Bioaccumulation of Benzo[a]pyrene Nonextractable Residues in Soil by *Eisenia fetida* and Associated Background-level Sublethal Genotoxicity (DNA Single-strand Breaks)

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Abstract

The potential for bioaccumulation and associated genotoxicity of nonextractable residues (NERs) of polycyclic aromatic hydrocarbon (PAHs) in long-term contaminated soils have not been investigated. Here were report research in which earthworms, *Eisenia fetida*, were exposed to a soil containing readily available benzo[a]pyrene (B[a]P) and highly sequestered B[a]P NERs aged in soil for 350 days. B[a]P bioaccumulation was assessed and DNA damage (as DNA single strand breaks) in earthworm coelomocytes were evaluated by comet assay. The concentrations of B[a]P in earthworm tissues were generally low, particularly when the soil contained highly sequestered B[a]P NERs, with biota-soil accumulation factors ranging from 0.6 – 0.8 kg_{OC}/kg_{lipid}. The measurements related to genotoxicity, that is percentage (%) of DNA in the tails and olive tail moments, were significantly greater ($p < 0.05$) in the spiked soil containing readily available B[a]P than in soil that did not have added B[a]P. For example, for the soil initially spiked at 10 mg/kg, the percentage of DNA in the tails (29.2%) of coelomocytes after exposure of earthworms to B[a]P-contaminated soils and olive tail moments (17.6) were significantly greater ($p < 0.05$) than those of unspiked soils (19.6% and 7.0, for percentage of DNA in tail and olive tail moment, respectively). There were no significant ($p > 0.05$) differences in effects over the range of B[a]P concentrations (10 and 50 mg/kg soil) investigated. In contrast, DNA damage after exposure of earthworms to B[a]P NERs in soil did not differ from background DNA damage in the unspiked soil. These findings are useful in risk assessments as they can be applied to minimise uncertainties associated with the ecological health risks from exposure to highly sequestered PAH residues in long-term contaminated soils.

Keywords: Benzo[a]pyrene Nonextractable Residues; Long-term Contaminated Soil; Biota-soil Accumulation Factor; Genotoxicity; Comet Assay; Risk Assessment

1. Introduction

The potential bioavailability of nonextractable residues (NERs) of hydrophobic organic compounds (HOCs), such as polycyclic aromatic hydrocarbons (PAHs), in soils to organisms has been widely discussed among regulators and risk assessors [1, 2], although supporting experimental investigations are sparse. PAHs are most commonly released into the environment by anthropogenic activities through incomplete combustion and pyrolytic processes. PAH deposition on soils is their major sink in the environment. Hence, PAHs are ubiquitous in soils and present at wide-ranging concentrations (from µg/kg to mg/kg, or even greater).

Over long periods of time, readily available HOCs age and become highly sequestered in soils [3-5]. Whether HOCs that are highly sequestered, or nonextractable by solvents, in soils pose risks to human and ecological health is a critical uncertainty in the risk assessment of long-term contaminated soils [6, 7]. This uncertainty needs to be minimised to support the widespread adoption of risk-based approaches that are based on reducing readily available contaminant concentrations, rather than reducing total concentrations, for the efficient management of long-term PAH-contaminated sites [8]. Considering that PAHs, e.g. benzo[a]pyrene (B[a]P) are pro-carcinogenic, studies that show extremely reduced potential for the remobilisation of highly sequestered NERs in soils, as well as minimal risks to human and ecological health following exposure, will be very useful for risk assessment purposes by reducing uncertainty.

Previous investigations reported the time-dependent remobilisation of B[a]P in soils, and the associated effects of soil properties and B[a]P concentrations, after the complete removal of total-extractable and readily available fractions [9, 10]. One of the key observations of these solvent-extractions based investigations was that the concentrations of B[a]P that were remobilised from B[a]P NERs in soils that had been aged up to 4 years, were extremely small [9, 10]. The lingering question is whether PAH NERs in long-term contaminated soils can be bioaccumulated in sufficient quantities that they become lethal, or sublethal by genotoxicity, to bioindicator organisms, such as earthworms.

61 The present study aims to evaluate the bioaccumulation of readily available B[a]P and highly
62 sequestered B[a]P NERs in a solvent-spiked soil (10 and 50 mg/kg) that was aged for almost 1 year,
63 as well as their potential to cause genotoxicity by DNA single strand breaks. *Eisenia fetida* was used
64 as the test exposure organism as it, lives in soils, is ubiquitous and accessible, and has been well-
65 studied for ecotoxicological purposes [11-13]. The widely used comet assay was employed to assess
66 DNA damage or DNA single strand breaks in earthworm coelomic leukocytes or coelomocytes after
67 earthworm exposure [14-17].

2. Materials and Methods

2.1. Chemicals and Reagents

Analytical grade B[a]P (>96% purity), methanol (HPLC grade), acetonitrile (HPLC grade), analytical grade acetone, 1-butanol (density = 0.81 g/mL, ≥ 99.4%), dichloromethane, toluene (99.8%), potassium hydroxide, and anhydrous sodium sulphate (Na_2SO_4), were purchased from Sigma-Aldrich Pty Ltd., Sydney, NSW, Australia. Ethyl acetate was purchased from Thermo Fisher Scientific, North Ryde, NSW, Australia.

2.2. Experimental Design

The selected soil was air-dried and sieved to pass through a 2 mm sieve. The soil was a sandy-clay-loam (21% clay, 62% sand) according to the USDA textural classification [9], and contained 7.5% total organic carbon as measured by LECO combustion after excess acid hydrolysis of soils. Two environmentally relevant B[a]P concentrations (to supply 10 and 50 mg/kg in soil) were prepared in acetone/toluene (2:1, v/v) and spiked into the soil, following a method described previously [18]. The solvent-spiked soils were aged for 350 d before being used for earthworm exposure. The soils were moistened to 30% of their water holding capacity. Prior to exposure to spiked soil, the lipid contents of the earthworms were determined gravimetrically after ultrasonic extraction (50 kHZ, 15 mins) of frozen earthworm tissue (fresh weight of 0.7 ± 0.1 g) in either 1 mL of ethyl acetate/acetone (4:6, v/v) [19], or acetone/hexane (1:1, v/v) [11]. Two treatments were used to evaluate B[a]P bioaccumulation in earthworms and its associated DNA damage after exposure to spiked soil. The first treatment had the solvent-spiked and aged soils with earthworms. The second treatment had same solvent-spiked soils that had been previously solvent-extracted to produce soils containing only B[a]P NERs [9, 18], as described below. For the second treatment, earthworms were exposed to the soils after allowing the solvent-extracted soils to equilibrate in the dark for 30 d [9]. Earthworms were also exposed to the unspiked soils (with solvent-spike only) and treated in a similar manner as the spiked soils. Overall, 6 microcosms (n = 3) were utilised for earthworm exposure. After exposure, the earthworms were depurated and prepared for measurements of PAH concentrations and DNA damage. In addition, the

PAH concentrations in soils before and after earthworm exposure were determined and biota-soil accumulation factors were calculated [20, 21].

2.3. Earthworm Exposure

Adult earthworms (*Eisenia fetida*) were purchased from Bunnings (Wallsend, Australia). Six earthworms (total fresh weight = 1.68 ± 0.23 g) were exposed to glass jars (300 mL) containing 60 g of soil [22]. The glass jars were covered with perforated transparent wraps and kept for 28 d (20.1 ± 0.01 ºC, 16/8 h light/dark cycles). The moisture content of soils was replenished weekly with MilliQ water, and no food was added throughout the exposure. After exposure in soil, the earthworms were removed, rinsed with MilliQ water, and allowed to depurate for 24 h on moistened filter paper. After depuration, the earthworms were cleaned and weighed. Coelomocytes were collected from one live earthworm of each jar for assessment of DNA damage, while the remaining worms were frozen at – 20 ºC until needed for the measurement of PAH concentrations.

2.4. Extraction and Analysis of PAHs

Frozen earthworms were mixed with Na_2SO_4 (1:7, g/g) in a glass mortar and ground with a glass pestle. The ground samples were then extracted with a mixture of 0.5 M potassium hydroxide and acetone/hexane (1:1, v/v) [11]. For the soils, 1 g of oven-dried (37 ºC) sample from each glass jar was extracted sequentially with butanol, and then with a mixture of dichloromethane/acetone (1:1, v/v) by ultrasonication [18]. The same extraction procedure was followed to produce soil containing B[a]P NERs only [9, 18], after which extracted soils were dried and moistened in preparation for earthworm exposure study. The solvent extracts from the soil and earthworm were then vacuum-concentrated and passed through 0.45 μm PTFE filters before HPLC analysis. The concentrations of B[a]P in the extracts were analysed with an Agilent 1100 Series HPLC equipped with a fluorescence detector (excitation wavelength of 230 nm and emission wavelength of 460 nm). Chromatographic separations were made with a reverse-phase C18 column (Agilent Eclipse PAH, 4.6 × 50 mm, 1.8 μm particle size) equipped with a Kinetex security guard cartridge (with a Krudkatcher in-line filter, 0.5 μm depth, 0.004 in., from Phenomenex, Lane Cove, NSW, Australia) that was thermostated on both sides at 37 °C. A sample of 10 μL was injected into the HPLC by an autosampler and isocratically eluted

with an acetonitrile/water mobile phase (85:15, v/v) at 1.0 mL/min. The total run time was 5 min, including a post-run of 30 seconds prior to subsequent injection, with needle rinses between successive injections.

2.5. Calculation of Biota-soil Accumulation Factor

Biota-soil accumulation factor (BSAF) (kg_{OC}/kg_{lipid}) was calculated from:

$$\mathrm{BSAF} = \frac{[\mathrm{Worm}] * F_{oc}}{[\mathrm{Soil}] * F_{lip}} \quad [1]$$

Where [worm] is the PAH concentration in earthworm tissue (μg/g fresh weight), F_{oc} is the fraction of organic carbon in soil (kg/g), [Soil] is the PAH concentration in soil (μg/g), and F_{lip} is the fraction of lipid in the earthworm tissues (kg/g fresh weight).

2.6. Non-invasive Extrusion of Earthworm Coelomocytes and the Assessment of DNA Damage by Comet Assay

A modified non-invasive procedure, originally described by Eyambe et al. [23], was followed to collect coelomic fluid through the dorsal pores of the earthworms [15]. The extrusion buffer consisted of 95% phosphate-buffered saline (PBS), 5% absolute ethanol, 2.5 mg/mL EDTA, and 10 mg/mL guaiacol glycerol ether, and the pH was adjusted to 7.3 ± 0.1 with 1 M NaOH. Individual earthworms were placed in a centrifuge tube containing 1 mL of the extrusion buffer, and extruded coelomic fluid containing coelomocytes was allowed to rest for 3 – 5 min at 20.1 ± 0.01 ºC. The coelomic fluid was then transferred into a 1.5 mL Eppendorf tube and centrifuged (6093*g*, 3 min). The supernatant was discarded and the residual cell pellet was washed twice with 1 mL PBS and centrifuged, before finally suspending the washed cells in PBS. The alkaline comet assay or single cell gel electrophoresis was conducted using manufacturer's recommended equipment and protocols (Trevigen Catalog # 4250-050-K, Gaithersburg, MD, USA). Briefly, an aliquot of 50 μL of cell suspension was mixed with 150 μL of low melting point agarose, and then 50 μL of the mixture was immediately coated onto the comet slides provided in the manufacturer's kit. The slides were then placed in a refrigerator (4 ºC) for 10 min. The slides were immersed in the provided lysis buffer and kept in the refrigerator overnight. The lysis buffer was drained, and the slides were immersed in freshly prepared alkaline

147 unwinding solution as described in the protocol for 1 h at 4 ºC. The unwinding solution was drained
148 and the slides were placed in an electrophoresis tank with the alkaline electrophoresis solution
149 according to the provided protocol. Electrophoresis was conducted for 40 min, at 300 milliampere and
150 21 volts. The electrophoresis solution was gently drained, and the slides were gently immersed (5
151 min, 2x) in MilliQ water and then in 70% ethanol for 5 min. The slides were oven-dried at 37 ºC for
152 15 min and then stored at room temperature prior to comet scoring. The slides were stained with a
153 fluorescent dye (SYBR Green) and kept in the dark until dried, and comet images were analysed with
154 a fluorescence microscope (Olympus IX73) at 10x magnification. The measures of DNA damage
155 were the percentage of DNA in tail and olive tail moment [17, 24, 25], and were obtained after scoring
156 comet images with the CometScore™ freeware (Tritek Corp., USA). Due to the different cell types in
157 the coelomic fluid of earthworms and heterogeneous responses to cellular perturbations (e.g. DNA
158 single strand breaks), only comets with similar size and shape were randomly selected for scoring [15].
159 The percentage of DNA in tail is the ratio of the total pixelated fluorescent intensity of the comet tail
160 to the total intensity of the overall comet, whereas the olive tail moment is the product of fraction of
161 DNA in tail and the distance between the centres of gravity of a comet's head and tail [26].

162 **2.7. Statistical Analysis**

163 The statistical software used for data analysis and graphing were SPSS Statistics (IBM 775 Corp,
164 version 24) and Origin (Microcal Software Inc., Northampton, MA, USA, version 8.5). No data
165 transformations were applied. Mann–Whitney U-test was used to compare DNA damage: in
166 earthworms exposed to the solvent-spiked soils and unspiked controls; between earthworms exposed
167 to 10 mg/kg and 50 mg/kg; and between earthworms exposed to solvent-spiked soils before re-
168 equilibration and earthworms exposed to pre-extracted soils after re-equilibration. The accepted level
169 of significance was $p < 0.05$.

3. Results and Discussion

3.1. Total-extractable B[a]P Concentrations in Soil

Prior to earthworm exposure to the 1-year aged spiked soils to which B[a]P was added at the rate of 10 mg/kg, the BuOH- and total-extractable B[a]P concentrations were 0.63 ± 0.06 and 1.62 ± 0.16 µg/g, and the corresponding results after addition of 50 mg/kg were 16.56 ± 3.45 and 28.92 ± 4.84 µg/g (Table 1). This indicates a 42 – 84% decrease in B[a]P total extractability after its initial addition to the soil, as well as the formation of large amounts of B[a]P NERs, particularly in the soils spiked at 10 mg/kg B[a]P. The amounts of highly sequestered B[a]P recovered by methanolic saponification of dichloromethane/acetone-extracted soils were 0.19 ± 0.01 (10 mg/kg) and 2.19 ± 0.03 (50 mg/kg) µg/g. Methanolic saponification and/or silylation may be used to recover PAH NERs that may be entrapped or occluded in soil micropores and referred to as Type I NERs [27]. While Type I NERs are highly sequestered in soils, they are regarded as having some potential for mobilisation and uptake by living organisms following exposure [27]. B[a]P sequestration on B[a]P extractability in aged soils are well known and have been described in detail elsewhere [9, 28].

184 **3.2. Accumulation of B[a]P in Earthworm Exposed to the Spiked Soil before Re-equilibration**

185 After 28 d of exposure, the tissue concentration of B[a]P in earthworms was only 5 – 13% of the
186 solvent (BuOH or total)-extractable B[a]P concentrations in soil (Table 1). BuOH-extractable and
187 total-extractable B[a]P concentrations in soil therefore overestimate the tissue concentrations of B[a]P
188 in earthworms, especially the total-extractable B[a]P concentrations. Total-extractable PAH
189 concentrations can over-predict actual PAH concentrations that can be bioaccumulated in earthworm
190 tissues by between one to four orders of magnitude [11]. However, approximately 41 – 53% of BuOH-
191 extractable B[a]P and 21 – 23% of total-extractable B[a]P were measured in the earthworm tissues
192 after normalising solvent-extractable and tissue B[a]P concentrations by the organic carbon content in
193 soil (7.5%) and by the lipid content in earthworm tissues (1.8%), respectively. Hydrophobic organic
194 contaminants, such as B[a]P, have affinity for lipids in earthworm tissues [11]. Bioaccumulation of
195 B[a]P in earthworm tissues may occur through gut uptake and passive transfer through the epidermal
196 layer of the skin [12]. Being an epigeic species, *Eisenia fetida* consumes less soil compared to endogeic
197 earthworm species. Hence, B[a]P in the tissues of *Eisenia fetida* is likely to be associated with its
198 uptake across the outer epidermis to a greater extent [12]. The tissue concentrations of PAHs in *Eisenia*
199 *fetida* that is due to its passive transfer from soil through earthworm's outer epidermis may be
200 predicted by PAH concentrations obtained from mild butanol extractions of PAH-contaminated soils
201 [29]. This may explain the lesser conservatism of the normalised butanol-extractable B[a]P
202 concentrations than the total-extractable concentrations, relative to the tissue concentrations of B[a]P
203 in the earthworm studied.

204 The mean weight loss was 25.8% and mortality across all soils was 9.9% and these could be attributed
205 to the effect of residual solvents in extracted soils, absence of food throughout the 28 d of exposure,
206 and the excessive earthworm populations per soil weight (units/g) utilised to meet the study
207 objectives.

208 The biota-soil accumulation factor in the soil spiked at 10 mg/kg B[a]P was slightly greater than the
209 soil spiked at 50 mg/kg B[a]P (Table 1); however, the biota-soil accumulation factors were not
210 significantly different ($p > 0.05$). Mixing and comminution of soils, resulting from burrowing and

feeding of earthworms in soils, may release PAHs sequestered in soils to varying extents. The amounts of PAHs released may depend on PAH concentration, soil properties, as well as the extent of sequestration. Based on equation 1, the results for biota-soil accumulation factors in Table 1 showed that larger amounts of B[a]P become extractable in soils spiked at higher concentrations relative to soils spiked at lower concentrations. For instance, from the total-extractable B[a]P concentration (28.9 mg/kg) in soil spiked at 50 mg/kg prior to earthworm exposure, it was observed that 46% of this concentration was subsequently removed after earthworm exposure (Table 1). However, for the 10 mg/kg B[a]P-spiked soil, it was observed that approximately 25% of the total-extractable B[a]P concentration (1.62 mg/kg) that was obtained prior to earthworm exposure was subsequently removed after exposure. These observations further confirmed that B[a]P was highly sequestered in the soils, particularly in soils spiked at 10 mg/kg, and this result is consistent with the very small tissue concentrations. Since biota-soil accumulation factor is a relative measure (equation 1), the slightly higher biota-soil accumulation factor in the soil spiked at 10 mg/kg B[a]P was therefore reasonable.

The biota-soil accumulation factors (0.6 – 0.8 kg_{OC}/kg_{lipid}) in this study were lower than those (1.6 kg_{OC}/kg_{lipid}) reported in a study that utilised freshly spiked soils for *Eisenia andrei* exposure [20], and 2.4 kg_{OC}/kg_{lipid} in a different study that utilised freshly spiked OECD soils that were aged for 25 d and exposed to *E. andrei* [21]. In contrast, the biota-soil accumulation factor in this study exceeded the range (approximately 0.03 – 0.16 kg_{OC}/kg_{lipid}) reported for ∑10PAHs in earthworms exposed to urban soils [20]. Overall, biota-soil accumulation factors from long-term aged field-contaminated soils will be smaller than those from freshly-spiked soils; however, this difference will also be influenced by the earthworm species studied and the methods utilised for the determination of lipid and PAH contents.

3.3. Accumulation of B[a]P NER in Earthworm Exposed to the Solvent-extracted Soil After Re-equilibration

After the re-equilibration of solvent-extracted soils and subsequent exposure of earthworms for 28 d, the tissue concentration of B[a]P was approximately 16% of the B[a]P NER concentration that was recovered by methanolic saponification prior to the re-equilibration of the soils (Table 1). Where normalised concentrations were considered, as described previously, the tissue concentration of B[a]P

238 in earthworms was approximately 65 – 66% of B[a]P NERs recovered by methanolic saponification
239 prior to re-equilibration (Table 1). Nonetheless, the tissue concentrations in the earthworms exposed
240 to solvent-extracted soils were extremely small, being 3 – 5 times smaller than tissue concentrations
241 in earthworms that were exposed to unextracted soils prior to re-equilibration. Considering that the
242 soil utilised in this study was high in organic carbon content (7.5%), the B[a]P spiked into soil is
243 expected to be highly sequestered and therefore bioaccumulated at low extents in earthworm tissues [5].
244 In contrast, soils with smaller organic carbon contents are likely to exhibit relatively higher
245 bioaccumulation than observed in this study, particularly for freshly spiked soils. Overall, the amount
246 of B[a]P NERs that may be released from soils will be influenced by soil properties and be reduced
247 over time [9], particularly for long-term contaminated soils.

248 **3.4. Potential for Earthworm Lethality and DNA Damage following Earthworm Exposure to**
249 **B[a]P and B[a]P NERs in Soil**

250 The concentrations of PAH residues in earthworm tissues that can cause irreversible damage to the
251 organism's membrane and result in death (the critical body residue) range from 50 – 200 mmol/kg
252 lipid [30]. The body residues of B[a]P, determined from the tissue concentrations of B[a]P (μg/g, fresh
253 weight) in earthworms exposed to unextracted soils prior to re-equilibration, were extremely low
254 ranging between 0.02 – 0.36 mmol/kg lipid (Figure 1). After re-equilibration, the body residues of
255 B[a]P in earthworms exposed to solvent-extracted soils were 3 – 5 times smaller still, further
256 indicating no potential for earthworm lethality. Even if the total-extractable B[a]P concentrations in
257 soils and the amounts of highly sequestered B[a]P residues were accumulated in the earthworm
258 tissues, earthworm deaths would still be unlikely since the critical body residue would be
259 approximately 7.0 mmol/kg lipid. While earthworm lethality is unlikely, chronic sublethal effects,
260 such as DNA damage, may result from the exposure of earthworms to the minute amounts of B[a]P
261 that can bioaccumulate in earthworm tissues.

262 The percentage of DNA in the tails of coelomocytes after exposure of earthworms to B[a]P-
263 contaminated soils and olive tail moments were significantly greater ($p < 0.05$) than those of unspiked
264 soils (Figure 1). For example, the percentage of DNA in the coelomocyte tails of earthworms exposed

to the unextracted soil spiked at 10 mg/kg B[a]P prior to re-equilibration was approximately 29.2 ± 2.0% (Figure 1A), whereas it was approximately 19.6 ± 2.3% in the unspiked control. Similarly, the olive tail moment in earthworm coelomocytes that were obtained from earthworms exposed to the unextracted soil (50 mg/kg) before re-equilibration was approximately 22.5 ± 5.1 (Figure 1B), whereas it was 7.0 ± 3.1 in the unspiked control. The significant differences show that B[a]P in the solvent-spiked soils were readily available to cause the breakage of DNA strands in earthworm coelomocytes. The DNA damage in the unspiked soils can be assumed to be induced by residual solvent that may be present even after venting of solvent from the soils, or by unavoidable background PAH concentrations which were below analytical detection limit [18]. Other studies with similar observations explained that unavoidable background may cause DNA damage in cells, even where exogenous DNA-damaging contaminants are absent [14, 16].

It is well known that electrophilic B[a]P metabolites, produced after cytochrome P-450 activation or free radical oxidation, are responsible for DNA damage through adduct formation or DNA strand breakage in earthworm coelomocytes [22, 31]. Where DNA damage in earthworms is substantial, internal repair mechanisms may be affected [14, 31]; as a result, earthworms may become susceptible to adverse metabolic and physiological effects that may impair immunity, growth, and reproduction [13].

In contrast, the DNA damage measured in the coelomocytes from earthworms exposed to pre-extracted and re-equilibrated soils were not significantly different ($p > 0.2$) from the DNA damage measured in coelomocytes from earthworms exposed to corresponding unspiked control (Figure 1). The DNA damage observed in earthworm coelomocytes before re-equilibration was significantly greater ($p < 0.05$) than that observed after re-equilibration. However, there was no significant difference in DNA damage between the 10 and 50 mg/kg B[a]P spiked soils before re-equilibration, as well as after re-equilibration. These findings show that B[a]P NERs in soil do not pose genotoxic risks or cause DNA damage, as measured by DNA single strand breaks, to the earthworms studied. For long-term contaminated soils with very highly sequestered B[a]P, potential genotoxic risks from exposure to B[a]P NERs will be extremely reduced or non-existent.

4. Conclusions

To the best of our knowledge, this study is the first report of biota-soil accumulation factor and DNA damage from exposure of earthworms to highly sequestered B[a]P NERs in spiked soil that was aged for almost 1 year. This study found that very minimal or no substantial bioaccumulation of B[a]P NERs in *Eisenia fetida* exposed to aged B[a]P-contaminated soil. In addition, no significant DNA damage was observed in earthworm coelomocytes following 28 d exposure relative to that in the unspiked soils. However, readily available B[a]P in solvent-spiked soils induced substantial DNA damage in earthworm coelomocytes relative to the unspiked soils. These findings are useful because they minimise the uncertainties associated with ecological health risk assessment of highly sequestered PAH residues in long-term contaminated soils.

5. Conflict of Interests

The authors declare no competing financial interest.

6. Acknowledgment

This work was supported by the joint support of the University of Newcastle (UoN), Australia through the UoN International Postgraduate Research Scholarships, and the Cooperative Research Centre for Contamination Assessment and Remediation of the Environment through the CRC CARE PhD Scholarships. AU acknowledges the kind assistance of Dr. Kannan Krishnan from the Global Centre for Environmental Remediation, UoN for providing guidance during fluorescence microscopy of comet assay slides.

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