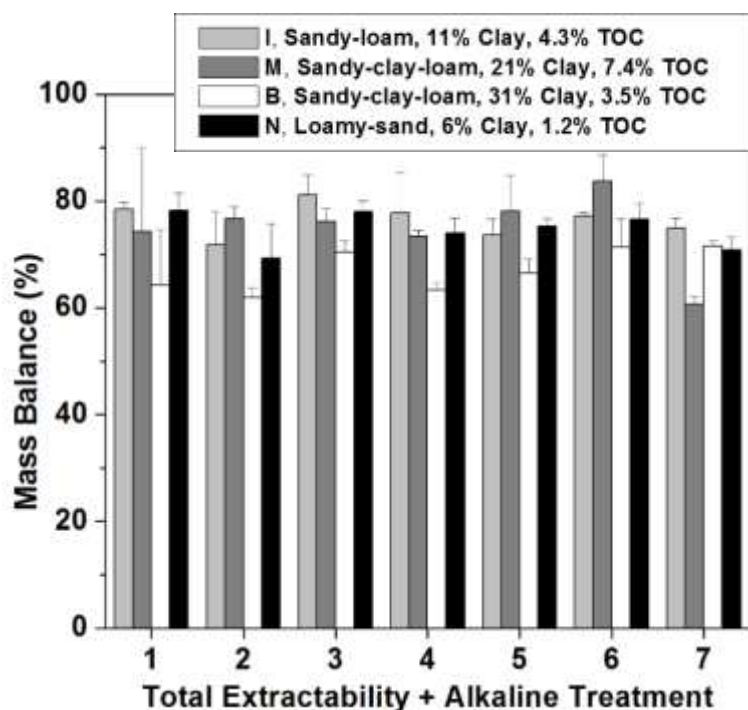


239
 240 **Figure 4.** Relationship between clay and recalcitrant organic carbon fractions in soils. The
 241 contents of total organic carbon (TOC) of acid-hydrolysed soils were determined by
 242 combustion at 1350 °C using LECO CNS analyser. Prior to combustion of acid-hydrolysed
 243 soils to determine the contents of hard organic carbon (hard OC), soft OC fractions were
 244 removed by wet oxidation with persulphate. The difference between TOC and hard OC
 245 contents estimated soft OC contents in the soils.

246 This relationship may indicate the association between recalcitrant OC fractions and fine-
247 sized soil particles which serve as sites for PAH sequestration in soils, thereby favouring
248 greater sequestration of B[a]P in soil B. A wider range of soils may be needed to validate the
249 observed relationships. Methanolic or non-methanolic alkaline treatments may partially
250 release occluded or sequestered fractions following hydrolysis of SOM^{6,35}, thereby
251 increasing mass recovery of PAHs spiked in soils²⁰. Hence, after the 7 different methanolic
252 and non-methanolic treatments (excluding '5 h MeKOH'), mass balance (%) of B[a]P after
253 180 d of aging in this study ranged from 71.8 ± 6.2 to 81.3 ± 3.7 , 60.7 ± 1.4 to 83.7 ± 4.9 ,
254 62.0 ± 1.7 to 71.6 ± 0.9 , and 69.3 ± 6.4 to 78.3 ± 3.3 for soils I, M, B, and N, respectively
255 (Figure 5). This showed that there was an increase of approximately 13 (soil I) to 48% (soil
256 B) of extractable B[a]P in pre-extracted soils following the alkaline treatments.

257 Overall, B[a]P mass balance in the soils after 180 d of aging were much reduced than after
258 fresh spiking and ranged from 61 to 84%. The fractions of the spiked B[a]P (50 $\mu\text{g/g}$) that
259 were not recovered (16 to 39%) after 180 d of aging were attributed to B[a]P NERs that were
260 highly sequestered in soils and non-extractable by the different alkaline treatments utilised. In
261 previous work using same soils to those used in this study, B[a]P recoveries after fresh
262 spiking ranged from 85% to 93%, and ¹⁴C-radioactivity in 160 d aged soils ranged from 88%
263 to 93% in the soils, except in soil B (77%)^{5,18}. This suggested that mass balance of B[a]P
264 spiked in soils decreased further by approximately 10 to 31% from 160 d to 180 d of aging.
265 The variations in B[a]P recovery may be attributed to the influence of different soil
266 properties. In a different study where Soxhlet extraction was used²⁸, total extractability of
267 B[a]P in 7 contrasting soils that were spiked at 10 $\mu\text{g/g}$ of B[a]P also ranged from 98 to 103%
268 after fresh spiking of B[a]P, and from 75 to 82 % after 200 d aging . Another study that used
269 a DCM-soxtec extraction technique and analysed ¹⁴C-B[a]P activity in extracts reported 83%
270 and 73% total extractability in sewage-sludge amended arable crop soil (10 $\mu\text{g/g}$ B[a]P) after

271 10 and 170 d of aging, respectively; the reported mass balances at 10 d and 259 d of aging
 272 were 94% and 77%, respectively ⁶. Furthermore, complete recovery of ¹⁴C-B[a]P was not
 273 achieved even after combusting spiked sterile soils that had been aged for 525 d ⁶.



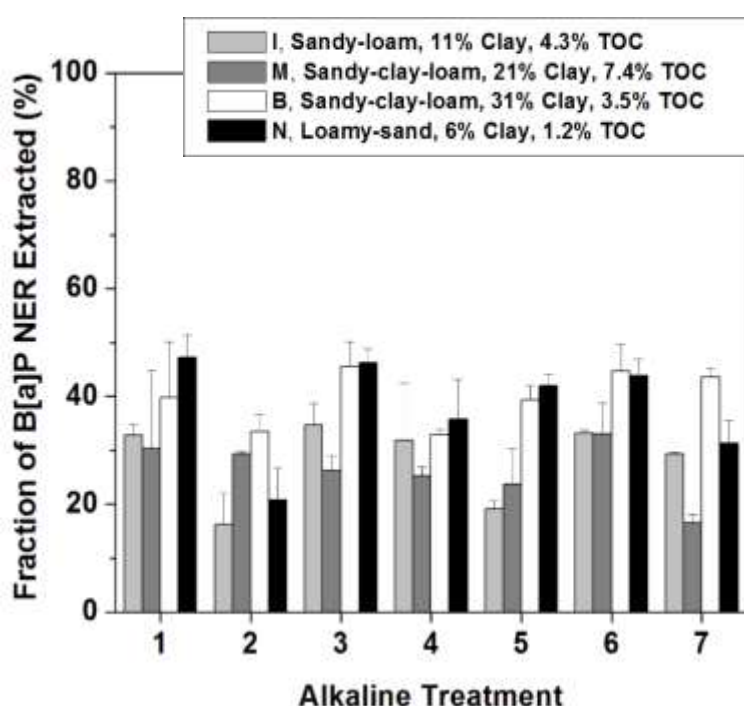
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275 **Figure 5.** Mass balance of B[a]P in soils after 180 d of aging. The different alkaline treatments
 276 exclude ‘5 h MeKOH’. 1 g soil was extracted with 3 mL DCM/Ace by ultrasonication (40 KHz,
 277 10 min, 3x). After extraction, combined supernatant was prepared for HPLC analysis. Pre-
 278 extracted soils containing B[a]P NERs were extracted with 10 mL alkaline solutions (Table 1).
 279 Mass balance is the sum of total-extractable B[a]P and B[a]P extractability by the different
 280 alkaline treatments. Values are means of duplicates ± standard deviations.

281 Overall, the total B[a]P extractability and mass balance as measured in this study were
 282 generally similar to the other cited studies where a range of solvents and extraction
 283 techniques were used. The differences in B[a]P extractability may be attributable to the
 284 influence of the contrasting properties of the soils utilised and has been discussed more
 285 extensively in our previous studies ^{27,34}.

286 **Comparing B[a]P Recoveries in Soils between the 7 Exhaustive Methanolic and Non-**
 287 **methanolic Alkaline Treatments.** Alkaline treatments ‘1’ (2 M NaOH), ‘3’ (2 M NaOH +

288 0.4 M NaF), and '6' (0.1 M NaOH + 0.4 M NaF + MeOH) showed relatively greater
 289 recoveries (%) based on the amounts of B[a]P spiked in soils (50 mg/kg) (Figure 6).
 290 Specifically, the fractions of B[a]P NERs recovered among the 7 treatments in each of soils I,
 291 M, B, and N ranged from $29.3 \pm 0.3 - 34.7 \pm 3.9\%$, $23.8 \pm 6.5 - 33.1 \pm 5.7\%$, $33.0 \pm 1.0 -$
 292 $45.6 \pm 4.6\%$, and $20.8 \pm 6.0 - 47.3 \pm 4.1\%$, respectively (Figure 6). The key result was that
 293 up to 50% of B[a]P NERs in the sandy-clay-loam and hard OC-rich soil B can be recovered
 294 by the exhaustive treatments after 180 d of aging.

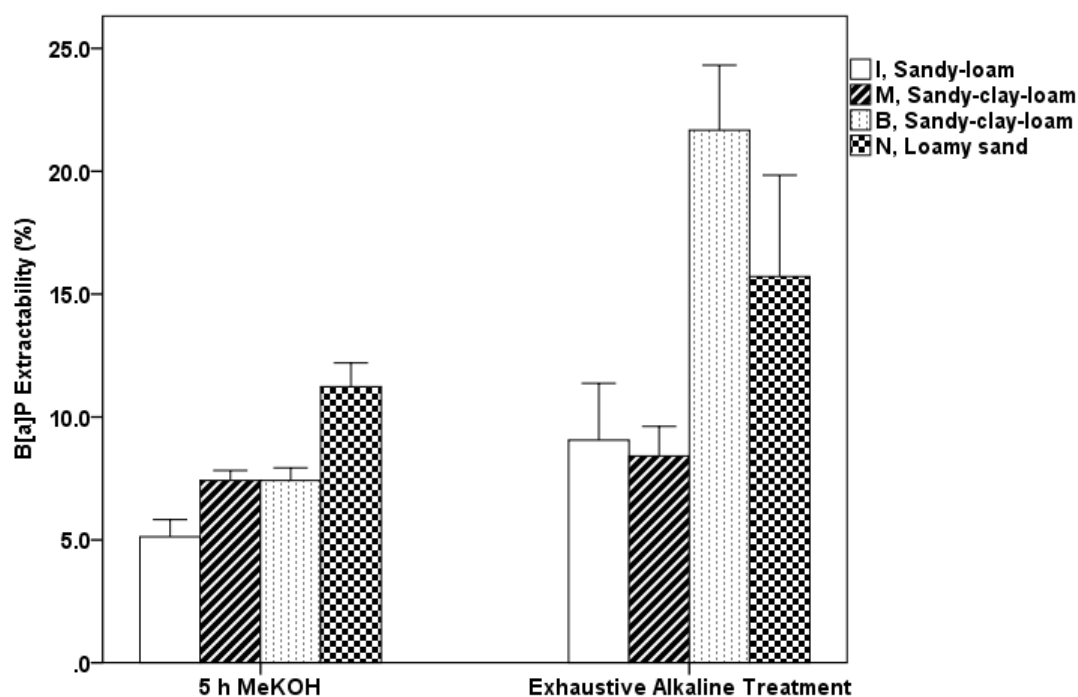


295 **Figure 6.** Fraction (%) of B[a]P NERs recovered by the 7 different alkaline treatments. The
 296 different alkaline treatments exclude '5 h MeKOH'. 1 g soil was extracted with 3 mL
 297 DCM/Ace by ultrasonication (40 KHz, 10 min, 3x). After extraction, combined supernatant
 298 was prepared for HPLC analysis. Pre-extracted soils containing B[a]P NERs were extracted
 299 with 10 mL alkaline solutions (Table 1). The fraction of B[a]P NER recovered is the percentage
 300 of B[a]P extracted by the different alkaline treatments relative to the amounts of B[a]P NER
 301 after DCM/Ace extraction. Values are means of duplicates \pm standard deviations.

303 Generally, recoveries of B[a]P NERs (%) in each soil did not differ ($p > 0.05$) between the 7
 304 different alkaline treatments (Figures 2 and 6). Mass balances of B[a]P were also not
 305 significantly different ($p > 0.05$) between the 7 different alkaline treatments ($p > 0.05$)
 306 (Figure 5). These observations generally reveal similar extraction capacities of the 7 different

307 alkaline treatments and suggest that the treatments access similar B[a]P NER fractions in
308 each soil. Therefore, the B[a]P recoveries by the 7 different alkaline treatments in each soil
309 were averaged (Figure 7), and further referred to as ‘exhaustive methanolic and non-
310 methanolic alkaline treatments’ for subsequent discussions.

311 **Exhaustive Methanolic and Mon-methanolic Alkaline Treatments versus Conventional**
312 **Methanolic Saponification.** A key observation after 180 d of aging in this study was that
313 recoveries of B[a]P in soils following exhaustive methanolic and non-methanolic alkaline
314 treatments were generally greater ($p < 0.05$) compared to those in soils subjected to ‘5 h
315 MeKOH’ (Figure 7), particularly in the sandy-clay-loam soil B. For instance, B[a]P
316 extractability in soil B following the exhaustive methanolic and non-methanolic alkaline
317 treatments was approximately 22% ($10.8 \pm 1.2 \mu\text{g/g}$); this corresponded to $39.9 \pm 5.2\%$ of
318 B[a]P NER after 180 d of aging. However, B[a]P extractability in soil B following ‘5 h
319 MeKOH’ was approximately 7% ($3.7 \pm 0.3 \mu\text{g/g}$); this corresponded to only $10.1 \pm 0.9\%$ of
320 B[a]P NERs after 180 d of aging.

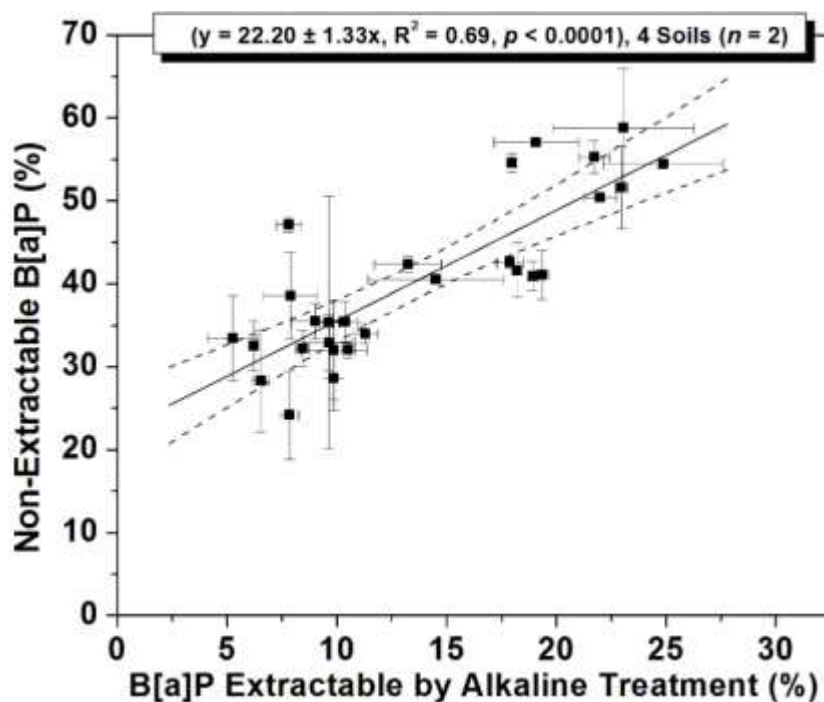


321 **Figure 7.** Comparison of B[a]P extractability between exhaustive alkaline treatments and
 322 methanolic saponification ('5 h MeKOH') of pre-extracted soils. Amounts of B[a]P recovered
 323 by the exhaustive alkaline treatments range from 8% ($4.2 \pm 0.6 \mu\text{g/g}$, soil M) to 22% ($10.8 \pm$
 324 $1.2 \mu\text{g/g}$, soil B), whereas amounts of B[a]P recovered by 5 h MeKOH range from 5% ($2.6 \pm$
 325 $0.4 \mu\text{g/g}$, soil I) to 11% ($5.6 \pm 0.5 \mu\text{g/g}$, soil B). 1 g soil was extracted with 3 mL DCM/Ace by
 326 ultrasonication (40 KHz, 10 min, 3x). After extraction, combined supernatant was prepared for
 327 HPLC analysis. Pre-extracted soils containing B[a]P NERs were extracted with 10 mL alkaline
 328 solutions (Table 1), or by conventional methanolic saponification. Values are means of
 329 duplicates \pm standard deviations.
 330

331 Our previous investigations using the same soils showed that B[a]P NERs increased with
 332 aging³⁴. Another study in which the same soils were aged for 160 d showed similar trends of
 333 increasing NER over time⁵. Also, B[a]P recovered by methanolic saponification of pre-
 334 extracted soils tended to decrease over time⁶. It was therefore expected that the amounts (%)
 335 of B[a]P NERs recoverable in soils after 180 d of aging would decrease or remain unchanged
 336 when compared to recoveries after shorter aging periods, using the same extraction
 337 techniques. Indeed, there was generally no significant difference ($p > 0.05$) between B[a]P
 338 NERs recovered in the same soils subjected to '5 h MeKOH' after 180 d of aging in this
 339 study, and after 33 d of aging in our previous investigations²⁷. The extraction efficiency of
 340 '5 h MeKOH' did not change substantially between 33 d and 180 d of aging; this showed that
 341 similar fractions of B[a]P NERs in soils were still being accessed.

342 The observation that the exhaustive methanolic and non-methanolic alkaline treatments
343 generally had greater B[a]P NER recoveries (%) at 180 d of aging than the often used '5 h
344 MeKOH' after the same aging period was interesting. This implied that additional B[a]P
345 NER fractions in soils were still accessible. The recoveries of B[a]P NERs in soil may
346 therefore be limited by the operational design of the methanolic or non-methanolic alkaline
347 treatment used which may affect understanding of B[a]P fate in soil. Based on the results of
348 this study, the conventional methanolic saponification of pre-extracted soils may be made
349 more exhaustive to increase recovery of B[a]P NERs, as well as for better estimation of total
350 B[a]P concentrations in soils. This may allow a better understanding of B[a]P fate in soils.
351 The differences in recoveries of B[a]P NERs from the 4 soils investigated indicate that soil
352 properties and the amounts of NERs in soils with similar contamination history may
353 influence the amounts of NERs than can be recovered by the alkaline treatments.

354 A significant correlation ($R^2 = 0.69$, $p < 0.001$, $n = 4$) was observed between the amounts of
355 B[a]P recovered by each of the 7 alkaline treatments in the pre-extracted soils, and the
356 corresponding amounts of estimated NERs in the soils at 180 d of aging (Figure 8). The
357 observed correlation suggests a potential relationship between B[a]P NERs in pre-extracted
358 soils with similar contamination history and amounts of B[a]P extractable by the alkaline
359 treatments. The quantification of the exact amounts of PAH NERs in soils is difficult. Very
360 useful but complex techniques involving ^{14}C - and ^{13}C - tracers have been used to quantify and
361 classify NERs in soil ⁷. However, the information obtained usually relates to ^{14}C - or ^{13}C -
362 activities from non-specific origins in soil, rather than non-extractable parent PAHs residues
363 or their metabolites. The position of the radio- or stable isotope- tracer on the PAH molecule
364 also influences the amount of activity attributed to NERs ³⁶.



365

366 Figure 8. Relationship between B[a]P NERs in soils and the amounts recovered by methanolic
 367 and non-methanolic alkaline treatments. The dotted lines represent 95% confidence limits.

368 In this study, quantification of B[a]P in soil relates only to extractable parent compounds. The
 369 potential relationship described earlier warrants further investigations using a wider range of
 370 soils, as it implies a potential to estimate the amounts of NERs in soils based on extractability
 371 by methanolic or non-methanolic alkaline treatments. In this regard, a combination of the
 372 alkaline treatments used in this study and ¹⁴C- or ¹³C-tracer approaches are suggested.

373 **Does the Enhanced Recovery of B[a]P NERs in Soils have Implications for**
 374 **Contaminated Land Risk Assessment and Decision-Making?**

375 Much of the current work on the risk assessments of PAHs in soils, as well as risk-based
 376 approaches to contaminated land management, focus on the bioavailable and/or bioaccessible
 377 fractions³⁷⁻³⁹. Bioavailable and bioaccessible fractions have been defined as fractions that are
 378 readily available and potentially available, respectively, to pose risks to human and
 379 environmental health following exposure⁴⁰. The measurement of these fractions may vary
 380 depending on the extraction techniques and conditions used, and the impact and significance

381 of the extracted fractions also vary depending on whether human or ecological health risk
382 assessment is the focus ^{8,12}.

383 However, little emphasis is placed on the impact and significance of PAH NERs in soils,
384 despite their uncertainty for long-term stability and potential to pose risks to human and
385 environmental health following exposure ^{8,11,41}. Our recent work in this regard provided
386 empirical data that showed that the amounts of B[a]P NERs that were potentially remobilisable,
387 after re-equilibrating long-term aged soils for 30 d, were small (less than 5% of the amount of
388 B[a]P spiked, 10 or 50 mg/kg) ³⁴. According to the study ³⁴, it was documented that the amounts
389 of B[a]P remobilised in the 4 soils utilised were generally below the B[a]P health investigation
390 levels (3 µg/g) from an Australian risk assessment perspective, and that the potential risks that
391 may be posed from exposure to the remobilised B[a]P were acceptable. The small amounts of
392 remobilised B[a]P may result from slow repartitioning of fractions that were entrapped,
393 occluded, adsorbed, or sequestered into readily available compartments in soils during re-
394 equilibration ³⁴. Further, B[a]P remobilisation was supported by the decrease in the fractions,
395 and absolute amounts, of B[a]P NERs in soils recovered by methanolic saponification after the
396 re-equilibration periods ³⁴. Therefore, fractions of B[a]P NERs in soils that are extractable by
397 methanolic saponification may indicate fractions that may be potentially remobilised.

398 Although the potential risks associated with remobilisable B[a]P fractions in the soils utilised
399 were generally acceptable from a risk assessment perspective, the use of extraction techniques
400 that do not completely recover B[a]P fractions that are potentially remobilisable may
401 underestimate the risks that may be associated with B[a]P NERs in soils. Since there was
402 generally no significant difference ($p > 0.05$) in B[a]P extractability from soils between the 7
403 different alkaline treatments in this study, it is proposed that any of the exhaustive alkaline
404 extraction would enhance the recovery of highly sequestered B[a]P in soils than the
405 conventional methanolic saponification, particularly alkaline treatments 1, 3, and 6. The

406 enhanced recovery is important for a better understanding of the fate of B[a]P NERs in soils
407 which is very useful for B[a]P risk assessments.

408 **CONCLUSION**

409 Non-extractable B[a]P residues in soils are currently not considered as important in risk
410 assessments as they are highly sequestered in soil and have very minimal potential to pose
411 risks or cause harm to human and environmental health. However, whether NERs have
412 potential to be remobilised in soils in the long-term, and the amounts that may be remobilised
413 are of concern, remain areas of interest in contaminated land risk assessment. A cost-effective
414 technique that could reliably estimate the amounts of NERs recoverable or remobilisable in
415 soils may be very useful for risk-based approaches to managing long-term PAH contaminated
416 soils. Our results demonstrate that 7 exhaustive alkaline extractions access similar B[a]P
417 NER fractions, and that their use resulted in enhanced recovery of B[a]P NERs in soils
418 compared to conventional methanolic saponification. The results may be indicative for other
419 PAHs, as B[a]P is usually the model PAH at most PAH-contaminated sites. Such exhaustive
420 alkaline treatments can provide more realistic estimations of total B[a]P concentrations based
421 on mass balance in spiked soils, and contribute to a better understanding of PAH fate in soils.

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