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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

4   **Jose L. Gomez-Eyles[a],*, Chris D. Collins[a] and Mark E. Hodson[a]**

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6   [a] University of Reading, School of Human and Environmental Sciences, Soil

7   Research Centre, Reading, RG6 6DW, Berkshire, United Kingdom.

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24   *****Corresponding author. Tel: +44 118 378 7903   Fax: +44 118 378 6666

25   Email address: j.l.gomezeyles@reading.ac.uk (J.L Gomez-Eyles)

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

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46   **Capsule**

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48   A novel method using isotope ratios to assess the ability of chemical methods to

49   predict PAH bioavailability to soil biota.

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2

1. Introduction

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

These chemical methods are normally validated in the literature by comparing how they approximate or correlate with the amount of organic compound accumulated by soil biota such as earthworms and to a lesser extent plants, or the amount degraded by microbes (Kelsey, et al., 1997; Tang and Alexander, 1999; Reid, et al., 2000; Liste and Alexander, 2002; Tang et al., 2002; Ten Hulscher, et al., 2003). However, recent studies have shown distinct differences between the PAHs extracted using some of these techniques and those accumulated in earthworms and plants (Hickman and Reid, 2005; Bergknut et al., 2007; Gomez-Eyles et al., 2010). It is important to realise 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. cyclodestrin 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

4

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

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

2. Experimental Section

2.1 Soil spiking and ageing

5

126

PAH-contaminated soil from a former gasworks site in the UK (Table 1) was passed

through a 2 mm sieve. The <2mm fraction was spiked using a single-step spiking/re-

hydration procedure (Reid et al., 1998) with a stock solution of deuterated PAHs

(Sigma Chemicals, Poole, UK) in acetone, to final concentrations of 30 mg kg^{-1} of

[$^2H_8$] naphthalene, [$^2H_{10}$] phenanthrene, [$^2H_{10}$] pyrene and 10 mg kg^{-1} of [$^2H_{12}$]

benzo(a)pyrene. After addition of the stock solution, the soil was left uncovered in a

fume cupboard for 24 h to ensure all the solvent had evaporated. After confirming

removal of the solvent by olfactory detection and checking for residual wetting in the

soil, the spiked soil was re-wetted to 60% of its water holding capacity. Samples of

the soil were taken immediately after re-wetting to determine initial PAH

concentrations. The remainder of the soil was used either in bioassays of 20 days

duration (see below) or transferred to loosely sealed amber glass jars and aged for 20

days at 20ºC.

140

The same procedure was followed using a control soil (Broughton Loam, Kettering,

UK) (Table 1), but this soil was spiked with fresh undeuterated PAHs as well as

dPAHs to the same final concentrations as above. Exposing plants and earthworms to

a soil freshly spiked with equal amounts of PAHs and dPAHs served as a control for

any potential preferential accumulation of one kind of PAH over the other. When

comparing ratios of dPAHs:PAHs between organisms and the chemical extractions

we assume there is no difference between the uptake processes or the metabolic fate

of dPAHs and PAHs within the organisms. Determining whether this assumption is

true is therefore important when using these ratios to evaluate the potential of the

chemical methods to predict the bioavailable fraction.

151

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 20ºC 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   ºC before being ground with 7 times their weight of dry sodium sulphate using a

198   pestle and mortar. Earthworms were then extracted following a saponiphication

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 ultrasonicating the mixture at 45 ºC 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 ºC). The GC

226   injector (220 ºC) was operated in a pulsed splitless mode, 1µl 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 ºC/min to 290 ºC, and held for 10 minutes. The MS was operated with

229   the ion source at 220 ºC and a damping flow of 0.3 ml min^{-1}.

230

231   **2.6 Statistical Analysis**

232

233   Statistical analysis was perfomed 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 biodegration 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

10

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 photodegration. 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).

11

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.

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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 bioavailablity 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 undeutrated 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 cylcodextrin 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 cylodextrin 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.,

16

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

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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   bioavailablity 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

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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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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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