

1 In Vitro Prediction of Polycyclic Aromatic Hydrocarbon Bioavailability of 14 Different  
2 Incidentally Ingested Soils in Juvenile Swine

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9 **Title Running Head**

10 Improving PAH bioavailability predictions

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18 **Abstract**

19 Predicting mammalian bioavailability of PAH mixtures from in vitro bioaccessibility  
20 results has proven to be an elusive goal. In an attempt to improve in vitro predictions of PAH  
21 soil bioavailability we investigated how energetic input influences PAH bioaccessibility by using  
22 a high and low energetic shaking method. Co-inertia analysis (COIA), and Structural Equation  
23 Modelling (SEM) were also used to examine PAH-PAH interactions during ingestion. PAH  
24 bioaccessibility was determined from 14 historically contaminated soils using the fed organic  
25 estimation of the human simulation test (FOREhST) with inclusion of a silicone rod as a sorption  
26 sink and compared to bioavailability estimates from the juvenile swine model. Shaking method  
27 significantly affected PAH bioaccessibility in the FOREhST model, with PAH desorption from the  
28 high energy FOREhST almost an order of magnitude greater compared to the low energy  
29 FOREhST. PAH-PAH interactions significantly influenced PAH bioavailability and when these  
30 interactions were used in a linear model, the model predicted benzo(a)anthracene  
31 bioavailability with an slope of 1 and  $r^2$  of 0.66 and for benzo(a)pyrene bioavailability has a  
32 slope of 1 and  $r^2$  of 0.65. Lastly, to confirm the effects as determined by COIA and SEM, we  
33 spiked low levels of benzo(a)anthracene into historically contaminated soils, and observed a  
34 significant increase in benzo(a)pyrene bioaccessibility. By accounting for PAH interactions, and  
35 reducing the energetics of in vitro extractions, we were able to use bioaccessibility to predict  
36 bioavailability across 14 historically contaminated soils. Our work suggests that future work on  
37 PAH bioavailability and bioaccessibility should focus on the dynamics of how the matrix of PAHs  
38 present in the soil interact with mammalian systems. Such interactions should not only include

39 the chemical interactions discussed here but also the interactions of PAH mixtures with  
40 mammalian uptake systems.

## 41 **Introduction**

42 Polycyclic aromatic hydrocarbons (PAHS) are carcinogenic compounds produced from  
43 incomplete combustion of organic material. Due to their relatively low solubility and vapour  
44 pressure PAHs will accumulate in soil over time and humans are exposed to PAHs through the  
45 incidental ingestion of PAH contaminated soil. The default assumption for exposure  
46 assessment is that all of the ingested PAHs have been solubilised and absorbed (i.e. 100%  
47 bioavailable) from the gastrointestinal tract, however a significant fraction of PAHs are strongly  
48 bound to soil constituents and are not released within the gastrointestinal tract.<sup>1, 2</sup>

49 PAH bioavailability from soil is estimated by monitoring uptake of PAHs into the  
50 bloodstream of a model organism, e.g. mice, swine or rats. Animals should, ethically, not be  
51 used for routine site assessments and thus, substantial effort has gone into developing in vitro  
52 bioaccessibility models to predict bioavailability. Current models for organic contaminants  
53 include Physiologically Based Extraction Test (PBET),<sup>3-5</sup> Colon-extended PBET,<sup>6</sup> Fed Organic  
54 Estimation human Simulation Test (FOREhST),<sup>2, 7</sup> Relative Bioaccessibility Leaching Procedure  
55 (RBALP),<sup>8</sup> as well as simulation of the human intestinal microbial ecosystem (SHIME).<sup>7</sup> To  
56 ensure that hydrophobic organic contaminant soil release is not limited to the compound  
57 solubility for the simulated intestinal fluids, a sorption sink such as C18 membranes,<sup>8, 9</sup> tenax  
58 beads,<sup>5</sup> ethyl vinyl acetate thin films,<sup>10</sup> and silicone rods<sup>11</sup> are incorporated into the models.  
59 These models can often predict the bioavailability of different PAHs within a soil,<sup>1</sup> but typically  
60 are not successful in estimating bioavailability between soils.

61 Juhasz et al.<sup>12</sup> noted that maximizing estimated bioaccessibility is not necessarily the  
62 most conservative measure of bioavailability (ie. bioaccessibility can be less than bioavailability).

63 Bioaccessibility is dependent upon the desorption conditions within the *in vitro* model, i.e.  
64 shaking method, temperature, desorption media, and desorption time.<sup>13, 14</sup> PAH release in *in*  
65 *vitro* models is linked to the activation energy of the desorption process<sup>15</sup> as well as organic  
66 matter composition.<sup>16, 17</sup> PAHs bind to either amorphous organic matter with non-competitive  
67 fast desorption kinetics or to carbonaceous geosorbents with competitive slow desorption  
68 kinetics.<sup>18</sup> A typical soil has both amorphous and carbonaceous geosorbents and regardless of  
69 carbon type, longer desorption times typically lead to greater desorption.<sup>14</sup> The RBALP model,  
70 which utilizes end-over-end rotation, can be coupled with a lipid sink and leads to high PAH  
71 release from soil.<sup>8</sup> Under such conditions, PAH bioaccessibility closely tracks PAH soil  
72 concentration but not PAH bioavailability<sup>8</sup>. *In vitro* models that use reduced energetic input,  
73 such as the TIM model,<sup>19</sup> will result in lower PAH release and perhaps this release is linked more  
74 closely to bioavailability. Our rationale for this hypothesis is that the current generation of *in*  
75 *vitro* models assumes that maximizing bioaccessibility will better predict bioavailability. While  
76 possible, our experience is that these *in vitro* approaches closely mirror chemical activity but  
77 not bioavailability. Hence, we modified the existing FOREhST model to reduce energetic inputs  
78 during extraction and compared this release to *in vivo* bioavailability results.

79 PAHs are present as mixtures and depending on the source of the PAHs, e.g. pyrogenic,  
80 petrogenic, etc., the relative ratios of each PAH will change.<sup>20</sup> The nature of this PAH mixture is  
81 a major factor influencing PAH bioaccessibility/bioavailability.<sup>21</sup> It is thought that these mixture  
82 effects occur because PAHs interact with other PAHs and influence their partitioning behavior.  
83 For example, phenanthrene solubility in various surfactants was enhanced in the presence of  
84 naphthalene yet reduced in the presence of pyrene.<sup>22</sup> Benzo(a)pyrene concentrations in gut

85 fluids increased in the presence of cholesterol (137%), phenanthrene (154%), lecithin (140%)  
86 and hexadeconal (232%).<sup>23</sup> Given that PAHs interact with each other, it is likely that linking PAH  
87 bioaccessibility to PAH bioavailability between soils requires that we explicitly link the matrices  
88 of PAH accessibility to PAH bioavailability.

89 Co-inertia analysis is statistical method developed to study the common structure of  
90 multiple sets of paired data.<sup>24</sup> Co-inertia analysis is a non-directional approach to identify  
91 individual variables within each matrix that influence the other corresponding matrix and is well  
92 suited to situations where the number of samples is low relative to the number of predictor  
93 variables. Here we use co-inertia to identify key PAHs in the bioaccessibility matrix that are  
94 influencing other PAHs in the bioavailability matrix. However, co-inertia analysis is largely an  
95 exploratory statistical approach, and thus we tested if these PAHs were significantly influencing  
96 bioavailability using structural equation modelling. Structural equation modelling (SEM) is well  
97 suited for assessing a hypothesis that links collinear variables in a causal network to predict a  
98 dependent variable.<sup>25</sup> Furthermore, unlike multiple regression approach, structural equation  
99 modelling explicitly accounts for collinearity and thus, allows one to estimate, not only the  
100 significance, but the strength of a relationship linking predictors (such as the bioaccessibility of  
101 single PAHs) to the bioavailability of a PAH.

102 Our goal here was to combine the concepts of bioaccessibility and bioavailability as  
103 outlined by Juhasz et al.<sup>12</sup> and Reichenberg and Mayer<sup>13</sup>, with explicit multivariate predictive  
104 approaches, to develop a numerical prediction of bioavailability based on a widely adopted  
105 bioaccessibility protocol. We then evaluated the robustness of this prediction by spiking PAHs

106 into water or soil and confirming that the drivers identified by the multivariate approaches  
107 were indeed occurring in in vitro settings.

## 108 **Materials and Methods**

### 109 **Soils**

110 A total of 14 PAH contaminated soils have been collected from the United Kingdom (n =  
111 12) and Sweden (n =2) as previously described by Cave et al.<sup>7</sup> and James et al.<sup>8</sup> Soil pH, organic  
112 carbon, and particle size were analyzed as previously described by Siciliano et al.<sup>26</sup>

### 113 **Sorptive sink**

114 Silicone rods, poly(dimethylsiloxane) (PDMS), are chosen to act as a PAH sorption sink as  
115 they have established partitioning properties for PAHs and have been previously used for in  
116 vitro bioaccessibility testing.<sup>4, 21</sup> The silicone rod (Altec, Cornwall, United Kingdom) has a  
117 diameter of 2.87-3.13 with a mass of 8.0 g m<sup>-3</sup>. To prepare the silicone rods for experimental  
118 use, the procedures of Gouliarmou et al.<sup>11</sup> are followed, where the silicone was cleaned by  
119 soaking once overnight with ethylacetate, three times overnight with methanol, 3 times  
120 overnight with acetone, and 4 times overnight with Milli-Q water.

### 121 **FOREhST**

#### 122 **Shaking Method / Energetic input**

123 To investigate the effects of energetic inputs two shaking methods were employed. The  
124 first is the standard high energy FOREhST where 125 mL glass bottles are rotated 30 rpm end-  
125 over-end inside of a water bath held at 37°C. The second method uses a less aggressive process  
126 to create a massaging motion that utilizes 2 – 1.5” rotating spherical balls moving back and  
127 forth horizontally (Supporting Information Figure S1). Modified polytetrafluoroethylene (PTFE)

128 bags (5"x4"- 5.0 Mil thick, Welch Fluorocarbon, Dover, New Hampshire) are used with this  
129 lower energy method, as their inherent flexibility allows for a massaging technique. In the low  
130 energy method, the FOREhST fluids are warmed up to 37°C prior to use and cool down to 28-  
131 32°C after 2 hours.

132 The FOREhST model described here follows the detailed procedures of Cave et al<sup>7</sup>. The  
133 FOREhST model is an adaption of the fed state methods developed by the RIVM - The  
134 Netherlands National Institute for Public Health and the Environment<sup>27</sup> and is intended for  
135 organic contaminants.<sup>7</sup> The fed state is the most conservative estimate of bioaccessibility for  
136 organic contaminants<sup>28</sup>. The compartments of the FOREhST model are saliva, gastric and  
137 intestinal, which consist of simulated fluids modeled to the physiochemical conditions present  
138 at each stage.

139 To each experimental unit, 0.3 g of contaminated soil is added, followed by  
140 approximately 0.8 g of HIPP creamy porridge™, 2.45 mL of deionized water, 50 µL sunflower oil,  
141 and 1 m silicone rod. Saliva fluid, 4.5 mL, is added to each unit and shaken for 5 min.  
142 Afterwards, 9 mL of gastric fluid is added and incubated for 2 hours. Finally, 9 mL of duodenal  
143 fluid and 4.5 mL of bile fluid were added, followed by an additional 2 hour incubation.

144 Post incubation, silicone rods are removed from the extraction units, washed with Milli-  
145 Q water and gently dried with lint free tissue paper. PAHs are extracted by soaking silicone  
146 rods in approximately 50 mL of acetone twice for 24 hours.<sup>11</sup> The 100 mL acetone was  
147 evaporated using nitrogen gas to near dryness, re-constituted into 1.8 mL of acetonitrile into 2  
148 mL HPLC vials and stored at -20°C until analysis.

#### 149 **Co-Solubility Experiments**



150 Phenanthrene (96%), pyrene (98%), and benzo(k)fluoranthene (99%) were obtained  
151 from Sigma Aldrich, while benzo(a)pyrene was obtained from MRI Global. Bioaccessibility  
152 experiments were conducted using de-ionized water and bile fluid. Bile fluid was prepared by  
153 dissolving 12.5 g L<sup>-1</sup> bile and 6 g L<sup>-1</sup> NaHCO<sub>3</sub><sup>-</sup> into de-ionized water. To each experimental unit, 1  
154 m of silicone rod is inserted into a 125 mL amber glass jar. To the jar, approximately 35 ± 5 mg  
155 of PAH is added, followed by 100 mL of either de-ionized water or bile fluid. Notably the  
156 solubility limits of these PAHs in water is less than 1.2 mg L<sup>-1</sup>.<sup>29</sup> The amber jar was then gently  
157 shaken on a horizontal shaker for 4 hours, the time was chosen to be representative of the  
158 gastric and intestinal transit time of the FOREhST model.

#### 159 **Low energy FOREhST spiking**

160 The low energy FOREhST was repeated for five soils and spiked with benzo(a)anthracene  
161 (99%, Sigma-Aldrich) or fluoranthene (99%, Sigma-Aldrich) dissolved in 100 µL of acetonitrile.  
162 The spiking consisted of five concentrations for each benzo(a)anthracene and fluoranthene.  
163 Soils were also spiked with 100 µL of clean acetonitrile as a solvent control. Spiking solution  
164 was added directly to the FOREhST media, in the mixture containing soil, water, food, saliva and  
165 silicone rod.

#### 166 **In vivo Swine Oral Bioavailability**

167 The oral bioavailability and area under the plasma concentration curve over a 48 hour  
168 time period (AUC48) of PAHs to swine has been previously reported by James et al.<sup>1</sup>

#### 169 **HPLC analysis**

170 PAHs were analyzed using an Agilent 1260 Infinity high pressure liquid chromatography  
171 coupled with fluorescence detection (HPLC-FD).<sup>30</sup> A 10 µL aliquot was injected onto an Agilent

172 PAH Pursuit Column (3  $\mu\text{m}$  particle size, 2mm internal diameter). The mobile phase consists of  
173 acetonitrile and water with a flow rate of 1.5 mL min<sup>-1</sup>. The acetonitrile:water gradient at the  
174 start of the run was 60:40 and gradually increases to 95:5 at 20 min, this gradient is held  
175 constant until the end of the run at 25 min. The column temperature is held constant at 25°C  
176 for the duration of the run. The fluorescence detector utilizes an excitation wavelength of 260  
177 nm and four emission wavelengths of 350, 420, 440 and 500 nm. Detection limits for  
178 anthracene is 0.70 pg  $\mu\text{L}$ , fluoranthene is 1.71 pg  $\mu\text{L}$ , pyrene is 0.43 pg  $\mu\text{L}$ , benzo(a)anthracene  
179 is 2.45 pg  $\mu\text{L}$ , chrysene is 5.27 pg  $\mu\text{L}$ , benzo(b)fluoranthene is 5.58, benzo(k)fluoranthene is 2.77  
180 pg  $\mu\text{L}$ , benzo(a)pyrene is 13.02 pg  $\mu\text{L}$ , dibenzo(a,h)- anthracene is 7.79 pg  $\mu\text{L}$ ,  
181 benzo(g,h,i)perylene is 1.78 pg  $\mu\text{L}$ , and indeno(1,2,3-cd)pyrene is 1.80 pg  $\mu\text{L}$ .

## 182 **Quality Assurance and Control**

183 To quantify the PAH recovery from soil, a sand matrix spike was added every 10 samples  
184 and the average recovery ranges from 77% to 94% with a standard deviation of 12%.

185 Benzo(b)chrysene is present at very low concentrations in all soils and was used as an internal  
186 standard and the recovery ranged between 90% to 110% with a standard deviation of 11%. For  
187 the in vitro digestors, a blank sample (no soil) is included every 8 samples. Average blank  
188 samples recovered a range of 0 to 120 pg from the high energy FOREhST, 110 to 810 pg from  
189 the low energy FOREhST, and 0 to 120 pg from the low energy FOREhST spiked with  
190 acetonitrile. Residual PAHs adhering to PTFE bags range from 0 to 1400 pg.

## 191 **Statistical Analysis**

## 192 **Co-Inertia Modeling**

193 Co-Inertia analysis (COIA) was performed using R software<sup>31</sup> and the “ade4”<sup>32</sup> package.  
194 Co-inertia analysis developed by Deledec and Chessel<sup>33</sup> was reviewed by Thioulouse<sup>24</sup> and  
195 compared with canonical correspondence analysis by Dray et al.<sup>34</sup> Co-inertia analysis is an  
196 alternative method to canonical correspondence analysis when number of samples is low  
197 relative to the number of predictor variables. Co-inertia analysis investigates the common  
198 structure of paired data tables by maximizing the covariance of the row scores between the  
199 tables. High co-inertia occurs when simultaneously high values (or inverse) occur in both  
200 tables, whereas low co-inertia occurs either when they vary independently or they do not vary.  
201 Thus, high scores indicate that parameters, such as a specific PAH, are concordant between two  
202 sets of data tables, whereas low scores indicate that these specific PAHs are discordant (or in  
203 other words, PAHs behaving dissimilarly between the two data tables, which in this case would  
204 be the soil concentration data table consisting of different soils versus different PAHs  
205 bioaccessibility compared to the data table of different PAH’s bioavailability).

206 PAHs have the ability to interact with each other and affect the solubility of each other,  
207 and in the environment PAHs are present as mixtures. When investigating the bioavailability of  
208 PAHs it is likely that the bioavailability of one PAH will affect the bioavailability of another, the  
209 same can be said for bioaccessibility; however the goal is to use bioaccessibility to predict  
210 bioavailability. In addition to bioaccessibility, other environmental variables such as soil PAH  
211 concentration, organic matter, soil texture, and soil metal concentrations may be used as  
212 predictive variables of PAH bioavailability. In one table there is bioavailability of individual  
213 PAHs, in columns, by soil samples, in rows. In another table there are the predictor variables,

214 including bioaccessibility of individual PAHs, as well as PAH concentration , organic matter, soil  
215 texture, and soil metal concentrations, in columns, by the same soil samples in rows.

216 The two data sets were first studied separately with Principal Components Analysis  
217 (PCA), and eventually analyzed as PCA-PCA COIA. In a PCA-PCA COIA, the two PCA's on the  
218 original two data sets reduces their dimensionalities by selecting the dominant components  
219 (axes). COIA uses the principle components from each data set and merges the complied data  
220 into a new multidimensional space such that the covariance between axes of each data set is  
221 maximized. The data tables are not transformed prior to analysis.

## 222 **Model Selection**

223 Co-inertia analysis provides the primary components for predicting PAH bioavailability.  
224 Using the results from co-inertia analysis, a general linear model is constructed consisting of the  
225 dependent variable,  $AUC_{48PAH}$ , being regressed on by  $FOREhST_{PAH}$ ,  $[soil]_{PAH}$ , and the top five  
226 variables as given by co-inertia analysis. Non-significant variables are then stepwise removed  
227 using the "stepAIC" function from the "MASS" package<sup>35</sup> with R software<sup>31</sup> until the best final  
228 model is chosen. The "stepAIC" function is combined with an anova to examine significant  
229 differences between model fits based on Akaike Information Criterion (AIC). Model residuals  
230 are plotted against predicted values and visually inspected as per Osborne et al.<sup>36</sup> to ensure  
231 homoscedasticity.

## 232 **Structure equation modelling**

233 Structure equation modelling was performed using R software<sup>31</sup> with the additional  
234 "laavan"<sup>37</sup> package. SEM is a statistical method akin to path analysis which allows for testing of  
235 hypotheses where the relationship is confounded by many variables inter-correlated. The

236 application of SEM is to determine the relative strength of the coefficient that each predictor  
237 variable has on the dependent variable in the presence of collinearity. After removing non-  
238 significant variables, SEM is used to account for collinearity between variables and to determine  
239 the path coefficients. The structure equation model is built similarly as outlined by James et al.<sup>1</sup>,  
240 where there is a high degree of collinearity between predictor variables, such as between  
241 bioaccessible FOREhST PAHs, they are set to co-vary. Where one predictor variable predicts  
242 another, such as total organic carbon predicting soil PAH concentration, the model reads PAH  
243 soil concentration is regressed on by total organic carbon. Finally, each predictor variable is  
244 included as a direct cause of AUC48<sub>PAH</sub>. A detailed SEM diagram is available in the supporting  
245 information (Supporting Information Figure S6)

## 246 **Results**

### 247 **Bioaccessibility**

248 In the standard high energy FOREhST model the PAH release correlates moderately to  
249 strongly ( $r^2$  between 0.43-0.62) with soil concentration (Figure 1), whereas no correlation was  
250 found between the low energy FOREhST and soil concentration (Supporting Information Figure  
251 S2). The average bioaccessibility (mean  $\pm$  standard deviation in parentheses) from the high  
252 energy FOREhST was 23% ( $8.0 \pm 9.5 \mu\text{g}$ ) for benzo(a)anthracene, 29% ( $9.2 \pm 8.1 \mu\text{g}$ ) for  
253 chrysene, 20% ( $11 \pm 7.9 \mu\text{g}$ ) for benzo(b)fluoranthene, 21% ( $4.4 \pm 2.7 \mu\text{g}$ ) for  
254 benzo(k)fluoranthene, and 13% ( $5.6 \pm 5.8 \mu\text{g}$ ) for benzo(a)pyrene while the average  
255 bioavailability from the low energy FOREhST was 3.7% ( $0.76 \pm 0.65 \mu\text{g}$ ) for benzo(a)anthracene,  
256 5.0% ( $1.2 \pm 0.96 \mu\text{g}$ ) for chrysene, 3.0% ( $0.99 \pm 0.58 \mu\text{g}$ ) for benzo(b)fluoranthene, 3.4% ( $0.38 \pm$   
257  $0.24 \mu\text{g}$ ) for benzo(k)fluoranthene, 1.6% ( $0.41 \pm 0.30 \mu\text{g}$ ) for benzo(a)pyrene.

258 **Bioaccessibility and Bioavailability**

259           Between soils, neither the low or high energy FOREhST model predicts in vivo AUC48  
260 juvenile swine exposure for individual PAHs (Supporting Information Figure S3). However,  
261 within a soil, both the low and high energy FOREhST predict in vivo AUC48 exposure between  
262 PAHs (Figure 2). Within a soil, desorption of PAHs is predictable likely due to the physiochemical  
263 properties of the PAH, as such they desorb from soil at a relative rate, however between soils,  
264 the PAH release cannot be predicted. The low energy FOREhST predicts exposure between  
265 PAHs with a slope of 1.9 ( $r^2 = 0.64$ ,  $p < 0.01$ ) while the high energy FOREhST predicts exposure  
266 between PAHs with a slope of 0.34 ( $r^2 = 0.81$ ,  $p < 0.005$ ). Notably, the energetic input does not  
267 appear to affect all PAHs equally. In Figure 2, the high energy FOREhST does not accurately  
268 predict anthracene, fluoranthene and pyrene AUC48, however the only outlier in the low  
269 energy FOREhST model is fluoranthene.

270 **Co-Inertia Analysis**

271           The PCA on PAH bioavailability reduces the data set to six principal components that  
272 explain 94.9% of the variance while the PCA on predictor variables (PAH bioaccessibility, PAH  
273 soil concentration, and soil properties) reduces the data set to five principle components that  
274 explain 90.2% of the variance (Supporting Information Table S3 and S4). COIA indicates that the  
275 primary variables predicting PAH in vivo exposure are FOREhST release of chrysene,  
276 fluoranthene, anthracene, and benzo(a)anthracene, followed by soil arsenic concentration, and  
277 then FOREhST release of pyrene and benzo(k)fluoranthrene. The relative rankings of the  
278 individual predictor variables are determined using the 'Strength' of the predictive vector as  
279 determined by the canonical weights of COIA (Supporting Information, Table S5).

280 **Model selection**

281 The top five variables given from COIA predicting PAH in vivo exposure are FOREhST  
282 release of chrysene, fluoranthene, anthracene, and benzo(a)anthracene, followed by soil  
283 arsenic concentration. We evaluated these variables as well as soil concentration and FOREhST  
284 release of the individual PAH (either benzo(a)anthracene or benzo(a)pyrene) for their ability to  
285 predict bioavailability. After removing non-significant predictor variables, the most  
286 parsimonious model for benzo(a)anthracene AUC48 includes FOREhST release of  
287 benzo(a)anthracene, fluoranthene and chrysene, while the most parsimonious model for  
288 benzo(a)pyrene AUC48 includes FOREhST release of benzo(a)pyrene and benzo(a)anthracene  
289 (Figure 3). COIA does not evaluate information criterion and thus, will identify multiple  
290 predictors, whereas stepwise regression eliminates predictors based on their information  
291 content. When combining significant predictor variables into a general linear model  $(B(a)A_{AUC48}$   
292  $\sim B(a)A_{FOR} + FLUO_{FOR} + CHR_{FOR})$ , the predicted AUC48 values were compared to observed AUC48  
293 resulting in a slope of 1.0,  $r^2$  of 0.66, and  $p < 0.0005$ . For benzo(a)pyrene, the general linear  
294 model predicts observed AUC48 with a slope of 1.0,  $r^2$  of 0.65, and  $p < 0.0005$ .

295 **Structure Equation Modelling**

296 Our hypothesized causal network linking bioaccessibility to bioavailability was congruent  
297 ( $P = 0.11$  for benzo(a)anthracene and  $P = 0.13$  for benzo(a)pyrene) with the data (Supporting  
298 Information Table S6). A non-significant P value for a SEM indicates the likelihood that a  
299 completely random models fits the data better than the hypothesized causal network. Other  
300 SEM fit values, e.g. CFI and RMSE, all indicate that the SEM represented the data reasonably  
301 well. A diagram detailing the specific relationship of SEM parameters is found in supporting

302 information (Supporting Information Figure S6). Only, PAH bioaccessibility and not soil organic  
303 carbon content were significant predictors of bioavailability (Supporting Information Table S5).  
304 The standardized coefficients, used for comparing within a model, predicting  
305 benzo(a)anthracene AUC48 given by structure equation modelling are -1.8 for FOREhST  
306 benzo(a)anthracene, -0.29 for FOREhST chrysene, and 2.5 for FOREhST fluoranthene. The  
307 standardized coefficients predicting benzo(a)pyrene AUC48 are -0.56 for FOREhST  
308 benzo(a)pyrene and 1.0 for FOREhST benzo(a)anthracene. The SEM coefficients suggest that  
309 benzo(a)anthracene and fluoranthene counter-act each other in predicting benzo(a)anthracene  
310 bioavailability. In contrast, benzo(a)anthracene and benzo(a)pyrene counter-act each other in  
311 predicting benzo(a)pyrene bioavailability

#### 312 **Co-solubility**

313 In the absence of soil (i.e. only water or bile), PAHs significantly decreased the  
314 bioaccessibility of other PAHs. The amount of benzo(a)pyrene solubilized in 100 mL of de-  
315 ionized water was  $94 \pm 14 \mu\text{g}$  (mean  $\pm$ SE), was reduced to  $39 \pm 15 \mu\text{g}$  in the presence of  
316 phenanthrene, significantly ( $p < 0.05$ ) reduced to  $15 \pm 4.8 \mu\text{g}$  in the presence of phenanthrene  
317 and pyrene, and  $13 \pm 6.1 \mu\text{g}$  in the presence of phenanthrene, pyrene and  
318 benzo(k)fluoranthrene (Figure 4). The amount of bioaccessible benzo(a)pyrene in 100 mL of  
319 simulated bile fluid was  $36 \pm 17 \mu\text{g}$ , and was significantly ( $p < 0.05$ ) reduced to  $8.0 \pm 2.5 \mu\text{g}$  in the  
320 presence of phenanthrene,  $1.3 \pm 2.1 \mu\text{g}$  in the presence of phenanthrene and pyrene, and  $8.1 \pm$   
321  $2.1 \mu\text{g}$  in the presence of phenanthrene, pyrene and benzo(k)fluoranthrene (Figure 4).

#### 322 **Low-Energy FOREhST of Spiked Field Contaminated Soils**



323 In contrast to the results in water and bile, PAH interactions in the presence of soil can  
324 increase the bioaccessibility of other PAHs. Benzo(a)anthracene was spiked into the low energy  
325 FOREhST model at 0, 0.38, 0.75, 1.5, 3.0 and 6.0  $\mu\text{g}$ , resulting in a significant increase ( $p < 0.05$ )  
326 in the amount of benzo(a)pyrene bioaccessibility when spiking 3.0 and 6.0  $\mu\text{g}$   
327 benzo(a)anthracene (Figure 5). Fluoranthene was spiked into the low energy FOREhST model at  
328 0, 2.3, 4.5, 9.0, 18 and 36  $\mu\text{g}$ , resulting in no significant difference in benzo(a)anthracene  
329 bioaccessibility (Figure 5).

### 330 **Discussion**

331 The FOREhST model successfully predicts 66% of the variance in benzo(a)anthracene  
332 and 65% of the variance in benzo(a)pyrene internal exposure across 14 soils polluted with a  
333 mixture of PAHs. To our knowledge, this is the first successful application of in vitro digester  
334 results to estimate PAH bioavailability across multiple soils. We achieved this by: (i)  
335 incorporating PAH-PAH interactions into the predictive algorithm, and (ii) altering the energetic  
336 input of the in vitro digestors. We were led to these modifications by building on key concepts  
337 outlined by Reichenberg and Mayer<sup>13</sup> that chemical activity, bioaccessibility and bioavailability  
338 are conceptually distinct. Specifically, bioaccessibility is a combination of chemical activity and  
339 solubility, and thus, human in vitro digestors should not be designed to solely estimate  
340 chemical activity because factors, other than chemical activity, can influence bioaccessibility.

341 At environmentally relevant concentrations, PAH-PAH interactions can influence  
342 bioaccessibility and bioavailability. Phenanthrene, pyrene, and benzo(a)pyrene were used  
343 based on previous work that demonstrated the importance of this PAH-PAH interactions.<sup>22, 23</sup>  
344 For example, Chun et al.<sup>22</sup>, attribute the change in PAH solubility from PAH-PAH interactions to

345 PAH-micelle interactions. Using benzo(a)pyrene and phenanthrene in both artificial sea water  
346 and *Arenicola marina* gut fluid, Voparil et al.<sup>23</sup> found that phenanthrene did not significantly  
347 change the benzo(a)pyrene concentration in the artificial sea water, whereas benzo(a)pyrene  
348 concentration was increased to 154% in *Arenicola marina* gut fluid in the presence of  
349 phenanthrene, alluding to the importance of the PAH-micelle interaction. Using phenanthrene,  
350 pyrene and fluoranthene with various surfactants and water, Prak et al.<sup>38</sup> found that PAH-  
351 micelle interactions was a significant factor but also that PAH-PAH interactions influenced the  
352 water solubility of fluoranthene.

353 Typically PAH-PAH experiments are in reduced mixtures of only 1 to 3 PAHs, e.g. Chun et  
354 al.<sup>22</sup>, Voparil et al.<sup>23</sup>, Prak et al.<sup>38</sup>, etc. In contrast, our dosed soils contained more than 11  
355 PAHs. Thus, an alternate numerical method was needed to incorporate PAH-PAH interactions  
356 because we were comparing two matrices, bioaccessibility and bioavailability which contained  
357 14 soils by 11 PAHs. Co-inertia analysis is one such method. We used co-inertia to link the  
358 matrix of PAH bioaccessibility with PAH internal exposure and identified that FOREhST release  
359 of chrysene, fluoranthene, anthracene, and benzo(a)anthracene were the principle components  
360 governing PAH uptake in vivo. For benzo(a)pyrene, we confirm that benzo(a)anthracene  
361 influences benzo(a)pyrene soil bioaccessibility. In contrast, fluoranthene does not increase  
362 benzo(a)anthracene soil bioaccessibility as predicted by statistical modelling bioaccessibility  
363 (Figure 5). Notably, PAH-PAH interactions are not limited to just desorption<sup>39</sup> and solubility<sup>22, 23,</sup>  
364 <sup>38</sup>, as PAH interactions are also relevant with cellular responses. DNA damage to HepG2 cells is  
365 modulated based on specific binary PAHs mixtures.<sup>40</sup> Furthermore, induction of PAH  
366 metabolizing enzymes, CYP1A1 and CYP1A2, are dependent upon exposure to specific PAHs.<sup>41</sup>

367           The collinearity of individual PAH bioavailabilities or bioaccessibilities likely reflects  
368 fundamental chemical-chemical interactions. PAHs with similar molecular weight, ring number,  
369 and structure have strong influences on each other. For example, Lui et al.<sup>42</sup> reports a  
370 significant correlation for PAH soil concentration of all 16 PAHs examined but a stronger  
371 correlation for PAHs of similar molecular weight. Similarly, the PAH ratio of compounds is used  
372 in PAH source appointment because ratios of similar PAHs are consistently found based on  
373 source.<sup>20, 43</sup> Although our results suggest the importance of chrysene, fluoranthene,  
374 benzo(a)anthracene, this may be limited to our sample set of 14 soils. In other soils, factors  
375 such as PAH source,<sup>20, 21, 43</sup> PAH concentration, sorption sink,<sup>10</sup> desorption media,<sup>13, 14</sup> soil  
376 physio-chemical properties,<sup>18</sup> dietary constituents<sup>23</sup>, and co-contaminants<sup>23</sup> may further  
377 influence the partitioning dynamic of PAHs and thus, may influence the equations describing  
378 the link between in vitro bioaccessibility and in vivo internal exposure.

379           Energetic input through shaking method is responsible for up to 99% of PAHs released  
380 from in vitro models. PAH release from the low energy FOREhST was between 0.66%  
381 (anthracene) and 31 % (fluoranthene) with an average of 19% between PAHs compared to the  
382 high energy FOREhST. PAH kinetic desorption from soil will be dependent upon the energy of  
383 the system, both kinetic and thermal, interacting with the PAH-soil binding media, amorphous  
384 organic matter and carbonaceous geosorbents. Given the limited desorption time of the  
385 FOREhST model, ie. 4 hours, the majority of desorbed PAHs were likely bound to the rapidly  
386 desorbing amorphous organic matter as opposed to the slowly desorbing recalcitrant  
387 carbonaceous geosorbents. However, these rapidly released PAHs from amorphous  
388 geosorbents may in turn, influence PAH release from carbonaceous geosorbents. For example,

389 White et al.<sup>39</sup> observed that freshly spiked anthracene or pyrene into soil leads to increased  
390 aged phenanthrene extraction by a mild solvent and increased biodegradation, suggesting that  
391 PAHs compete for and interact at the slow desorption sites of carbonaceous geosorbents.  
392 Within the FOREhST model, it is uncertain if a similar interaction is occurring between the  
393 rapidly desorbed PAHs competing with the recalcitrant PAHs to influence bioaccessibility. If  
394 this interaction is occurring, this may explain the energetic disparity between the low and high  
395 energy FOREhST with PAH desorbed from amorphous organic matter in the high energy  
396 FOREhST increasing PAH desorption rate from the carbonaceous geosorbents. In either case  
397 the high energy FOREhST desorbs PAHs at a rate such that there is a strong correlation to soil  
398 PAH concentration, and we've repeatedly observed that soil concentration does not correlate  
399 with bioavailability.<sup>1, 8</sup> As a product of the desorption kinetics and desorption time of in vitro  
400 models, energetic input becomes a dominant factor linking PAH desorption from soil in an in  
401 vitro model to mammalian PAH uptake into the blood stream. Yet oddly, this factor has not  
402 been identified in the round robins of in vitro digester performance that have been performed  
403 previously.<sup>14, 19</sup> Notably, the energetic input does not appear to affect all PAHs equally. In  
404 Figure 2, the high energy FOREhST does not accurately predict anthracene, fluoranthene and  
405 pyrene AUC48, whereas only fluoranthene is not predicted in the low energy FOREhST model.  
406 Suggesting that for these relatively lower molecular weight PAHs, energetic input is not a  
407 dominant factor.

408           When considered as single contaminants, PAH bioaccessibility and bioavailability is  
409 strongly linked to soil characteristics.<sup>10, 44, 45</sup> When considered as a mixture, PAH-PAH  
410 interactions dominate. Our work suggests that future work on PAH bioavailability and

411 bioaccessibility should focus on the dynamics of how the matrix of PAHs present in the soil  
412 interact with mammalian systems. Such interactions should not only include the chemical  
413 interactions discussed here but also the interactions of PAH mixtures with mammalian uptake  
414 systems.

#### 415 **Acknowledgements**

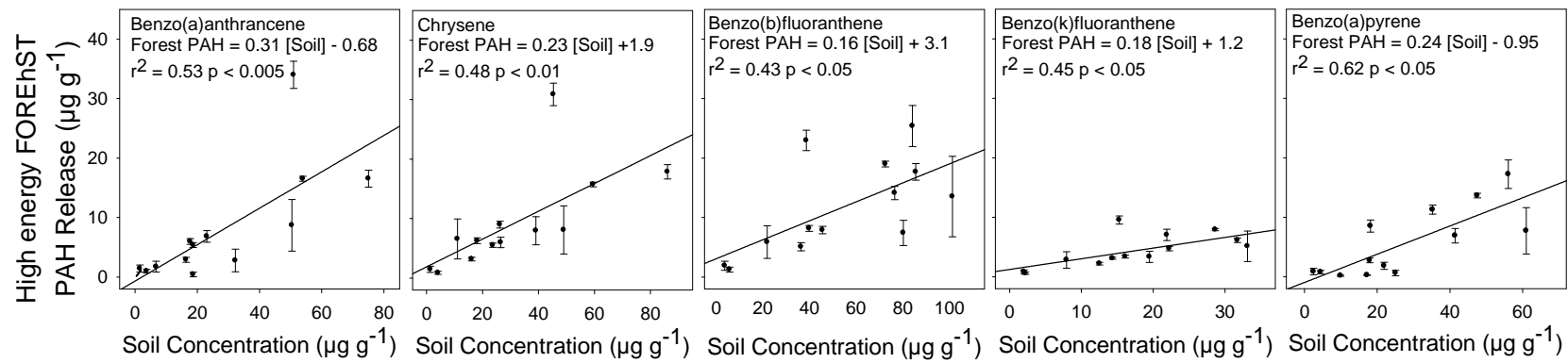
416 This work was supported by a NSERC Discovery Grant to SDS. This work was approved by the  
417 University of Saskatchewan's Animal Research Ethics Board, and adhered to the Canadian  
418 Council on Animal Care guidelines for humane animal use. Swedish soils were provided by  
419 Staffan Lundstedt and Paul White. Special thanks to Philipp Mayer for scientific input.

#### 420 **Supporting Information Available**

421 The supporting information contains five figures and six tables:

- 422 - The first figure is a diagram describing the low energy FOREhST method.
- 423 - The second figure compares low energy FOREhST bioaccessibility and soil  
424 concentration for five PAHs.
- 425 - The third figure displays the correlation between swine area under the curve (AUC48)  
426 and PAH release for both the low and high energy FOREhST models for five PAHs.
- 427 - The fourth figure displays phenanthrene and benzo(a)pyrene solubility in water and bile  
428 fluids with mixtures consisting of one to four PAHs.
- 429 - The fifth figure is the output from co-inertia analysis.
- 430 - The sixth figure is a SEM diagram detailing the relationships between multiple predictor  
431 variables of AUC48.

- 432 - The first table contains the low energy FOREhST PAH release for 11 PAHs: anthracene,  
433 fluoranthene, pyrene, benzo(a)anthracene, chrysene, benzo(b)fluoranthene,  
434 benzo(k)fluoranthene, benzo(a)pyrene, dibenzo(ah)anthracene, benzo(ghi)perylene, and  
435 indeno(123,cd)pyrene.
- 436 - The second table contains the high energy FOREhST PAH release for 11 PAHs
- 437 - The third table contains PCA output of co-inertia analysis for PAH exposure (AUC48)
- 438 - The fourth table contains the PCA output of co-inertia analysis for predictor variables of  
439 PAH exposure.
- 440 - The fifth table contains canonical weights and calculated strength of predictor variables
- 441 - The sixth table summarizes output of SEM results for benzo(a)anthracene and  
442 benzo(a)pyrene

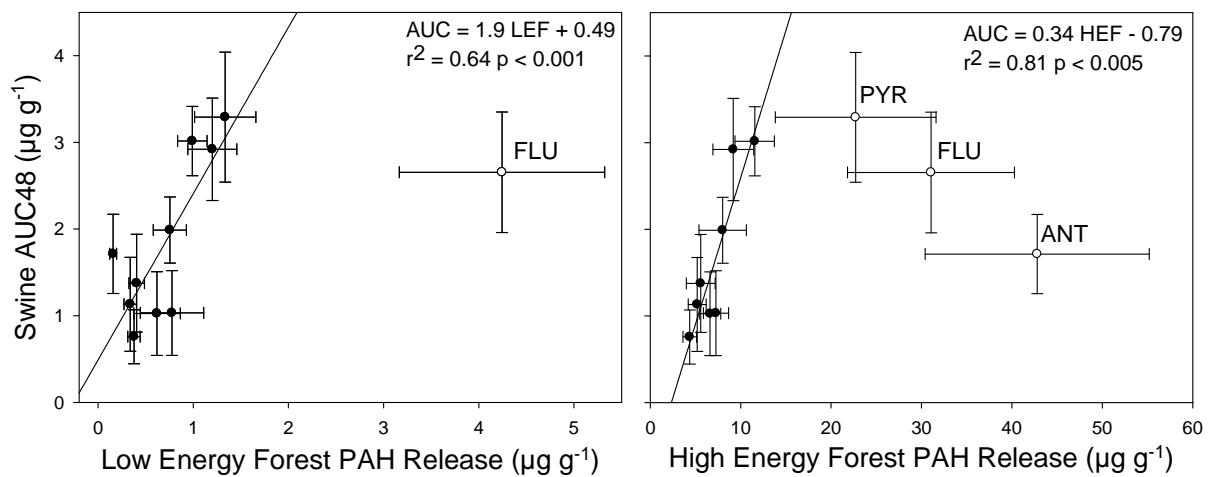


443

444 Figure 1. Comparison between FOREhST PAH release and soil concentration of five PAHs in 14 soils historically contaminated with

445 hydrocarbons. Lines indicate line of best fit. Data points represent the mean ( $n=3$ ) for FOREhST PAH release and error bars

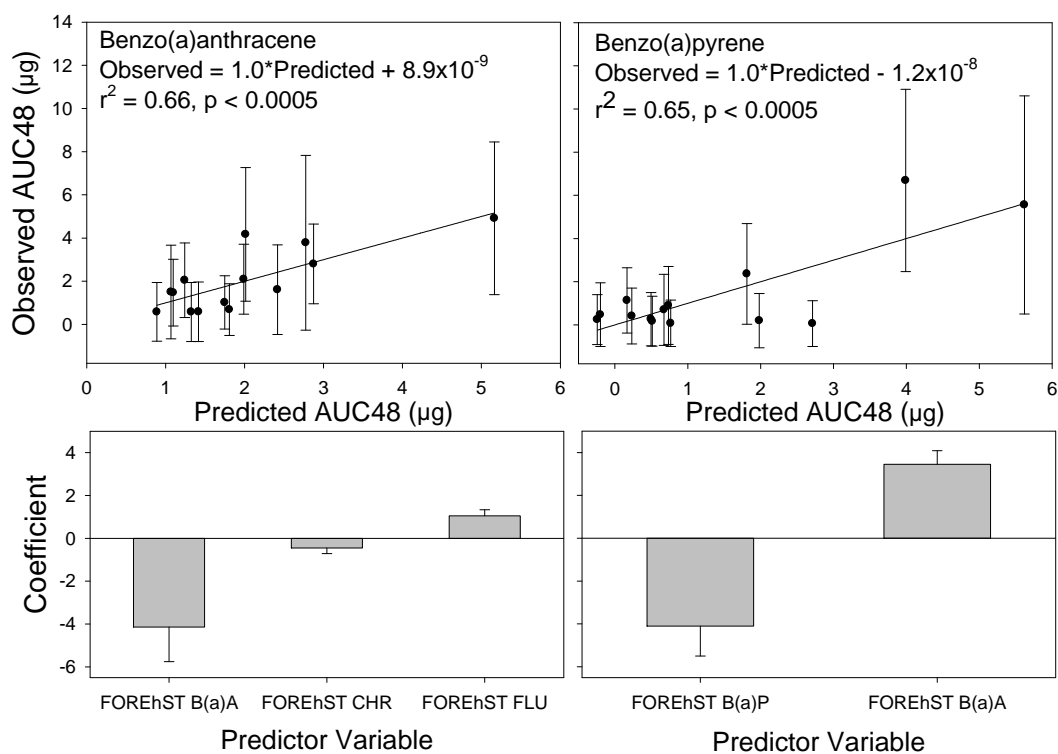
446 represent the standard error of this mean.



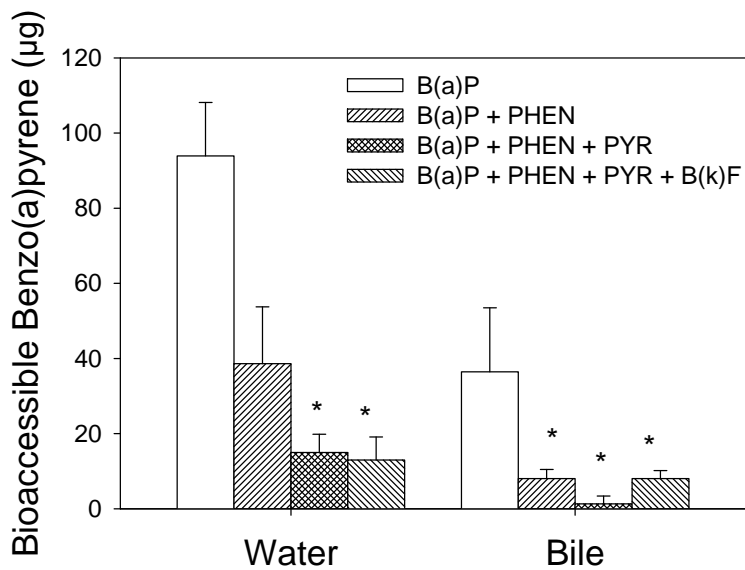
447

448 Figure 2. Regression between in vivo swine PAH area under the plasma concentration curve  
 449 over 48 hours in units of  $\mu\text{g}$  PAH recovered in plasma per gram of soil ingested (AUC48) against  
 450 in vitro FORE(h)ST PAH release in Low energy (left) and High energy (right). Each data point  
 451 represents the mean bioavailability of a single PAH from 14 soils historically contaminated with  
 452 PAHs and error bars are the standard error of this mean. Abbreviations are as follows: ANT is  
 453 anthracene, FLU is fluoranthene, PYR is pyrene.



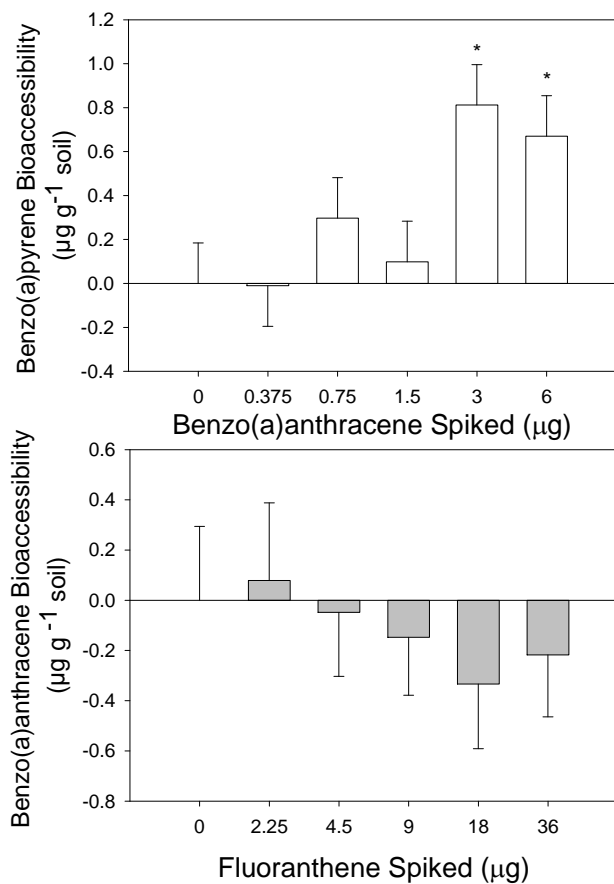


454  
 455 Figure 3. Comparison of observed AUC48 (area under the 48 hr plasma concentration curve)  
 456 versus linear model predicted AUC for benzo(a)anthracene and benzo(a)pyrene PAHs (top) and  
 457 the corresponding coefficient for each predictor variable (bottom). Coefficients are determined  
 458 using structure equation modelling. Data points for observed AUC48 represent the mean of 6  
 459 measurements while error bars represent the standard error of this mean. Abbreviations are  
 460 as follows: B(a)A is benzo(a)anthracene, CHR is chrysene, FLU is fluoranthrene, and B(a)P is  
 461 benzo(a)pyrene.



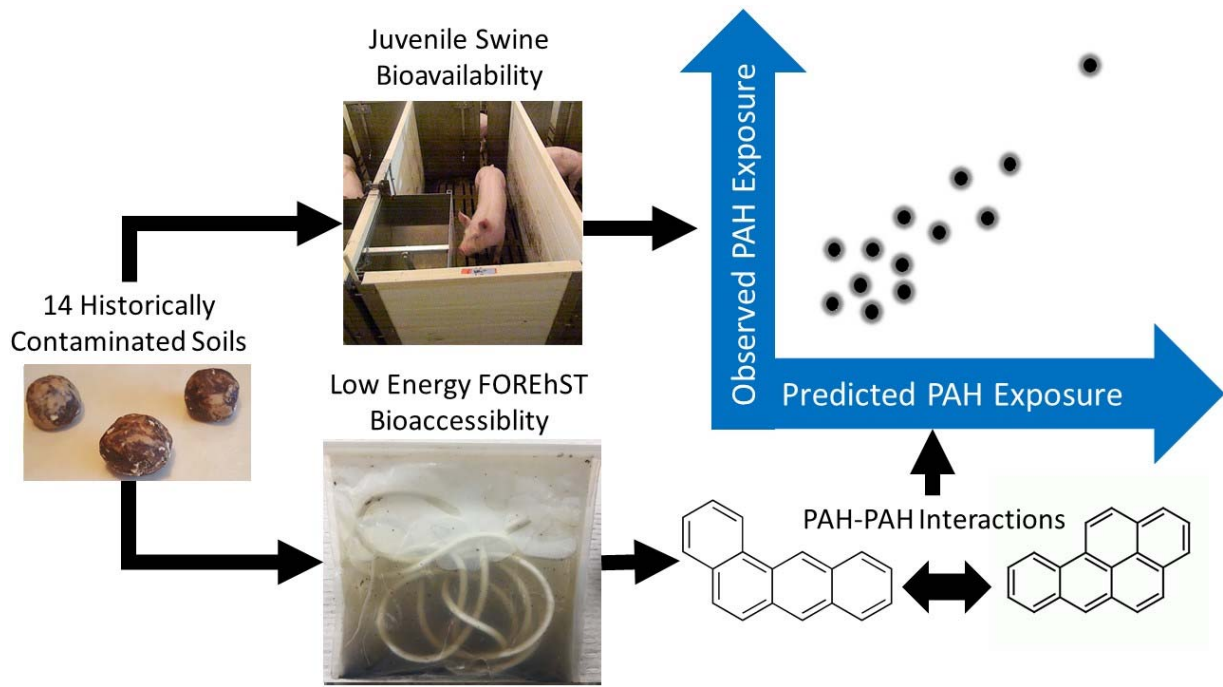
462

463 Figure 4. Bioaccessible fraction of benzo(a)pyrene in either water or bile in the presence of  
 464 other PAHs. Approximately 30 mg of each PAH is added to the respective treatment which is  
 465 above the solubility limit for the PAHs. Abbreviations are as follows: B(a)P is benzo(a)pyrene,  
 466 PHEN is phenanthrene, PYR is pyrene, and B(k)F is benzo(k)fluoranthene. \* indicates a  
 467 significant ( $p < 0.05$ ) difference from bioaccessibility in the presence of only benzo(a)pyrene, i.e.  
 468 only benzo(a)pyrene by itself.



469  
 470 Figure 5. Top – Amount of benzo(a)pyrene released from soil in FOREhST fluids in the presence  
 471 of increasing amounts of benzo(a)anthracene. Bottom – Amount of benzo(a)anthracene  
 472 released in the presences of increasing amounts of fluoranthene. Each bar is the mean release  
 473 from 5 soils and error bars represent the error of this measurement with the entire experiment  
 474 duplicated. . ‘\*’ denotes a significant difference from acetonitrile control spike at  $p < 0.05$ .

475 **Table of Content Art**



476

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