

*Relative proportions of polycyclic aromatic hydrocarbons differ between accumulation bioassays and chemical methods to predict bioavailability*

Article

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26 **Abstract**

27

28 Chemical methods to predict the bioavailable fraction of organic contaminants are  
29 usually validated in the literature by comparison with established bioassays. A soil  
30 spiked with polycyclic aromatic hydrocarbons (PAHs) was aged over six months and  
31 subjected to butanol, cyclodextrin and tenax extractions as well as an exhaustive  
32 extraction to determine total PAH concentrations at several timepoints. Earthworm (*E.*  
33 *fetida*) and rye grass root (*L. multiflorum*) accumulation bioassays were conducted in  
34 parallel. Butanol extractions gave the best relationship with earthworm accumulation  
35 ( $r^2 \leq 0.54$ ,  $p \leq 0.01$ ); while cyclodextrin, butanol and acetone-hexane extractions all  
36 gave good predictions of accumulation in rye grass roots ( $r^2 \leq 0.86$ ,  $p \leq 0.01$ ). However,  
37 the profile of the PAHs extracted by the different chemical methods was significantly  
38 different ( $p < 0.01$ ) to that accumulated in the organisms. Biota accumulated a higher  
39 proportion of the heavier 4-ringed PAHs. It is concluded that bioaccumulation is a  
40 complex process that cannot be predicted by measuring the bioavailable fraction  
41 alone.

42

43 **Keywords**

44 bioavailability, polycyclic aromatic hydrocarbons, earthworms, plants, accumulation

45

46 **Capsule**

47 The ability of chemical methods to predict PAH accumulation in *E. fetida* and *L.*  
48 *multiflorum* was hindered by the varied metabolic fate of the different PAHs within  
49 the organisms

50

51 **1. Introduction**

52

53 As organic compounds age in soil they become less available for uptake by  
54 organisms, and are thus less likely to have toxic effects or be degraded by soil  
55 microorganisms (Alexander, 2000). The biological effects of a contaminant are  
56 therefore not related to its total concentration, but to the bioavailable fraction. This is  
57 the fraction of the contaminant that is biologically available for uptake.

58

59 Regulators and the public are used to a system where total concentrations are  
60 considered as well founded and definitive values, although there are now new  
61 approaches for ecological risk assessment where bioavailability data, obtained from  
62 the results of bioassays, have a more important role (Harmsen, 2007). Bioassays only  
63 respond to the bioavailable fraction of contaminants and have the advantage of being  
64 able to consider site-specific effects of mixtures of contaminants and their metabolites  
65 (Jensen and Mesman, 2007). Although they are the most established method of  
66 quantifying bioavailability, their application may be time consuming and laborious, so  
67 a large number of theoretically more time and cost-efficient chemical methods for  
68 predicting bioavailability have been published in the scientific literature (Kelsey et al.,  
69 1997; Reid et al., 2000; Ten Hulscher et al., 2003).

70

71 The most frequent approach to evaluate chemical methods for the prediction of  
72 polycyclic aromatic hydrocarbon (PAH) bioavailability is by comparing how they  
73 approximate or correlate with the amount of organic compound accumulated by soil  
74 biota such as earthworms and to a lesser extent plants, or the amount degraded by  
75 microbes (Kelsey et al., 1997; Tang and Alexander, 1999; Reid et al., 2000; Liste and

76 Alexander, 2002; Tang et al., 2002; Ten Hulscher et al., 2003; Hickman and Reid,  
77 2005; Bergknut et al., 2007). When correlating chemical predictors of bioavailability  
78 to bioassays it is important to consider that the bioavailability being measured is  
79 specific to the organism used in that particular bioassay, and also to be aware that the  
80 determination of earthworm or plant accumulation does not necessarily measure  
81 contaminant bioavailability, but rather measures an interaction end-point between the  
82 organism and the compound (Hickman and Reid, 2005).

83

84 Earthworms are appropriate model organisms for bioavailability as they live in  
85 intimate contact with the soil, have a thin and permeable cuticle and consume large  
86 volumes of soil (Jager et al., 2005). They have been used in many studies as reference  
87 systems for organic compound bioavailability due to their importance in the terrestrial  
88 food chain, their potential to accumulate contaminants and ease of handling in the  
89 laboratory (Kelsey et al., 1997; Tang and Alexander, 1999; Liste and Alexander,  
90 2002; Tang et al., 2002; Ten Hulscher et al., 2003; Van der Wal et al., 2004a;  
91 Hickman and Reid, 2005; Bergknut et al., 2007). Considerably less work has been  
92 carried out using plant accumulation as a reference system (Tang and Alexander,  
93 1999; Tao et al., 2006a).

94

95 This study aims to compare how a range of chemical methods (namely extractions  
96 using butanol, cyclodextrin or tenax), frequently tested in isolation, predict PAH  
97 bioavailability using two different accumulation bioassays (earthworms and plants) as  
98 reference systems. It is important to consider different reference systems as  
99 bioavailability has been shown to vary between different organisms (Kelsey et al.,  
100 1997; Stroo et al., 2000).

101

## 102 **2. Methods**

103

### 104 **2.1 Soil spiking and ageing**

105

106 A 2 mm sieved Kettering Loam soil (Broughton Loam, Kettering, UK) (Table 1) was  
107 spiked using a single-step spiking/re-hydration procedure (Reid et al., 1998) with a  
108 stock solution of naphthalene and acenaphthene (2-ringed PAHs), fluorene and  
109 phenanthrene (3-ringed PAHs) and fluoranthene and pyrene (4-ringed PAHs) (Sigma  
110 Chemicals, Poole, UK) in acetone, to final concentrations of approximately 90 and  
111 450 mg kg<sup>-1</sup> total PAH with equal concentrations of each PAH. After addition of the  
112 stock solution, the soil was left uncovered in a fume hood for 24 h to ensure all the  
113 solvent had evaporated. After checking for removal of the solvent by olfactory  
114 detection and checking for residual wetting in the soil, the spiked soil was re-wetted to  
115 60% of its water holding capacity, transferred to 10 loosely sealed amber glass jars (5  
116 for each concentration) and aged for 6 months at 20°C. After 0, 1, 2, 3 and 6 months,  
117 2 jars (1 of each concentration) were emptied for use in the different chemical  
118 extractions and bioassays. A non-spiked 2mm sieved Kettering Loam soil was used  
119 as a control in all soil extractions and bioassays.

### 120 **2.2 Soil extractions**

121

122 To determine the total amount of PAHs in the soil, five replicate 2 g portions of soil  
123 were agitated in 10 ml of 1:1 by volume acetone/hexane mixture for 1.5 hours on an  
124 end over end shaker. After extraction the samples were left to settle for 30 min, and  
125 then 2 ml of solution were placed in a test tube containing 0.1 g of dry sodium

126 sulphate before transferring to gas chromatography (GC) vials for analysis. This  
127 method was adapted from a mechanical shaking method previously reported to give  
128 better recoveries than a Soxhlet extraction (Song et al., 2002). This adapted method  
129 was also found to give better recoveries than a soxhlet extraction in a preliminary  
130 study. Native PAH concentrations in the control soils were below the method  
131 detection limit ( $0.5 \text{ mg kg}^{-1}$ ).

132

133 Three different kinds of butanol extraction were carried out; a vortex extraction where  
134 10 g of soil were mixed in 10 ml of butanol solvent and agitated for 50 s (Swindell  
135 and Reid, 2006) or 120 s (Liste and Alexander, 2002) and then left to settle for 30  
136 minutes, and a shake (Reid et al., 2004) where 10 g of soil were mixed with 15 ml of  
137 butanol and placed on an end over end shaker for 12 hours and then left to settle for  
138 30 minutes. All butanol extractions were replicated 5 times and analysed using GC-  
139 FID. The method detection limits were  $0.10 \text{ mg kg}^{-1}$  and  $0.15 \text{ mg kg}^{-1}$  for the butanol  
140 mix and shake respectively.

141

142 Cyclodextrin extractions (Stokes et al., 2005) were carried out in triplicate by mixing  
143 1.5 g of soil with a 25 ml solution of 60-mM HPCD (Sigma Aldrich, Poole, UK) in  
144 deionised water and agitating the mixture for 20 hours using an orbital shaker (Orbital  
145 Shaker SO1, Bibby Sterilin Ltd, Stone, Staffordshire, UK) at 200 rpm. The mixture  
146 was then centrifuged at 2500 rpm using a Mistral 3000i centrifuge (MSE Sanyo-  
147 Gallenkamp, Leicester, UK) for 30 minutes and the supernatant discarded. The  
148 resulting soil pellet was shaken with 25 ml of deionised water for 10 s, centrifuged  
149 again and the supernatant was again discarded to remove any remaining HPCD  
150 solution. The soil pellet was then exhaustively extracted using the acetone/hexane

151 mechanical shaking extraction described above. GC analysis of this exhaustive  
152 extraction measured the PAHs remaining in the soil after HPCD extraction. The  
153 method detection limit was 0.67 mg kg<sup>-1</sup>.  
154  
155 Tenax extractions (Ten Hulscher et al., 2003) were also carried out in triplicate by  
156 mixing 1.4 g of soil and 1 g of Tenax TA<sup>®</sup> beads (60/80 mesh, 177-250 µm, Sigma  
157 Aldrich) in 70 ml of deionised water and placing them on an end over end shaker for 6  
158 hours. The beads were separated from the soil, rinsed with distilled water to remove  
159 soil particulates and solvent extracted by ultrasonication in 10 ml of hexane for  
160 1 hour. The solvent samples were then analysed by GC-FID. The method detection  
161 limit was 0.71 mg kg<sup>-1</sup>.

162

163 Chemical extractions were carried out in months 0, 1, 2, 3 and 6 except the Tenax  
164 extractions that were only carried out at months 0, 2 and 6.

165

### 166 **2.3 Earthworm bioassays**

167

168 Earthworms (*Eisenia fetida*) were obtained from Blades Biological (Cowden, UK).  
169 Only adult worms with a clitellum were used in the bioassays. Five worms were  
170 exposed to 300 g of the spiked soil (after 0,1,2,3 and 6 months of ageing) at 20°C for  
171 14 days. After exposure, the worms were rinsed with water and kept on wet filter  
172 paper for 24 h for depuration of their guts. They were then weighed and frozen at -20  
173 °C before being ground with 7 times their weight of dry sodium sulphate using a  
174 pestle and mortar. Tissues were then extracted following a saponification method to  
175 remove fat from the earthworms (Contreras-Ramos et al., 2008). This consisted of

176 adding 10ml of 0.5M KOH and 10 ml of a 1:1 acetone/hexane solvent mixture to the  
177 ground earthworm and ultrasonicated the mixture at 45 °C for 1 hour. The solvent  
178 layer was then cleaned on a deactivated silica column, pre-eluted with 5ml of 1:1  
179 acetone/hexane. The sample was then eluted with a further 5 ml of 1:1 acetone/hexane  
180 before being concentrated down to 1 ml by nitrogen blowdown prior to analysis by  
181 GC/MS.

182

#### 183 **2.4 Plant bioassays**

184

185 Rye grass (*Lolium multiflorum*) was grown for 4 weeks in the soil (after 0,1,2 and 3  
186 months of ageing) in a temperature controlled greenhouse. After 4 weeks the plants  
187 were harvested and the roots separated from the soil. Root samples were rinsed with  
188 deionised water, wiped with tissue paper and freeze-dried (Super Modulyo 12K  
189 Freeze Dryer, Edwards, Crawley, West Sussex, UK) overnight. The dried roots were  
190 then ground, homogenized and weighed prior to ultrasonication for 2 hours in 10 ml  
191 of dichloromethane. The extracts were then concentrated down to 1 ml by nitrogen  
192 blowdown and passed through 0.45 µm filters obtained from Chromacoal Ltd  
193 (Welwyn Garden City, UK) before being transferred to GC vials. Solutions were  
194 analysed by GC/MS.

#### 195 **2.5 GC-FID analysis**

196

197 Soil extraction samples were all analysed using an Agilent 6890N Network GC  
198 system equipped with a HP5 capillary column (dimensions: 30 m x 320 µm x 0.25  
199 µm; Agilent Technologies Inc, Santa Clara, USA), operating with helium as a carrier  
200 gas. The oven was configured to 50 °C, and held for 1 minute, then ramped to 280 °C

201 at a rate of 15 °C min<sup>-1</sup>, and held for 8 minutes. The injector and the FID were held at  
202 300 °C.

203

## 204 **2.6 GC-MS analysis**

205

206 Plant and earthworm samples were all analysed using an Agilent 7890A Network GC  
207 system equipped with an HP5 capillary column (dimensions: 30 m x 250 µm x 0.50  
208 µm; Agilent Technologies Inc), operating with helium as a carrier gas and coupled to  
209 an Agilent 5975C mass spectrometer (MS) through a heated transfer line (250 °C).

210 The GC injector (300 °C) was operated in a pulsed splitless mode, 1µl aliquots were  
211 injected using an autosampler, and the GC oven was programmed to hold 45 °C for  
212 2.25 min then raise the temperature by 40 °C/min to 300 °C, which was held for 6  
213 minutes. The MS was operated in single ion monitoring (SIM) mode with electric  
214 impact ionization.

215

216

217

218

219

## 220 **2.7 Statistical Analysis**

221

222 Chemical extractions and bioassays were compared using General Linear Regressions  
223 in Genstat Release ver. 7 (Lawes Agricultural Trust, Rothamsted Experimental  
224 Station).

225

## 226 **3.0 Results**

227

### 228 **3.1 PAH loss from spiked soil**

229

230 The loss of 2 and 3-ringed PAHs during the ageing period was more rapid than that of  
231 the heavier 4-ringed PAHs as measured by the mechanical acetone hexane extraction  
232 (Figure 1). All the naphthalene was depleted after 2 months and only pyrene and  
233 fluoranthene remained in the soil in month 6 at both concentrations. The initial rate of  
234 PAH loss was significantly greater for the 450 mg kg<sup>-1</sup> concentration where 25% of  
235 the original spike remained after 1 month compared to 50% in the 90 mg kg<sup>-1</sup>  
236 treatment (p<0.01). All the naphthalene was lost from both soils over the following  
237 month and less than 5% of the original spike of the other 2-3 ring PAHs remained  
238 after month 3. Less than 20% of the original amount of pyrene and fluoranthene  
239 remained in the soil spiked with 90 mg kg<sup>-1</sup> and less than 10% in the 450 mg kg<sup>-1</sup> soil  
240 after 6 months.

241

### 242 **3.2 Soil extractions – Total PAH**

243

244 The acetone hexane extraction extracted significantly more PAHs than any of the  
245 chemical methods used to predict bioavailability at all five time points (p<0.01)  
246 (Figure 2). All the extractions were significantly different from each other over the 6  
247 month period (p<0.05). Raising the contact time in the butanol extractions from 50s to  
248 120s and 120s to 12h led to a significant increase in the total amount of PAHs  
249 extracted (p<0.05).

250

### 251 3.3 Bioassay data

252

253 There was no significant difference between the total amount of PAHs extracted from  
254 the earthworms exposed to either soil after 0 and 1 months ( $p < 0.01$ ) (Figure 3). After  
255 month 1 there was a significant decline in PAH accumulation in the earthworms  
256 exposed to the soil spiked with a total of  $450 \text{ mg kg}^{-1}$  PAH at each successive time  
257 point ( $p < 0.01$ ). There was no significant decline in earthworm accumulation in the  $90$   
258  $\text{mg kg}^{-1}$  soil between month 2 and 3 but there was between months 1 and 2 and  
259 months 3 and 6 ( $p < 0.01$ ).

260

261 The only significant decline in the total amount of PAH accumulated in the rye grass  
262 roots was between months 0 and 1 in the soil spiked with  $450 \text{ mg kg}^{-1}$  PAH ( $p < 0.01$ )  
263 (Figure 4).

264

### 265 3.4 Comparing chemical extractions with bioassay data

266

267 General Linear Regression suggests that 12 h butanol extractions explain a larger  
268 proportion of the variation in total PAH accumulated in earthworm tissue than any  
269 other chemical extraction (Table 2). The  $r^2$  values are generally higher for the heavier  
270 4-ringed PAHs (fluoranthene and pyrene) than for the 3-ringed PAHs (fluorene and  
271 phenanthrene).  $r^2$  values for acenaphthene are deceptively high as it virtually  
272 disappears from the soil after month 2. Regression analysis was not possible for  
273 naphthalene as it was not detected in earthworm tissue.

274

275 Only cyclodextrin extractions explain a larger proportion of the variation in total PAH  
276 accumulated in plant tissue than the acetone hexane extraction (Table 3). Naphthalene  
277 values are not included as it was only detectable in the soil in months 0 and 1.

278

279 Comparisons between plants and earthworms should not be made using these values  
280 as plants were not sampled in the month 6 time point. Tenax extractions are not  
281 included in these tables as they were not performed throughout all time points either.

282

### 283 **3.5 Composition of accumulated and extracted PAHs**

284

285 For the month 0 soils there was a significantly larger percentage contribution of 4-  
286 ringed PAHs in both the earthworm and plant accumulation bioassays relative to the  
287 chemical extractions ( $p < 0.01$ ), with the proportion of 4-ringed PAHs being less than  
288 40% in all chemical extractions (Figure 5). The soils still contained a substantial  
289 amount of 2 and 3-ringed PAHs. There was also a significantly higher contribution of  
290 2-ringed PAHs in the Tenax and cyclodextrin extractions than in any of the other  
291 extractions or bioassays ( $p < 0.05$ ).

292

293 On month 2 however, when the soils contained a substantially smaller amount of the 2  
294 and 3-ringed PAHs, there was no significant difference between the PAH profiles of  
295 earthworms and butanol extractions in the soil spiked with  $90 \text{ mg kg}^{-1}$  PAH ( $p < 0.05$ )  
296 (Figure 5). All other extractions had significantly different compositions than the  
297 earthworms ( $p < 0.05$ ), but they were substantially closer than in month 0. The  
298 proportion of 4-ringed PAHs was higher than 60% for the acetone hexane and tenax  
299 extractions and higher than 90% in all others. There was no significant difference

300 between the acetone hexane and plant extractions in the soil spiked with 450 mg kg<sup>-1</sup>  
301 PAH (p<0.05), there was a different between all others (p<0.01), but again they were  
302 substantially closer than in month 0.

303

#### 304 **4. Discussion**

305

##### 306 **4.1 PAH loss from spiked soil**

307

308 The low-molecular weight PAHs exhibited the highest loss rates. These PAHs are  
309 susceptible to abiotic processes like volatilization (Park et al., 1990). This together  
310 with biodegradation is most likely responsible for the rapid loss of the 2-ringed PAHs  
311 in the first month. For the remainder of the PAHs, biodegradation is likely to have  
312 been the main loss process. There is a broad inverse relationship between the rate of  
313 biodegradation and the number of rings in the PAH (Bossert and Bartha, 1986; Wild  
314 and Jones, 1993) which is consistent with only the 4-ringed PAHs being detectable in  
315 the 6 month old soils.

316

317

##### 318 **4.2 Soil extractions**

319

320 The non exhaustive extractions only recovered a fraction of the PAHs extracted by the  
321 acetone hexane extraction at all time points. This has been reported in other papers  
322 where the fraction recovered by these non exhaustive extractions has been related to  
323 the bioavailable fraction (Kelsey et al., 1997; Reid et al., 2000). However, the  
324 different extraction techniques generally extracted different amounts of PAHs over

325 the different time points. Differences between different chemical methods to predict  
326 PAH bioavailability have also been found in a previous study, where a number of  
327 chemical extractions were compared using PCA (Bergknut et al., 2007).

328

329 The fact that increasing the contact time of the butanol extractions significantly  
330 increased the amounts of PAHs extracted has important implications when trying to  
331 measure the bioavailable fraction as will be discussed in the following section.

332 Differences in extraction between the varying contact times were not as pronounced  
333 in previous studies. Swindell and Reid (2006) found a vortexing time of 50s to be  
334 appropriate as an approximation for the rapidly desorbing fraction and that increasing  
335 it to 120s as in Liste and Alexander (2002) made very little difference to the value  
336 obtained.

337

### 338 **4.3 Comparing chemical methods with the earthworm accumulation bioassay**

339

340 The regressions show that the exhaustive extraction using acetone hexane does not  
341 provide the best prediction of PAH accumulation in earthworms. Butanol extractions  
342 had the better regression results. This is in line with earlier studies where mild  
343 solvents were initially proposed as chemical methods to predict bioavailability  
344 (Kelsey et al., 1997; Liste and Alexander, 2002), although much higher  $r^2$  values  
345 ( $>0.90$ ) have been found in other studies with butanol (Tang and Alexander, 1999).  
346 The extraction with the longest contact time 12h, between the solvent and the soil,  
347 showed the best correlations. Different contact times and mild solvents of varying  
348 strength have been found to correlate differently with different bioassays and other  
349 chemical methods to predict bioavailability (Kelsey et al., 1997; Tang and Alexander,

350 1999; Liste and Alexander, 2002; Tang et al., 2002; Swindell and Reid, 2006;  
351 Bergknut et al., 2007). A more rigorous defence of the extraction time chosen is  
352 required.

353

354 Some studies have found butanol and other mild solvents to provide a poor indication  
355 of earthworm bioavailability (White et al., 1997; Johnson et al., 2002). Johnson et al.  
356 (2002) suggest that butanol may be a good mimic of the passive uptake of chemicals  
357 by organisms through their outer epidermis, but that it is less effective at predicting  
358 the amount absorbed through the gut as here the soil structure and extraction  
359 conditions are altered. Gut uptake could be higher than passive uptake through the  
360 outer epidermis (Landrum, 1989), although this may not be the case with *E. fetida* as  
361 it is an epigeic earthworm species and therefore consumes less soil than endogeic  
362 earthworm species. This may be the reason for butanol having a relatively good  
363 correlation in this study and strong correlations in the previously mentioned studies  
364 where *E. fetida* was used as the test species, but not with the endogeic *Aporrectodea*  
365 *longa* used in Johnson *et al.* (2002). It is therefore important to be aware of these  
366 differences between species as results in investigations like this one are greatly  
367 influenced by the choice of species.

368 Cyclodextrin extractions only slightly improved the prediction of PAH accumulation  
369 relative to the acetone hexane extraction. There are some studies indicating that  
370 cyclodextrin extractions of organic pesticides are a good indicator of earthworm  
371 bioavailability (Hartnik et al., 2008), but most studies using PAHs indicate a poor  
372 correlation with earthworm accumulation (Hickman and Reid, 2005; Bergknut et al.,  
373 2007). Reasons for this include that earthworms have complex accumulation  
374 mechanisms, and that they can access compounds from both the aqueous and the solid

375 phase (Gevao et al., 2001), suggesting the simple aqueous to hydrophobic sink model  
376 provided by cyclodextrin or Tenax extractions may not account for the complexity of  
377 the system. However, it is also important to consider that the lower sensitivity of these  
378 methods due to the dilution stages and smaller masses of soil used in the extractions  
379 relative to the butanol extractions could be another reason for their poorer  
380 predictability.

381

382 The butanol and cyclodextrin extractions account for a larger percentage variance in  
383 the amount of PAHs accumulated in the earthworm tissue than the acetone hexane  
384 extractions, but there is still a large proportion of the variation in accumulated PAHs  
385 unaccounted for by these extraction methods.

386

#### 387 **4.4 Comparing chemical methods with the plant accumulation bioassay**

388

389 The chemical methods to predict bioavailability did not improve the description of the  
390 variation in plant accumulation provided by the acetone hexane extraction. Other  
391 attempts to compare plant accumulation with extractions using this solvent mixture  
392 were not found, but a good correlation with hexane extractions was also observed by  
393 Tao *et al.* (2006a). Here the amount of PAHs extracted by the water and hexane  
394 fractions of a sequential extraction scheme using an accelerated solvent extraction  
395 system was found to correlate well with accumulation in wheat roots. Tang and  
396 Alexander (1999) found that a number of mild solvent extractions including butanol  
397 correlated strongly ( $r^2 > 0.89$ ) with anthracene accumulation in wheat and barley roots,  
398 but no direct indication of how an exhaustive extraction compared with this was  
399 given. Tenax extractions have been shown to have potential to predict toxicity to

400 plants as measured by the emergence of lettuce seedlings (Cofield et al., 2008), but no  
401 studies have attempted to correlate either cyclodextrin or tenax extractions with plant  
402 accumulation to date. Further investigation is required in this field as non exhaustive  
403 methods to predict bioavailability should theoretically provide a better indication of  
404 bioavailability to plants than exhaustive ones. Predicting the amount of PAHs that will  
405 accumulate in plants is important from a human health perspective, as food ingestion  
406 is the main source of human exposure to PAHs, with the major dietary contributions  
407 being cereals and vegetables (Phillips, 1999).

408

409 It should be noted that in this study and in the one by Tao *et al.* (2006a), the plant  
410 roots were only rinsed with water prior to analysis. It is therefore possible that the root  
411 extractions included some PAHs sorbed to the root surface and therefore not strictly  
412 accumulated within the roots (Tao et al., 2006b).

413

#### 414 **4.5 Composition of accumulated and extracted PAHs**

415

416 The PAH profile of the earthworms and plants was different from the profile obtained  
417 by the soil extractions. Bergknut *et al.* (2007) observed a higher proportion of 5- and  
418 6-ringed PAHs accumulated in earthworms than those extracted by a series of  
419 chemical extractions using mild solvents and cyclodextrins amongst others. The  
420 higher octanol-partitioning coefficient ( $K_{ow}$ ) of these heavier PAHs was given as the  
421 reason for their increased accumulation. A strong negative correlation ( $r^2=0.93$ )  
422 between  $\log K_{ow}$  and PAH elimination rate from earthworm tissue (Matscheko et al.,  
423 2002) and the fact that earthworms have been found to promote the degradation of the  
424 more readily biodegradable PAHs (Ma et al., 1995) may have contributed to the

425 increased accumulation of the heavier 4-ringed PAHs in the earthworms of this study.  
426 Similar studies have also shown that PAHs with higher  $K_{ow}$  accumulate more in plant  
427 roots than those of lower  $K_{ow}$  (Gao and Ling, 2006), confirming earthworm and plant  
428 accumulation are not only controlled by the bioavailable fraction of the contaminant  
429 but also by contaminant characteristics. The greater proportion of heavier PAHs  
430 accumulated by the plants and worms is highly significant from a risk assessment  
431 point of view as these are generally the more toxic/carcinogenic/mutagenic  
432 components. If a soil were to be extracted with a surrogate chemical assay the wrong  
433 bioavailability/toxicity profile might be assumed. The same overall PAH  
434 concentration as that of a bioassay may be obtained but hidden in that is the greater  
435 proportion of the heavier and more toxic PAHs.

436

437 Tenax extractions have been found to provide good predictions of bioaccumulation of  
438 PCBs and some organic pesticides in oligochaetes (You et al., 2006; Landrum et al.,  
439 2007). This was not the case for a number of PAHs including phenanthrene, and the  
440 authors believed the most logical reason for this was that some PAHs are readily  
441 biotransformed by some oligochaetes unlike most chlorinated compounds. Similarly  
442 measuring the desorption of two pesticides into the aqueous phase using cyclodextrin  
443 extractions has been found to predict pesticide uptake into earthworms, but not  
444 pesticide bioaccumulation within the earthworm tissues (Hartnik et al., 2008).

445 Differences in bioaccumulation rates between compounds cannot necessarily be  
446 explained by differences in the bioavailable fraction and are most likely due to  
447 different metabolic fate in the organisms (Hartnik and Styrishave, 2008). Chemical  
448 methods to predict bioavailability therefore cannot account for biological factors, like  
449 elimination or biotransformation, which affect the accumulation of chemicals. This is

450 probably one of the main reasons for the bioavailable fraction predicted by methods  
451 such as cyclodextrin extractions to correlate strongly with microbial mineralisation  
452 (Reid et al., 2000; Hickman and Reid, 2005; Allan et al., 2006; Papadopoulos et al.,  
453 2007), but only correlate weakly with earthworm accumulation (Hickman and Reid,  
454 2005). The fact that the composition of the PAHs accumulated in plants and  
455 earthworms also differed despite them being exposed to exactly the same soil  
456 reiterates this point. PAHs will have different metabolic fates in different organisms  
457 and it will be hard if not impossible to develop a chemical method that can mimic soil  
458 biota to this level.

459

460 Some authors have used the Equilibrium Partitioning (EP) theory to account for the  
461 different biota to sediment accumulation factors (BSAF) of different contaminants  
462 (Krauss and Wilcke, 2001; Van der Wal et al., 2004b; Kreitinger et al., 2007). Van der  
463 Wal *et al.* (2004b) for example used EP theory considering contaminant  $K_{ow}$  and pore  
464 water concentrations as measured by SPME fibres, to relate the bioavailable fraction  
465 as measured by the SPME fibres to accumulation in earthworms. Measuring the  
466 bioavailable fraction of a contaminant in this way and then combining it with EP  
467 theory to obtain a prediction of earthworm accumulation may be a better way of  
468 predicting earthworm and plant accumulation. However, Bergknut *et al.* (2007) found  
469 poor correlations between PAH accumulation in earthworms and PAHs extracted by  
470 SPME fibres using the method considering EP theory as proposed in Van der Wal *et*  
471 *al.* (2004b). Using contaminant  $K_{ow}$  on its own to predict accumulation may  
472 therefore not be sufficient and other factors like organism specific uptake and  
473 detoxification mechanisms may need to be included in the calculation. More research  
474 into this issue is vital as being able to predict the uptake of PAHs by plants and

475 earthworms has important implications both for human health and the environment  
476 due to their accumulation potential up the food chain and their carcinogenicity.

477

## 478 **5.0 Conclusion**

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480 Using accumulation bioassays to assess the capability of chemical methods to predict  
481 the bioavailability of readily biotransformable or biodegradable PAHs is not a fair test  
482 of their potential as bioavailability indicators. Even if they do provide a good estimate  
483 of the bioavailable fraction other processes influence the accumulation of  
484 contaminants in soil biota, including the physicochemical properties of the  
485 contaminant and the characteristics of soil biota themselves. Modelling these  
486 contaminant properties and soil biota uptake, biotransformation and elimination  
487 mechanisms may be the best way of predicting the amount of contaminant  
488 bioaccumulated in soil biota using the bioavailable fraction measured by chemical  
489 methods.

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665 Table 1. Chemical and physical properties of the Kettering loam soil.

pH	Organic Matter (%)	Sand (%)	Silt (%)	Clay (%)
7.1	5.0	66.9	21.74	11.76

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687 Table 2. Results of General Linear Regressions between the total and individual  
688 amount of PAHs extracted by the acetone hexane shake (AH), the cyclodextrin  
689 extraction (CD), the 50s butanol mix (BM50s), the 120s butanol mix (BM120s) and  
690 the 12h butanol shake (BS12h) relative to the amounts accumulated in the earthworm  
691 *E. fetida*.

	AH	CD	BM50s	BM120s	BS12h
Acenaphthene r <sup>2</sup> p	0.81 <0.01	0.78 <0.01	0.80 <0.01	0.82 <0.01	0.86 <0.01
Fluorene r <sup>2</sup> p	0.03 0.30	0 <sup>a</sup>	0 <sup>a</sup>	0.02 0.31	0.06 0.24
Phenanthrene r <sup>2</sup> p	0 <sup>a</sup>	0 <sup>a</sup>	0 <sup>a</sup>	0.01 0.34	0.20 0.11
Fluoranthene r <sup>2</sup> p	0.55 0.01	0.40 0.03	0.63 <0.01	0.59 0.01	0.47 0.02
Pyrene r <sup>2</sup> p	0.55 0.01	0.48 0.02	0.76 <0.01	0.62 <0.01	0.47 0.02
Total PAH r <sup>2</sup> p	0.48 0.02	0.51 0.03	0.54 0.01	0.60 0.01	0.64 <0.01

692 <sup>a</sup> Residual variance exceeds variance of response variate

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703 Table 3. Results of General Linear Regressions between the total and individual  
704 amount of PAHs extracted by the acetone hexane shake (AH), the cyclodextrin  
705 extraction (CD), the 50s butanol mix (BM50s), the 120s butanol mix (BM120s) and  
706 the 12h butanol shake (BS12h) relative to the amounts accumulated in the rye grass  
707 (*L. multiflorum*) roots.

	AH	CD	BM50s	BM120s	BS12h
Acenaphthene					
r <sup>2</sup>	0.84	0.85	0.76	0.75	0.94
p	0.01	0.01	0.01	0.02	<0.01
Fluorene					
r <sup>2</sup>	0.64	0.68	0.68	0.66	0.62
p	0.04	0.03	0.03	0.03	0.04
Phenanthrene					
r <sup>2</sup>	0.07	0.11	0.09	0.06	
p	0.31	0.27	0.29	0.32	0 <sup>a</sup>
Fluoranthene					
r <sup>2</sup>	0.80	0.84	0.70	0.36	
p	0.01	0.01	0.03	0.12	0 <sup>a</sup>
Pyrene					
r <sup>2</sup>	0.73	0.78	0.57	0.19	
p	0.02	0.01	0.05	0.21	0 <sup>a</sup>
Total PAH					
r <sup>2</sup>	0.95	0.97	0.93	0.86	0.82
p	<0.01	<0.01	<0.01	0.01	0.01

<sup>a</sup> Residual variance exceeds variance of response variate

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