Bioaccessibility of Arsenic and Lead in Polluted Soils Using Three In-vitro Gastrointestinal Simulation Models

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Abstract. In human health risk assessment (HHRA), oral ingestion of soil can be a major route of exposure to many immobile soil contaminants. Development and validation of in vitro assays is currently being undertaken to avoid overestimation of pollutants absorbed by the human body when calculating total pollutant concentrations in HHRA. In this study, arsenic (As) and lead (Pb) bioaccessibility in polluted Australian soils (n = 6) was assessed using three in vitro assays: a physiologically based extraction test (PBET), Solubility/Bioavailability Research Consortium Assay (SBRC) and Unified Bioaccessibility Research Group Of Europe Method (UBM). In vitro results were compared among these three assays and the possible causes of their differences were discussed. A bioaccessibility-corrected HHRA was then conducted. Bioaccessibility varied greatly among metal(loid)s and methods, and extending the three assays from the gastric to the intestinal phase generally resulted in decreased As and Pb bioaccessibility. Using these bioaccessibility values, both hazard index (HI) and carcinogenic risk (CR) were calculated, and were found to be higher than threshold values in most samples, indicating a potential health risk to local inhabitants.

1. Introduction

Soil is the major sink for trace elements released into the environment due to its high metal-scavenging potential [1]. For that reason, soil contamination is now receiving increasing research attention in terms of restoring soil ecosystems and sustainable use. High concentrations of arsenic (As) and lead (Pb) have been observed in soils around mining and smelting sites, orchards, agricultural lands, and other contaminated areas. As a class 1 carcinogen (i.e., carcinogenic to humans), As has adverse health effects, including dermatological and cardiovascular effects, pulmonary disorders, and reproductive and neurological effects [2]. As a heavy metal, Pb toxicity is strongest in the nervous system; it can severely damage the brain and kidneys and ultimately causes death at high levels [3].

In vivo animal models (e.g., swine and mouse) have been employed to determine the relative bioavailability of As and Pb in soils. Due to the operating time, cost and ethical issues of such methods, in vitro assays were developed to assess oral bioaccessibility of metal(loid)s in soils. Oral bioaccessibility is defined as the fraction of the contaminant released from the food matrix into the digestive juice chime that is available for absorption [4]. In recent years, numerous in vitro assays, including the physiologically based extraction test (PBET), Solubility/Bioavailability Research
Consortium Assay (SBRC) and Unified Bioaccessibility Research Group of Europe Method (UBM), have been applied to assess As and Pb bioaccessibility in contaminated soils. Bioaccessibility results vary significantly with soil/solution ratio, gastric and intestinal pH, solution composition and other factors, and so should be compared within and among in vitro assays.

Within assays, As and Pb bioaccessibility showed large variability between the gastric phase (GP) and intestinal phase (IP). Juhasz et al. found that for the SBRC and in vitro gastrointestinal extraction (IVG) assays, As bioaccessibility in contaminated soils was reduced in the IP, corresponding to reduced soluble Fe from the gastric to IP and suggesting that dissolved As is absorbed by amorphous Fe through surface complexation or ligand exchange with surface hydroxyl functional groups [5]. As reported by Li et al., Pb bioaccessibility also sharply decreased, to 0.01–20%, on SBRC, PBET, IVG and UBM assays, possibly due to Pb sorption to solids at the higher pH of the IP [6]. In addition to variability within assays, there is significant variability in As and Pb bioaccessibility values among assays. Juhasz et al. compared As bioaccessibility in 12 As-contaminated soils using the SBRC, PBET, standardized German In Vitro (DIN) and IVG assays [5]. Their results showed that As bioaccessibility, based on the GP in SBRC, is significantly higher than in the PBET, DIN and IVG, mainly due to variability in gastric pH. Li et al. compared Pb bioaccessibility among 12 Pb-contaminated soils using SBRC, PBET, UBM and IVG [6]. Lead bioaccessibility was found to vary among assays, with SBRC (3.0–99%) producing significantly higher values than other assays (0.46–84%) in the GP. However, the reasons for these differences within and among assays remain unclear due to a paucity of studies comparing in vitro assays, and future research should address the trends and causes of this variability.

The main objective of this study was to investigate As and Pb bioaccessibility within and among in vitro assays in contaminated soils. In six samples from Australia, total As and Pb concentrations and bioaccessibility were assessed with the SBRC, PBET and UBM assays, after which a bioaccessibility-corrected HHRA was conducted to estimate the potential health risk to local inhabitants.

2. Materials and methods

2.1. Contaminated soils
Surface soil samples (0–20 cm) containing elevated concentrations of As and Pb were collected from six locations in Australia, including sites in Victoria (TP39, GA12, GA13, CS005) and New South Wales (BHK1) associated with mining activities, and in South Australia (SH15) associated with nonferrous slag application [7]. Following collection, the soils were oven dried (105 °C), sieved to obtain the ingestible fraction (particle size, < 250 μm), homogenized through end-over-end rotation (45 rpm) for 24 h, and then stored at 20 °C until determination of total metal(loid) concentrations and bioaccessibility assessment through three in vitro assays.

2.2. Total concentrations
Total metal(loid) concentrations were analyzed by pre-digesting 0.2 g of each soil sample (n = 3) overnight with 5 mL aqua regia (1:3, 70% HNO3: 36.5% HCl), followed by digestion in a MARS-6 microwave (CEM, USA) following USEPA method 3051A. Dissolved metal(loid)s were separated from the solid residue via syringe filtration (0.45 μm), stored at 4 °C and analyzed by inductively coupled plasma mass spectrometry (ICP-MS) (7500ce; Agilent, USA) following EPA Method 6020A.

2.3. Three in vitro assays
SBRC is the simplest and most commonly used assay for evaluating the bioaccessibility of As and Pb [8]. This assay contains two phases: gastric and intestinal. The gastric solution consists of 0.4 M glycine adjusted to pH 1.5 using HCl. Soil is added to the GP at a solid-to-solution ratio of 1:100. Once the soil has been added and the pH is within the desired gastric range, the solution is rotated end-over-end for 1 h. Then, 4 mL of GP solution is collected and centrifuged for 15 min at 4,500 rpm. For the small IP, 1.75 g/L bile and 0.5 g/L pancreatin are added and the solution is adjusted to a pH of 7 ±
0.20. Once intestinal-phase conditions have been achieved, the sample is placed back on the end-over-end shaker for 4 h, after which 4 mL small intestinal solution is collected and centrifuged for 15 min at 7,500 rpm. Following collection of gastric and IP samples, the solutions are filtered (0.45 μm filter) and diluted to 50 mL with 1 N HNO₃ for ICP-MS analysis.

PBET is also a two-phase method, and is commonly used for the evaluation of As, Pb and Cd [9]. The PBET method differs from SBRC in terms of the solution composition, soil/solution ratio and GP pH. For this assay, 0.2 g of soil was extracted in triplicate using 40 mL per L of simulated gastric fluid consisting of 1.25 g pepsin, 0.5 g citrate, 0.5 g malate, 420 μL lactic acid, 500 μL acetic acid, 1 mL decanol and 1 L ultrapure water. Gastric pH was adjusted to 2.5 using HCl and a pH meter. After applying the same end-over-end mixing process for 1 h, 4 mL of supernatant liquor was centrifuged for 15 min at 4,500 rpm. For the small IP, 70 mg/L bile salt and 20 mg/L pancreatin were then added and the pH was adjusted to 7. The small intestinal solution was subsequently treated in the same way as for the SBRC assay and finally diluted to 50 mL with 1 N HNO₃ for ICP-MS analysis.

In contrast to these assays, UBM includes a saliva phase prior to gastro-intestinal extraction, which simulates dissolution processes in the mouth. This method yields results that show strong correlations with swine data for As, Cd and Pb [10]. The test is carried out on 0.4-g dried and sieved (< 250 μm) samples. For each solid sample, two bioaccessible extracts are collected: one at the end of the GP and another at the end of the gastro-IP. Four types of digestive fluid are required: saliva (S), gastric fluid (G), duodenal fluid (D) and bile (B). All fluids are prepared the day use in the UBM extraction procedure. Each fluid constitutes two solutions, one inorganic (I) and one organic (O), in addition to specific enzymes. The soil/solution ratio is 1 : 37.5 and the GP pH is 1.2. The small IP pH is 6.5.

### 2.4. Data analysis

The bioaccessibilities (%) of As and Pb were calculated using the following equation [4]:

\[
\text{Bioaccessibility (\%) = \frac{\text{contaminant mobilized from soil during digestion(\mu g)}}{\text{contaminant present in soil before digestion(\mu g)}} \times 100\%}
\]  

(1)

Human health risk assessment (HHRA) for exposure to heavy metals in these contaminated soils via oral ingestion was conducted based on the hazard index (HI) and cancer risk estimation. The general exposure equations used in this study are based on the recommendations of Health Canada and the US Environmental Protection Agency (EPA) [11, 12]. Because As exhibits strong carcinogenic potential in humans, the carcinogenic risk (CR) for As was calculated, while noncarcinogenic risks were calculated for As and Pb. For exposure assessment, the chronic daily intake (CDI) of individual metals from incidental ingestion of soil was calculated using the following equation:

\[
\text{CDI} = \frac{C \times I_r \times E_f \times E_D}{W_{AB} \times T_A}
\]

(2)

where C is the total concentration of the soil metal (mg/kg) and I_r is the ingestion rate. According to US EPA data, I_r for soil was set as 100 mg/day [13]. E_f is the exposure frequency (days/year). According to the US EPA, E_f for soil is 210 days/year; E_D is the exposure duration (30 years); W_{AB} is adult body weight (70 kg); and T_A is the average time. For noncarcinogens, T_A = E_D; for carcinogens, T_A = 70 years [13].

The noncarcinogenic and CRs for individual metals were calculated using the following equations:

\[
\text{Hazardous Quotient = (CDI \times OBA)/ RfD}
\]

(3)

\[
\text{Carcinogenic risk = CDI \times OBAxSF}
\]

(4)

where OBA is the oral bioaccessibility of the metal(loid)s, RfD is the reference dose and SF is the slope factor. According to the US EPA, the toxicity response (dose response) to As is 0.0003 mg/(kg-day) and the SF is 1.5 per mg/kg/day for As [14]. However, the use of a single parameter for Pb risk assessment was rejected because this cannot account for different risk scenarios, according to land use and the population likely to be exposed, for example. Thus, a Pb risk parameter was developed based on the quantitative relationship between blood-Pb and soil-Pb concentrations [15].
In a previous study [16], an RfD value of 0.0035 mg/(kg·day) for Pb was chosen for HHRA, so the same value was applied in this study.

As exposure to two or more pollutants may result in additive or interactive effects, a hazard quotient (HQ) can be calculated to generate the HI for a specific receptor/pathway combination, using the following equation:

$$HI = \sum HQ_i$$

(5)

If HI exceeds 1, noncarcinogenic effects are possible, with a probability that tends to increase as the value of HI increases. An HQ > 10 is considered high and chronic risk. Cancer risk exceeding 0.0001 is considered sufficiently large to warrant remediation efforts [17].

2.5. Quality assurance

Appropriate quality assurance procedures and precautions were followed to ensure the reliability of this research. All experimental reagents used were of analytical reagent grade. Milli-Q water was used throughout the study. Reagent blanks were used to correct instrument readings. Experiments were carried out in triplicate, including acid digestion and in vitro assay experiments. One standard reference soil sample, NIST 2710a, and one blank sample were used in each experiment. The recovery rate of total NIS T2710a concentrations are 111.6% for As and 88.4% for Pb, which are within the acceptable range.

3. Results and discussion

3.1. Total As and Pb concentrations

Total As and Pb concentrations are presented in table 1. Total concentrations of As varied between 74 and 16,000 mg/kg, while those of Pb varied between 140 and 9,400 mg/kg. According to the Environment Protection Authority of Australia (2012), all total As concentrations measured herein were markedly higher (3.7–790 times) than the threshold value. Only one sample, TP39, had a Pb concentration below the threshold of 140 mg/kg, while all others clearly exceeded the threshold (2.2–31 times). The total high concentrations of As and Pb indicated that these soils were severely contaminated and were therefore ideal for estimating gastro-intestinal bioaccessibility and conducting HHRA.

| sample name | As (mg/kg) | Pb (mg/kg) |
|-------------|------------|------------|
| SH15        | 970        | 650        |
| TP39        | 3000       | 140        |
| BHK1        | 74         | 9400       |
| GA13        | 890        | 1300       |
| GA12        | 520        | 950        |
| CS005       | 16000      | 970        |
| Guideline   | 20         | 300        |

3.2. As and Pb bioaccessibility values based on three in vitro assays

Figure 1 presents the bioaccessibility data of As and Pb in six Australian soils based on three assays. Bioaccessibility varied among the in vitro assays employed. The As bioaccessibility in the GP varied from 5.5% to 54% among the three assays. According to the SBRC method, As bioaccessibility in GP varied from 11% to 54%; with PBET, it varied from 5.5% to 44%, while with UBM, it was 28% to 53%; this was higher than with the other two methods for the same four soil samples (SH15, BHK1, GA13 and GA12). In the small IP, As bioaccessibility varied from 5.6% to 49%.
Using the SBRC method, As bioaccessibility in IP varied from 11% to 40%, while with PBET, it varied from 5.6% to 49%; with UBM, it was 10% to 44%, which was higher than with the other two methods for the same four soil samples. Sample CS005 generally had the highest bioaccessibility value regardless of the assay used, and it also had the highest total As concentration. Samples SH15 and TP39 also had significantly elevated As bioaccessibility. Compared to GP, As bioaccessibility in IP was generally lower. This is because the higher pH in the IP of 7.0 may result in precipitation of dissolved Fe as amorphous Fe oxides, reducing the As concentration via adsorption and/or co-precipitation [18].

Pb bioaccessibility in GP varied among the three assays, from 2.6% to 77%. With the SBRC method, Pb bioaccessibility in GP varied from 9% to 77%. Using PBET, it varied from 2.6% to 39%. Meanwhile, with UBM, it was 12% to 63%. The PBET method had markedly lower values than the other methods. In IP, Pb bioaccessibility varied from 0.01% to 20%. Using the SBRC method, Pb bioaccessibility in IP varied from 0.5% to 14%; with PBET, it varied from 0.01% to 16%, while with UBM it was 3% to 19%. Pb bioaccessibility in IP was markedly lower than that in GP, which may be due to the higher pH of the small intestinal compartment, which causes chemical precipitation of the metals. The bile salts and pancreatin added to simulated small intestinal solution may also contribute to the decrease in bioaccessibility [19].
3.3. Comparison among three in vitro assays
Compared to PBET, the SBRC assay provided higher As bioaccessibility in the GP. In the IP, dissolved As was probably absorbed by precipitated iron oxides, causing a sharp decrease in the small IP of the SBRC assay. According to a previous study [5], in vitro assessment (SBRC, IVG, PBET and DIN) of the same soils showed that As bioaccessibility varied depending on the methodology employed. When the correlation between in vivo As relative bioavailability and in vitro As bioaccessibility was assessed, As relative bioavailability could be predicted using the GP or IP of the SBRC, IVG, PBET and DIN assays with varying degrees of confidence ($R^2 = 0.53–0.75$), and the in vitro assay encompassing the SBRC GP provided the best prediction of in vivo As relative bioavailability. The standardized in vitro assay in Europe, the UBM assay, has been shown to generally estimate higher As bioaccessibility values in both the GP and small IP compared to the other two assays. Despite this difference, comparison of the in vitro and in vivo results indicated that the correlation between As bioaccessibility and As relative bioavailability was similar irrespective of the in vitro phase used for determination [20]. The UBM that incorporated all phases provided the best in vivo–in vitro correlation (slope = 1.08; $R^2 = 0.59$), and thus was considered the most suitable batch method for assessing contaminant bioaccessibility developed to date.

The disparity in Pb bioaccessibility among methods was caused by differences in assay parameters. Gastric pH was observed to strongly influence Pb solubility in soils [21], and the lower gastric pH used in SBRC (pH 1.5) compared to PBET (pH 2.5) contributed to the higher Pb bioaccessibility estimated using SBRC. However, the pH used in UBM (pH 1.2) could not explain its bioaccessibility results, which were lower than those of SBRC. Differences in other assay parameters (e.g., soil/solution ratio) and gastric fluid components (e.g., chyme composition) were probably responsible for the UBM results. According to Van de Wiele et al., less Pb is dissolved under lower soil/solution ratio conditions [22]. Low soil/solution ratios may lead to underestimation of Pb bioaccessibility in soils due to limited metal solubility [21]. Compared to SBRC (1 : 100), UBM uses a lower soil : solution ratio of 1 : 37.5, which might inhibit Pb dissolution from soils, causing lower Pb bioaccessibility. Additional components used in the gastric fluids of the three methods (pepsin and mucin for UBM, glycine for SBRC, pepsin for PBET) may also have contributed to the variability in Pb bioaccessibility [23].

3.4. Human health risk assessment (HHRA)
Table 2 presents the HQ, HI and CR of the Australian soil samples. CR was only calculated for As.

4. Conclusions
This study focused on the bioaccessibility of As and Pb in contaminated soils from Australia through application of three in vitro assays. HHRA was then conducted to estimate the potential health risk to local inhabitants. The results indicated that the total concentrations of As and Pb in contaminated Australian soil samples from mining-impacted locations and a nonferrous slag application site were all higher than the Environment Protection Authority of Australia threshold values, aside from Pb in sample TP39, collected in a mining-impacted location. This finding indicates severe contamination, which requires bioaccessibility testing as well as HHRA. Bioaccessibility varied greatly among different metal(loid)s, and also showed marked variance in the GP and small IP. Compared to PBET, the SBRC assay had a higher As bioaccessibility value in the GP, while in the small IP, dissolved As was likely absorbed by precipitated iron oxides, causing a sharp decrease in the small IP of this assay. In contrast, the PBET assay exhibited an increase in As bioaccessibility from the GP to the IP for most soils. The UBM assay generally had higher As bioaccessibility values in both the GP and IP compared to the other two assays, and comparison of in vitro and in vivo results indicated that the correlation between As bioaccessibility and As relative bioavailability was similar irrespective of the in vitro phase used for its determination. Thus, UBM was considered the most suitable batch method for assessing contaminant bioaccessibility. Pb bioaccessibility in the IP was generally much lower than that in the GP with all methods. However, Pb bioaccessibility in GP varied widely among the
three methods: PBET values were always lowest, while four samples had their highest values with the UBM method. The disparity in Pb bioaccessibility among methods was caused by differences in assay parameters (e.g., pH, soil/solution ratio, and gastric fluid components). HHRA of contaminated Australian soils indicated very high risk to human health, regardless of the assay method applied. Use of a single parameter for Pb risk assessment was rejected in this study, with a parameter based on the quantitative relationship between blood-Pb and soil-Pb concentrations being used instead. Pb risk assessment should be further explored and discussed in future studies.

Table 2. Hazard quotient (HQ), hazard index (HI) and carcinogenic risk (CR) of the Australian fine soil samples (values exceeding thresholds are in bold).

| Sample | SBRC | PBET | UBM | Sample | SBRC | PBET | UBM |
|--------|------|------|-----|--------|------|------|-----|
| HQ     | GP 1.3 | 1.2  | 1.4 | HQ     | GP 0.41 | 0.27 | 0.68 |
|        | IP 0.74 | 0.70 |    |        | IP 0.30 | 0.34 | 0.59 |
|        | SH15   | 2.0  | 1.9 | HI     | 0.62   | 0.61 | 1.3 |
|        | GP 2.4E-04 | 2.3E-04 | 2.7E-04 |        | GP 7.1E-05 | 5.2E-05 | 1.3E-04 |
|        | IP 1.4E-04 | 1.4E-04 | 2.3E-04 |        | IP 5.2E-05 | 6.6E-05 | 1.1E-04 |
|        | CR (total) | 3.8E-04 | 3.7E-04 | 5.0E-04 | CR (total) | 1.2E-04 | 1.2E-04 | 2.5E-04 |
| HQ     | GP 4.4  | 3.4  | 2.4 | HQ     | GP 0.27 | 0.22 | 0.70 |
|        | IP 2.4  | 1.7  | 0.83|        | IP 0.17 | 0.27 | 0.46 |
|        | HI     | 6.8  | 5.1 | HI     | 0.44   | 0.49 | 1.2 |
|        | GA13   | 8.5E-04 | 6.6E-04 | 4.7E-04 | GA12   | 5.3E-05 | 4.2E-05 | 1.4E-04 |
|        | IP 4.7E-04 | 3.3E-04 | 1.6E-04 |        | IP 3.3E-05 | 5.3E-05 | 8.9E-05 |
|        | CR (total) | 1.3E-03 | 9.9E-04 | 6.3E-04 | CR (total) | 8.6E-05 | 9.4E-05 | 2.3E-04 |
| HQ     | GP 0.04 | 0.01 | 0.06| HQ     | GP 23   | 19    | 22  |
|        | IP 0.02 | 0.01 | 0.04|        | IP 17   | 21    | 12  |
|        | HI     | 0.06 | 0.02 |0.10    | HI     | 40    | 40  |34  |
|        | CS005  | GP 7.9E-06 | 2.2E-06 | 1.1E-05 |        | GP 4.5E-03 | 3.7E-03 | 4.3E-03 |
|        | CR     | GP 4.3E-06 | 2.2E-06 | 7.1E-06 |        | IP 3.3E-03 | 4.1E-03 | 2.2E-03 |
|        | CR (total) | 1.2E-05 | 4.4E-06 | 1.8E-05 | CR (total) | 7.8E-03 | 7.7E-03 | 6.6E-03 |

Acknowledgement
The author would like to acknowledge Educational Unit for Studies on Connectivity of Hills, Humans and Oceans (CoHHO) for financial support on this research.

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