

1 ***In vivo* Validation of the Unified BARGE Method to assess the Bioaccessibility of Arsenic**
2 **Antimony, Cadmium and Lead in soils**

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12 **ABSTRACT**

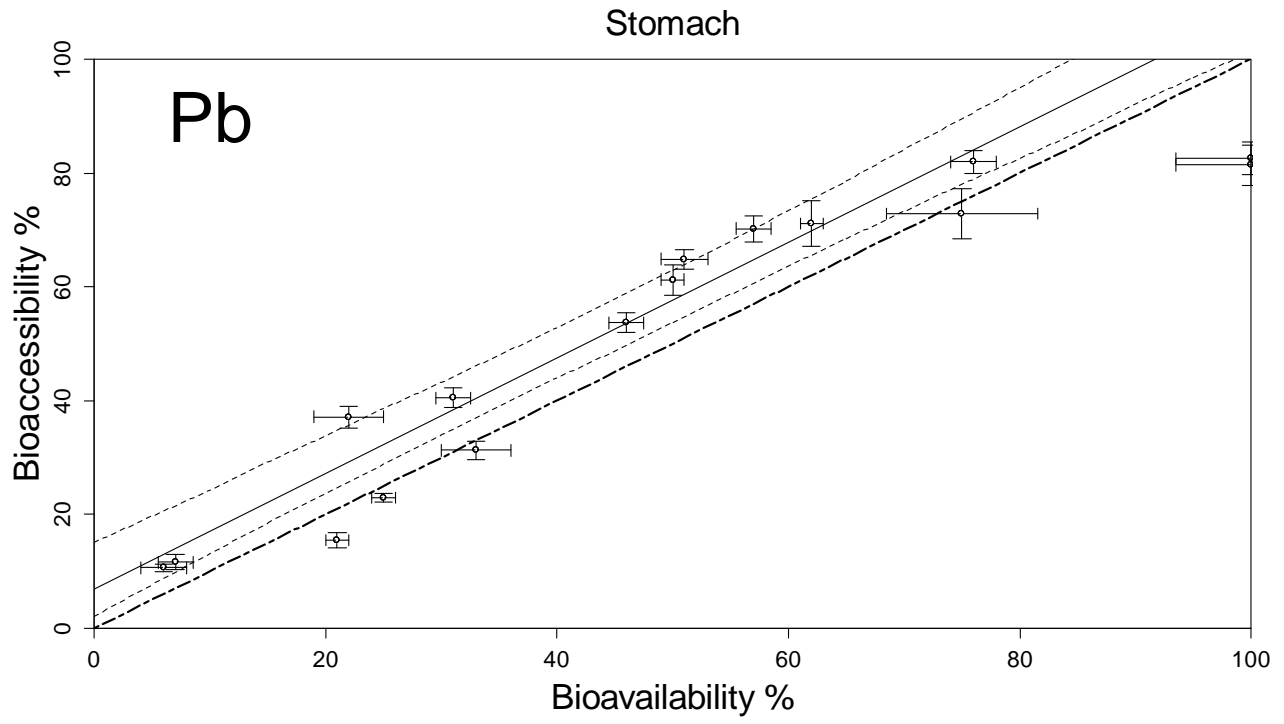
13 The relative bioavailability of arsenic, antimony, cadmium and lead for the ingestion pathway was
14 measured in 16 soils contaminated by either smelting or mining activities using a juvenile swine
15 model. The soils contained 18 to 25000 mg kg⁻¹ As, 18 to 60000 mg kg⁻¹ Sb, 20 to 184 mg kg⁻¹ Cd
16 and 1460 to 40214 mg kg⁻¹ Pb. The bioavailability in the soils was measured in kidney, liver, bone
17 and urine relative to soluble salts of the four elements. The variety of soil types, the total
18 concentrations of the elements and the range of bioavailabilities found were considered to be
19 suitable for calibrating the *in vitro* Unified BARGE bioaccessibility method. The bioaccessibility
20 test has been developed by the BioAccessibility Research Group of Europe (BARGE) and is known
21 as the Unified BARGE Method (UBM). The study looked at 4 end points from the *in vivo*
22 measurements and two compartments in the *in vitro* study ('stomach' and 'stomach & intestine').
23 Using benchmark criteria for assessing the 'fitness for purpose' of the UBM bioaccessibility data to
24 act as an analogue for bioavailability in risk assessment, the study shows that the UBM met criteria

25 on repeatability (median relative standard deviation value < 10%) and the regression statistics
26 (slope 0.8 to 1.2 and r-square >0.6) for As, Cd and Pb. The data suggest a small bias in the UBM
27 relative bioaccessibility of As and Pb compared to the relative bioavailability measurements of 3%
28 and 5% respectively. Sb did not meet the criteria due to the small range of bioaccessibility values
29 found in the samples.

30 **Keywords: Relative Bioavailability, Arsenic, Cadmium, Lead, Antimony, swine model,**
31 **bioaccessibility, soil**

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33 **TOC/Abstract Art**



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37 INTRODUCTION

38 Soils contaminated by potentially harmful elements (PHE), such as cadmium (Cd) and lead (Pb) constitute a
39 potential risk to human health [1, 2]. Other important PHE are the metalloids arsenic (As) and antimony
40 (Sb). These elements are distributed through the environment as a result of both natural and
41 anthropogenic activities such as mining or smelting [3, 4]. Once released into the environment, soils
42 often serve as a sink for these PHE and the question of human exposure to such elements must then
43 be addressed. Indeed, As and Sb were recognized as priority pollutants by the US-EPA in 1979,
44 because of their contribution to cancer development, genotoxicity and apoptosis in mammals [5, 6].
45 Ingestion is one of the major routes of soil exposure to these contaminants by children. [7-9].
46 Exposure is currently assessed using the total soil concentration of individual contaminants.
47 However, several *in vivo* studies, using diverse animals such as monkeys, juvenile swine, rabbits
48 and rodents, have demonstrated that only a fraction of a contaminant, the bioavailable fraction, is
49 absorbed following oral administration [10-16]. In the literature, the juvenile swine model is
50 considered to be a good physiological model for gastrointestinal (GI) absorption of contaminants in
51 children [17]. Recently, in the particular case of As, the swine model was described as being a
52 particularly accurate representation of human physiology [18]. Bioavailability is defined as the
53 fraction of an ingested dose that crosses the GI epithelium and becomes available for distribution to
54 internal target tissues and organs [19, 20]. Absolute bioavailability is directly determined in the
55 blood plasma and consists in comparing the concentration in the plasma following an intra-venous
56 injection and an oral administration [13, 19-21]. However, this method is not easily achievable due
57 to both experimental issues linked to blood sampling and to analytical limitations such as the
58 generally low concentrations in the blood compared to quantification limits [22]. Thus, *in vivo*
59 protocols have been developed to estimate the relative bioavailability (RBA). This is measured as
60 the uptake of the contaminant in the target organ from the soil matrix relative to the uptake from a
61 readily soluble salt of the contaminant (reference matrix) [16, 19, 23]. Several studies have
62 established that either absolute or relative bioavailability of soil metals were below 1 and are

63 dependent on soil edaphic properties (*e.g.* pH, granulometry) and the soil metal speciation [10, 15,
64 16, 20]. Consequently, human exposure to soil bound contaminants can be overestimated when the
65 bioavailability is not considered. The BioAccessibility Research Group of Europe (BARGE) [24]
66 have developed an *in vitro* test, the Unified BARGE Method (UBM), to measure the
67 bioaccessibility of soil contaminants. So far, a preliminary study [25] suggests that the UBM
68 bioaccessibility data are correlated to *in vivo* bioavailability data. However, problems with the soils
69 used in the study (they contained unusually high content of mining slag) require that a more
70 rigorous and robust validation of the UBM against *in vivo* data is essential before the UBM can be
71 used as a routine tool in risk assessment. The aim of this study is to measure the relative
72 bioavailability of As, Sb, Cd and Pb in soil using a juvenile swine model, for 16 soils contaminated
73 by either smelting or mining activities and to use the data from these soils to validate the UBM. So
74 far, most other studies have focused on a single element and not on multi-contaminated soil samples
75 which are commonly found together on contaminated lands. Moreover, no study has been carried
76 out on the human bioavailability of Sb.

77 The first part of this study is to measure the relative bioavailability (RBA) of As, Cd, Pb and Sb
78 from selected contaminated soils using a swine model. Whilst this data gives some insight into the
79 fraction of inorganic contaminants that is bioavailable, risk assessors need specific information
80 about each site being studied. However, due to the high number of sites with soils contaminated
81 with As, Cd, Pb and/or Sb, it is not possible to determine the bioavailability in each case, as *in vivo*
82 experiments are time-consuming, costly and ethically problematic [19]. To address this, numerous
83 *in vitro* protocols have been designed to simulate the human digestive processes using artificial
84 digestive fluids to determine the bioaccessible fraction of contaminants, *i.e.* the fraction of the PHE
85 content of the soil released into solution within the GI system which is then potentially available for
86 absorption, and have been comprehensively reviewed [26, 27]. The underlying hypothesis is that the
87 bioaccessibility reflects the bioavailability of a soil contaminant and allows for a more accurate

88 estimation of the exposure concentration compared to the total soil concentration of the
89 contaminant. However, from one *in vitro* test to another, the bioaccessibility can greatly vary for the
90 same soil sample [28-31]. Consequently, before such assays can act as a surrogate measurement for
91 relative metals bioavailability, a correlation between *in vitro* bioaccessibility and *in vivo*
92 bioavailability is necessary, for both regulatory and scientific acceptance. The objective of this
93 research was to carry out a more robust validation study to demonstrate the physiological accuracy
94 of the UBM for As, Cd, Pb and Sb.

95 **MATERIALS AND METHODS**

96 **Soil collection sample preparation and chemical analysis**

97 Full details of the soil collection, sample preparation and chemical analysis of the soil and swine
98 samples are given in the supporting information.

99 **Determination of *in vivo* relative bioavailability**

100 The RBA of As, Pb, Cd and Sb were determined for each soil sample using readily soluble forms of
101 the contaminants, sodium arsenate (NaH_2AsO_4), Pb-acetate ($(\text{CH}_3\text{COO})_2\text{Pb}$), Cd-chloride (CdCl_2)
102 and potassium antimonate (KSbO_3). These reference matrices were chosen to estimate the RBA as
103 they had been used in previous RBA studies for As and Sb [14, 21, 32] and Pb and Cd [11, 33].

104 The RBA of all elements studied were determined in four end points: urine; bone (metacarpal IV);
105 liver; and kidney. The number of swine is 15 for the reference groups and 9 for the soil groups,
106 leading to a total of 159 swine. Full details of the methodology are given in the supporting
107 information.

108 **Dose Response Curve and RBA calculation**

109 For a given contaminant, each soil and reference matrix, a dose-response curve was established by
110 plotting the concentration in the target end point as a function of the administered dose. Before
111 calculating the RBA, three conditions needed to be verified [34]:

- 112 • That the response was linear for the soil and reference dose;
- 113 • That the intercepts for all of the lines were equal (*i.e.* had a common intercept);
- 114 • That the response at the zero level (called “blanks” *i.e.* the 3g of moistened feed without any
115 soil or reference dose; for details see the SI) was less than or equal to the common intercept
116 value of the lines.

117 These assumptions were verified using SAS 9.1 (SAS Institute, Cary, NC, USA) using a
118 standard methodology for animal bioavailability studies [34].

119 For each linear response, the slope value and the standard deviation were determined for each value.
120 The RBA was calculated as the ratio of the soil to the reference matrix slope values, when the
121 difference between the two slope values was significant ($P < 0.05$) [34]. In the case of a non-
122 significant difference between the two slope values, the RBA was assumed to be 100%.

123 **Unified BARGE Method**

124 Bioaccessibility measurements were performed on five replicates of each soil and reference matrix
125 (Na-arsenate, K-antimoniate, Pb-acetate and Cd-chloride) using the UBM. A full description of the
126 method is given in the supporting information.

127 **Bioaccessibility calculation**

128 The following equations are used to calculate bioaccessible concentration for the ‘stomach’ and
129 ‘stomach & intestine’ phases and the bioaccessible fraction in the soil.

$$130 \text{BA}_s = V_s \times C_e \times d / m \quad \text{i)}$$

131 $BA_{s\&int} = V_{s\&int} \times C_e \times d / m$ ii)

132 $BAF_s = 100 \times BA_s / T_e$ iii)

133 $BAF_{s\&int} = 100 \times BA_{s\&int} / T_e$ iv)

134

135 Where:

136 BA_s = Bioaccessible concentration for the ‘stomach’ phase in the soil (mg kg^{-1})

137 $BA_{s\&int}$ = Bioaccessible concentration for the ‘stomach & intestine’ phase in the soil (mg kg^{-1})

138 V_s = Volume of fluid used in the ‘stomach’ phase extraction including any pH adjustments (mL)

139 $V_{s\&int}$ = Volume of fluid used in the ‘stomach & intestine’ phase extraction including any pH
140 adjustments (mL)

141 C_e = Measured concentration of the contaminant **e** in the diluted extract solution (mg L^{-1})

142 d = Dilution applied to the extract solution prior to analysis

143 m = Mass of soil used in the extraction (g)

144 T_e = Total concentration of the contaminant **e** in the soil (mg kg^{-1})

145 BAF_s = The ‘stomach’ phase bioaccessible fraction (%)

146 $BAF_{s\&int}$ = The ‘stomach & intestine’ phase bioaccessible fraction (%)

147 **Statistical analysis**

148 The statistical analysis was carried out using the R programming language [35]. The regression
149 analysis was carried out using Siegels’s repeated medians method [36] as implemented in the
150 “mblm” R statistical analysis package [37].

151 **RESULTS AND DISCUSSION**

152 **Animal health over the time frame of the experiment**

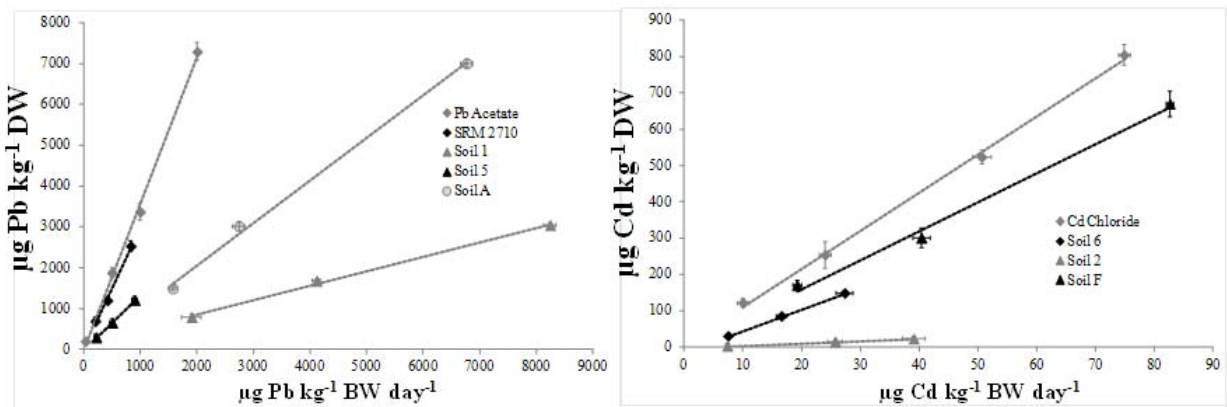
153 During the 14 runs of the *in vivo* experiments, the animals exposed to As, Cd, Pb and Sb contamination
154 remained healthy, continued to consume their feed, grew normally and none died. The mean BW of the
155 swine at the beginning of experiment was 9.5 ± 1.2 kg (n=168 swine) and, at the end 16.8 ± 1.5 kg (n=168
156 swine). Moreover, there was no correlation between the several exposure doses for each contaminant and the
157 final BW of each swine ($r^2 = 0.12$, $p > 0.05$, n=168). Similarly, for the different target end point (kidney, liver
158 and metacarpal IV), there was no impact of exposure doses on their final weight.

159 **Dose-response curves**

160 To ensure comparability between the dose response curves for the soluble salt and for the soils the
161 concentration of the soluble salt dose was designed to give a response which encompassed those
162 obtained for each element in each soil for each end point (see Figures 1 and 3)

163 *Metals - Cd and Pb*

164 The concentrations of Cd and Pb in the end points resulting from dosing with the reference matrix
165 were all above quantification limits. For the soils, Pb concentrations were all above the
166 quantification limits, whereas for Cd some concentrations were below. When the concentrations
167 were measurable, the dose-response curves for both soils and reference matrix fitted to a linear
168 model ($p < 0.05$) (example plots in Figure 1) except for soils 8 and 9 for the Pb response in the liver
169 (Figure 2). A similar pattern in the dose-response curves (Figure 1) has been previously reported
170 for both Pb and Cd [11, 16, 33].

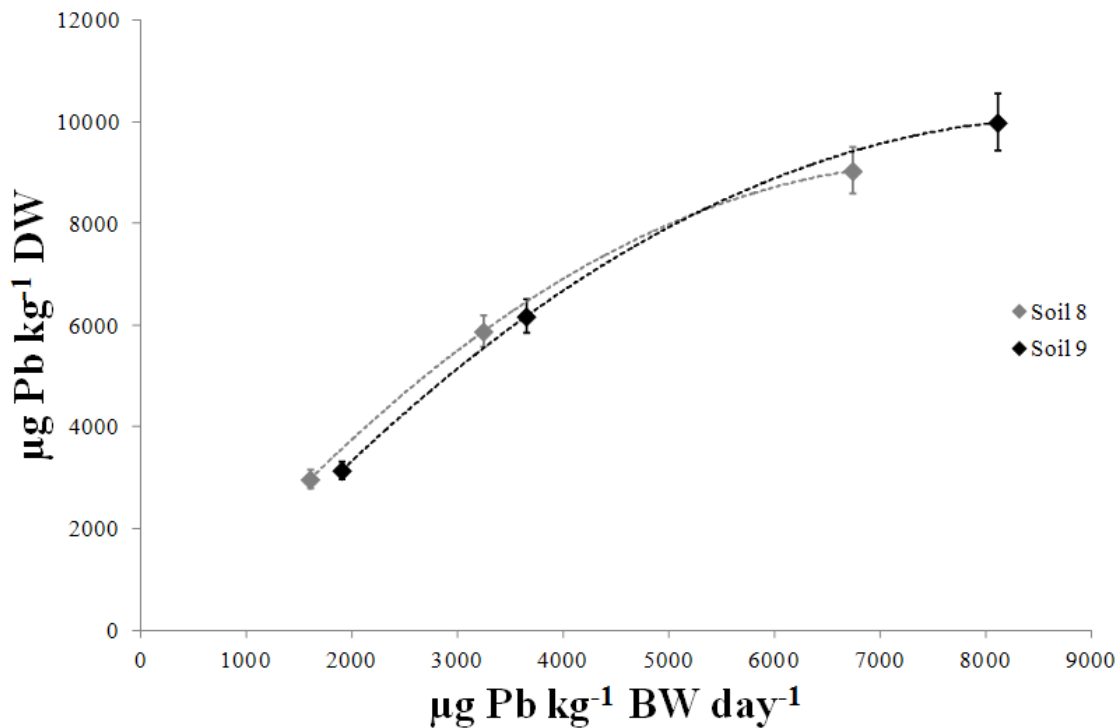


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172

173 *Figure 1 Examples of linear dose-response curves for Cd (kidney) and Pb (liver)*

174



175 *Figure 2 Non-linear dose-response curve for Pb in liver*

176

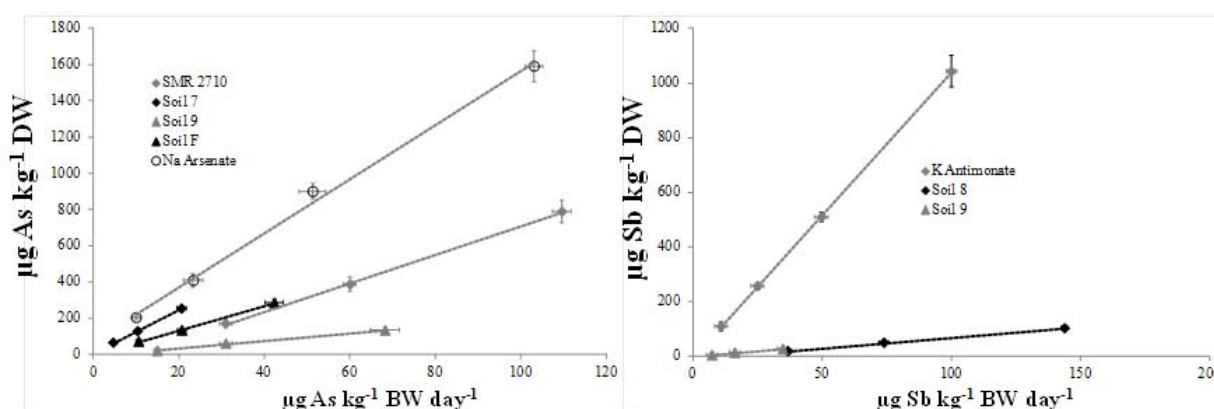
177 The repeatability of each response was also evaluated by calculating the relative standard deviation
 178 (RSD) n=3 data for each end point. Where RSD is calculated as the mean value divided by the
 179 standard deviation expressed as a percentage) For Pb, the RSD values were the lowest for Pb-

180 acetate (less than 1%) and the highest for soils F and G (around 20%). For Cd, the RSD values were
 181 lowest for Cd-chloride (less than 1%) and highest for soil 2 (around 30%)

182 *Metalloids – As and Sb*

183 Arsenic was quantified in each end point, giving linear models ($p < 0.05$). For Sb, however, apart
 184 from soils 1 and 2 with high total Sb content (Table S1 of the supporting information), dose
 185 response data could only be obtained for urine. The dose-response curve for this end point fitted a
 186 linear model ($p < 0.05$) (Figure 3). Example dose response curves for both As and Sb are given in
 187 Figure 3.

188 The repeatability of the response was also evaluated by calculating its RSD for each end point. For
 189 As, the lowest RSD value was obtained for the reference matrix (around 0.6%) and the highest
 190 value was obtained for the soil 7 (70 %). For Sb, the lowest RSD was obtained for urine on the
 191 reference material (less than 1%). The soils ranged from 15% to 50%. This reflects the difficulty of
 192 obtaining reproducible values of Sb concentrations due to the combined effect of relatively low
 193 concentrations (apart from soil 1 and 2) and low bioavailability of this element.



194
 195 *Figure 3 Examples of linear dose response curves for As and Sb in urine*

196 **Relative bioavailability of Pb and Cd**

197 The Cd and Pb RBA values and associated uncertainties are given in the supporting information
198 (Table S4). Cd-RBA could not be calculated for soils 1, 4, 10 and A, C, E in any of the end points
199 because the concentrations were below the quantification limits. The Pb-RBA could not be
200 calculated in soils 8 and 9 from the liver results, as the dose-response curves for these end points
201 were not linear. For soils E and F (kidney, bone and urine) and for soil D (bone and urine) there was
202 no significant difference between the slopes obtained for the reference matrix and the contaminated
203 soil for Pb. In these cases, the RBA was 100% meaning that Pb in these soils is as bioavailable as
204 Pb-acetate for the purposes of oral exposure.

205 The RBA values were consistent among the end points (Table S2) and were reproducible between
206 the replicates. This confirms the robustness of the juvenile swine *in vivo* model to estimate the RBA
207 of Pb and Cd in contaminated soils. The RBA values are within the range of other juvenile swine
208 studies with the same soluble reference compounds [11, 15, 16, 33].

209 Both Pb and the Cd showed a similar range of RBA values with a good coverage of the % RBA
210 range with minimum to maximum values of 6-100% RBA for Pb and minimum to maximum values
211 of 9-89% RBA for Cd. This is a fundamental pre-requisite to use these data in correlation studies
212 [22, 38].

213 **Relative bioavailability of As and Sb**

214 RBA values for As and Sb estimated from each end point and each soil samples are given in the
215 supporting information (Table S3).

216 The RBA for As could not be calculated for any of the target compartments for soils 5, D and E and
217 the RBA of Sb could not be calculated for soils 3, 5, D and E as the concentrations of the elements
218 in the end points were below the quantification limits. This reflects a strong decrease of both As and
219 Sb bioavailability compared to the reference matrix.

220 For Sb, the RBA could be calculated from kidney, liver and bone only for soils 1 and 2, with the
221 highest Sb content (Table S1). For these two soils, the RBA values of Sb were consistently low
222 among the target end points, (<4%). For the other soils, the RBA was measured only in urine and
223 did not exceed 11%. For As the average minimum to maximum % RBA range was 3-74%.

224 The results obtained for Sb are critical for the overall objective of this study as a fundamental
225 criterion of such a validation study is to have values of RBA evenly spread between the minimum
226 and maximum interval for the overall data set [22, 38]. This is probably due to a particularly low
227 overall bioavailability of Sb irrespective of the soil properties. Unfortunately, no previous study on
228 Sb has been published in the literature for comparison with the data produced here. The low average
229 % RBA range for Sb (2-6%) is unlikely to be suitable for validation of *in vitro* bioaccessibility tests.

230 For As (Table S3), however, the RBA values are evenly dispersed over the RBA range (3-100%).
231 Moreover, the RBA values are similar to the range of values obtained by several authors on soils
232 contaminated by both mining and smelting activities. For instance Rodriguez et al [39] reported
233 values ranging between 3% and 43%, and Juhasz et al [13] 7%-75%. A major factor that explains
234 the variation observed among the soil samples is the solid phase distribution of As within the soil
235 which differs according to soils type and physico-chemical properties [13].

236 **Bioaccessibility of As, Sb Cd and Pb in the reference matrix**

237 The BAF of each PHE in the soluble salts used to measure the *in vivo* RBA were determined using
238 the UBM procedure (As in Na-arsenate, Sb in K-antimonate, Pb in Pb-acetate and Cd in Cd-
239 chloride). These soluble salts were spiked to give a 1 mg kg⁻¹ concentration of each of the elements
240 in the final 'stomach' or 'stomach & intestine' extract. This allowed the calculation of the relative
241 bioaccessibility (RBAc), to allow a direct comparison with the RBA values. For the cations in the
242 'stomach' phase, the BAF values were 99 ± 2% and 98 ± 3% for Pb-acetate and Cd-chloride,
243 respectively. For the anions the As BAF was 95 ± 3% and the Sb BAF was 93 ± 5%. This showed

244 that all four elements were either indistinguishable or within 2% of being 100% bioaccessible for
245 the reference compounds in this compartment. In contrast, in the ‘stomach & intestine’ phase the
246 cations had much reduced BAFs with Pb and Cd giving values of $66 \pm 3\%$ and $68 \pm 3\%$ with As
247 and Sb BAFs of $92 \pm 4\%$ and $90 \pm 2\%$ respectively. The lower recoveries of Pb and Cd can be
248 explained by the fact that the behaviour of these elements is strongly pH dependent. In the higher
249 pH environment of the ‘stomach & intestine’ phase these metals can precipitate from solution, be
250 reabsorbed onto the soil and complexed by pepsin [40, 41]. This is not observed in the case of
251 elements (such as As and Sb) that form anions in solution and is consistent with previous studies
252 [42].

253 **Relative bioaccessibilities of As, Cd, Pb and Sb in the contaminated soils**

254 The RBAC was estimated as the ratio of the soil bioaccessibility to the reference matrix
255 bioaccessibility (%) for each phase and each element and are tabulated in the supporting
256 information (Tables S4 and S5). When individual t statistic 95% confidence intervals were
257 calculated for Cd and Pb the data indicated that, in general, the ‘stomach & intestine’
258 bioaccessibility is not significantly different from the ‘stomach’ phase bioaccessibility except for
259 soils 5, 7, C and E for Pb and soil 5 for Cd where the ‘stomach phase’ gives a significantly higher
260 bioaccessibility. For Cd and Pb the ‘stomach phase’ bioaccessibility is usually significantly higher
261 than the GI bioaccessibility for these elements [29]. This is because of the behaviour of Pb and Cd
262 is strongly pH dependent with lower solubility in the higher pH environment of the GI
263 compartment. In this instance, however, the bioaccessibility results have been calculated relative to
264 the bioaccessibility of the soluble salts (Pb-acetate and Cd chloride) which also show reduced
265 solubility at high pH. Taking measurement relative to the soluble salts therefore corrects for the
266 lower absolute Pb and Cd bioaccessibilities in the ‘stomach & intestine’ phase.

267 For the mining soils, RBAC of Pb and Cd ranged from 9% to 75% and from 7% to 70%,
268 respectively. For the smelting soils, the relative Pb bioaccessibility ranged from 40% to 90% and
269 the relative Cd bioaccessibility ranged from 28% to 87%. These values are in similar to values
270 reported in the literature [16, 42, 43].

271 For As and Sb no difference was observed between the two phases, apart from soil 2 for As. The
272 values of As RBAC ranged between 3% and 11% for the mining soils and between 11% and 74% in
273 the smelting soils. Thus, it seems that the bioaccessibility seems to be influenced by the source of
274 contamination, being higher in the smelting contaminated soils. This might be due to the difference
275 in the solid phase distribution of As within the soil constituents between the mining and smelting
276 soils. In the mining soils As appears to be associated with iron oxides and sulphide minerals and
277 consequently has a low bioaccessibility [44-46].

278 For Sb, RBAC was always 20% lower than RBA and no significant difference was observed
279 between mining and smelting soils. This overall low bioaccessibility might be explained by the
280 association of Sb and soil bearing phases like iron oxy-hydroxides, sulphides and refractory soil
281 constituents [47-50] that are not easily dissolved by the artificial digestive solutions used during the
282 UBM.

283 **Correlation between relative bioavailabilities and bioaccessibilities**

284 For a given contaminant, the bioavailability theoretically results from three steps:

- 285 • the dissolution of the contaminant in the lumen that is determined as the bioaccessibility
286 (BAC);
- 287 • the absorption of the contaminant through the GI membrane (ABS);
- 288 • the metabolism of the contaminant within the internal media (this is assumed to be
289 negligible for trace elements) [42].

290 The RBA can be determined as from the following formula [42]:

$$291 \quad RBA = RBAC \times ABSR \quad v)$$

292 Where:

- 293 • RBAC: the relative bioaccessibility of the contaminant, *i.e.* the soil:reference matrix
294 bioaccessibility of the contaminant
- 295 • ABSR: the relative absorption of the contaminant.

296 If the RBA is properly reflected by the RBAC, then the bioaccessibility should be the limiting factor
297 [38, 42]. As such, ABSR should be close to 1, meaning that the absorption step is independent from
298 the initial form of the contaminant that is ingested. In this case, RBA should be equal to RBAC, *i.e.*
299 the slope of the regression between RBA and RBAC should be equal to 1. The slopes of the
300 regression between RBA and RBAC were calculated for each target compartment and for the two
301 phases of the UBM.

302

303 *Regression of the UBM relative bioaccessibility against in-vivo relative bioavailability*

304 An earlier study comparing UBM data against *in vivo* bioavailability on test soils, [25] set out a
305 series of benchmark criteria that should be met by the *in vitro* and *in vivo* data and any subsequent
306 mathematical regression relationship in order for the *in vitro* methodology to supply “fit for purpose
307 data” for risk assessments. The first criterion is that the median repeatability on the bioavailability
308 data should be better than 20% RSD.

309

310 Figures S1 and S2 in the supplementary information show boxplot summaries of the repeatability
311 (RSD of the RBA replicate measurements) of the bioavailability the four end points of the sixteen
312 soils for As, Cd and Pb.

313 Sb has not been included since the bioavailability data were of not of sufficient quality to carry out
314 a correlation with bioaccessibility data.

315 For both Cd and Pb the median repeatability values are well within the benchmark (Figure S1 in the
316 supplementary information). The repeatability values for As values are higher for all end points
317 with the kidney end point benchmark value of 20.6% and the liver end point only just above at
318 22.5% (Figure S1). Although not strictly met, it is considered that a median value of 20.6% vs the
319 ideal criteria of 20% for the two compartments was considered acceptable for the kidney end point
320 and should not to compromise the use of the UBM for As in a soil risk assessment.

321 The second benchmark relates to the bioaccessibility repeatability (within-laboratory variability)
322 and reproducibility (between-laboratory variability). The former should have a median value of
323 10% RSD and the latter a median value of 20% RSD.

324 Only within-laboratory data are available for this study so, only the repeatability can be tested.
325 Figure S2 in the supporting information shows boxplots of the repeatability (RSD of the RBAC
326 replicate measurements) for each of the elements studied.

327

328 In this case, Sb values have been included as since robust results were obtained for this element
329 from the UBM bioaccessibility test. Figure S2 shows that the median repeatability values for the
330 UBM are all below the 10% benchmark for all elements in both the 'stomach' and 'stomach &
331 intestine' compartments. The median reproducibility values are very similar for each compartment
332 although the spread of values is consistently higher in the 'stomach & intestine' compartment.
333 Median repeatability values are all very similar at c. 5-7% RSD but As shows higher variability in

334 values compared the other three elements. This is a similar pattern to the *in vivo* data shown in
335 Figure S1.

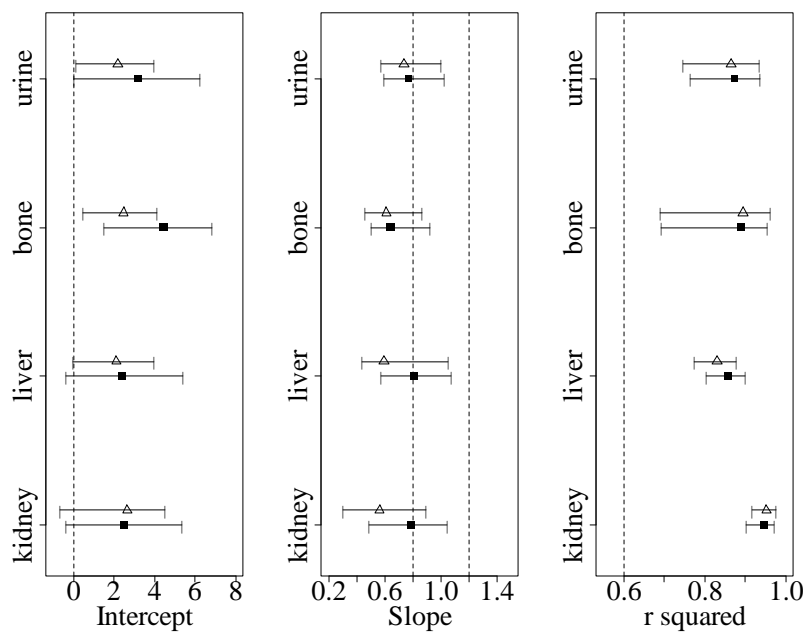
336 The next set of benchmark criteria relate to the statistical parameters associated with linear
337 regression fits to the relationship between RBA and RBAC. Since there is significant error (median
338 RSD of up to 30% for bioavailability and 8% for bioaccessibility, Figures S1 and S2) on both the
339 bioaccessibility and bioavailability data (Tables S2-S5), ordinary linear regression is not appropriate
340 as it assumes that there are errors only on the 'y' co-ordinate. In this study a repeated medians
341 approach [36] is used, which makes no assumptions about errors and is robust to outliers. The
342 method has been applied using a monte-carlo approach varying each point over a normal
343 distribution described by its mean value and standard deviation. The advantage of this is that it
344 produces a distribution of values for the descriptive statistics for the regression (intercept, slope and
345 r square) so that 95% confidence intervals can be calculated and can then be judged against a
346 benchmark value. Wragg et al [25] suggested that the benchmark criteria should be:

347 i) The intercept is not significantly different from 0;

348 ii) The slope should be between 0.8 and 1.2;

349 iii) The r square value (measure of the scatter around the line) should be greater than 0.6.

350 Using this methodology, the linear regressions of relative bioaccessibility against relative
351 bioavailability were calculated using the data from the supporting information (Tables S2-S5). All
352 data were included in the calculation apart from the RBA values which could not be calculated
353 because the absolute concentration of the elements in the target organ was below detection limit or
354 because the dose response curves were not linear. Summary statistics, in the form of a mean value
355 of the intercept, slope or r square value and their associated 95% confidence intervals for each
356 element regression for each end point and each stomach compartment are shown in Figures 4-6.



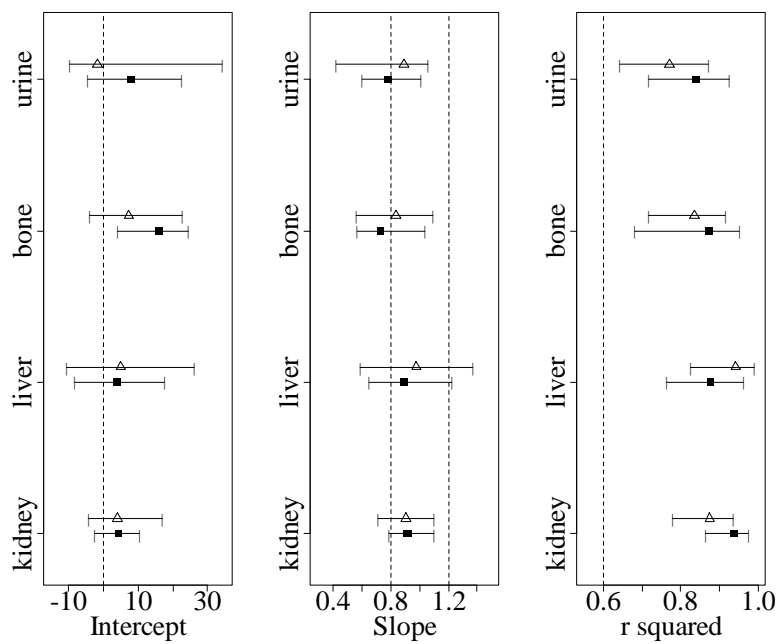
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358 *Figure 4 Summary of the RBA vs RBAC regression statistics for the four end points for As. Black*
 359 *squares show data for the 'stomach' phase and white triangles for the 'stomach & intestine' phase.*
 360 *Error bars represent 95% confidence limits dotted lines show benchmark values.*

361

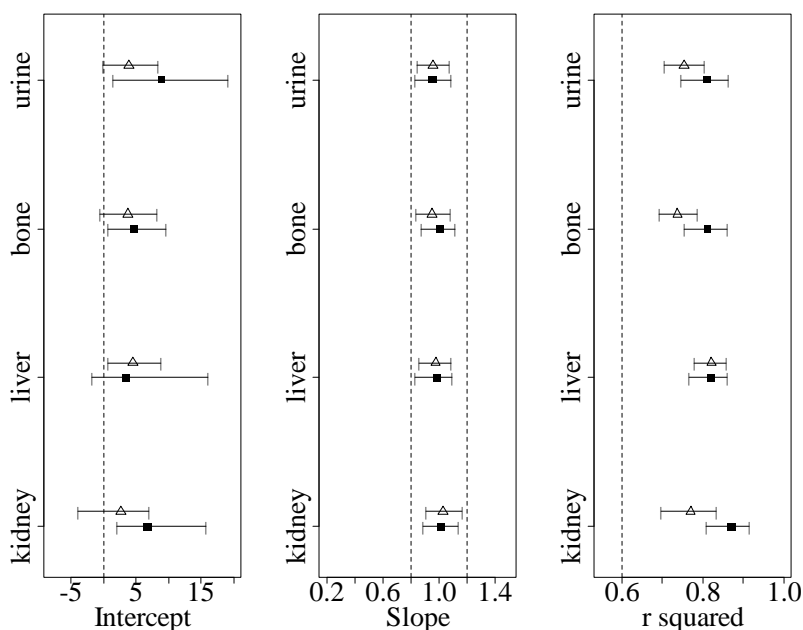
362 Examination of Figures 4 to 6 shows that, for all elements in all endpoints the slope and the r square
 363 values all meet the benchmark criteria. For Cd (Figure 5) the intercepts only shows one incidence
 364 out of eight where the intercept is positive (bone in the stomach compartment). For both As and Pb
 365 (Figures 4 and 6), however, there are five incidences out of eight where the intercepts are shown to
 366 be >0. This suggests there is a small bias in the RBAC measurement for these elements compared to
 367 the RBA (3% RBAC for As and 5% RBAC for Pb averaged over all endpoints and compartments).
 368 The plots also confirm that there is no significant difference between the 'stomach' and 'stomach &
 369 intestine' compartments for all three elements and all four end points.

370



371

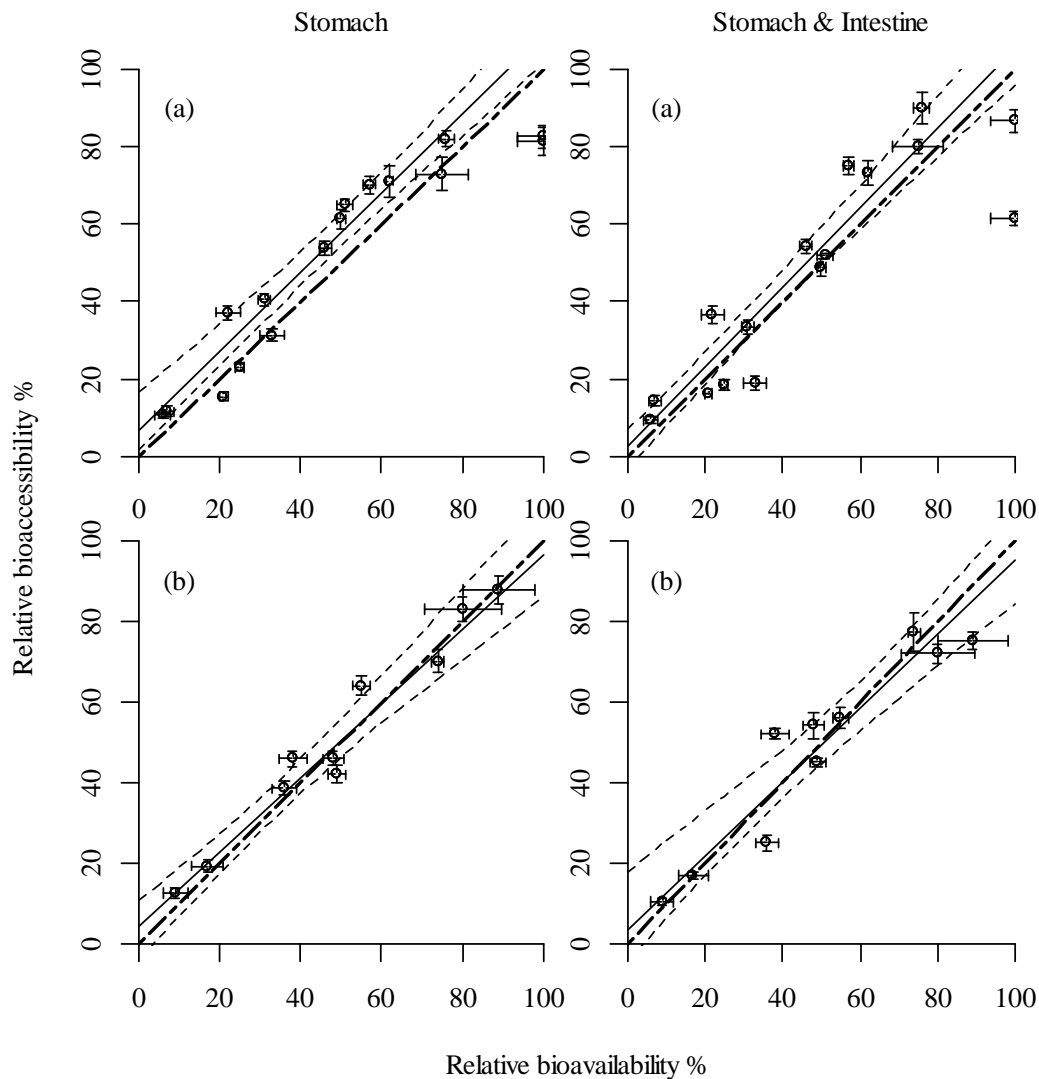
372 *Figure 5 Summary of the RBA vs RBAC regression statistics for the four end points for Cd. Black*
 373 *squares show data for the ‘stomach’ phase and white triangles for the ‘stomach & intestine’ phase.*
 374 *Error bars represent 95% confidence limits, dotted lines show benchmark values*



375

376 *Figure 6 Figure 3 Summary of the RBA vs RBAC regression statistics for the four end points for Pb.*
 377 *Black squares show data for the ‘stomach’ phase and white triangles for the ‘stomach & intestine’*
 378 *phase. Error bars represent 95% confidence limits, dotted lines show benchmark values.*

379



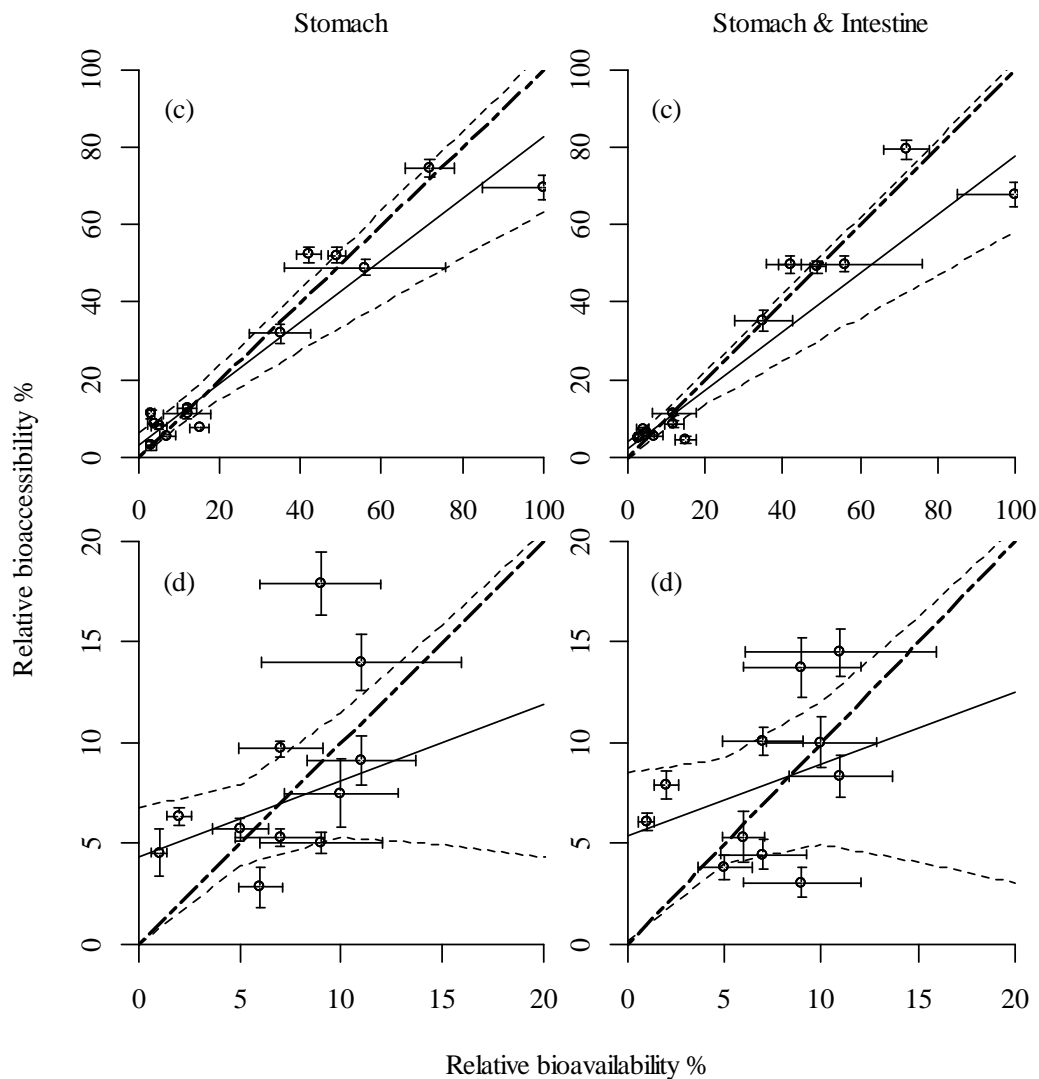
380

381 *Figure 7 correlation plots for RBAC against RBA for (a) Pb and (b) Cd for the 'stomach' and*
 382 *'stomach & intestine' phases for the kidney endpoint. Bold dashed dotted line is the line of*
 383 *equivalence, dashed lines are the 95% confidence intervals and the solid lines is the best line of fit*

384

385 For the four target end points selected for this study (kidney, urine, bone and liver), the r square
 386 value for the RBAC and RBA regressions were all significantly different from 0 both for the
 387 'stomach' and the 'stomach & intestine' phases. Since the slopes of regressions are all close to 1, it

388 appears that the RBAc is actually the limiting factor of the RBA. This confirms the ability of the
 389 UBM test to assess the bioaccessibilities of As, Cd and Pb in the contaminated soils studied. The
 390 bioaccessibility as measured by UBM better reflects the external exposure to soil contaminants
 391 following an oral ingestion than the total concentration.



392

393 *Figure 8 correlation plots for RBAc against RBA for (c) As and (d) Sb for the 'stomach' and*
 394 *'stomach & intestine' phases for the urine end point. Bold dashed dotted line is the line of*
 395 *equivalence, dashed lines are the 95% confidence intervals and the solid line is the best line of fit.*

396 Figures 7 and 8 show example RBAC plots versus RBA showing how the fitted regressions are very
397 close to the ideal 1:1 relationship for As (Figure 8 a and b), Cd and Pb (Figure 7) but with evidence
398 for small positive intercepts in As in the 'stomach' phase (Figure 8 a) and Pb in the 'stomach' phase
399 (Figure 7 a) . For Sb, however, the low bioavailabilities and bioaccessibilities that were measured
400 for the soils sampled in this work meant that the correlations could only be studied in the 0%-20%
401 area. In these conditions, the UBM test could not be validated for Sb due to a lack of statistical
402 significance which is clearly illustrated in Figure 8 c) and d). The 95% confidence interval in the
403 line of best fit is far too wide to provide a useful relationship between RBAC and RBA, which could
404 be used by a risk assessor.

405 The juvenile swine model has been shown to produce RBA values that are consistent within the
406 target end points for As, Cd and Pb for the 16 soils studied. The variety of soil types, the range of
407 total element values are representative of the total concentrations of these elements that would
408 normally be considered for bioaccessibility testing [25]. The RBA values for all three of these
409 elements covered at least 70% of the RBA range making them highly suitable for calibrating *in*
410 *vitro* testing protocols.

411 For Sb, however, the RBA values were approximately 10% or less for all soils and it was difficult
412 to measure the amount of Sb absorbed into the target end points, apart from urine, for all but soils 1
413 and 2 which are grossly contaminated with Sb (>50000 mg kg⁻¹, Table S1). The small RBA range
414 covered will not make this data set suitable for calibrating Sb bioaccessibility measurements from *in*
415 *vitro* testing methods.

416 Whilst it would be impossible to show that the UBM has been validated for all soil types, this study
417 has concentrated on soils with anthropogenic contamination (combined with their natural PHE
418 content) which are likely candidates for human health risk assessment. The study has used soils
419 from a variety of spatial locations with a range of physicochemical properties and which exhibit a

420 good range of PHE bioaccessibilities. These results provide strong evidence that, through a
421 pragmatic choice of soils, the UBM provides a robust tool for use in risk assessment of As, Cd and
422 Pb. The study suggests the ‘stomach’ compartment alone is a good analogue of *in vivo*
423 bioaccessibility but this need to be confirmed by use of the method on a wider variety of soils.

424 This study has addressed many of the issues arising from a preliminary inter-laboratory trial of the
425 UBM [25] showing that a specifically designed *in vivo* study with soils relevant to European
426 conditions along with better control on pH in the ‘stomach’ phase that the UBM produces
427 bioaccessibility data that is a very good analogue of juvenile swine bioavailability measurements
428 for As, Cd and Pb. The one point that this study has not yet addressed is the inter-laboratory
429 reproducibility that was problematic in the study of Wragg et al [25]. A further follow up study on
430 inter-laboratory performance is required to provide the last piece of evidence that the method can be
431 used as a routine test in risk assessment studies.

432 **Supporting Information Available**

433 The supporting information contains details of the procedures used to determine the bioavailability
434 and bioaccessibility of the PHEs and the methods used for the preparation, analysis and quality
435 control of the PHEs in the soil and swine samples. In addition, tabulations of the soil properties and
436 the bioavailability of the bioaccessibility of the PHE in each of the soils tested are provided along
437 with box and whisker plots of the repeatability of the bioavailability and bioaccessibility
438 measurements. This information is available free of charge via the Internet at <http://pubs.acs.org>.

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