

Using deuterated PAH amendments to validate chemical extraction methods to predict PAH bioavailability in soils

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1 **Using deuterated PAH amendments to validate chemical extraction**
2 **methods to predict PAH bioavailability in soils**

3

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26 **Abstract**

27

28 Validating chemical methods to predict bioavailable fractions of polycyclic aromatic
29 hydrocarbons (PAHs) by comparison with accumulation bioassays is problematic.
30 Concentrations accumulated in soil organisms not only depend on the bioavailable
31 fraction but also on contaminant properties. A historically contaminated soil was
32 freshly spiked with deuterated PAHs (dPAHs). dPAHs have a similar fate to their
33 respective undeuterated analogues, so chemical methods that give good indications of
34 bioavailability should extract the fresh more readily available dPAHs and historic
35 more recalcitrant PAHs in similar proportions to those in which they are accumulated
36 in the tissues of test organisms. Cyclodextrin and butanol extractions predicted the
37 bioavailable fraction for earthworms (*Eisenia fetida*) and plants (*Lolium multiflorum*)
38 better than the exhaustive extraction. The PAHs accumulated by earthworms had a
39 larger dPAH:PAH ratio than that predicted by chemical methods. The isotope ratio
40 method described here provides an effective way of evaluating other chemical
41 methods to predict bioavailability.

42

43 **Keywords**

44 Bioavailability; polycyclic aromatic hydrocarbons; earthworms; plants; deuterated

45

46 **Capsule**

47

48 A novel method using isotope ratios to assess the ability of chemical methods to
49 predict PAH bioavailability to soil biota.

50

51 **1. Introduction**

52

53 Prolonged contact times between organic contaminants and soil decrease the
54 bioavailability of these compounds for uptake by organisms or for degradation by
55 microorganisms in a process often referred to as ‘ageing’ (Belfroid et al., 1995;
56 Alexander, 2000; Northcott and Jones, 2001). Thus measuring the total concentration
57 of organic contaminants present at contaminated sites may lead to over conservative
58 risk assessments as only the bioavailable fractions can cause toxic effects. Recently,
59 approaches for ecological risk assessment have been developed where bioavailability
60 data, obtained from the results of bioassays are used (Harmsen, 2007). These
61 bioassays only respond to the bioavailable fraction of contaminants (Jensen and
62 Mesman, 2007), but their application can be time consuming and laborious. As a
63 result a number of more time- and cost-efficient chemical methods for predicting
64 bioavailability have been published in the scientific literature (Kelsey et al., 1997;
65 Reid et al., 2000; Ten Hulscher et al., 2003).

66

67 These chemical methods are normally validated in the literature by comparing how
68 they approximate or correlate with the amount of organic compound accumulated by
69 soil biota such as earthworms and to a lesser extent plants, or the amount degraded by
70 microbes (Kelsey, et al., 1997; Tang and Alexander, 1999; Reid, et al., 2000; Liste
71 and Alexander, 2002; Tang et al., 2002; Ten Hulscher, et al., 2003). However, recent
72 studies have shown distinct differences between the PAHs extracted using some of
73 these techniques and those accumulated in earthworms and plants (Hickman and Reid,
74 2005; Bergknut et al., 2007; Gomez-Eyles et al., 2010). It is important to realise
75 however, that these methods are meant to provide a measure of bioavailability not

76 bioaccumulation. Apart from being influenced by the bioavailability of the
77 contaminant, the final concentration of an organic contaminant accumulated within a
78 soil organism will also depend on the metabolic fate of the contaminant within the
79 organism and the partitioning properties of the contaminant. Assessing chemical
80 methods by comparing the concentration of a PAH they extract, with that accumulated
81 in a soil organism is therefore not a fair test of their ability to predict PAH
82 bioavailability (Gomez-Eyles et al., 2010).

83

84 An alternative way of assessing the ability of chemical methods involves predicting
85 accumulation concentrations from concentrations measured by chemical methods and
86 accounting for contaminant partitioning properties (Jonker et al., 2007; van der
87 Heijden and Jonker, 2009). However these calculations do not account for differences
88 in the metabolic fate of different contaminants and carry significant assumptions.
89 When using passive sampling methods, like solid phase micro-extraction (SPME)
90 fibres, these assumptions include using contaminant K_{ow} values as approximations for
91 bioconcentration factors. When using mild solvent extractions (e.g. butanol) or
92 depletive sampling extractions (e.g. cyclodextrin or tenax extractions) even further
93 assumptions have to be made by using generically derived K_{oc} values (van der Heijden
94 and Jonker, 2009). The latter is a very substantial assumption considering field
95 contaminated soils have been shown to have K_{oc} values several orders of magnitude
96 above generically derived ones (Hawthorne et al., 2002; Jonker, et al., 2007).

97

98 We propose a novel method to evaluate the ability of chemical extractions to predict
99 PAH bioavailability to earthworms and plants that can account for differences in
100 bioaccumulation concentrations caused by different contaminant properties. This

101 method follows the same principle used in a previous study on the effect of ageing in
102 sediments on PAH accumulation at the top levels of aquatic food chains (Moermond
103 et al., 2007). Here we spike a soil historically contaminated with PAHs, with
104 deuterated PAHs (dPAHs) enabling a comparison of the extraction and uptake of
105 freshly spiked PAHs and aged historic PAHs by chemical methods and accumulation
106 bioassays. dPAHs have been used as internal standards in many studies involving
107 PAHs as they have very similar properties to their respective undeuterated analogue
108 PAHs (Bucheli et al., 2004; Bergknut, et al., 2007). They should therefore also have
109 the same metabolic fate and partitioning properties as their respective undeuterated
110 analogue PAHs. Consequently, a method that correctly predicts the fraction of PAHs
111 available to earthworm and plants should extract the freshly spiked dPAHs and the
112 aged historic PAHs in a similar ratio to that in which they are accumulated within
113 earthworm and plant tissues. Comparing the ratio in which the chemical method
114 extract the PAHs with that in which it accumulates in the soil organism, enables a fair
115 assessment of these chemical methods to measure bioavailability. This cannot be
116 achieved by simply comparing the concentration of a compound accumulated in a soil
117 organism with that extracted by the chemical method.

118

119 This investigation aims to use this novel method to evaluate the ability of butanol and
120 cyclodextrin extractions, two of the most widely reported methods, to predict PAH
121 bioavailability to earthworm and plants in soils.

122

123 **2. Experimental Section**

124

125 **2.1 Soil spiking and ageing**

126

127 PAH-contaminated soil from a former gasworks site in the UK (Table 1) was passed
128 through a 2 mm sieve. The <2mm fraction was spiked using a single-step spiking/re-
129 hydration procedure (Reid et al., 1998) with a stock solution of deuterated PAHs
130 (Sigma Chemicals, Poole, UK) in acetone, to final concentrations of 30 mg kg⁻¹ of
131 [²H₈] naphthalene, [²H₁₀] phenanthrene, [²H₁₀] pyrene and 10 mg kg⁻¹ of [²H₁₂]
132 benzo(a)pyrene. After addition of the stock solution, the soil was left uncovered in a
133 fume cupboard for 24 h to ensure all the solvent had evaporated. After confirming
134 removal of the solvent by olfactory detection and checking for residual wetting in the
135 soil, the spiked soil was re-wetted to 60% of its water holding capacity. Samples of
136 the soil were taken immediately after re-wetting to determine initial PAH
137 concentrations. The remainder of the soil was used either in bioassays of 20 days
138 duration (see below) or transferred to loosely sealed amber glass jars and aged for 20
139 days at 20°C.

140

141 The same procedure was followed using a control soil (Broughton Loam, Kettering,
142 UK) (Table 1), but this soil was spiked with fresh undeuterated PAHs as well as
143 dPAHs to the same final concentrations as above. Exposing plants and earthworms to
144 a soil freshly spiked with equal amounts of PAHs and dPAHs served as a control for
145 any potential preferential accumulation of one kind of PAH over the other. When
146 comparing ratios of dPAHs:PAHs between organisms and the chemical extractions
147 we assume there is no difference between the uptake processes or the metabolic fate
148 of dPAHs and PAHs within the organisms. Determining whether this assumption is
149 true is therefore important when using these ratios to evaluate the potential of the
150 chemical methods to predict the bioavailable fraction.

151

152 **2.2 Soil extractions**

153

154 To determine the total amount of PAHs in the soils five replicate 4 g portions of soil
155 were agitated in 10 ml of 1:1 by volume acetone/hexane mixture for 2 hours on an
156 orbital shaker (Orbital Shaker SO1, Bibby Sterilin Ltd, Stone, Staffordshire, UK) at
157 250 rpm. After extraction the samples were left to settle for 30 min, and then 2 ml of
158 solution were placed in a test tube containing 0.1 g of dry sodium sulphate before
159 transferring to gas chromatography vials for analysis (LOD=0.05 mg kg⁻¹). This
160 method was adapted from a mechanical shaking method previously reported to give
161 better recoveries than a Soxhlet extraction (Song et al., 2002).

162 Two different kinds of butanol extraction were carried out; a vortex extraction where
163 10 g of soil were mixed in 15 ml of butanol solvent and agitated for 120 s (Liste and
164 Alexander, 2002), and a shake (Reid et al., 2004) where 10 g of soil were mixed with
165 15 ml of butanol and placed on a rock and roll shaker for 12 hours. All butanol
166 extractions were passed through 0.45 µm polytetraflouroethylene (PTFE) filters
167 obtained from Chromacoal Ltd (Welwyn Garden City, UK) and were replicated 5
168 times before analysis by GC/MS. The method detection limits were 0.01 mg kg⁻¹ and
169 0.015 mg kg⁻¹ for the butanol mix and shake respectively.

170

171 Cyclodextrin extractions (Stokes et al., 2005) were carried out in replicates of 5 by
172 mixing 1.5 g of soil with a 25 ml solution of 60-mM HPCD (Sigma Aldrich, Poole,
173 UK) in deionised water and agitating the mixture for 20 hours using an orbital shaker
174 at 250 rpm. The mixture was then centrifuged at 2500 rpm using a Mistral 3000i
175 centrifuge (MSE Sanyo-Gallenkamp, Leicester, UK) for 15 minutes and the

176 supernatant discarded. The resulting soil pellet was shaken with 25 ml of deionised
177 water for 10 s, centrifuged again and the supernatant was again discarded to remove
178 any remaining HPCD solution. The soil pellet was then exhaustively extracted using
179 the acetone/hexane mechanical shaking extraction described above. GC/MS analysis
180 of this exhaustive extraction measured the PAHs remaining in the soil after HPCD
181 extraction (LOD=0.07 mg kg⁻¹).

182

183 All soil extractions were carried out after 20 days, once the earthworm and plant
184 exposures had concluded. The extractions were carried out on both the soil that had
185 been left in loosely sealed amber glass jars and also on the soil that had been used in
186 the bioassays. An exhaustive acetone hexane extraction was also carried out on day 0
187 to determine the initial concentration of PAHs in the soils.

188

189 **2.3 Earthworm bioassays**

190

191 Earthworms (*Eisenia fetida*) were obtained from Blades Biological (Cowden, UK).
192 Only adult earthworms with a clitellum were used in the bioassays. Five earthworms
193 were exposed to 250 g of the spiked soils at 20°C for 20 days in loosely sealed amber
194 glass jars; 20 days was selected for consistency with the plant bioassays. After
195 exposure, the earthworms were rinsed with water and kept on wet filter paper for 24 h
196 to allow them to clear their guts. They were then cleaned, weighed and frozen at -20
197 °C before being ground with 7 times their weight of dry sodium sulphate using a
198 pestle and mortar. Earthworms were then extracted following a saponification
199 method to remove fat from the earthworms (Contreras-Ramos et al., 2008). This
200 consisted of adding 10ml of 0.5M KOH and 10 ml of a 1:1 acetone/hexane solvent

201 mixture to the ground earthworm and ultrasonicated the mixture at 45 °C for 1 hour.
202 The solvent layer was then cleaned on a deactivated silica column, pre-eluted with
203 5ml of hexane. The sample was then eluted with a further 5 ml of hexane before being
204 concentrated down to 1 ml under a stream of nitrogen prior to analysis by GC/MS.
205 Extraction efficiencies for all PAHs ranged between 80.2-103.5%.

206

207 **2.4 Plant bioassays**

208

209 Rye grass (*Lolium multiflorum*) was grown for 20 days in the soils in a temperature
210 controlled greenhouse. The plants were harvested and the roots separated from the
211 soil. Root samples were rinsed and ultrasonicated with deionised water to ensure
212 complete removal of soil particles from the roots. The cleaned roots were freeze-dried
213 (Super Modulyo 12K Freeze Dryer, Edwards, Crawley, West Sussex, UK) overnight.
214 Once dried, the roots were ground, homogenized and weighed prior to ultrasonication
215 for 2 hours in 10 ml of dichloromethane. The extracts were then concentrated down to
216 1 ml under a stream of nitrogen and passed through 0.45 µm filters before being
217 transferred to GC vials. Solutions were analysed by GC/MS. Extraction efficiencies
218 for all PAHs ranged between 84.7-100.3%.

219

220 **2.5 GC-MS analysis**

221

222 All samples were analysed using a Thermo Trace GC Ultra system equipped with a
223 Thermo TR-5MS capillary column (dimensions: 30 m x 250 µm x 0.25 µm; Thermo
224 Scientific, Runcorn, UK) operating with helium as a carrier gas, coupled to a Thermo
225 ITQ 1100 mass spectrometer (MS) through a heated transfer line (300 °C). The GC

226 injector (220 °C) was operated in a pulsed splitless mode, 1 µl aliquots were injected
227 using an autosampler, and the GC oven was programmed to hold 60 °C for 3 min then
228 ramped at 15 °C/min to 290 °C, and held for 10 minutes. The MS was operated with
229 the ion source at 220 °C and a damping flow of 0.3 ml min⁻¹.

230

231 **2.6 Statistical Analysis**

232

233 Statistical analysis was performed using R 2.9.2 (R Development Core Team).
234 Differences between the ratios of dPAH: PAH accumulated in the organisms and
235 those extracted by the different chemical methods were tested by performing an
236 ANOVA after general linear modelling of the data. The general linear model was
237 given a gamma distribution to account for the data being expressed as ratios.

238

239 **3. Results and Discussion**

240

241 **3.1 PAH loss from the spiked soils**

242

243 The loss of the freshly spiked 2 and 3-ringed PAHs and dPAHs (naphthalene and
244 phenanthrene) during the 20 days of exposure was more rapid than that of the freshly
245 spiked 4 and 5-ringed PAHs and dPAHs (pyrene and benzo(a)pyrene), as measured by
246 the mechanical acetone hexane extraction, in both the gasworks and Kettering loam
247 soils. This is consistent with previous reports that have shown a broad inverse
248 relationship between the rate of biodegradation and the number of rings in the PAH
249 (Bossert and Bartha, 1986; Wild and Jones, 1993). Low-molecular weight PAHs are
250 also more susceptible to abiotic processes like volatilisation (Park et al., 1990). The

251 loss of the freshly spiked 2 and 3-ringed PAHs during the 20 day exposures were
252 significantly lower in the gasworks soil than in the Kettering loam ($p < 0.01$). The two
253 soils were not characterised in sufficient detail to provide conclusive reasons for this,
254 but it was probably occurred due to differences in physicochemical properties and
255 microbial activities between the soils.

256

257 There was no significant difference in the loss of the dPAHs relative to their
258 undeuterated analogues in all Kettering loam treatments ($p < 0.01$). This is to be
259 expected as deuterated organic compounds are known to have very similar chemical
260 and physical properties to their undeuterated analogues. However, there was a
261 significantly smaller loss of naphthalene and phenanthrene from the soil used in the
262 plant bioassays compared to loss from the soil kept in amber glass jars and the soil
263 used for the earthworm bioassays ($p < 0.01$). This was despite the plant bioassay soil
264 being left uncovered and in the light. These conditions are theoretically more
265 conducive to abiotic loss processes such as volatilization or photodegradation. This
266 could indicate that most losses in this soil were due to biodegradation, and that the
267 relatively higher soil moisture in the loosely sealed amber glass jars may have
268 provided better conditions for microbial activity. There was a significantly larger
269 decrease in the pyrene and benzo(a)pyrene concentrations in the Kettering loam used
270 in the earthworm and plant bioassays relative to the soil that had not been exposed to
271 any organisms ($p < 0.01$). Earthworms have been previously found to promote the
272 degradation of PAHs (Ma et al., 1995) and a number of plant species have been
273 shown to increase hydrocarbon degradation, although rye grass in particular had a
274 smaller effect than others and has been shown to even decrease rhizosphere PAH
275 degradation (Phillips et al., 2006; Phillips et al., 2008).

276

277 The loss of historic PAHs from the gasworks soils was higher than previously
278 anticipated for a soil with contamination that had been ageing for decades. We
279 hypothesise that introducing some freshly available dPAHs may have stimulated the
280 microbial activity in the soil and induced the catabolism of some historic PAHs
281 (Bauer and Capone, 1988; Reid et al., 2002). There was a greater loss of the freshly
282 spiked deuterated naphthalene than that of its historic counterpart in both the soil that
283 was not exposed to any organisms and the soil that was exposed to plants ($p < 0.01$).
284 However, this was generally not the case for the other dPAHs and their non-
285 deuterated PAH counterparts. Faster degradation of the fresh and theoretically more
286 available PAHs might have been expected, but the reduced losses relative to those in
287 the Kettering loam coupled with the hypothesised induced catabolism of the historic
288 PAHs may have prevented this from happening.

289

290

291

292

293 **3.2 Comparing ratios of dPAH:PAH between chemical methods and earthworm**

294 **bioassays**

295

296 The ratios of dPAH to PAHs in the spiked gasworks soil are highly variable compared
297 to those in the spiked Kettering loam (Figure 1). Note naphthalene is not included in
298 these figures due to the low concentrations left in the soil after 20 days. However, it
299 should be noted that the gasworks soil was not spiked with exactly the same
300 concentration of dPAHs as the concentration of historic PAHs in the soil. The acetone

301 hexane extraction therefore gives an indication of the actual ratio of dPAH:PAH in the
302 soil.

303

304 Low concentrations of phenanthrene and deuterated phenanthrene accumulated in the
305 earthworms exposed to the gasworks soil, resulting in highly variable accumulation
306 ratios. Differences between the dPAH:PAH ratios accumulated in the earthworms and
307 those extracted by the chemical methods are therefore not statistically significant.

308 However, there are highly significant differences in the ratios of dPAH:PAH
309 accumulated in the earthworms exposed to the gasworks soil compared to those
310 extracted by the chemical methods for the heavier 4-5 ring PAHs (pyrene and
311 benzo(a)pyrene) ($p < 0.001$). The ratios can be up to 6 times bigger in earthworm
312 tissues relative to some chemical methods when considering benzo(a)pyrene. This
313 implies that the benzo(a)pyrene fraction bioavailable to earthworms differs
314 significantly to that predicted by the chemical methods. Earthworms accumulate an
315 increasingly higher proportion of the fresh dPAHs with increasing PAH size.

316 Although the mode of toxicity of benzo(a)pyrene to earthworms is non-polar narcosis
317 it is a proven human carcinogen and as such is the main risk driver for many
318 contaminated sites in the UK. Heavier PAHs have been shown to have relatively
319 higher potencies as aryl hydrocarbon receptor agonists (Barron et al., 2004), and
320 benzo(a)pyrene has a relative carcinogenic potency several order of magnitude higher
321 than other PAHs like phenanthrene (Pufulete et al., 2004). Therefore it is important
322 for chemical methods to correctly assess the bioavailability of benzo(a)pyrene. A large
323 number of investigations that attempt to validate the use of chemical methods to
324 predict bioavailability often only use smaller 3-4 ringed PAHs like phenanthrene as
325 models (Kelsey, et al., 1997; Tang and Alexander, 1999; Reid, et al., 2000; Liste and

326 Alexander, 2002), so care must be taken when extrapolating these results to the
327 heavier more recalcitrant and toxic PAHs in soil.

328

329 It was expected that the dPAH:PAH ratios for the Kettering loam bioassays and
330 chemical extractions would be at or close to unity as the 2 different kinds of PAHs
331 were added on the same day and in equal concentrations to the soil. The results
332 corroborate this, indicating that dPAHs have a similar behaviour to that of their
333 analogue undeuterated counterparts. It is therefore safe to assume that any differences
334 between the ratio of dPAH:PAH accumulated by the earthworms or plants and the
335 ratios in the chemical extractions from the gasworks soil are because they are
336 accessing different pools of PAHs and not because of any inherent difference in the
337 uptake rate or metabolism of dPAHs and PAHs. This confirms that dPAH
338 amendments can provide a good indication of the ability of a chemical method to
339 predict the bioavailable fraction.

340

341 The fact that earthworms did not show signs of preferential accumulation of the
342 dPAHs relative to the PAHs in the Kettering loam therefore confirms that the
343 increased relative accumulation of the dPAHs from the gasworks soil is due to the
344 higher availability of these freshly spiked dPAHs to earthworms relative to the
345 historic PAHs. The chemical methods to predict bioavailability should have reflected
346 this by extracting dPAHs and PAHs in a similar ratio to that accumulated in the
347 earthworms. The concentrations of the different PAHs and dPAHs extracted by the
348 different chemical methods were examined to determine whether the reason for their
349 smaller dPAH:PAH ratios in the extractions relative to those in the earthworm were
350 due to chemical methods extracting less dPAHs than those accumulated in the

351 earthworms, more of the historic PAHs than those accumulated in the earthworms, or
352 a combination of the two. The concentrations in the acetone hexane extractions, the
353 butanol mix and the cyclodextrin extractions indicated that the lower ratios were
354 caused by a combination of both factors, whereas the butanol shake extractions had
355 extracted higher concentrations of the historic PAHs. The concentrations of the
356 dPAHs in both butanol extractions were similar but the 12 hour shake extracted even
357 more of the historic PAHs, suggesting the increased contact time enabled the
358 extraction of the more recalcitrant historic PAHs. Earthworms were therefore found to
359 accumulate smaller amounts of historic PAHs than was predicted by any of the
360 chemical methods. This is probably due to the lower chemical activity of historic
361 PAHs relative to the freshly spiked dPAHs. Extraction methods like the ones used in
362 this study involve shaking which maximises chemical potential gradients and
363 minimises the kinetic constraints. This is not the case in the earthworm bioassays,
364 where there will be a kinetic limitation of PAH uptake into the earthworms. Methods
365 that provide a measure of the chemical activity of a substance, which is related to its
366 energetic state (Reichenberg and Mayer, 2006), could therefore give a better
367 indication of accumulation in soil organisms. Cyclodextrin and butanol extractions
368 give a measure of the bioaccessible concentration, which is the portion of the total
369 concentration that is or can become bioavailable (Alexander, 2000). This could
370 explain why some studies have found poor correlations between the amounts of PAHs
371 accumulated in earthworms and those extracted by butanol or cyclodextrin extractions
372 (Hickman and Reid, 2005; Bergknut, et al., 2007; Gomez-Eyles, et al., 2010). There
373 are a number of studies however in which butanol and cyclodextrin extractions
374 provide a better indication of the bioavailable fraction of an organic contaminant than
375 exhaustive extraction methods (Kelsey, et al., 1997; Liste and Alexander, 2002;

376 Hartnik et al., 2008). This is also true in this investigation as despite being
377 significantly smaller than the ratio of dPAH:PAH accumulated in the earthworms, the
378 ratios of dPAH:PAH extracted by the cyclodextrin and 120s butanol extractions are
379 still closer to the bioassay values than the dPAH:PAH ratio of the exhaustive acetone
380 hexane extraction.

381

382 **3.3 Comparing ratios of dPAH:PAH between chemical methods and plant** 383 **bioassays**

384

385 The ratios of dPAH:PAH accumulated in the rye grass roots exposed to the gasworks
386 soil are closer to those extracted by the chemical methods relative to the ratios
387 accumulated in the earthworm tissues for pyrene and benzo(a)pyrene (Figure 2).

388 Again most of the significant differences occur with the heavier 4-5 ringed PAHs. For
389 pyrene all chemical extractions remove a significantly higher proportion of the
390 historic PAHs except for the 120s butanol extraction ($p < 0.05$). The acetone hexane
391 and 12 hour butanol extraction also extracted a significantly higher proportion of the
392 historic benzo(a)pyrene than that which accumulates in the plant roots ($p < 0.01$). This
393 is not the case for the cyclodextrin and the 120s butanol extraction. The 120s butanol
394 extraction and in some cases the cyclodextrin extraction therefore generally provide a
395 better indication of the fraction of PAHs available to plants than the more exhaustive
396 acetone hexane extraction. It is hard to validate these results in the literature as few
397 investigations have been carried out attempting to relate chemical methods to predict
398 bioavailability to plant accumulation, although in a previous investigation we found
399 that a number of chemical methods did not improve the description of the variation in
400 plant accumulation provided by an acetone hexane extraction (Gomez-Eyles, et al.,

401 2010). Tang and Alexander (1999) however found that a number of mild solvent
402 extractions including butanol correlated strongly with anthracene accumulation in
403 wheat and barley roots. No direct indication of how an exhaustive extraction
404 compared with this was given.

405

406 Plants accumulated a much lower proportion of the freshly spiked dPAHs than the
407 earthworms did. This could have occurred as plant roots are relatively static compared
408 to earthworms. When exposed to the spiked gasworks soil they are likely to deplete
409 the more readily available dPAHs surrounding them. The earthworms on the other
410 hand are more mobile and are therefore likely to come across areas of soil they have
411 not explored before. When exposed to these areas of soil, they will preferentially
412 accumulate a higher proportion of the more bioavailable dPAHs before they move on
413 to another area of soil where they will do the same. Differences in dPAH:PAH ratios
414 between plants and earthworms could also be due to the earthworm tissues being more
415 lipophilic than the root tissues causing more of the readily available dPAHs to
416 partition into their tissues. Other reasons could include differences in the PAH uptake
417 mechanisms between the two organisms.

418

419 **4.0 Conclusions**

420

421 In this investigation there are large differences between the ratios of dPAH:PAH
422 accumulated in plants relative to those accumulated in earthworms suggesting there
423 cannot be one sole chemical method to predict bioavailability. Factors like the
424 behaviour of different soil biota within the soil or their different lipid contents have an
425 important role in determining what fraction of a contaminant may or may not be

426 available to them. It is extremely challenging if not impossible to develop a chemical
427 method that is able to mimic soil organisms at a level in which differences between
428 species can be accounted for. Although in some cases the ratios extracted by the
429 chemical methods differ substantially from those accumulated in the earthworm
430 tissues, results from this investigation do suggest that cyclodextrin and short butanol
431 extractions extract a fraction of the PAHs which is closer to that bioavailable to
432 earthworms and plants than that extracted by an exhaustive extraction. Deuterated
433 PAH amendments could be used to evaluate the ability of other methods, like Tenax
434 extractions (Ten Hulscher, et al., 2003), solid-phase microextraction (SPME) fibres
435 (Van der Wal et al., 2004), poly-oxymethylene solid-phase extractions (POM-SPE)
436 (Jonker and Koelmans, 2001), persulphate oxidations (Cuypers et al., 2000) or super
437 critical carbon dioxide extractions (Kreitinger et al., 2007), to predict PAH
438 bioavailability to different soil biota. We believe that using this isotope ratio method
439 can enable the comparison of methods that give an indication of the chemical activity
440 of a contaminant (e.g.SPME or POM) with those that give an indication of
441 contaminant accessibility (e.g. Tenax or cyclodextrin). This is of particular interest as
442 previously comparisons between methods have been made by comparing correlations
443 between chemical methods and bioaccumulation assays, or by using equilibrium
444 partitioning calculations to make predictions. In the former approach the correlations
445 are largely affected by the partitioning and metabolism of the contaminant within the
446 organism whilst the latter approach involves substantial assumptions, particularly
447 when using measurements from mild solvent and depletive sampling extractions. We
448 also suggest using a representative 5-ringed PAH like benzo(a)pyrene in tests of
449 chemical extractions due to the importance of this class of PAH in risk assessment. It
450 is therefore of particular importance that the fraction of the benzo(a)pyrene extracted

451 by the chemical methods examined in this investigation was the one that differed most
452 substantially from that accumulated in the earthworms.

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454

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617 Table 1. Chemical and physical properties of the soils.

| | pH | Total Organic Carbon (%) | Sand (%) | Silt (%) | Clay (%) |
|----------------|-----|--------------------------|----------|----------|----------|
| Kettering loam | 7.1 | 1.99 | 66.9 | 21.7 | 11.8 |
| Gasworks soil | 7.4 | 10.6 | 81.1 | 16.7 | 2.24 |

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