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

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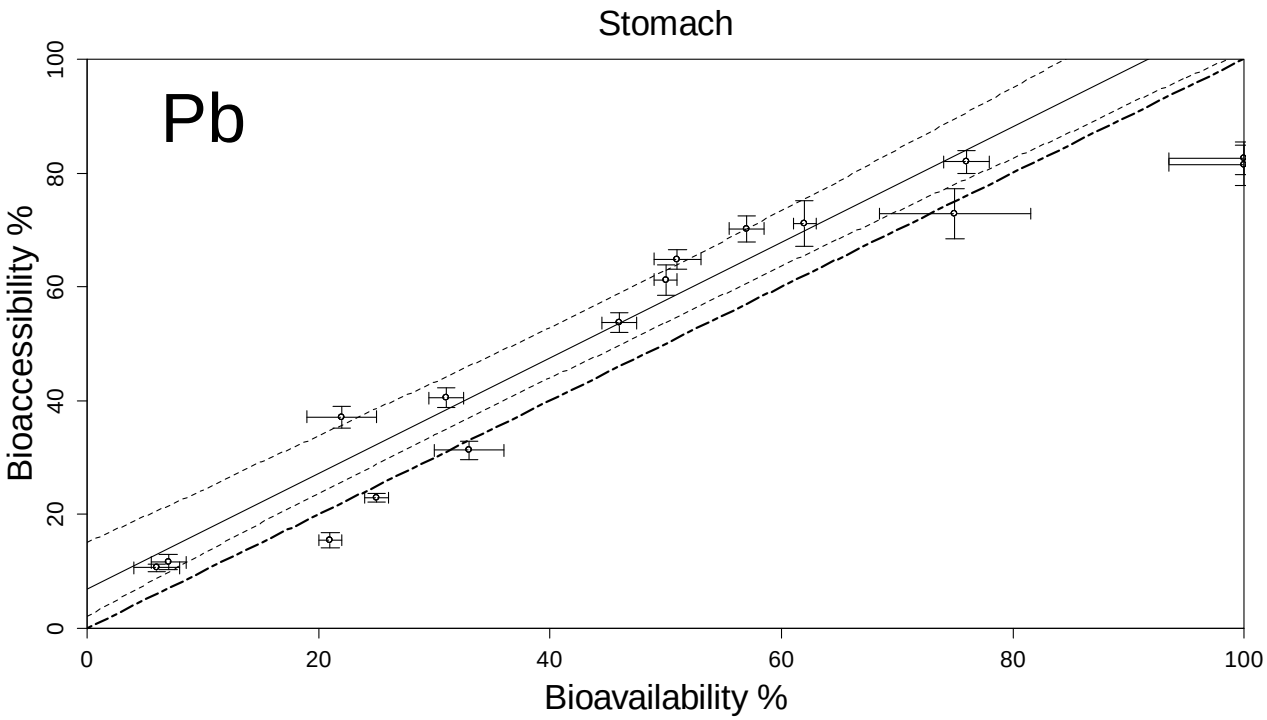
ABSTRACT

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

on repeatability (median relative standard deviation value < 10%) and the regression statistics (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 relative bioaccessibility of As and Pb compared to the relative bioavailability measurements of 3% and 5% respectively. Sb did not meet the criteria due to the small range of bioaccessibility values found in the samples.

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

33 **TOC/Abstract Art**



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INTRODUCTION

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

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

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

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

MATERIALS AND METHODS

Soil collection sample preparation and chemical analysis

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

Determination of *in vivo* relative bioavailability

The RBA of As, Pb, Cd and Sb were determined for each soil sample using readily soluble forms of the contaminants, sodium arsenate (NaH_2AsO_4), Pb-acetate ($(CH_3COO)_2Pb$), Cd-chloride ($CdCl_2$) and potassium antimonate ($KSbO_3$)). These reference matrices were chosen to estimate the RBA as they had been used in previous RBA studies for As and Sb [14, 21, 32] and Pb and Cd [11, 33].

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

Dose Response Curve and RBA calculation

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

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

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

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

Unified BARGE Method

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

Bioaccessibility calculation

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

$$BA_s = V_s \times C_e \times d / m \qquad \text{i)}$$

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**

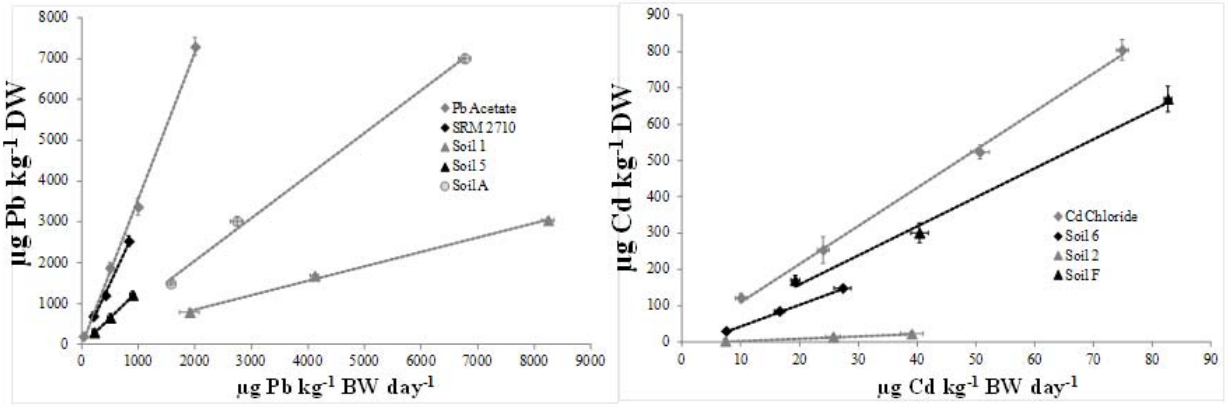
153 During the 14 runs of the *in vivo* experiments, the animals exposed to As, Cd, Pb and Sb contamination
154 remained healthy, continued to consume their feed, grew normally and none died. The mean BW of the
155 swine at the beginning of experiment was 9.5 ± 1.2 kg (n=168 swine) and, at the end 16.8 ± 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 metacarple 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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173 *Figure 1 Examples of linear dose-response curves for Cd (kidney) and Pb (liver)*
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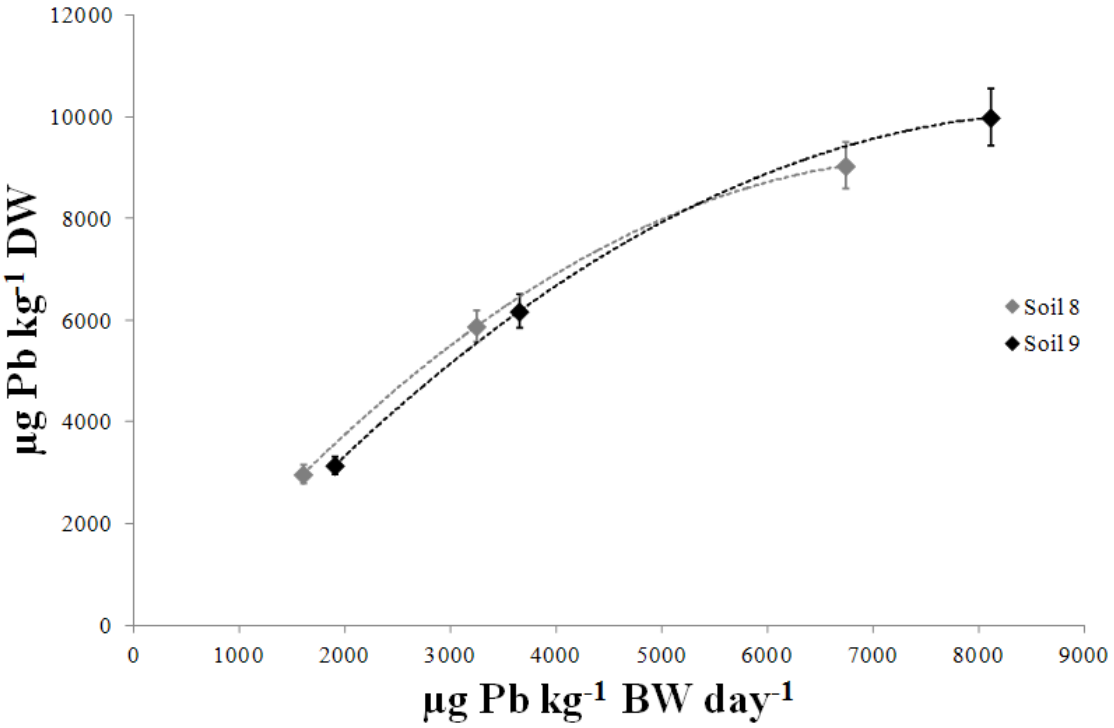


175
176 *Figure 2 Non-linear dose-response curve for Pb in liver*

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-

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

Metalloids – As and Sb

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

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

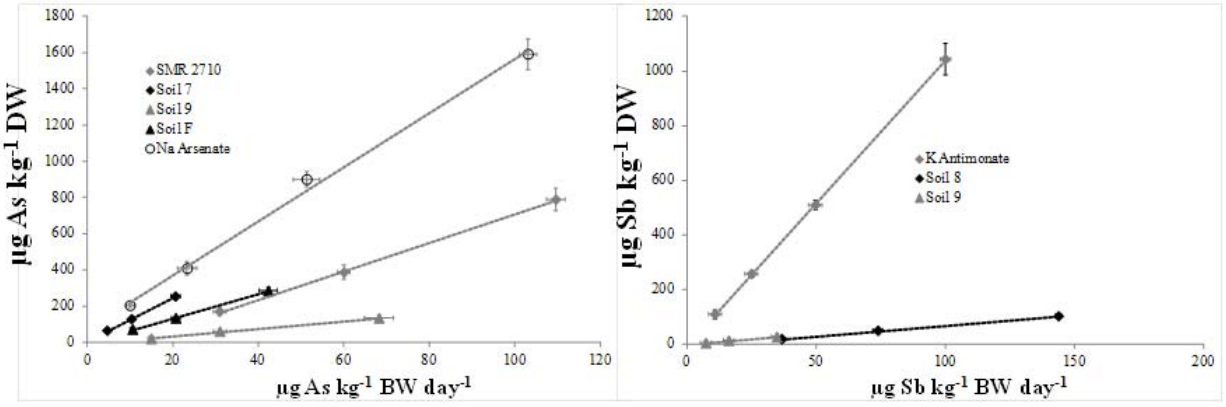


Figure 3 Examples of linear dose response curves for As and Sb in urine

Relative bioavailability of Pb and Cd

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

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

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

Relative bioavailability of As and Sb

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

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

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

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

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

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

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

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

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

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

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

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

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

Correlation between relative bioavailabilities and bioaccessibilities

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

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

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

$$RBA = RBAc \times ABSR \quad v)$$

Where:

- RBAc: the relative bioaccessibility of the contaminant, *i.e*. the soil:reference matrix bioaccessibility of the contaminant
- ABSR: the relative absorption of the contaminant.

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

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

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

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

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

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

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

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

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

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

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

i) The intercept is not significantly different from 0;

ii) The slope should be between 0.8 and 1.2;

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

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

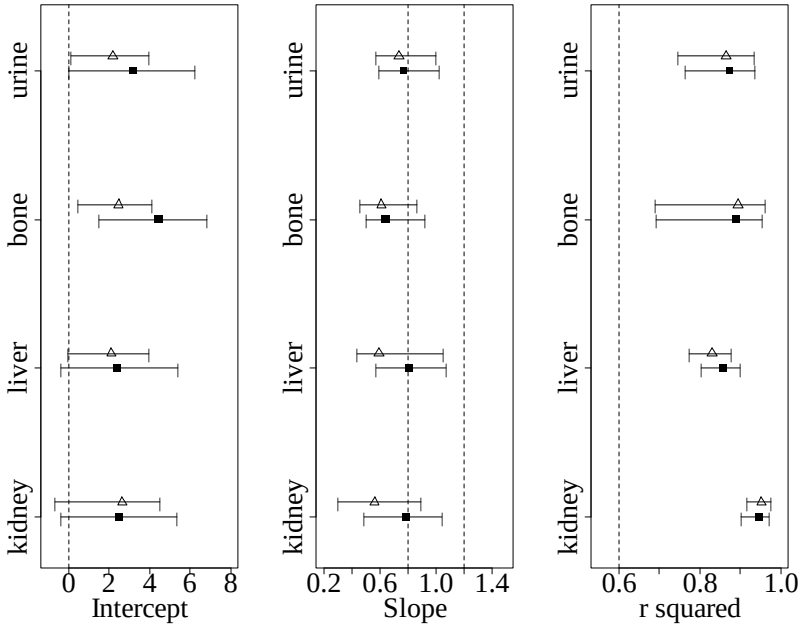
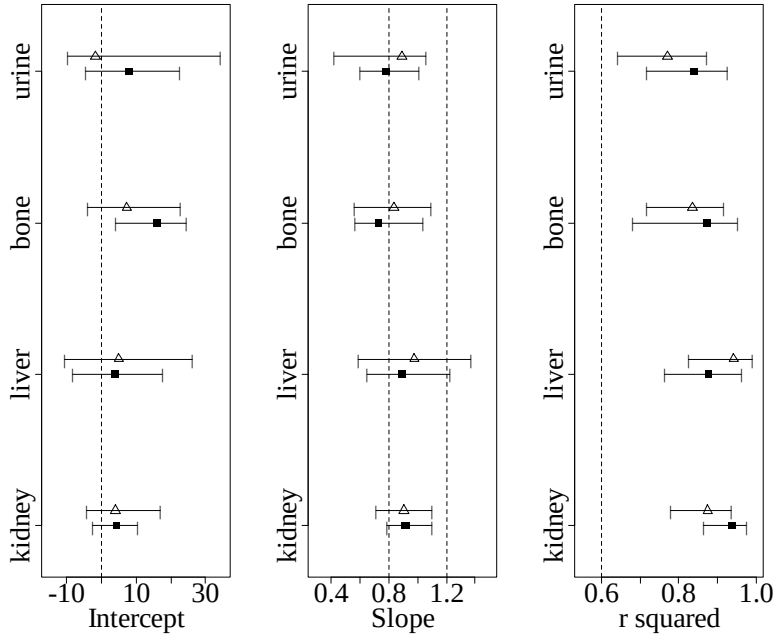


Figure 4 Summary of the RBA vs RBAc regression statistics for the four end points for As. Black squares show data for the 'stomach' phase and white triangles for the 'stomach & intestine' phase. Error bars represent 95% confidence limits dotted lines show benchmark values.

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



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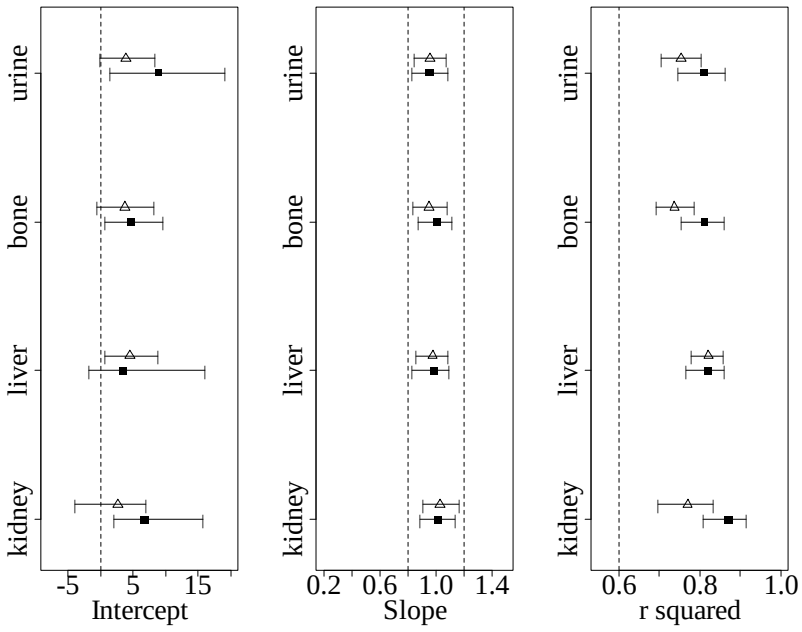
372 *Figure 5 Summary of the RBA vs RBAc regression statistics for the four end points for Cd. Black*
373 *squares show data for the 'stomach' phase and white triangles for the 'stomach & intestine' phase.*
374 *Error bars represent 95% confidence limits, dotted lines show benchmark values*



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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.*

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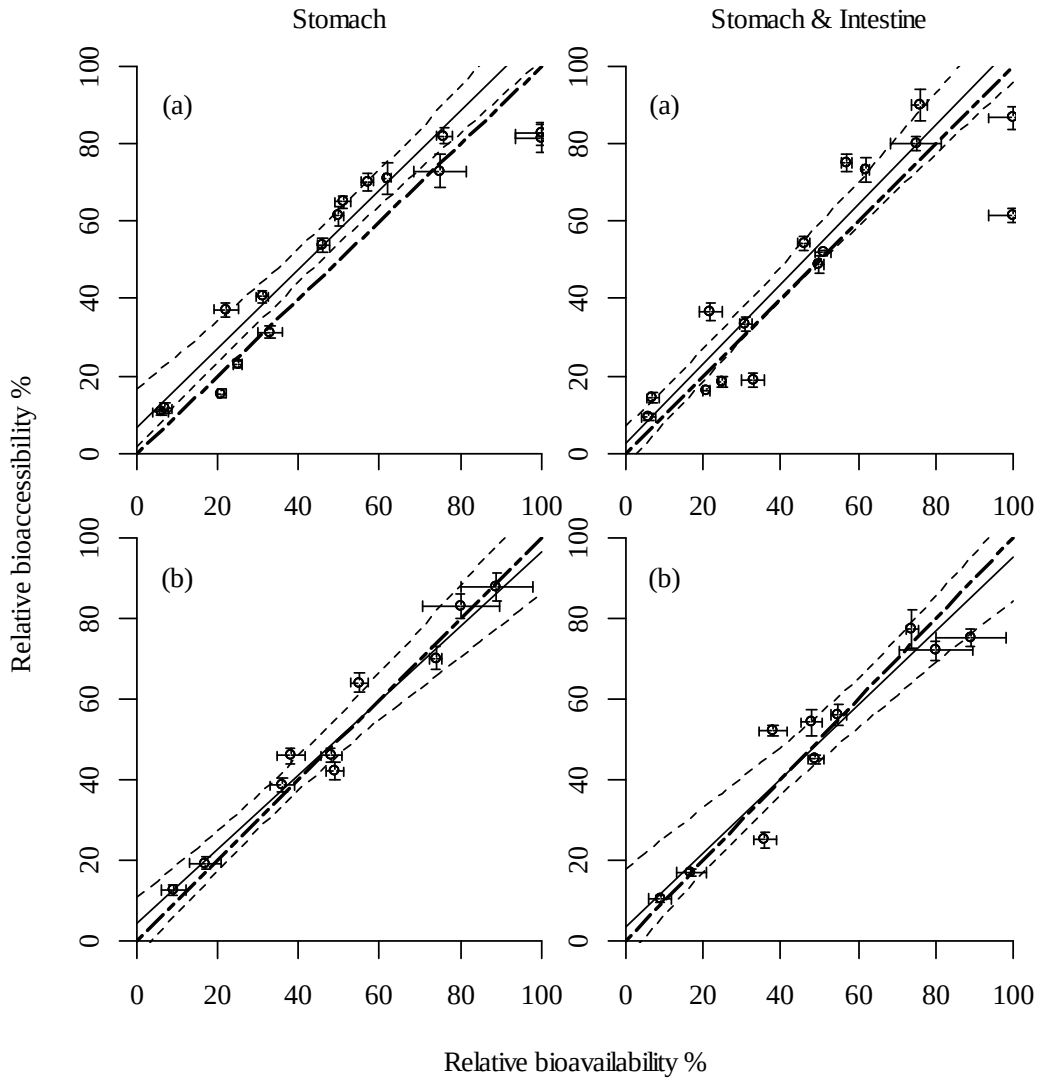

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381 *Figure 7 correlation plots for RBAc against RBA for (a) Pb and (b) Cd for the 'stomach' and*
382 *'stomach & intestine' phases for the kidney endpoint. Bold dashed dotted line is the line of*
383 *equivalence, dashed lines are the 95% confidence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

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

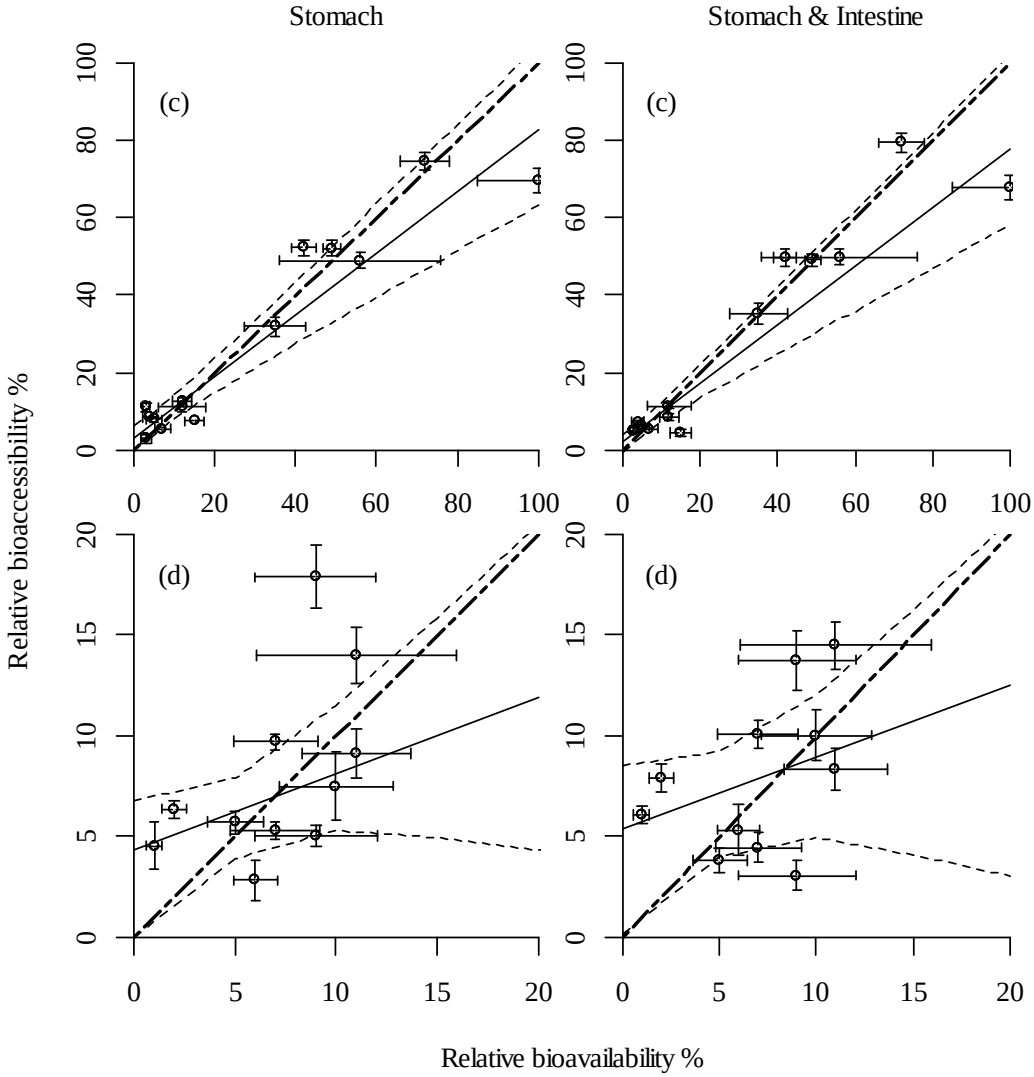

Figure 8 correlation plots for RBAc against RBA for (c) As and (d) Sb for the 'stomach' and 'stomach & intestine' phases for the urine end point. Bold dashed dotted line is the line of equivalence, dashed lines are the 95% confidence intervals and the solid line is the best line of fit.

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

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

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

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

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

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

Supporting Information Available

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

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