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Validation of the Predictive Capabilities of the Sbrc-G in Vitro Assay for Estimating Arsenic Relative Bioavailability in Contaminated Soils

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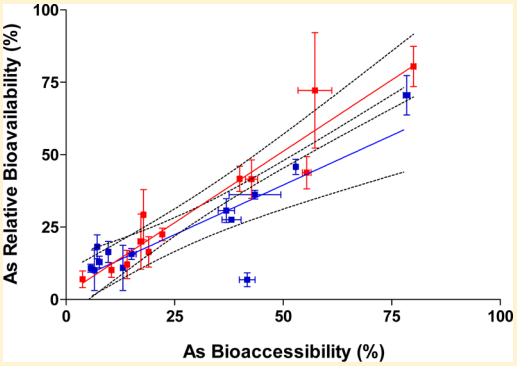
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Supporting Information

ABSTRACT: A number of bioaccessibility methodologies have the potential to act as surrogate measures of arsenic (As) relative bioavailability (RBA), however, validation of the in vivo-in vitro relationship is yet to be established. Validation is important for human health risk assessment in order to ensure robust models for predicting As RBA for refining exposure via incidental soil ingestion. In this study, 13 As-contaminated soils were assessed for As RBA (in vivo swine model) and As bioaccessibility (Solubility Bioaccessibility Research Consortium gastric phase extraction; SBRC-G). In vivo and in vitro data were used to assess the validity of the As RBA-bioaccessibility correlation previously described by Juhasz et al. (2009). Arsenic RBA and bioaccessibility in the 13 soils ranged from 6.8 ± 2.4% to 70.5 ± 6.8% and 5.7 ± 0.3% to 78.4 ± 0.8% respectively with a strong linear relationship (R^2 = 0.75) between in vivo and in vitro assays. When the As in vivo-in vitro correlation was compared that of Juhasz et al. (2009), there was no significant difference ($P > 0.05$) indicating that the relationship between As RBA and As bioaccessibility was consistent thereby demonstrating its validation. For these data, a grouped linear regression model was developed (R^2 = 0.82) with a slope and *y*-intercept of 0.84 and 3.56 respectively. A number of cross validation methodologies (2-fold, repeat random subsampling, leave one out) were utilized to determine the performance of the linear regression model. Residuals and prediction errors ranged from 5.4 to 9.4 and 6.9−12.2 respectively illustrating the capacity of the SBRC-G to accurately predict As RBA in contaminated soil.



INTRODUCTION

Arsenic (As) is ubiquitously distributed throughout the soil environment and may be present at elevated concentrations as a result of both geogenic and anthropogenic processes.[1−6] In the U.S., As was ranked the most common inorganic constituent in the National Priority List of Sites[7] while in other jurisdictions As is considered a priority pollutant[8] due to the well documented deleterious effects of As exposure on human health.[9,10]

When considering exposure to As, chemical daily intake may be quantified by considering the magnitude, frequency and duration of exposure. In the context of incidental soil ingestion, the magnitude of exposure will be influenced by the concentration of As in the soil matrix but also by its bioavailability (i.e., the proportion of the total soil-borne As concentration that is absorbed into the systemic circulation following ingestion). Arsenic relative bioavailability (RBA) may be influenced by a number of physiological parameters in addition to physicochemical properties of As and/or the contaminated soil.[11−18] In the absence of site-specific data, the default value for As RBA in the U.S. was 100%. It was assumed that As bioavailability in the soil matrix was equivalent to that in the exposure medium (i.e., water) to derive the toxicity value. However, after the compilation and review of As RBA values in soil, the USEPA revised the default value to 60%.[19] Acknowledging that the default value may not reflect all variables that may influence As RBA at a given site, the U.S. Environmental Protection Agency[19] recommended that "*site-specific assessment should still be performed where such assessments are deemed feasible and valuable for improving the characterization of risk at the site*".

While a number of different in vivo methodologies are available for the assessment of As RBA in soil,[11,12,15,17] their use for refining exposure at contaminated sites is cost prohibitive. As a consequence, in vitro bioaccessibility methodologies, which mimic key processes in the gastrointestinal tract, have been proposed as a surrogate measure for the prediction of As RBA.[11,12,14,20−24] Bioaccessibility methodologies determine the amount of As that is solubilized in gastrointestinal fluid and is therefore potentially available for absorption into the systemic circulation. A number of studies have determined that there is a

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strong correlation between As RBA, determined using swine or mouse in vivo assays, and As bioaccessibility, determined using the in vitro gastrointestinal extraction method (IVG),[20−22] Solubility, Bioaccessibility Research Consortium method (SBRC)[11,12,14,22] and the Unified Bioaccessibility Research Group of Europe Method (UBM).[23,24] However, as detailed by Juhasz et al.,[25] these relationships are yet to be validated using samples outside of the empirical data set used in the correlation study. Validation of As in vivo-in vitro relationships is important for human health risk assessment in order to ensure robust models for predicting As RBA for the refinement of exposure via incidental soil ingestion.

In previous studies, the relationship between As RBA and As bioaccessibility was determined in soils contaminated with As via a number of different sources.[14,22] A linear regression model was developed for predicting As RBA (swine assay encompassing area under the blood As concentration time curve) using As bioaccessibility data derived from gastric phase extraction using the Solubility Bioaccessibility Research Consortium (SBRC-G) assay. The relationship between As RBA and As bioaccessibility was strong (R^2 = 0.75), with a slope close to 1 (0.99) and a small y-intercept (0.66).[22] This study aimed to validate the As in vivo-in vitro relationship from Juhasz et al.[22] through the assessment of an independent data set using the aforementioned swine assay and SBRC-G assay. Predictive performance was assessed using a number of validation methodologies and compared to validation criteria as detailed in Juhasz et al.[25] and Wragg et al.[26]

MATERIALS AND METHODS

As-Contaminated Soils. An independent set of As-contaminated soils (n = 13) were collected from a variety of locations in Australia and the U.S. where soils had been impacted as a result of herbicide application or mining activities. Soils were oven-dried (105 °C) then sieved with the <250 μm soil particle size fraction retained for characterization and As RBA and bioaccessibility assessment. Soil physicochemical properties were determined in triplicate for each sample. Total metal/metalloid concentration was determined using ICP-AES following digestion using aqua-regia and the USEPA 3051 dissolution procedure.[27] A certified reference material (NIST 2711) was included in the digestion and analysis for quality assurance and quality control. Citrate dithionite extractable Fe was determined using the method of Holmgren.[28]

Assessment of As Bioaccessibility. Arsenic bioaccessibility in contaminated soils was determined using the gastric phase of the SBRC assay as detailed by Kelley et al.[29] Briefly, As-contaminated soil (in triplicate) was added to SBRC-G (0.4 M glycine, pH 1.5) at a soil:solution ratio of 1:100. Suspensions were incubated at 37 °C, 40 rpm on a Ratek suspension mixer for 1 h. During mixing, the pH was monitored and adjusted where necessary (with concentrated HCl) to maintain the pH at 1.5 ± 0.05. After 1 h, sample extracts were collected (10 mL) and filtered (0.45 μm) prior to analysis by ICP-AES or ICP-MS.

Arsenic bioaccessibility was determined by dividing the amount of As (μg) extracted from the soil following SBRC-G extraction by the amount of As (μg) in the contaminated soil prior to in vitro assessment (see Supporting Information (SI), eq 1).

Assessment of As Relative Bioavailability. Arsenic RBA in contaminated soils was determined using an in vivo swine model. Animal experimental protocols were approved by the SA Pathology/AHN Animal Ethics Committee (application number 177/12). Female "Large White" swine, weighing ∼20 kg, were used for As RBA assays according to Rees et al.[17] Further details regarding the in vivo methodology can be found in the SI. Arsenic-contaminated soils (<250 μm particle size fraction) were administered as soil slurries via gavage in 150 mL of As free water to replicate animals (n = 4), whereas sodium arsenate (100 μg As kg bw^{-1}) was administered as the reference dose. Blood samples were routinely taken over a 26 h period following dosage. Blood serum was digested using a CEM Mars6 microwave, according CEM's blood digestion application note, prior to analysis by ICP-MS

Arsenic RBA was assessed using pharmacokinetic analysis encompassing areas under the blood As concentration (AUC) time curves following zero correction and dose normalization (see SI; eq 2).[14,22]

Quality Assurance and Quality Control. The accuracy of the aqua-regia digestion method was confirmed by a quantitative average As recovery of 97 ± 6 mg kg^{-1} (n = 4) from NIST 2711 (105 ± 8 mg As kg^{-1}). During the determination of As concentration in soil digests, in vitro extracts and blood samples, duplicate analysis, spiked sample recoveries and check samples (50 μg As L^{-1}) were included. The average deviation between duplicate samples (n = 38) was 2.7% (0.12−12.0%), the average recovery from spiked samples (n = 38) was 100.5% (91.8−106.0%) whereas check sample recoveries (n = 42) ranged from 93.1 to 114.0% (105.6% average recovery).

Validation of the As In Vivo−In Vitro Correlation. Previously, the correlation between As RBA (swine assay; AUC analysis) and As bioaccessibility (SRBC-G) was determined by Juhasz et al.[14,22] These studies determined that the SBRC-G assay has the potential to be utilized as a surrogate method for the prediction of As RBA. In order to validate the previously established in vivo-in vitro relationship, a number of strategies were employed to determine the As RBA prediction accuracy of SBRC-G (using SPSS 22.0, Excel 2013 and GraphPad Prism 5) as described in the following sections. It is important to note that a prerequisite for adopting this validation approach is that the methods utilized for determining As RBA (swine assay utilizing AUC analysis) and As bioaccessibility (SBRC-G) are the same, which was the case for this study and Juhasz et al.[22]

2-Fold Cross Validation. Data from this study and Juhasz et al.[22] were utilized as independent data sets to assess As RBA prediction accuracy. Each data set was assigned as either the training set or the holdout (or test) set. The training set was utilized to construct the in vivo−in vitro linear regression model while the holdout set was used to test the strength of the relationship. Initially, data from Juhasz et al.[22] was utilized as the training set while data from this study was utilized as the test set. Subsequent analyses allocated data from this study to the training set and data from Juhasz et al.[22] to the test set.

Repeat Random Subsampling Cross Validation. A holdout validation method with random subsampling was also utilized to assess As RBA prediction accuracy. In this approach, data from Juhasz et al.[22] and this study were combined resulting in a data set of 25 soils with corresponding in vivo and in vitro data. For holdout validation assessment, the combined data set was randomly divided into two mutually exclusive subsets; approximately $^2/_3$ of the data was allocated to the training set (n = 17) while the remaining $^1/_3$ was allocated to the test set (n = 8). As with the 2-fold cross validation methodology, the

Table 1. Selected physico-chemical properties of soils (< 250 μm particle size fraction) used in this study

		major and minor elements[a]					
sample	As source	As (mg kg^{-1})	Al (g kg^{-1})	Fe (g kg^{-1})	Mg (g kg^{-1})	Mn (mg kg^{-1})	P (mg kg^{-1})
S1	herbicide	275 ± 10	52.4 ± 1.7	89.6 ± 3.3 (86.9)[b]	9.6 ± 0.8	1110 ± 84	2567 ± 104
S2	herbicide	210 ± 11	44.2 ± 2.9	60.6 ± 3.8 (55.3)	20.1 ± 2.5	887 ± 95	2350 ± 137
S3	herbicide	81 ± 1.8	26.7 ± 0.5	60.3 ± 0.9 (13.4)	33.1 ± 0.6	2109 ± 23	1067 ± 12
S4	herbicide	358 ± 10	65.1 ± 2.4	96.2 ± 2.7 (84.1)	8.0 ± 0.6	1094 ± 45	1710 ± 47
S5	herbicide	200 ± 3.3	29.0 ± 0.5	52.8 ± 1.4 (42.4)	22.9 ± 2.9	834 ± 24	2393 ± 35
S6	herbicide	215 ± 5.5	73.9 ± 5.5	107 ± 5.1 (102.5)	5.0 ± 0.6	1354 ± 93	2340 ± 87
S7	herbicide	981 ± 21	77.8 ± 5.2	141 ± 4.1 (39.5)	12.1 ± 0.5	1144 ± 62	2294 ± 53
S8	herbicide	1221 ± 23	66.9 ± 0.7	96.8 ± 0.2 (91.3)	8.7 ± 0.5	1030 ± 8.7	3787 ± 29
S9	mining	949 ± 55	7.1 ± 0.5	27.1 ± 1.6 (5.5)	8.8 ± 0.5	528 ± 30	718 ± 60
S10	mining	1126 ± 120	2.9 ± 0.3	18.4 ± 2.0 (4.6)	5.4 ± 0.6	515 ± 33	578 ± 60
S11	mining	1695 ± 128	2.4 ± 0.2	18.2 ± 1.5 (0.5)	4.8 ± 0.4	243 ± 16	375 ± 26
S12	mining	1306 ± 104	10.4 ± 1.0	24.6 ± 2.0 (0.8)	9.2 ± 0.8	384 ± 22	628 ± 52
S13	mining	2270 ± 71	14.0 ± 0.6	32.1 ± 1.2 (0.9)	11.9 ± 0.5	479 ± 20	838 ± 36

[a]Values represent the mean and standard deviation of triplicate analysis. [b]Values in parentheses indicate citrate dithionite extractable Fe (reactive Fe).

training set was utilized to construct the in vivo-in vitro linear regression model while the holdout set was used to test the strength of the relationship. The holdout method was repeated 10 times with randomly selected training and holdout sets and the estimated accuracy was calculated by averaging the runs.[30]

Leave One out Cross Validation. As the name suggests, leave one out cross validation involves combining all but one data point from this study and Juhasz et al.[22] and utilizing these as the training set with the remaining data point used as the test set. This analysis is repeated so that all data points act as the test set with the estimated accuracy calculated by averaging the runs

As it appeared that one soil (S3) biased the results, cross validation analysis was also repeated with this sample withheld to determine its influence on As RBA prediction accuracy. The suitability of SBRC-G for predicting As RBA was based on the identification of a linear regression model that had little model bias (i.e., mean bias error close to zero), little error (i.e., low root mean square error) and the correspondence was as close as possible (i.e., the slope of the line of best fit was close to unity). If the resulting analysis indicated that As RBA prediction accuracy was high, a grouped model (slope and *y*-intercept) was calculated. Mean bias error and root mean square error were calculated according to eqs 3 and 4 respectively in the SI.

RESULTS AND DISCUSSION

As-Contaminated Soils. Table 1 shows selected physicochemical properties of the soils used in the study. The total concentration of As in the <250 μm soil particle size fraction ranged from 81 to 2270 mg As kg^{-1} with mean values of 1469 and 443 mg As kg^{-1} for mining and herbicide impacted soils, respectively. Considerable differences in Al, Fe, and P concentrations were also observed between mining and herbicide impacted soils. Herbicide impacted soils contained significantly higher ($P > 0.05$) concentrations of Al (1.9−32.0-fold) and Fe (1.6−7.7-fold) compared to mining impacted soils due to their volcanic nature. As detailed by Cutler et al.,[31] the concentration of Al and Fe in volcanic soils develop over time as a result of the weathering of parent material (basalt). Soils with elevated Al and Fe concentrations result from their enrichment in ash dominated profiles compared to the lithic-rich profile.[31] In contrast, the mining impacted soils (gray slime tailings) had moderate Al and Fe concentrations within the range typically found in the goldmining region of Victoria.[22] The herbicide impacted soils also contained elevated P (1.3−10.1-fold higher) compared to the mining impacted soils. Background levels of P in these soils typically range from 1000 to 1500 mg kg^{-1},[32] however, higher P concentrations may result from the application of phosphate-based fertilizers such as bonemeal, superphosphate, triple superphosphate and monoammonium phosphate.[31]

Assessment of As Bioaccessibility. The SBRC gastric phase is a commonly utilized in vitro assay for the assessment of As bioaccessibility.[11,14,22,29,33] As detailed in Figure 1, As

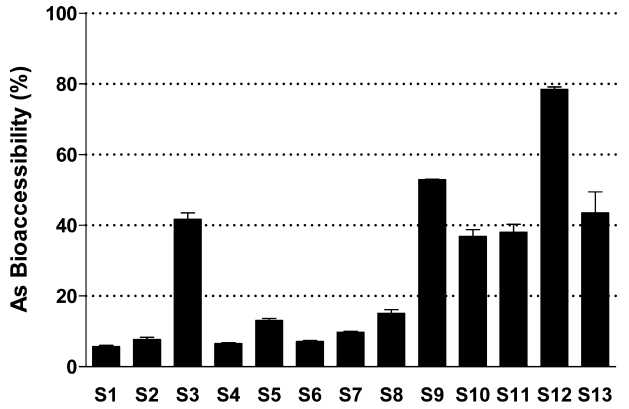


Figure 1. As bioaccessibility in contaminated soils determined using the gastric phase of the SBRC assay. Values represent the mean and standard deviation of triplicate analyses.

bioaccessibility in individual soils was variable with values ranging from 5.7 ± 0.3% (S1) to 78.4 ± 0.8% (S12). The disparity in As bioaccessibility may arise from differences in As speciation and mineralogy in addition to other physicochemical properties of the contaminated matrix (e.g., Fe and P).[14,34−37] Although XANES analysis identified that As in contaminated soils (predominantly arsenate) was present as mineral sorbed species (SI Figure S1), the disparity in As bioaccessibility may be attributed to differences in Fe, namely reactive Fe, in the respective soils. Beak et al.[36] determined that As bioaccessibility may be significantly reduced in the presence of ferrihydrite due to the strong binuclear bidentate bonding of As with Fe oxide surfaces. Table 1 shows that the concentration of reactive Fe,

determined using citrate dithionite extraction, varied between samples with significantly higher values determined for herbicide impacted soils compared to mining impacted soils. As detailed by Cutler et al.,[31] reactive Fe in soil profiles of this nature (Acrudoxic Hydrudands) may constitute up to 80% of the total Fe concentration. In contrast, the mining-impacted soils contained lower concentrations of total and reactive Fe (Table 1) resulting in weaker association of As with minerals phases.

For 12 of the 13 soils used in this study, analysis of the within-laboratory repeatability of SBRC-G resulted in relative standard deviations (RSDs) ranging from 0.4 to 8.5% (mean = 4.4). A higher value was determined for S13 (13.7%) presumably due to greater variance in pH drift between replicate samples. Reanalysis of the SBRC-G data from Juhasz et al.[22] also confirmed the repeatability of SBRC-G with RSDs ranging from 0.3 to 6.8% (mean = 2.1). Low within-laboratory repeatability values (RSDs < 5.1%) following the assessment of As bioaccessibility in NIST 2710 standard reference material have been reported by Koch et al.[38] during a round robin study by 14 laboratories. In contrast, greater variability in within-laboratory repeatability (RSDs 26.7−35.7%) have been reported for other round robin studies utilizing different in vitro assays and As-contaminated materials.[26]

Assessment of As Relative Bioavailability. Mean As RBA in contaminated soils varied from 6.8 ± 2.4% to 70.5 ± 6.8% (Figure 2). These values fall within the As RBA range that has previously been reported for mining and herbicide impacted soils when assessed using an in vivo swine model.[12,14,19−21,24] Arsenic RBA was significantly lower (6.8−18.2%) in herbicide impacted soils compared to values measured in mining impacted soils (27.5−70.5%). It is well established that As speciation is a key parameter that influences As RBA.[11,19,39] Low As RBA is associated with As bearing minerals such as scorodite and arsenopyrite while high As RBA is associated with Ca−Fe arsenate minerals.[37] Although discrete As minerals were not present in As in herbicide impacted soils, XANES analysis identified that As was present as mineral sorbed species (SI Figure S1). The low As RBA in these soils was attributed to similar mechanisms which limited As bioaccessibility, that is, the strong binuclear bidentate bonding with Fe oxide surfaces.[36] Although determined using in vitro models, Yang et al.,[34] Rodriguez et al.[35] and Meunier et al.[37] proposed that the low As bioaccessibility in contaminated soils was a result of the association of As with reactive Fe oxide minerals. In contrast, total and reactive Fe was considerably lower in mining impacted soils with a corresponding increase in As RBA (Table 1).

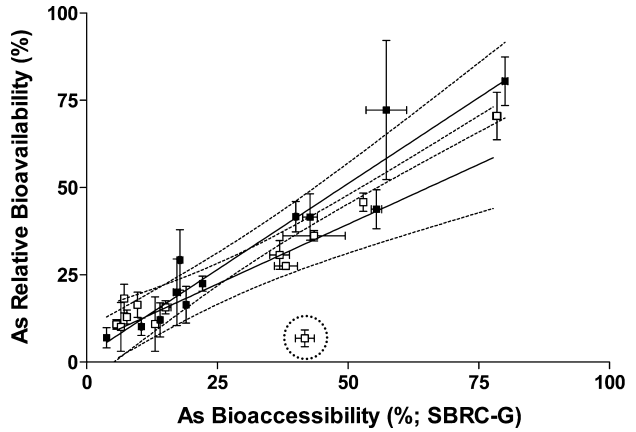


Figure 2. Relationship between As relative bioavailability (determined using an in vivo swine assay) and As bioaccessibility (determined using SBRC-G) for soils used in this study (□; n = 13) and for soils from Juhasz et al.[22] (■).

Correlation of As Relative Bioavailability and As Bioaccessibility. In order to determine the suitability of SBRC-G for predicting As RBA, the relationship between in vivo and in vitro data was ascertained and the As RBA-bioaccessibility linear regression model developed. Table 2 outlines the slope, y-intercept and goodness of fit for the linear regression model developed for the 13 independent mining and herbicide impacted soils. Comparison of in vivo and in vitro data indicated that there was a strong correlation between As RBA and SBRC-G with a goodness of fit of 0.75 (Table 2). The As RBA-bioaccessibility linear regression model detailed in Table 2 is similar to previously published correlations. When assessed using swine and SBRC-G, Juhasz et al.[14] and Brattin et al.[12] reported the slope of the in vivo−in vitro correlation to be 0.99 (R^2 = 0.75) and 0.62 (R^2 = 0.72) respectively. Similarly, using a mouse model for the assessment of As RBA, Bradham et al.[11] derived a slope of 0.72 (R^2 = 0.92) for the in vivo−in vitro (SBRC-G) correlation. As there are notable differences in As RBA methodologies (single versus multiple doses; AUC versus steady state urinary excretion) and physiological parameters associated with the in vivo models (i.e., swine versus mouse), these factors may contribute to variation in derived As RBA values and as a consequence influence in vivo−in vitro correlations.

Although a strong correlation was observed between As RBA and As bioaccessibility, one sample (S3) deviated significantly from the in vivo-in vitro relationship as highlighted in Figure 2. In S3, As bioaccessibility was 6.1-fold higher than As RBA. Presumably, the large difference in As bioaccessibility and As RBA for S3 arose from factors that influence As absorption that are not accounted for by the in vitro assays. For example, a number of studies have demonstrated the efficacy of Mn[40−42] and Mg[41,43,44] surfaces to sorb As. As S3 contained elevated concentrations of both Mn and Mg, dissolution and precipitation processes for these elements in the gastrointestinal

Table 2. Comparison of Linear Regression Models from This Study and Juhasz et al.[22] for Predicting As Relative Bioavailability Using SBRC-G

assay	linear regression model (this study)			linear regression model (Juhasz et al.[22])			comparison of models[a]		grouped model[b]	
	slope	y-inter.[c]	R^2	slope	y-inter.	R^2	slope	y-inter.	slope	y-inter.
SBRC-G	0.69	5.24	0.75	0.99	1.89	0.92	P = 0.06	P = 0.14	0.84	3.56

[a]Comparison of the slopes and y-intercepts of in vivo-in vitro models developed in this study and Juhasz et al.[22] P values are provided for a confidence level of 95%. [b]If there was no significant difference in the linear regression models, a grouped model (slope and y-intercept) was calculated, that is, As relative bioavailability (%) = slope x SBRC-G (%) + y-intercept. [c]y-intercept.

Table 3. Two-Way Cross Validation Analysis to Determine the Accuracy and Precision of As Relative Bioavailability Predictions Using SBRC-G

training set					holdout set			
data set[a]	n[b]	slope[c]	MBE[d]	RMSE[e]	data set[f]	n[b]	MBE[d]	RMSE[e]
this study	13	0.80	2.05	9.38	Juhasz et al.[22]	12	7.76	10.83
this study	12[g]	0.88	2.41	5.37	Juhasz et al.[22]	12	5.24	8.82
Juhasz et al. (2009)	12	1.03	0.62	6.52	this study	13	−4.07	12.23
					this study	12[g]	−1.42	7.37

[a]Data set used to construct the training set linear regression model. [b]Number of paired As relative bioavailability-SBRC-G data points. [c]Slope of the in vivo-in vitro linear regression models (with zero *y*-intercept) for the respective data set. [d]Mean bias error. [e]Root mean square error. [f]Data set (holdout set) used to test the strength of the in vivo-in vitro relationship. [g]S3 was omitted from the 2-fold cross validation analysis.

tract may influence (decrease) the As concentration that is available for absorption. In addition, it is known that phosphate can decrease the intestinal absorption of As^V as these elements share the same transport mechanism.[45] Potentially, the high P:As molar ratio in S3 may decrease As RBA due to the dynamic nature of the in vivo system (due to P competition) which will not be accounted for in batch in vitro assays. Other interactions may also influence the release and/or absorption of As from S3. Regardless of the reason, the results obtained for this sample were atypical for the soils analyzed in this study. As a consequence, during the validation analysis, the influence of including and excluding S3 was investigated to determine its impact on error estimates.

Validation of As Relative Bioavailability and As Bioaccessibility Relationships. Although a number of researchers have demonstrated a strong correlation between As RBA and As bioaccessibility,[11,12,14,20,22−24] validation of these relationships is yet to be established. Validation in this case refers to testing the predictive performance of a previously established in vivo-in vitro linear regression model by using independent data outside of the data set used to establish the initial correlation. As detailed in the Materials and Methods, a number of different strategies were employed to "validate" the As RBA-bioaccessibility (SBRC-G) relationship (2-fold, repeat random subsampling, leave one out cross validation analysis). In addition, the influence of S3 on error estimates was also determined.

Figure 2 shows a comparison of the relationship between As RBA and As bioaccessibility for the 13 soils used in this study and 12 soils from Juhasz et al.[22] Initially, model performance was assessed by comparing in vivo-in vitro correlations (n = 13 and 12 respectively) to determine whether there was a significant difference ($P < 0.05$) in the slopes and *y*-intercepts. Table 2 shows a comparison of linear regression models for predicting As RBA using in vitro assays and a grouped model if there was no significant difference between data from the current study and that of Juhasz et al.[22] For SBRC-G, there was no significant difference ($P > 0.05$) between the linear regression model developed in this study and from Juhasz et al.[22] indicating the consistency and validity of the relationship between As RBA (swine assay incorporating AUC analysis) and As bioaccessibility (SBRC-G) (Table 2). The similarity in the in vivo−in vitro relationships satisfies the predominant validation criterion detailed by Juhasz et al.[25] For these data, a grouped linear regression model was developed with a slope and *y*-intercept of 0.84 and 3.56 respectively (i.e., As RBA = 0.84 × SBRC-G + 3.56). Further analysis determined that the p-value for the test of significance of a zero *y*-intercept was 0.26 indicating that the *y*-intercept was not significantly different from zero.

Alternative strategies were also employed to validate the As RBA-bioaccessibility relationship. As the *y*-intercept was not significantly different from zero, training models were generated with a zero *y*-intercept. Initially, 2-fold cross validation analysis was undertaken as this type of analysis has the advantage of maximizing the size of the training and holdout sets and each point is utilized for both training and testing purposes.[46] As a consequence, both data sets (this study and Juhasz et al.[22]) were utilized for training and testing with and without the inclusion of S3 (the outlier sample). The accuracy of model predictions was determined by calculating mean bias error (MBE; a measure of the overall bias or systematic error) and root-mean-square error (RMSE; the difference between values predicted by the model and the values actually observed). Residuals and prediction errors were calculated for samples used for the estimation and for holdout samples, respectively.

Table 3 shows 2-fold cross validation error measures and slopes of the lines of best fit for training and holdout sets when SBRC-G was utilized for predicting As RBA. Residuals for the As RBA-SBRC-G training set (data from this study; 9.38) and prediction errors for the holdout set (data from Juhasz et al.;[22] 10.83) were low indicating the accuracy of the in vitro assay for predicting As RBA. Similarly, when training and holdout sets were reversed, low residuals (6.52) and prediction errors (12.23) were also observed (Table 3). When the As RBA-SBRC-G model was constructed with data from this study, As RBA predictions using SBRC-G values from Juhasz et al.[22] were higher (on average) than the true value as indicated by the MBE (7.76) (SI Figure S2). In contrast, using SBRC-G values from this study and the As RBA-SBRC-G model from Juhasz et al.[22] predicted As RBA values were on average lower than the true value (MBE = −4.07) (SI Figure S2) which was reflective of the difference in the slopes of the linear regression models (0.80 versus 1.03).

As highlighted in Figure 2, one data point (S3) exerted leverage over the data set by influencing the linear regression. Inclusion of this data point influenced the RMSE for the training set in addition to both MBE and RMSE for the holdout set. To investigate its impact, S3 was omitted from the data set -and the 2-fold cross over analysis repeated. As expected, omitting S3 from the model estimation process, resulted in a reduction in RMSE for both training (from 9.38 to 5.37) and holdout sets (from 10.83 to 8.82 and 12.23 to 7.37) resulting in an improved model fit (Table 3; SI Figure S2).

To further test the strength of the As RBA-SBRC-G relationship, an alternative holdout validation analysis was undertaken using a combined data set comprising in vivo and in vitro data from this study and Juhasz et al.[22] Approximately two-thirds (n = 17) of the data was randomly selected to construct the in vivo-in vitro linear regression model (training

Table 4. Repeat Random Sub-Sampling and Leave One out Cross Validation Analysis to Determine the Accuracy and Precision of As Relative Bioavailability Predictions Using SBRC-G

	training set							holdout set				
		slope[b]		MBE[c]		RMSE[d]			MBE[c]		RMSE[d]	
analysis	n[a]	mean	z[g]	mean	st dev	mean	st dev	n[e]	mean	st dev	mean	St Dev
repeat random subsampling cross validation[f]	17	1.03	0.02	0.11	1.06	7.94	2.23	8	1.30	3.16	11.24	3.71
	16[h]	1.02	0.03	−0.76	0.74	6.43	0.68	8	−1.89	2.46	6.91	1.70
leave one out cross validation[i]	24	0.92	0.01	1.27	0.18	9.09	0.57	1	1.22	9.70	9.58	3.05
	23[h]	0.96	0.01	1.48	0.15	6.52	0.25	1	1.46	6.94	6.95	1.82

[a]Number of paired As relative bioavailability-SBRC-G data points used for in vivo-in vitro linear regression model construction. [b]Slope of the in vivo-in vitro linear regression models (with zero y-intercept). [c]Mean bias error. [d]Root mean square error. [e]Number of paired As relative bioavailability-SBRC-G data points used to test the strength of the in vivo-in vitro relationship. [f]The cross validation method was repeated 10 times utilizing randomly selected training (∼2/3 of the data) and holdout (∼1/3 of the data) sets from this study and Juhasz et al.[22] Values reported (slope, MBE and RMSE) represent the mean and standard deviation of 10 analyses. [g]95% confidence interval for the mean of the slope of best fit for the training set. [h]S3 was omitted from the cross validation analysis. [i]All but one of the paired As relative bioavailability-SBRC-G data points from this study and Juhasz et al.[22] was used for in vivo-in vitro linear regression model construction while the remaining data point was used to test the strength of the relationship. Values reported (slope, MBE and RMSE) represent the mean and standard deviation of 24 (S3 omitted) or 25 (complete data set from this study and Juhasz et al.[22]) analyses.

set) while the remaining data (holdout set; $n = 8$) was used to test the strength of the relationship. Replication ($n = 10$) of the random allocation of data to training and holdout sets was undertaken to determine the variability in error measures and the slopes of the lines of best fit. The number of random data allocations was set to 10 as previous model accuracy estimations using cross validation analysis suggest that additional folds do not offer any advantage.[30] In addition, to determine the influence of S3 on prediction accuracy, analysis was undertaken with this data point included and excluded (Table 4).

Table 4 shows mean repeat random subsampling validation error measures and mean slopes of the lines of best fit for 10 randomly selected allocations of training and holdout sets for As RBA prediction using SBRC-G. As observed for the 2-fold cross validation analysis, mean residuals for the training set (7.94 ± 2.23) and prediction errors for the holdout set (11.24 ± 3.71) were low (not significantly different between the 2-fold cross validation methodologies; $P > 0.05$) indicating the accuracy of SBRC-G for predicting As RBA. However, due to the larger data set used to construct the As RBA-SBRC-G linear regression model compared to the 2-fold validation approach, MBE was reduced in the repeat random subsampling validation analysis for both training (0.11 ± 1.06) and holdout sets (1.30 ± 3.16) (Table 4). The low MBE in the holdout set indicates that predicted As RBA values were on average similar to the true or measured values. When S3 was omitted from the model estimation process, error measures were reduced for both training and holdout sets although only significantly ($P < 0.01$) for the holdout set (SI Figure S3). Mean bias error was also influenced by the omission of S3 although predicted As RBA values were again on average similar to the true value (Table 4). As detailed by Kohavi et al.,[30] utilizing this validation approach may represent a pessimistic estimator as only a portion of the data is given to the inducer for training. However, if additional data is held for testing, this may influence (i.e., increase) the bias of the estimation.

To test the influence of testing numbers on model bias, As RBA-SBRC-G model performance was assessed using leave one out cross validation. All but one data point from this study and Juhasz et al.[22] were combined and utilized as the training set with the remaining data point used as the test set. This process was repeated so that all data points acted as the test set with the estimated accuracy calculated by averaging the runs. The leave one out cross validation approach is almost unbiased, but has high variance as more of the data is held for testing.[47] However, this analysis provides an approach for identifying individual samples which exhibit higher variance from model predictions (SI Figure S4) which may be the focus of future research efforts to determine the mechanisms influencing model prediction of As RBA. For example, the model significantly overpredicts As RBA for S3 and under-predicts measured As RBA for samples #2, #24 (from Juhasz et al.[22]), and S6. However, as detailed in Table 4, training and holdout set RMSEs (9.09 ± 0.57 and 9.58 ± 3.05, respectively) were similar to those calculated for repeat random subsampling validation analysis. Although holdout set MBEs were similar for leave one out (1.22) and repeat random subsampling (1.30) validation analyses, as expected higher variance was observed for the leave one out analysis resulting in standard deviations of the mean of 9.70 compared to 3.16 (Table 4). When S3 was omitted from the model estimation process, RMSE was significantly reduced ($P < 0.001$) for both training (9.09 ± 0.57 to 6.25 ± 0.25) and holdout sets (9.58 ± 3.05 to 6.95 ± 1.82). Holdout set MBE was also influenced by the omission of S3 resulting in a reduction in the variance for MBE (Table 4).

Application of in Vivo-in Vitro Linear Regression Models for Predicting As Relative Bioavailability. Site-specific As RBA data may be used to refine exposure for the incidental soil ingestion pathway.[48] Exposure refinement has the potential to influence human health risk assessment which in turn may impact site-specific remediation targets. This was demonstrated by Walker and Griffin[49] at the Anaconda Superfund Site where As RBA data, determined using cynomolgus monkeys, reduced the risk from exposure to As-contaminated soil by approximately an order of magnitude (1.7×10^{-4} to 4.0×10^{-5}). However, the routine use of As RBA data for exposure adjustment is limited due to the prohibitive costs of in vivo assays. This limitation may be overcome through the use of As RBA-bioaccessibility linear regression models for the prediction of As RBA using As bioaccessibility data derived from simple, rapid, inexpensive in vitro assays. The SBRC-G assay represents a robust, reproducible (with low within-laboratory repeatability RSD) in vitro approach for

predicting As RBA.[11,12,22] As detailed in Table 2, a grouped model encompassing all data from this study and Juhasz et al.[22] (n = 25) could be utilized to predict As RBA using SBRC-G data. This model satisfies a number of validation criteria[25,26] including a correlation coefficient of >0.8 (0.91), a slope of >0.8 and <1.2 (0.84) and a small y-intercept (3.56). In addition, as the y-intercept was not significantly different from zero (P = 0.259), a model with a zero y-intercept and a slope of 0.92 ± 0.05 (SE; r = 0.94) could equally be utilized. As both the aforementioned As RBA predictive models included data from the "outlier" sample, error measures could be reduced (see Tables 3 and 4) by the exclusion of S3 from the predictive model. This would result in an As RBA-SBRC-G linear regression model with a slope of 0.96 ± 0.04 (zero y-intercept) and a correlation coefficient of 0.96. Irrespective if S3 is included or excluded from the As RBA-SBRC-G linear regression model, the slopes of the zero y-intercept models are not significantly different from a slope of unity. When S3 was included, the 95% Confidence Interval for the slope was 0.82 to 1.02, while when S3 was excluded it was 0.89 to 1.03. In both cases, the P-value would be greater than 0.05 for the Null Hypothesis of the slope equaling unity.

■ ASSOCIATED CONTENT

Supporting Information

Details of methodologies (As bioaccessibility, As RBA), As speciation in contaminated soil (Figure S1) and measured versus mean SBRC-G predicted As RBA for different cross validation methodologies (Figures S2–S4) is available free of charge via the Internet at http://pubs.acs.org

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Notes

The authors declare no competing financial interest.

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