In Vitro Bioaccessibility Assessment as a Tool to Predict the Toxicity of Bioremediation Products

N. Jebril1,2, R. Boden2 and C. Braungardt3

1Department of Biology, College of Sciences for Women, University of Babylon, Iraq.
2School of Biological and Marine Sciences, Plymouth University, Drake Circus, Plymouth PL4 8AA, United Kingdom.
3School of Geography, Earth and Environmental Sciences. Plymouth University, Drake Circus, Plymouth, PL4 8AA, United Kingdom.

*Corresponding author's e-mail: nadia.tawfiq@uobabylon.edu.iq

Abstract. The demand for the development of bioremediation processes designed to maintain healthy environments has increased; however, evaluation of the toxicity of its products is needed. Therefore, the toxicity of the Cd-loaded beads of the bioremediation approach developed in this paper was evaluated by using in vitro human gastrointestinal simulation (BARGE method). Cd-loaded beads were obtained from adsorption experiments of Cd from artificial groundwater (AGW) and natural river water (NRW, Walkham River, England) using Ca-alginate beads containing live cells of the mutant Brevibacillus agri C15 CdR and its wild type B. agri C15, in batch flasks. The results showed that the Ca-alginate beads containing the mutant adsorbed a significant concentration of Cd (1700 mmolal), related to its adsorption capacity. Cd-loaded beads had higher concentrations of Ca and Na (2030 ± 40 and 4300 ± 18 molal, respectively), related to its composition. The effects of the gastrointestinal simulation showed that Ca had the highest bioaccessible concentrations from Cd-loaded beads of all tested elements (Al, Ca, Co, Cu, Fe, K, Mg, Na, and Zn) from (1280 ± 13.00 molal); while some other elements were not detected at the end of the gastrointestinal system. Cd bioaccessibility was significantly lower in the Cd-loaded beads containing the mutant (0.17 and 0.14 molal in the gastric and gastrointestinal phases, respectively), compared to the wild type (0.23 and 0.19 molal, respectively). The bioaccessible fractions (BAFs) of Cd were significantly lower in the Cd-loaded beads containing the mutant at the gastric and gastrointestinal phase, with the mean of 4.85 % and 2.95 %, respectively. The low percentages of BAFs of Cd suggested that the products of the bioremediation process developed in this project might not be relevant as a human health risk.

1. Introduction:
Entrainment is used as a method to improve the stability and bioavailability of some bioactive materials, such as bacteria. A variety of natural matrixes have been used as entrainment carriers such as cellulose, starch, pectin, agarose gel, collagen, carrageenan, chitin, chitosan, agar-agar, and alginate. Alginate is naturally extracted from brown seaweed and bacteria. The gelation of alginate in calcium forms ca-alginate beads with a shape that is generally known as a cylindrical shape with rounded ends. Ca-alginate beads are used as an entrainment method in pharmaceutical [1], plant [2], medical [3] and adsorption [4] applications. Ca-alginate beads possess some unique properties, such as being cost-effective, non-toxic, biodegradable [5] and the design and development of bioremediation processes using Ca-alginate beads take advantage of their adequate metal – binding capacity [6]. The evaluation of designed bioremediation processes typically focuses on the estimation of their efficiency. However, the adsorbed concentrations of contaminants within the Ca-alginate beads may present an environmental or toxicological challenge. Therefore, the Ca-alginate beads produced by the bioremediation processes must be investigated, as their ultimate purpose is to reduce environmental contamination and toxicity in terms of using absorbent material and its secondary products. To evaluate the toxicity of the bioremediation products, bioaccessibility determination instead of the total
concentration is necessary. A range of methods are available, each of them representing a different and operationally defined, evaluation of bioavailability to either specific receptors (e.g., bioassay [7]) or biogeochemical setting as a proxy (e.g., redox condition [8]). Useful Ca-alginate beads are defined as absorbents that have no toxicity or health risk in case of accidental ingestion. For this purpose, they should be appraised using in vivo or in vitro assays to interpret their toxic effects under the gastrointestinal system of the human body. The bioaccessibility of metal in used Ca-alginate beads could be defined as the fraction of the adsorbed metal released from the beads into the gastrointestinal system to be accessible for intestinal adsorption. This metal fraction contributes to evaluating the beads’ toxicity. In vitro assays are being used as a standard regulatory purpose rather than in vivo assays, due to their operability, efficiency, and accessibility. Most importantly, in vitro assays are the most effective way to determine the bioaccessibility when it is impossible to assess the bioaccessibility of a matrix in oral human studies and to reduce the need for animal models due to ethical considerations. Specifically, the bioaccessibility can be determined through an in vitro human gastrointestinal simulation. This approach mimics the physiological conditions of the stomach and the small intestine in the human digestive system, while also taking into consideration the retention time and pH of each section of the system [9]. Various in vitro human gastrointestinal simulation assays have been used to evaluate the bioaccessibility of metals in the soil, including the physiological based extraction test (PBET), in vitro gastrointestinal (IVG), solubility bioaccessibility research consortium (SBRC) and the bioaccessibility research group of Europe (BARGE, called unified bioaccessibility method (UBM)) [10]. The BARGE method was designed to determine bioaccessibility for the assessment of the health risk to humans from metal contamination in soils and secondary products, such as food [11, 12]. By this method, the bioaccessible fraction of Cd has been determined previously in soils [13], particulate matter [14], fish [15] and food [16], and showing the possibility for determining the bioaccessible fractions in different products. Therefore, this study aims to evaluate the risk to human health arising from the products of the bioremediation process developed in [19] by estimating the bioaccessible fraction (BAFs) of Cd of Cd-loaded beads using the BARGE method. The objectives of this study were firstly to achieve Cd adsorption on the beads from AGW and NRW by Ca-alginate beads containing live cells of the mutant B. agric C15 Cd<sup>6</sup> or its wild type B. agric C15, in batch flasks. Secondly, the determination of the adsorbed concentration of Cd was estimated after aqua regia acid digestion using ICP-MS or ICP-OES, in addition to the elements of Al, Ca, Co, Cu, Fe, K, Mg, Na, and Zn. Thirdly, the element bioaccessibility within beads in the gastrointestinal tract was estimated using synthetic simulation solutions for gastric and gastrointestinal digestion. Finally, the BAFs of Cd was calculated from the total (after aqua regia acid digestion) and bioaccessible (after the gastric or gastrointestinal digestion) concentrations.

2. Materials and Methods:

2.1 Preparation of Ca-alginate beads and adsorption of cadmium

Ca-alginate beads containing either living cells of B. agric C15 [17] or B. agric C15 Cd<sup>6</sup> [18] were prepared using the entrapment method of calcium alginate gel, as described in [19]. In order to compare bead properties after adsorption of Cd from AGW or NRW, the beads of each strain were divided into two parts. One part was used for Cd adsorption obtained from AGW. The second part of the beads was used for Cd adsorption obtained from NRW (Walkham River). Preparation of AGW was according to [20] and NRW was collected from Walkham River (tributaries of Tamar River) at Magpie Bridge near Horrabridge, Plymouth, England, UK. The Walkham River was chosen on the basis that no major pollution was recorded [21]. The water sample was sterilized using membrane filtration (0.22 μm, Fisher brand) to avoid any interference. The water was analysed using ICP-MS for its chemical composition. The pH was measured, and the hardness was calculated as an average hardness (mmol/L), hardness as CaCO₃ (ppm), hardness as degree Deutsche Härte (°dH, CaO), and hardness as mg-CaCO₃/L (°Clark). The hardness of the Walkham River was classified according to the US Geological Survey (USGS) as soft water in mg-CaCO₃/L, mmol/L, °dH, and CaCO₃ ppm. To determine dissolved organic carbon (DOC), the dry ashing technique was used by placing a bottle (25 mL) in a muffle furnace at a temperature of 450 °C overnight. The river sample was preserved in the cleaned bottle and stored at ~ 20 °C to determine DOC using a Total Organic Carbon analyser (TOC-V) (Shimadzu TOC5000A). The elemental composition of AGW and NRW are provided in Table 1.
Table (1) the composition of AGW and NRW (Walkham River) was used in this experiment. ± presents the standard error of the mean (SEM) (n = 3 replicates).

| Element | AGW: concentration, unit | NRW: concentration, unit |
|---------|--------------------------|--------------------------|
| Al      | ** 2 ± 0.03 μM           | 2 ± 0.03 μM              |
| Ca      | 1.75 mM                  | 83 ± 0.2 μM              |
| Cl      | 1.75 mM                  | *                        |
| Cd      | ** 0.1 ± 0.02 nM         | 31 ± 0.8 nM              |
| Cu      | ** 103 μM                | 4 ± 0.1 μM               |
| K       | ** 2 ± 0.02 μM           | *                        |
| Mg      | 448 μM                   | 58 ± 0.6 μM              |
| Na      | 1.14 mM                  | 202 ± 6 μM               |
| NO      | ** 0.4 ± 0.06 nM         | *                        |
| Pb      | ** 448                   | *                        |
| DOC     | ** 0.037 ± 0.03 mg/L     | 8 ± 0.05 nM              |
| Zn      | ** 7.00                  | 6.78 ± 0.03              |

DOC: Dissolved organic matter.
*Not measured.
** No component added.

The batch adsorption experiments were carried out in flasks (250 mL Erlenmeyer flasks) to adsorb Cd into Ca-alginate beads, before the determination of the bioaccessible element fractions in loaded Cd-beads using the BARGE method. To acquire a constant adsorption level of Cd by the beads, the adsorption was carried out in a single batch experiment. The tests were performed separately for Cd adsorption from 100 mL of AGW or NRW, respectively, with a nominal concentration of 10 μM of Cd(NO₃)₂·4H₂O (Sigma-Aldrich) using the beads (100 g). The flasks were covered with aluminium foil and incubated at room constant temperature (22 °C) for five days, which were stirred twice a day using a glass rod.

2.2 Ca-alginate bead samples and pretreatment
After five days of the adsorption period, the Ca-alginate beads were collected from each batch using a plastic sieve, rinsed quickly with ddH₂O, and transferred into acid-washed and deionised ceramic drying boats. The bead samples were then weighted and dried to constant mass at 90 °C (Gallenkamp OV-160). The dry Cd-loaded beads were then ground and sieved to a fraction (< 250 μm particle size, using a mesh sieve) that is expected to be available for children’s hand-to-mouth contact [22].

2.3 Aqua regia acid digestion of Ca-alginate beads
The element concentration contained in the dried, sieved beads (0.6 g, n = 3, from each batch) was determined using an aqua regia acid digestion [23]. The results were used to determine the total concentrations of elements and to calculate the percentage of BAFs of Cd, which results from the in vitro human gastrointestinal simulation. The accuracy of the acid digestion for the beads was evaluated using a spike recovery test, according to the method described by [24] as there is no certified reference material for Ca-alginate beads. The spike recovery test was carried out by spiking the dried, sieved beads with Cd (0.6 g, n = 3 samples). The amount of 50 mg Cd was added to the samples (Ca-alginate beads without bacterial mass) as 0.6 mL of 89.28 mM Cd to 0.6 g of the beads in a Falcon tube (50 mL). The beads were mixed with the Cd using a class rode and left for 48 h, mixing frequently. Then, the beads were dried at room constant temperature (22 °C) before the aqua regia acid digestion. Comparably, aqua regia acid digestion was carried out with the same dried sieved bead before being spiked with Cd. The spike recovery was determined using the equation:

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\text{Spike recovery (\%)} = \frac{\text{Cd measured in spiked sample} - \text{Cd measured in unspiked sample}}{\text{Cd spiked}} \times 100
\]

The Cd measured in the spiked sample is the total amount of Cd determined at the end of the spike recovery test. In contrast, the Cd concentration determined in the unspiked sample is the total amount of Cd measured before the addition of Cd, and the Cd spiked is the nominated amount of Cd spiked into the beads.
2.4 Elements analyses:
The total concentrations of Al, Ca, Cd, Co, Cu, Fe, K, Mg, Na, and Zn in aqua regia acid digestion extracts of Ca-alginate beads before in vitro human gastrointestinal digestion and digestive fluids after the gastrointestinal digestion were analysed using ICP-MS (Thermo Scientific, X Series 2) or ICP-OES (Thermo Scientific, iCAP 700 Series). The instruments’ limited detection (LOD) are reported in Table (as five times the standard deviation (SD) of multiple (n =10) analysis of the lowest calibration standard. Table 2 also presents the results of the procedural blank samples (without any beads, n = 3 blanks). The digestive fluids after the gastrointestinal digestion were analysed for determining the total concentrations of elements.

Table 2 The LOD (derived from 5 X SD of n = 10 analyses) and procedural blank (n = 3), and analytical techniques for each element.

| Element | Analytical technique | LOD   | Procedural blank ± SEM | Unit |
|---------|----------------------|-------|------------------------|------|
| Al      | ICP-MS               | 0.13  | < LOD                  | μM   |
| As      | ICP-OES              | 0.01  | 0.02 ± 0.008           | mM   |
| Ca      | ICP-OES              | 0.01  | < LOD                  | mM   |
| Cd      | ICP-MS               | 0.02  | 0.003 ± 0.0004         | μM   |
| Co      | ICP-OES              | 0.06  | < LOD                  | mM   |
| Cu      | ICP-OES              | 0.01  | < LOD                  | mM   |
| Fe      | ICP-OES              | 0.3   | < LOD                  | μM   |
| Pb      | ICP-OES              | 0.01  | < LOD                  | mM   |
| K       | ICP-OES              | 0.9   | < LOD                  | μM   |
| Mg      | ICP-OES              | 0.8   | < LOD                  | μM   |
| Na      | ICP-OES              | 0.04  | < LOD                  | mM   |
| Zn      | ICP-OES              | 0.06  | < LOD                  | mM   |

The recovery of the aqua regia acid digestion of the British geological society reference soil (BGS-102, Wragg, 2009) was evaluated for Cd and other elements. The accuracy was low for some elements (Table 3): Ca (65.5%), Co (60%), Cu (67.5%) and Fe (48%). However, significant recovery percentages (p<0.05) of the other elements were as follows: K (100%), Cd (95%), and Zn (96%). The good accordance between the measured and the certified values for K, Cd, and Zn indicates that losses during experimental or analytical procedures were not responsible for the poor recovery of Ca, Co, Cu, and Fe.

Table 3 Results of aqua regia acid digestion, certified value, and recovery percentage of soil BGS-102. The recoveries were estimated from the mean of the recorded, and certified values and ± presents the standard error of the mean (SEM) (n = 3 experimental replicates). *Significant differences between recoveries.

| Element | Concentration (molal) | Recovery (%) |
|---------|-----------------------|--------------|
|         | Recorded value        | Certified value |
| Ca      | 459 ± 0.2             | 700 ± 7.5    | 65.5 ± 1.8 |
| Co      | 0.40 ± 0.005          | 0.67 ± 0.001 | 60 ± 3.0  |
| Cu      | 0.27 ± 0.002          | 0.40 ± 0.02  | 67.5 ± 2.2|
| Fe      | 115 ± 13.5            | 236 ± 14.3   | 48 ± 1.7  |
| K       | 30.3 ± 0.6            | 30 ± 2       | 100 ± 1.4*|
| Cd      | 2.35 ± 0.04*          | 2.45 ± 1.60* | 95 ± 1.8* |
| Zn      | 2.8 ± 0.05            | 2.9 ± 0.03   | 96 ± 1.3* |
Results of the Cd spike test (nominated amount of Cd = 50 mg) for the Ca-alginate beads was with the recovery of 00.08 ± 0.2 %, which was calculated from the Cd amount measured in spiked beads (50.08 ± 0.2 mg) and Cd amount measured in unspike beads (0.04 ± 0.0002 mg).

2.5 In vitro human gastrointestinal evaluation of Cd-loaded beads

The BARGE-in vitro human gastrointestinal simulation was carried out to determine the BAFs of Cd in the products, Ca-alginate beads, of the bioremediation process. The in vitro simulation consisted of two digestion phases, gastric and gastrointestinal. Four synthetic gastric -intestinal digestive fluids (saliva, gastric, duodenal, and bile) were prepared separately in sterile ddH₂O from analytical reagents in two different solutions, inorganic (I) and (I) according to masses and volumes specified in the BARGE protocol [10]. Then, the solution I and solution O of each type of fluid (i.e., saliva, gastric, etc.) were mixed, and the appropriate enzymes were added into the final volume of 500 mL. The fluids were stirred for at least three h using magnetic stirring (IKA-WERKE R015), and the pH of each fluid was adjusted with a pH meter (Thermo Scientific Orion Star Plus, calibrated at pH 4.0, 7.0 and 10.0). The fluids were used within 24 h of the day of preparation. For the gastric phase, 0.6 g of dried, sieved bead was added to a polypropylene tube (50 mL, n = 4, from each batch), salivary fluid at pH 6.5 ± 0.5 and gastric fluid at pH 1.1 ± 0.1, were added sequentially and shaken by hand for 10 s. The pH at this step was adjusted with NaOH (1 M) or HCl (~37 %) to 1.20 ± 0.05 (pH meter as above), followed by 10 s of shaking by hand, repeating three times until the pH remained stable. Then, the tubes were incubated at 37 °C for an hour and shaken using an end-over-end rotator (Grant-bio, PTR-60). After the incubation time, the pH was checked to be < 1.50. Two tubes from this phase were centrifuged for 15 mins at 4500 g; then, the supernatants were collected by prudently pipetting and acidified with 0.5 mL of concentrated HNO₃ (> 68 %). The procedural blank sample of the gastric phase (G₀) was the salivary and gastric fluids without any beads (n = 2 blanks). The other two tubes of gastric phases were then processed to the intestinal phase by the addition of the duodenal fluid at pH 7.4 ± 0.2 and bile fluid at pH 8.0 ± 0.2. The pH at this phase was adjusted to 6.30 ± 0.5, as mentioned above. Then, the tubes were incubated at 37 °C for 4 h and shaken using the end-over-end rotator. The pH of the samples was noted at the end of the incubation, followed by the centrifugation at 4500 g for 15 mins. The supernatants were collected to be acidified with 1.0 mL of concentrated HNO₃ (> 68 %). The procedural blank sample of the gastrointestinal phase (I₀) consisted of the salivary, gastric, duodenal, and bile fluids, respectively, without any beads (n = 2 blanks). Soil BGS-102 was used to validate the analytical chemistry.

2.6 Cadmium speciation and precipitation during BARGE experiments

The speciation and potential for precipitation of Cd within during the BARGE experiments were evaluated with thermodynamic equilibrium calculations. To this end, the inorganic chemical speciation at 22 °C was calculated using the geochemical speciation software Visual MINTEQ, version 3.1 [25]. The input files contained the inorganic components and concentrations of the gastric (pH 1.5) and intestinal fluids (pH 6.3), respectively, as well as Cd, Al, and Zn concentrations after incubation of the Ca-alginate beads containing the mutant and exposed to AGW, as specified in Table 4. The organic components contained in the BARGE fluids are assumed to form complexes that maintain Cd in solution and therefore, the inorganic speciation provides a worst-case scenario for precipitation. Oversaturated solids were allowed to precipitate, pH was fixed, ionic strength was calculated, the experiment was open to the atmosphere, and activity corrections were performed after Davies. The redox potential was fixed at E₀H = -200 mV, 0 mV +200 mV and 400 mV in separate calculations.

Table (4) the input files for Visual MINTEQ contained the inorganic components and their concentrations in gastric and intestinal BARGE fluids. The inorganic components of the BARGE solutions were entered, as well as the determined concentrations of Cd, Al, and Zn after incubation of the Ca-alginate beads containing the B. agri C15 or B. agri C15 Cd⁶ and exposed to AGW.
Mg$^{2+}$ & * & 0.9 \\
Na$^+$ & 47100 & 255200 \\
NO$_3^-$ & 2100 & * \\
NH$_4^+$ & 6100 & * \\
PO$_4^{3-}$ & 9600 & * \\
SO$_4^{2-}$ & 37500 & 150000 \\
* No component added.

2.7 Statistical analysis
The results were subjected to statistical analysis using one-way analysis of variance (ANOVA), followed by Tukey post hoc test in IBM SPSS statistics 22 software. This analysis helped to evaluate whether total and bioavailable concentrations of each element were significantly different. The bioaccessible fractions of Cd of each bead in each adsorption matrix were compared between gastric and gastrointestinal phases. SigmaPlot (version 13) was used to illustrate the data of bar charts. The data were expressed as mean ± standard error of the mean (SEM).

2.8 Calculation of bioaccessibility index for cadmium
The outcome of in vitro human gastrointestinal digestion on the Cd-loaded beads was expressed in terms of the BAF using the equation:

$$\text{BAF (\%)} = \frac{\left(\frac{\text{Cd}_{\text{bioaccessible}} \cdot \text{[Cd mol per kg beads]}}{\text{Cd}_{\text{total}} \cdot \text{[Cd mol per kg beads]}}\right) \times 100}{\text{ }}$$

The Cd$_{\text{bioaccessible}}$ is the total Cd concentration in digestive fluids at the end of both gastric and gastrointestinal phases. In contrast, the Cd$_{\text{total}}$ is the total Cd concentration in the bead before using in vitro digestion (measured from the aqua regia acid digestion). The results of this experiment were expressed as molal.

3. Results and Discussion

3.1 Adsorption of cadmium onto Ca-alginate beads
The adsorption of Cd by the beads was determined by measuring the concentrations of Cd, adsorbed from the different matrices (five days), after extraction using aqua regia acid digestion of the dried sieved beads. The adsorption experiments revealed higher total concentrations of Cd were adsorbed from water by the Ca-alginate beads containing live cells of B. agri C15 CdR (1700 mmolal), than by those containing live cells of B. agri C15 (1100 mmolal). The results showed no significant differences (Error! Reference source not found., $^*$p<0.05) in Cd concentrations adsorbed from the different matrixes of AGW and NRW.

3.2 Total concentrations of elements in Ca-alginate beads
Concentrations of Ca and Na (2030 ± 40 and 4300 ± 18 molal, respectively) in both types of beads and both adsorption matrixes were higher than other elements analysed (Figures 2A, 3A), and this is related to Ca and Na being components released from the alginate beads. There were no significant differences between Ca concentrations in Ca-alginate beads containing B. agri C15 used in either AGW or NRW (Error! Reference source not found.). In contrast, the concentrations of Ca in the Ca-alginate beads containing live cells of B. agri C15 CdR used in NRW was significantly higher ($^*$p<0.05) than those used in AGW. Similarly, the concentration of K in the Ca-alginate beads containing B. agri C15 was significantly higher when used in NRW ($^*$p<0.05) than AGW (Figure 2B). In Ca-alginate beads containing B. agri C15 Cd$^+$, the K concentration was similar in the beads used in either water (Figure 3B, $^*$p<0.05). The total concentrations of Al, Cd, Co, Cu, Fe, and Zn in both types of Ca-alginate beads were at least one order of magnitude below those of Ca (Figures 2B, C, 3B, C). More Al, Cd, and Fe recorded in Ca-alginate beads containing B. agri C15 Cd$^+$ (Figure 3C), compared to the Ca-alginate beads containing B. agri C15 (Figures 2C), and more Mg detected significantly in the adsorption from AGW than from NRW (Figures 2B, 3B, $^*$p<0.05) for either strain.
Figure (1). Concentrations of Cd adsorbed from AGW or NRW, with a nominal concentration of 10 μM Cd, onto Ca-alginate beads containing live cells of *B. agri* C15 or *B. agri* C15 Cd<sup>R</sup>. The adsorption concentrations were measured after *aqua regia* acid digestion of the dried sieved beads. # Significant differences between strains.

Figure (2) The total element concentrations of (A) Ca and Na, (B) K and Mg, and (C) Al, Cd, Co, Cu, Fe and Zn in the dried sieved Ca-alginate beads containing live cells of *B. agri* C15 after adsorption of Cd from AGW or NRW, determined after *aqua regia* acid digestion. The concentrations are mean, and the error bars indicate the standard error of the mean (*n* = 3). The concentrations were subjected to two-way ANOVA, Tukey post hoc test, and a different letter indicates a significant difference in the element concentrations between water, AGW and NRW.
Figure (3). The total element concentrations (A) Ca and Na, (B) K and Mg, and (C) Al, Cd, Co, Cu, Fe and Zn in the dried sieved Ca-alginate beads containing live cells of *B. agri* C15 Cd^6+ after adsorption of Cd from AGW or NRW, determined after *aqua regia* acid digestion. The concentrations are mean, and the error bars indicate the standard error of the mean (*n* = 3). The concentrations were subjected to two-way ANOVA, Tukey post hoc test, and a different letter indicates a significant difference in the element concentrations between water, AGW, NRW.

3.3 In vitro bioaccessibility of cadmium in Ca-alginate beads of two phases of the human gastrointestinal system

The bioaccessibility of Cd and other elements adsorbed by Ca-alginate beads from either AGW or NRW was evaluated by determining the element concentrations in different Ca-alginate beads in each digestion phase (gastric and gastrointestinal phases). As was the case for *aqua regia* acid digestion, Figures 4A, 5A, 6A, and 7A show that the Ca concentrations dominated the element distribution in gastric and gastrointestinal phases. Generally, higher element concentrations were recorded in the gastric phase than the gastrointestinal phase (Na, K, and Mg, Figure 5A and B; K and Mg, Figure 6B; Al, K and Mg, Figure 7B). Lowest total concentrations were recorded for Al, Co, Cu, Fe, Mg, Na, and Zn, and these were not detectable in either of the gastric or of the gastrointestinal phase.
Figure (4). The total (determined after *aqua regia* acid digestion) and the bioavailable (determined after the gastric and the gastrointestinal digestions) of the element concentrations of (A) Ca and Na, and (B) Al, Co, Cu, K, Fe, Mg and Zn in the dried sieved Ca-alginate beads containing live cells of *B. agricola* C15 after adsorption of Cd from AGW. All concentrations are mean, and the error bars indicate the standard error of the mean (*n* = 2). The concentrations were subjected to two-way ANOVA, Tukey post hoc test, and a different letter indicates a significant difference in the element concentrations between digestions.

Figure (5). The total (determined after *aqua regia* acid digestion) and the bioavailable (determined after the gastric and the gastrointestinal digestions) of the element concentrations of (A) Ca and Na, and (B) Al, Co, Cu, K, Fe, Mg and Zn in the dried sieved Ca-alginate beads containing live cells of *B. agricola* C15 after adsorption of Cd from NRW. All concentrations are mean, and the error bars indicate the standard error of the mean (*n* = 2). The concentrations were subjected to two-way ANOVA, Tukey post hoc test, and a different letter indicates a significant difference in the element concentrations between digestions.
Figure (6) The total (determined after *aqua regia* acid digestion) and the bioavailable (determined after the gastric and the gastrointestinal digestions) of the element concentrations of (A) Ca and Na, and (B) Al, Co, Cu, K, Fe, Mg and Zn in the dried sieved Ca-alginate beads containing live cells of *B. agr* C15 CdR after adsorption of Cd from AGW. All concentrations are mean, and the error bars indicate the standard error of the mean (*n* = 2). The concentrations were subjected to two-way ANOVA, Tukey post hoc test, and a different letter indicates a significant difference in the element concentrations between digestions.

Figure (7) The total (determined after *aqua regia* acid digestion) and the bioavailable (determined after the gastric and the gastrointestinal digestions) of the element concentrations of (A) Ca and Na, and (B) Al, Co, Cu, K, Fe, Mg and Zn in the dried sieved Ca-alginate beads containing live cells of *B. agr* C15 CdR after adsorption of Cd from NRW. All concentrations are mean, and the error bars indicate the standard error of the mean (*n* = 2). The concentrations were subjected to two-way ANOVA, Tukey *post hoc* test, and a different letter indicates a significant difference in the element concentrations between digestions. The concentrations of Cd in the dried sieved beads digested *aqua regia* ranged between 0.83 and 1.7 molal (Figure 8A and B, respectively), and much lower Cd concentrations in the gastric (0.23 – 0.17 molal) and gastrointestinal (0.19 – 0.14 molal) phases were only slightly different from each other.
Figure (8) The total (determined after *aqua regia* acid digestion) and the bioavailable (determined after the gastric and the gastrointestinal digestions) of the concentrations of Cd in the dried sieved Ca-alginate beads containing live cells of (A) *B. agri* C15 and (B) *B. agri* C15 CdR after adsorptions of Cd from AGW and NRW. All concentrations are mean, and the error bars indicate the standard error of the mean (*n* = 2). The concentrations were subjected to two-way ANOVA, Tukey post hoc test, and a different letter indicates a significant difference in the element concentrations between digestions.

The BAFs of Cd was significantly higher (Figure 9, *p* < 0.05) in Ca-alginate beads containing *B. agri* C15 than *B. agri* C15 CdR. The BAFs of Cd from Ca-alginate beads containing *B. agri* C15 CdR were lower for both gastric and gastrointestinal phases than beads containing *B. agri* C15, and lower when used for Cd adsorption from NRW than from AGW.

Figure (9) BAFs of Cd in the Ca-alginate beads containing live cells of (A) *B. agri* C15 and (B) *B. agri* C15 CdR from different adsorptions, in two phases of human gastrointestinal, the gastric and gastrointestinal phases. All percentages are mean, and the error bars indicate the standard error of the mean (*n* = 2). *Significant difference in the percentages of BAFs. # Significant difference between strains (A and B).

The BAFs of Cd in the secondary products of an adsorptive water remediation process, using *B. agri* C15 and *B. agri* C15 CdR entrapped in calcium alginate gel, were estimated to evaluate the effects of accidental ingestion by humans. The Ca-alginate beads were exposed to Cd within AGW or NRW for five days, after which Cd adsorption, as shown in previous study [19], has been confirmed. *Aqua regia* acid digestion of the sieved dried beads showed effective disintegration of the solid, with releases of high total concentrations of Ca and Na, which are the main components of the Ca-alginate beads, followed with the release of total...
concentrations of K and Mg due to their concentrations in AGW or NRW. Al, Cu, Fe, and Zn were recorded in lower concentrations in the digested Cd-loaded beads. These elements were not integral components of the bead structure and were not added to the AGW or recorded in the NRW (Table 2). The Cd concentrations released from the beads determined after *aqua regia* acid digestion were between 0.83 and 1.7 molal, and the significant concentration recorded from Ca-alginate beads containing cells of *B. agri* C15 Cd®, which were related to its adsorption capacity. The binding strength of metals with the Ca-alginate beads determines their bioaccessibility in the gastrointestinal system. Metal has a weaker binding strength to Ca-alginate beads, which causes a higher bioaccessibility and, consequently, a risk to human health upon ingestion. There was no statistically significant difference between AGW and NRW in the bioaccessible concentrations determined, and hence, the two matrices will not be discussed separately in the following paragraphs. It was important to determine the bioaccessible concentrations of Cd as well as of the elements, which are the main component in Ca-alginate beads, mainly Ca and Na. Studies have reported the relationship between calcium and sodium and hypertension in humans, whereas potassium is well known for its effects on electrolyte concentrations in cardiac electrophysiology [26]. For all elements in the Cd-loaded beads, the total concentrations (*aqua regia*) were higher than the bioaccessible concentrations. Under the effects of gastrointestinal simulation on element bioaccessibility, higher element concentrations were released in the gastric phase, than in the intestinal phase. Al, Co, Fe, K, Na, and Zn were not detected in either of the gastrointestinal simulation on element bioaccessibility, higher element concentrations were released in the gastric phase, than in the intestinal phase. Al, Co, Fe, K, Na, and Zn were not detected in either of the gastrointestinal or of the gastrointestinal phase. These results are comparable with previous studies, which showed that the BAFs of Al, Cu, Fe, and Zn in infant cereal samples were <LOD, 0.84%, 28%, and 1.1%, respectively [27]. The bioaccessible concentrations of Ca, K and Mg, were low in the intestinal phase than in the gastric phase, and these results are analogous with the study of evaluation of these elements in linseed and sesame, which showed that significant effects of the intestinal phase on these elements [28]. These differences in element bioaccessibility are associated with the characteristics of the Ca-alginate beads and digestive fluids. The elements were more prone to release under the stomach conditions (pH 1.5); as hydrogen ion concentrations increase, the acidic environment in the gastric phase increases the solubility of the divalent cations [29]. On the other hand, the human gastrointestinal phase is a less acidic (pH 6.3) environment, which may provide lower solubility. Thermodynamic equilibrium calculations (VMINTEQ 3.11) provided no evidence that precipitation took place in either gastric (pH 1.5) or intestinal fluids (pH 6.3) after BARGE over a wide range of redox conditions (Eh -200 to +400 mV). The calculated speciation shows Cd²⁺ (~37%) and CdCl⁻ (~43%) as dominant species in the gastric experiment, owing to the high stability constant of Cd-chloro complexes and high Cl⁻ concentration in the solution. In the intestinal experiment, Cd-sulfate complexes (total of 67%) dominated, due to high sulfate concentrations in the solution, with ~13% present as free Cd²⁺ ion. This indicates that re-adsorption of elements from the synthetic digestive fluids may have caused the reduction of the estimated bioaccessible concentrations and a decrease in intestinal absorption, rather than precipitation.

In confirmation of the above results, the BAFs of Cd in the gastric portions were higher (14.3 – 6.2%) than in the intestinal phase (4.85 – 2.95%). These results are comparable with previous studies, which showed that the BAFs of Cd decreased from the gastric phase (83.6%) to the intestinal phase (19.9%) [30]. The BAFs of Cd varied in the human gastrointestinal main due to the differences in the pH, as noted above. The differences in the pH change the solubility of Cd, and its solubility depends on its potential precipitation mineralogy. So far, few bioaccessibility studies have investigated the impact of mineralogy on BAFs of metals. Recently, Xing et al. [14] reported that the PbS and Pb₃(PO₄)₂ contribute to the low bioaccessibility of Pb due to their low solubility product constants (*K*ₚₛ) and consequently, precipitation from the synthetic digestive fluids. Olsson et al. [31] observed that the BAFs of As increased in the gastric phase due to the presence of sulphides, sulphates, and hydroxides of As, which have high *K*ₚₛ values, preventing precipitation and increasing their bioaccessibility. Results from this study suggest that the high BAFs of Cd in the gastric phase were possibly due to the formation of the mineral CdS (greenockite and hawleyite) in response to the acidic conditions. The *K*ₚₛ of CdS is high (8 ×10⁻¹⁹) [32], which is enough to contribute to the increase in Cd bioaccessibility. Conversely, when the pH was raised in the intestinal phase, the BAFs of Cd were lower, possibly due to the formation of minerals with low *K*ₚₛ in addition to CdS such as Cd(OH)₂ (7.2 ×10⁻¹⁹), causing the precipitation and the decreasing of Cd bioaccessibility. In addition, the intestinal phase was extracted after the gastric phase. Hence, the total Cd concentration remaining in the experiment had been lowered, compared to the start of the experiment. The BAFs of Cd resulting from using Ca-alginate beads containing the *B. agri* C15 Cd® were lower for both gastric and intestinal phases than the *B. agri* C15. This suggests that Ca-alginate beads containing cells of *B. agri* C15 Cd® bond Cd more firmly due to the coordination of Cd with functional groups on its cell surface that were not available in *B. agri* C15.
4. Conclusion
Once Cd removal has been achieved, non-hazardous waste (Cd-loaded beads) must be managed through land disposal units such as landfills, land treatment or land farming, and this disposal way puts the human under the risk from Cd toxicity [33]. Newly designed bioremediation processes must be evaluated for the toxicity of their absorbent materials and their secondary products, to satisfy the desire to use eco-friendly methods, and especially for regulatory toxicological values. Among the in vitro human gastrointestinal stimulation assay, the BARGE method plays a significant role in overcoming a current limitation of performing ecotoxicological experiments evaluating human health risks of contaminants in soils or secondary products. In this study, BAFs of the bioremediation products were evaluated using the BARGE method, which has been validated for Cd for the gastric and gastrointestinal extraction phases. It is the standard method for estimating the human risk from contaminated soil, tested on children aged ten years, and based on the BAFs of Cd in vitro human gastrointestinal simulation. BAFs of Cd significantly decreased at the end of the gastrointestinal system, and researchers use this reduction as a guide that oral exposure to Cd (within beads) does not pose a risk to human health. This method was considered in this study without food, indicating the worst incident situation for the possible absorption. This study suggested that BAFs of Cd could be figured as a real scenario when children accidentally ingest Ca-alginate beads, which contain Cd. The BARGE procedure has been applied to the Cd-loaded beads; however, this procedure can be applied to Cd-loaded beads and mixed with the soil to understand the accidental scenario of children’s hand-to-mouth contact. It would be possible to determine the Cd mineralogy in gastric and gastrointestinal phases under the BARGE assay [31] to investigate the differences of the BAFs of Cd in the gastrointestinal system between the strains and matrices.

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