Title: **Freeze-thaw action increased As in vivo and in vitro bioavailability in soils from derelict industrial sites**

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Abstract: Arsenic is a metalloid with carcinogenic properties and has been classified as a Category I carcinogen by the International Agency for Research on Cancer (IARC). Freeze-thaw processes affect the migration and transformation of soil heavy metals, as well as adsorption/desorption and redox reactions. However, there is limited research directly addressing the impact of freeze-thaw processes on the bioavailability of soil heavy metals. In this study, we focused on As and selected As-contaminated soil samples from three types of legacy sites in heavy industrial areas. Under controlled freeze-thaw experimental conditions, we utilized both in vivo and in vitro bioavailability measurement methods to investigate whether and how freeze-thaw processes affect the bioavailability of soil As. The results of this study showed that freeze-thaw processes reduced soil pH ($P<0.05$), CEC, SOM, and particle size, with decreases of 0.33, 1.2 cmol/kg, 5.2 g/kg, and 54 μm, respectively. However, it increased weight specific surface area (BET) ($P<0.05$), with an increase of 300 m^2/kg. Freeze-thaw processes increased the proportions of exchangeable ($P<0.05$), carbonate-bound, and iron-manganese oxide-bound As ($P<0.05$), but reduced the proportions of organic-bound and residual As ($P<0.05$). Freeze-thaw processes significantly increased the relative bioavailability and bioaccessibility of As, with increases of 32±9.6% and 13±0.23%, respectively. Soil pH, SOM, BET and electronic conductivity (EC) were identified as main factors contributing to the increased bioavailability of As due to freeze-thaw processes. These results provide new insights and evidence for refining the assessment of human health risks associated with heavy metal contamination in polluted soils.

Keywords: Arsenic contaminated soils; freeze-thaw cycles; gastrointestinal simulation method; mouse-based model; environmental risk

Environmental Implication

Research on the assessment of the bioavailability of pollutants in freeze-thaw soils remains limited. It has been shown that freeze-thaw can enhance the mobility of heavy metals, increase their bioavailability, and raise the risk of releasing heavy metals into the environment. Therefore, in-depth research on the effects of freeze-thaw on the bioavailability of soil heavy metals in human beings, revealing the change of soil heavy metal bioavailability in human beings under freeze-thaw conditions and its key factors, has important practical value and theoretical significance for the refinement of the human health risk assessment of soil heavy metals.

1. Introduction

Arsenic is a metalloid with carcinogenic properties and has been classified as a Category I carcinogen by the International Agency for Research on Cancer (IARC) [1]. Arsenic primarily enters the soil through anthropogenic activities, such as mining and coal combustion, and its associated human health issues have received widespread attention from various sectors [2, 3]. The main pathways for soil As to enter the human body are inhalation, ingestion, and dermal contact. Currently, unintentional ingestion of soil As through the "soil-human" pathway has become one of the primary exposure routes, especially for children who ingest soil during outdoor activities by sucking on their fingers or engaging in hand-to-mouth behaviors [4, 5]. In recent years, research on the human health risks of soil As has mainly focused on indirect As contamination risks through the food chain (soil-plant-human) [6], with less attention paid to the direct ingestion of contaminated soil. It is worth noting that previous studies have shown that soil particles with diameters < 250 μm are more likely to be ingested by humans [7]. The average daily intake for adults is 100 mg, while that for children can be as high as 200 mg [4]. Moreover, with the acceleration of urbanization, a large number of contaminated industrial sites have been relocated, leaving behind substantial amounts of polluted soil, posing a significant threat to the health of surrounding residents [8-10]. Therefore, it is necessary to conduct health risk assessments for As in urban contaminated sites.

Reliable analyses of human health risk assessments for the ingestion of As-contaminated soil are predominantly based on the estimation of bioavailability of As in soil. Currently, methods for assessing the bioavailability of soil heavy metals mainly include in vivo animal experiments and in vitro simulation experiments [11-17]. In vivo bioassays primarily involve the use of various animal models, exposing them to contaminated environments, circulating the contaminants within their bodies, and finally measuring the heavy metal content in their blood, gastrointestinal tract, and lymphatic tissues. Animal models commonly used for heavy metal determination include primates, pigs, rabbits, and mice. Related studies have shown that the mouse model has considerable advantages in measuring heavy metal bioavailability in vivo [18]. However, due to issues related to research duration, cost, and ethics, its large-scale application in heavy metal contamination site risk assessments is limited [18, 19]. At present, commonly used in vitro simulation methods include gastrointestinal simulation techniques [12, 20-23]. Gastrointestinal simulation methods measure the dissolution rate of soil contaminants in human gastrointestinal organs, which represents the bioaccessibility of the contaminants. According to surveys, there are more than 10 gastrointestinal simulation methods internationally. Related studies have demonstrated that Soluble Bioavailability Research Consortium (SBRC) [21], the Physiologically Based Extraction Test (PBET) [21, 24], and the Unified BARGE Method (UBM) [17, 25] exhibit good predictive performance for As bioavailability.

Freeze-thaw, as a natural phenomenon, is commonly found in climate change phenomena at high altitudes, high latitudes, or temperate regions, which essentially involves the freezing and thawing of soil water. It is a comprehensive ecological process involving changes in soil physical, chemical, and biological properties, affecting processes such as the migration and transformation of soil heavy metals, adsorption and desorption, and redox reactions[26, 27]. The physical effects produced by freeze-thaw accelerate the fragmentation of large particulate solid media, enhance soil water release and moisture permeability coefficients, and are accompanied by the dissolution and migration of soluble components during the melting process [28, 29]. Existing studies have shown that freeze-thaw can enhance the mobility of metal(liod)s, increasing the risk of their release into the environment [30, 31]. For example, a study on the effect freeze-thaw cycles on phosphorus forms and content of animal manure suggested that freeze-thaw cycles increased the mobility of phosphorus particle [32]. Hou et al (2020) demonstrated that freeze-thaw cycles increased soil cadmium and lead content [33]. Subsequently, Hou et al (2022) further found that damaged soil aggregates caused by freeze-thaw aging during the winter nongrowth period is the reason of remobilized soil cadmium [34]. A latest study based on four gastrointestinal methods have showed freeze-thaw cycles does effect on arsenic and lead bioavailability, yet this study is only based on in vitro methods [35]. Although direct evidence of the impact of freeze-thaw on the bioavailability of soil heavy metals is lacking, recent studies have found that the freeze-thaw processes reduce soil pH and cation exchange capacity, and with the increase of freeze-thaw cycles, the desorption of heavy metal ions also increases [31, 32, 36, 37]. Meanwhile, with the intensification of global climate change, changes in temperature and precipitation will directly alter freeze-thaw patterns, increasing the uncertainty of soil heavy metal bioavailability and their release risk [38]. Meng et al (2020) showed that freeze-thaw process might accelerate the process of increasing bioavailability levels of heavy metals caused by chemical, biological and physical process [39]. Therefore, it is necessary to further investigate the impact of freeze-thaw on the bioavailability of soil heavy metals to humans.

Based on the aforementioned research progress, this study selected As-contaminated soil samples from legacy sites in the northeastern heavy industrial area of China and investigated soil As bioavailability using a combination of in vivo and in vitro methods. Thus, this study aims to (1) investigate whether freeze-thaw affects As bioavailability; (2) explore how freeze-thaw affects As bioavailability in terms of soil physicochemical properties and As speciation. The research results provide a theoretical basis for assessing human health risks of soil heavy metals in permafrost regions.

2. Materials and Methods

2.1 Soil sample and processing

In this study, seven sites were selected for sample collection in the polluted industrial sites in northeast China. After entering the sample sites, the known pollution areas or areas close to the pollution sources were selected. Three typical types of industrial pollution sites in northeast China were selected: machinery manufacturing, steel coking, non-ferrous smelting. Five point samples were collected by the diagonal method within a 40m grid of the geographical centre of each site. The manual drilling method was adopted for sampling. The sampling depth was 0-20 cm of topsoil (impurities on the surface were removed to avoid plant residues and crushed stones). A total of seven soil samples were taken at each site. The samples were put into sealed bags, with < 1 kg taken at each location. The samples were air dried at room temperature, plant residues and other sundries removed, and gently sieved to pass 2 mm and 250 μm mesh sizes. The treated samples were then divided into two parts – the 2 mm soil samples for determination of soil physical and chemical properties and sequential extraction experiments, the 250 μm soil samples for gastrointestinal simulation and animal in vitro and in vivo experiments, respectively.

2.2 Freeze-thaw experiment design

Both of 2 mm and 250 μm sieved soil samples were used in the freeze-thaw experiment. The freeze-thaw experiment consisted of two treatment groups, the freeze-thaw group and the control group. For the freeze-thaw group, the soil freeze-thaw experiment was set to -15 °C to 6 °C, based on local meteorological data of the sampling area. The number of freeze-thaw cycles was set to 12 [40]. The freeze-thaw simulation experiment was carried out in a high and low temperature freezer, and seven soil samples were incubated with 48 hours as a freeze-thaw cycle, freezing temperature set at -5 ℃ for 24 hours, thawing temperature set at 15 ℃ for 24 hours and moisture content set at 60%. The control soils were maintained at room temperature (15-20 ℃) without freeze-thaw treatments. Total incubation period of each treatment was 24 days. After the freeze-thaw experiment, freeze-thaw soils and control soils were divided into four parts for soil physical and chemical properties, sequential extraction, in vivo experiment, and in vitro experiment, respectively.

2.3 Determination of soil properties and As speciation

The 2 mm soils were selected to determine the physicochemical properties (Table 1) and As speciation of all soil samples. Soil pH was determined by potentiometry; The cation exchange capacity (CEC) was determined by barium chloride sulfuric acid forced exchange method; The soil organic matter content (SOM) was determined by potassium dichromate titration; The soil particle size (PS) was determined by TopSizer laser; The soil conductivity (EC) was determined by conductivity meter; and the soil weight specific surface area (BET) was determined by BET method. The United States Environmental Protection Agency method (USEPA 3050B) was used to digest the soil, and inductively coupled plasma mass spectrometry (ICP-MS) used to determine the total concentration of As, Fe and Mn. In this study, the Tessier five-step extraction method was used to investigate soil heavy metal speciation. This consists of five fractions, namely exchangeable (F1), carbonate-bound (F2), iron-manganese oxide-bound (F3), organic matter-bound (F4), and residual (F5).

Table 1 Characteristics of seven control soils used in the study.

Samples	Soil 01	Soil 03	Soil 04	Soil 06	Soil 07	Soil 08	Soil 09
pH	7.40	7.25	7.48	7.55	7.32	7.50	7.49
EC (μs/cm)	59	77	48	53	214	80	45
CEC (cmol/kg)	7.5	6.1	6.9	1.3	3.1	9.3	13

Samples	Soil 01	Soil 03	Soil 04	Soil 06	Soil 07	Soil 08	Soil 09
SOM (g/kg)	34	30	20	26	25	61	87
BET (m^2/kg)	93	190	82	52	49	90	300
PS (μm)	95	110	110	200	220	120	63
Total As (mg/kg)	77	150	930	700	540	450	130
Total Fe (mg/kg)	38000	37000	49000	130000	94000	54000	38000
Total Mn (mg/kg)	800	890	3900	3700	1800	1900	910

Notes: pH: soil pH, EC: Soil electronic conductivity, BET: Weight specific surface area, PS: Particle size, Total Fe: Total concentration of Fe, Total Mn: Total concentration of Mn.

2.4 Relative bioavailability of As determined by in vivo experiments

The < 250 μm soil fraction was used as the test soil. In vivo assays including various animal models (eg, primates, pigs, dogs, rabbits, rodents) have been used to quantify the relative bioavailability of As in soil [13, 18, 23]. As they are similar to humans in terms of As metabolism, nutritional requirements, skeletal development, etc., the mouse bioassay has been considered as a suitable surrogate model for assessing relative As bioavailability for human health risk assessment [18, 22]. In this study, the commonly used mouse model was selected [21, 22, 41]. The concentration of As in the targeted organs (liver, kidney and femur) of mice was measured in the mouse in vivo experiment, and the relative bioavailability of As calculated, and the change of the relative bioavailability of As under the freeze-thaw soils and control soils is analyzed. Sodium arsenate mixed with contaminated soils was used as the standard reference [13] for constructing the standard curve of As exposure-dose.

In this experiment, the relative bioavailability of As in control and freeze-thaw soil was determined by the multiple feeding method with steady state exposure of the mouse model [21, 22, 41]. (1) Mouse domestication and food preparation scheme: 18-22 g female Balb/C mice were used to determine the relative bioavailability of As. Before the experiment, the mice were put into plastic cages and domesticated in animal rooms with 12 hours of light and night, 20-22 ℃ of temperature and 50% of humidity. During the domestication, the mice ate and drank freely. After 7 days, the mice were randomly assigned to the metabolic cage, one cage for each mouse, and three mice as a group. Each group of mice was exposed to a soil/reference substance (sodium arsenate), which was mixed with food in a 1:50 weight ratio. Set up three treatment groups: blank control, reference material and soil. (2) Mouse pollution exposure and sample collection: after the end of domestication, the mice were fasted overnight, weighed, and then allowed to eat food mixed with soil/reference materials. Each mouse ate a food ball every day for 2 weeks (14 days). During the process, the mice freely drank water. During the exposure, each mouse consumed about 4-5 g food every day. After two weeks (14 days) of exposure to the mixed food, the mice fasted for one night, weighed again, and then took mouse liver and kidney and femur samples, quickly stored them in the 80 ℃ refrigerator or liquid nitrogen, frozen for 4 hours, and dried them in a freeze dryer. (3) Determination and calculation of the relative bioavailability of As: the liver, kidney and femur samples taken out were freeze-dried and digested using the USEPA 3050B method, and the As concentration in the liver, kidney and femur samples was determined using ICP-MS. Mice with normal food intake were used as blank control, and 3 replicates were set for each treatment. The relative bioavailability of As is calculated as follows:

$$As\ RBA\ (\%) = \left(\frac{Con_{As_{Soil}}}{Con_{As_{Na_3AsO_4}}} \times \frac{Dose_{Na_3AsO_4}}{Dose_{As_{Soil}}}\right) \times 100\%$$

Where: Con_{AsSoil} represents the total As concentration measured in the liver, kidney and femur of mice after exposure to soil; $As_{Na_3AsO_4}$ represents the total As concentration measured in the liver, kidney and femur of mice after exposure to sodium arsenate. $DoseAs_{Soil}$ represents the dose of As contaminated soil; $Dose_{Na_3AsO_4}$ represents the exposure dose of sodium arsenate.

2.5 Relative bioavailability of As determined by in vitro experiments

The < 250 μm soil fraction was used as the test soil in this experiment. In this study, the UBM (Unified BARGE Method) was selected to determine the bioaccessibility of As in soil. This includes gastric phase experiment and intestinal phase experiment. The reliability of in vitro simulation methods in predicting bioavailability has been confirmed by the analysis of bioavailability measured by in vivo experiments and bioavailability results measured by in vitro experiments [17, 25]. Refer to the research of Denys et al. [42] and Juhasz et al. [21] for experimental components and parameters. The detailed information on the experimental parameters of UBM was provided in Table S1.

2.6 Statistical analysis

The mean value and standard error were used to characterize the relative bioavailability and bioavailability of As in control and freeze-thaw soils. t test was used to compare the differences in soil properties, As speciation fractions, As bioavailability between the freeze-thaw and control soils and determine the variation of As bioavailability between in vivo and in vitro experiments. Spearman correlation analysis was used to analyze the correlation between soil properties, As speciation fractions and As relative bioavailability, As bioaccessibility. All statistics and figures were carried out in OriginPro 2021.

3 Results and Discussion

3.1 Change of soil properties in control soils and freeze-thaw soils

Soil physicochemical properties are important indicators for evaluating soil conditions and analyzing soil changes. In order to understand the impact of freeze-thaw on the physicochemical properties of As-contaminated soil, this study measured the physicochemical properties of both control and freeze-thaw soils, comparing the changes in these properties under control and freeze-thaw soils.

In the control soils, the pH of the seven As-contaminated soil samples was 7.25-7.55, the SOM was 20-87 g/kg, the CEC was 1.30-12.60 cmol/kg, the PS was 63-220 μm, the BET was 49-190 m^2/kg, and the EC was 48-210 μs/cm. The soil samples in this study were collected from the northeastern region of China, and the background values of conventional soil physicochemical properties, such as acidity and alkalinity, were investigated. The selected soil samples represent the basic characteristics of the soil in the northeast of China [43].

In the freeze-thaw soils, the pH of the seven As-contaminated soil samples ranged between 6.73-7.48, the SOM was 14-79 g/kg, the CEC was 0.6-8.5 cmol/kg, the PS was 12-210 μm, the BET was 55-850 m^2/kg, and the EC was 55-190 μs/cm (Table S2). Comparing the soil physicochemical properties of the seven As-contaminated soils under control and freeze-thaw condition (Table 2), it was found that pH, CEC, SOM, PS, EC and BET all changed. The pH (P<0.05), CEC, SOM, and PS in the freeze-thaw soils all showed a decreasing trend, decreasing by 0.33, 1.2 cmol/kg, 5.2 g/kg, and 54 μm, respectively; while BET (P<0.05) and EC showed a significant increase in all seven freeze-thaw soils, increasing by 300 m^2/kg and 16 μs/cm, respectively. These results confirm that freeze-thaw affects soil physicochemical properties [31, 44-46], but the changing trends of different properties varied. It is worth to note that the decreased PS and increased BET (P<0.05) with the effect of freeze-thaw treatment. This result is due to the fact that freeze-thaw reduce the stability of soil aggregates, and the expansion of ice crystals generated in the soil voids during freeze process breaks up the inter-particle associations and breaks up large soil aggregates into smaller ones [45, 46]. Increased EC might relate to the soil water-salt movement caused by freeze-thaw cycles, because soil freeze causes a decrease in soil water potential in the freeze zone, which leads to soil water salts moving towards the frozen layer, resulting in a significant increase in soil water salts after thawing and increase the concentration of solutes in the soil solution [47, 48]. Besides of the change of soil physical structure, freeze-thaw action increased pH (P<0.05) and CEC. Their changes often are interrelated. Freeze-thaw processes promotes soil nitrification and the release of dissolved organic acids, resulting in a decrease in soil pH, and the decrease in soil pH reduces the negative charge carried on the surface of soil colloids, which causes a decrease in soil CEC [49]. In addition, changes in the phase state of moisture during freeze-thaw action lead to the contraction of organic matter, causing the destruction of binding sites with solid particles and increasing the release of organic matter; however, the destruction of soil particle size during freeze-thaw action produces more fine particles, clay particles, and organic colloids with a large specific surface area, which have a stronger adsorption of organic matter and other organic substances, and also lead to the redistribution of organic matter in the soil solution or solubilization [50, 51]. Thus freeze-thaw action has weak effect on the content of total organic matter.

Table 2 Differences in the soil properties and speciation between the freeze-thaw and the control soils. Values in bold show a significant difference between freeze-thaw and control soils.

Freeze-thaw vs. Control	**pH**	**CEC (cmol/kg)**	**SOM (g/kg)**	**BET (m^2/kg)**	**PS (μm)**	**EC (μs/cm)**	**F1 (%)**	**F2 (%)**	**F3 (%)**	**F4 (%)**	**F5 (%)**
Soil 01	-0.27	-1.8	-2.9	570	-73	79.8	0.51	0.32	0.78	1.4	-3.0
Soil 03	-0.52	-1.4	-7.2	319	-78	-15.5	0.39	-0.03	0.85	1.3	-2.5
Soil 04	0	0.33	-6.3	436	-72	13.3	0.16	0.06	0.02	-1.1	0.87
Soil 06	-0.13	-0.73	-7.8	3.5	12	20	1.1	0.05	1.1	-0.84	-1.4
Soil 07	-0.56	-0.34	-3.8	51	-79	-21.8	0.18	0.47	0.19	-0.27	-0.55
Soil 08	-0.52	-0.09	-0.79	169	-36	28.8	0.60	1.2	0.26	-0.49	-1.6
Soil 09	-0.3	-4.1	-7.7	553	-51	9.9	0.25	0.98	0.92	0.85	-3.0
Mean	-0.33	-1.2	-5.2	300	-54	16	0.46	0.44	0.58	0.11	-1.6
P	**0.01***	0.54	0.70	**0.02***	0.15	0.59	**0.01***	0.06	**0.001****	0.77983	**0.02***

Notes: Values with minus and plus symbols show a reduction and increase in the treated soils versus the control soils, respectively. F1: exchangeable forms, F2: carbonate bound forms, F3: Fe-Mn oxide bound forms, F4: organic bound forms, F5: residual forms. * P≤0.05, ** P≤0.001. The meanings of soil properties see the notes of Table 1.

3.2 Changes of As speciation in control soils and freeze-thaw soils

The bioavailability of As in soil is not only related to its total content but also closely associated with its speciation [52-54]. Soil As speciation refers to the physical or chemical forms of As existing in soil in various binding forms. When exogenous As enters the soil, it can interact with soil solution or soil matrix (such as organic matter, minerals, etc.) through dissolution, adsorption, complexation, and redox reactions to form different chemical forms. This study found that the average proportion of exchangeable As (F1) in control soils was 5.66% (4.74%-6.16%), carbonate-bound As (F2) was 8.61% (8.25%-9.13%), iron-manganese oxide-bound As (F3) was 18.3% (18.03%-18.78%), organic compound-bound As (F4) was 19.5% (18.18%-20.88%), and residual As (F5) was 47.94% (45.24%-50.18%). In freeze-thaw soils, the average proportion of F1 was 6.1% (5.72%-6.24%), F2 was 9.07% (8.78%-9.38%), F3 was 18.88% (18.66%-19.28%), F4 was 19.61% (19.04%-20.01%), and F5 was 46.34% (45.69%-47.18%) (Figure 1 and Table S3). These results indicate that the residual form has the highest proportion in the seven As-contaminated soils, followed by organic matter-bound, iron-manganese oxide-bound, carbonate-bound, and exchangeable forms. Overall, the mobility of As in the seven contaminated soil samples, the biotoxicity, and the risk of contamination were low, regardless of whether the soil was freeze-thaw soil or control soil.

The analysis of the changes of As speciation in As-contaminated soils under control and freeze-thaw condition (Table 2) showed that there were significant differences in the F1, F3, and F5 under the control and freeze-thaw soils ($P<0.05$). The proportion of F1 in the soil after freeze-thaw was significantly lower than that under control soils, decreasing by 1.59%; while the proportions of F1 and F3 in the soil after freeze-thaw were significantly higher than those under control soils, increasing by 0.46% and 0.58%, respectively. There were no significant differences in F2 and F4 under control and freeze-thaw soils. These results indicate that freeze-thaw affects As speciation, with a significant decrease in residual As and a significant increase in exchangeable and carbonate-bound As. Increased exchangeable As might be caused by the decrease of pH before and after freeze-thaw action. A decrease in soil pH reduces the negative charge on the surface of soil organic matter and oxides, which in turn reduces the adsorption of As ions by the soil and increases the solubility of As [55], resulting in an increase in the amount of As in the exchange form. In addition, increased exchangeable As could be from the transformation of other four forms. When soil pH decreases, As in the carbonate-bound form and iron-manganese oxide-bound form is readily released into the environment with increased mobility and biological activity. Although the organic compound-bound and residual As are relatively stable, changes in soil redox conditions can lead to oxidative decomposition of organic matter, and increases in soil specific surface can promote desorption of residual As, which in turn can lead to the release of As. It is worth noting that although As in overall situation of seven soil samples is low mobility, freeze-thaw action increased the percentage of exchangeable As. The environmental behavior of soil heavy metals is inseparable from their speciation. Exchangeable As is sensitive to environmental changes and prone to migration and transformation, therefore freeze-thaw action increase the environmental risk of arsenic in contaminated soils. .

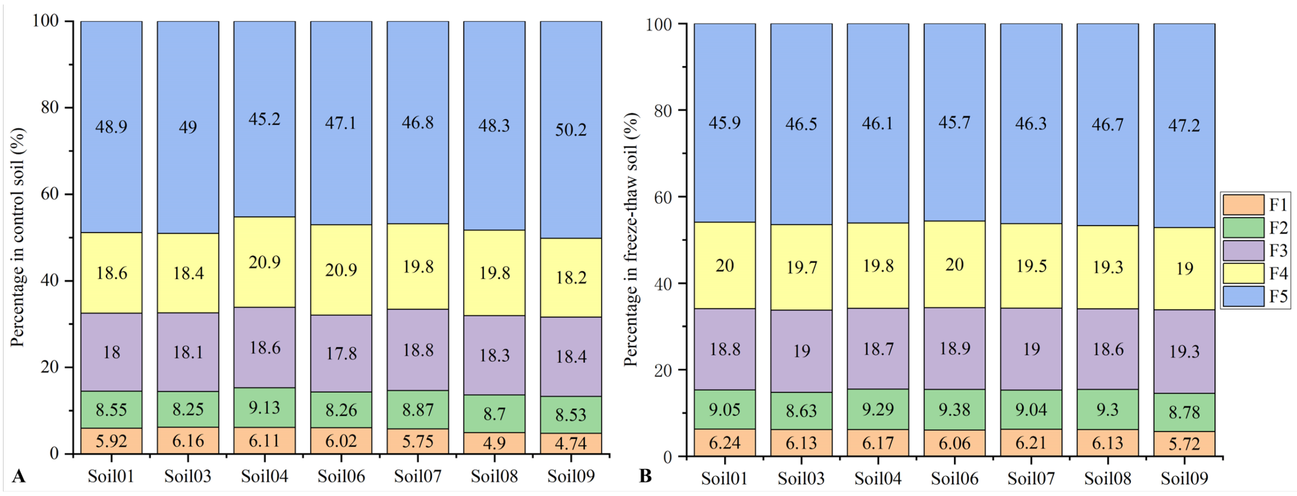

Figure 1 Distribution of five forms of As speciation in control soils (A) and freeze-thaw soils (B). The meaning of F1, F2, F3 and F4 are shown in Table 2.

3.3 Change of As bioavailability determined by in vivo and in vitro assays in control soils and freeze-thaw soils

In control soils, the changes in the relative bioavailability and bioaccessibility of As were investigated. The average relative bioavailability of As in the seven soils was 40±6.2% (15%-68%), the average bioaccessibility of As in the gastric and intestinal phases combined was 55±0.98% (50-57%), the average bioaccessibility in the gastric phase was 45±0.74% (41-46%), and the average bioaccessibility in the intestinal phase was 11±0.63% (9.3-13%).

In the freeze-thaw soils, the average relative bioavailability of As was 72±6.8% (47-106%), the average bioaccessibility of As in the gastric and intestinal phases combined was 68±1.2% (62-72%), the average bioaccessibility in the gastric phase was 54±0.91% (50-57%), and the average bioaccessibility in the intestinal phase was 14±0.81% (12-17%) (Table 3). Comparing the bioaccessibility and relative bioavailability of As under control and freeze-thaw soils (Table 4), it was found that the relative bioavailability of As after freeze-thaw increased significantly by 32±9.6% (P<0.05), and the bioaccessibility of As in the combined gastric and intestinal phases, gastric phase, and intestinal phase increased by 13±0.23% (P<0.05), 9.8±0.17% (P<0.05), and 3.2±0.18% (P>0.05), respectively. These results suggested that variation of As bioavailability was found in different soil samples, which is explained by the specific characteristics of soil properties and As speciation [25]. Even so, As bioavailability of freeze-thaw and control soils determined by two methods in this study is consistent with previous studies (2%-80%) [20, 23, 25]. The same change trend of in vivo and in vitro methods also confirmed that UBM method is a robust method to estimate As bioavailability [25, 42]. As bioavailability also differed in freeze-thaw and control soils. Both in vivo experiments with mice and in vitro gastrointestinal simulations using the UBM method consistently demonstrated that freeze-thaw increased the bioavailability of As. This result is consistent with the findings of four in vitro simulation methods reported by Sun et al. [35]. We infer that the change in As bioavailability is indirectly caused by the change in soil physical and chemical properties due to freeze-thaw action. This inference is mainly based on the significant differences in soil physicochemical properties and speciation between control and freeze-thawed soils in this study.

Table 3 As bioavailability of control soils determined by in vivo and in vitro methods.

Treatment	Method	S01 (%)	S03 (%)	S04 (%)	S06 (%)	S07 (%)	S08 (%)	S09 (%)
Control soil	UBMG	41±3.9	44±5.8	45±1.5	45±1.1	46±4.9	46±2.0	46±2.6
	UBMI	9.3±1.5	11±1.6	14±0.79	12±1.1	8.7±0.39	9.4±0.34	12±0.44
	UBM	50	55	57	56	54.7	56	57
	FLK	15±11	33±2.7	38±4.3	33±7.6	45±4.3	68±3.8	47±14
Freeze-thaw soil	UBMG	50±1.9	55±0.67	54±1.0	55±0.79	56±1.0	57±1.07	56±1. 6
	UBMI	12±0.49	14±0.26	17±0.35	15±0.13	11±0.22	12±0.22	15±0.44
	UBM	62	68	71	70	67	69	71
	FLK	68±1.2	783±4.2	106±1.7	61±6.4	47±6.1	69±5.9	75±6.5

Notes: UBMG: As bioavailability of control and freeze-thaw soils determined by gastric phase of UBM; UBMI: As bioavailability of control and freeze-thaw soils determined by intestinal phase of UBM; UBM: As bioavailability of control and freeze-thaw soils determined by gastric and intestinal phases of UBM; FLK: As bioavailability of control and freeze-thaw soils determined by combined organs (femur, liver and kidney) of in vivo mouse method. Different letters represent for the significant difference among four methods.

Table 4 Comparison of As bioavailability between control and freeze-thaw soils determined by in vivo and in vitro methods.

Method	Control soil (%)	Freeze-thaw soil (%)	Difference (%)
UBMG	45±0.74AB/a	54±0.91A/b	9.8±0.17
UBMI	11±0.63C/a	14±0.82B/a	3.2±0.18
UBM	55±0.98A/a	68±1.2C/b	13±0.23
FLK	40±6.2B/a	72±6.8C/b	32±9.6

Notes: Mean As bioavailability of seven soil samples are shown in the table. Difference is calculated by As bioavailability of freeze-thaw soils minus As bioavailability of control soils. The meaning of UBMG, UBMI, UBM, FLK are shown in Table 3. Different capital letters represent for the significant difference among four methods.

Different lowercase letters represent for the significant difference between control soils and freeze-thaw soils.

To further analyze the mechanism of changes in As bioavailability, this study used Spearman correlation to investigate the relationships between the differences in As bioavailability (relative bioavailability and bioaccessibility) of control and freeze-thaw soils, and the differences in soil environmental factors and As speciation (Table 5). The analysis results showed that the difference in As relative bioavailability was significantly correlated with soil pH and carbonate-bound fraction (F2). The difference in gastric-phase As bioaccessibility was significantly correlated with pH, BET, and EC. The difference in intestinal-phase As bioaccessibility was significantly correlated with pH and SOM, and the bioaccessibility of As in both gastric and intestinal phases was significantly correlated with SOM and organic compound-bound fraction (F4). The analysis of the relationship between As bioavailability and soil physical and chemical properties also showed that EC, SOM, and BET have a strong correlation with the relative bioavailability and bioaccessibility under control and freeze-thaw soils (Table S4). This result validated the aforementioned inference and further indicated that soil pH, SOM, BET and EC are probably the main factors affecting As bioavailability due to freeze-thaw action. Increased As bioavailability after freeze-thaw action might be mainly from the increased exchangeable As from the transformation of other forms. These transformations are caused by the adsorption and desorption of As with soil properties. Therefore, the freeze-thaw action essentially causes changes in chemical properties due to physical changes in the soil [49, 50].

Table 5 Spearman correlation of the difference value of relative bioavailability, bioaccessibility, the difference of soil properties, and the difference value of five speciation fractions.

Correlation	UBMG-D		UBMI-D		UBM-D		FLK-D	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*
pH-D	**-0.76**	0.05**	**0.86**	0.01***	0.63	0.13	**0.72**	0.07*
CEC-D	0.36	0.43	0.04	0.94	0.29	0.53	-0.04	0.94
SOM-D	0.21	0.64	**-0.68**	0.09*	**-0.68**	0.09*	-0.21	0.64
BET-D	**-0.71**	0.07*	0.14	0.76	-0.18	0.7	0.5	0.25
PS-D	0.43	0.34	-0.11	0.82	0.14	0.76	-0.61	0.15
EC-D	**-0.78**	0.04**	0.02	0.97	-0.58	0.17	0.32	0.49
F1-D	0.21	0.64	-0.11	0.82	-0.18	0.7	-0.29	0.53
F2-D	0.29	0.53	-0.5	0.25	-0.36	0.43	**-0.68**	0.09*
F3-D	-0.07	0.88	0.25	0.59	0.14	0.76	-0.11	0.82
F4-D	-0.18	0.7	-0.46	0.29	**-0.71**	0.07*	0.04	0.94
F5-D	0.21	0.64	0.14	0.76	0.36	0.43	0.11	0.82

Notes: * $P \le 0.1$, ** $P \le 0.05$, *** $P \le 0.01$. D: Difference between freeze-thaw soil and control soil.

Freez-thaw action are essentially phase change processes of water in the soil, and when the liquid water phase becomes ice, there is a corresponding increase in volume. The formation of ice squeezes the surrounding particles and breaks up the large soil particle aggregates. Freeze-thaw cycles continuously break up large soil aggregates into smaller ones. Due to the active transportation of water, the unfrozen part of the water is constantly migrating to the frozen ice surface, further freeze makes the soil divided into layers and webs by the ice, and a large number of small particles are produced by the reduction of soil capillary pressure during the freeze process. [50]. Increased BET and decreased PS also revealed the physical effect of freeze-thaw cycles on soil. However, the physical action might not continuously increase soil BET and PS with the increase of freeze-thaw cycles. Ma et al (2019) [45] explored soil aggregate size changes with freeze-thaw cycles by setting 1-30 freeze-thaw cycles. They found that soil aggregates initially decreased with the increase of freeze-thaw cycles, and the magnitude of aggregate decrease remained basically unchanged until the freeze-thaw cycles reached 20. In our study, the freeze-thaw cycle was set at 12, which is still some way from 20 cycles, so the range of changes in arsenic bioavailability in the soil may still increase with increasing freeze-thaw cycles. The environmental risk of arsenic is closely related to its bioavailability, so mastering the threshold of arsenic under special environmental conditions is a key issue for assessing its toxicity risk and environmental remediation. Due to the limitations of objective factors, such as human and physical, the threshold of arsenic bioavailability under freeze-thaw cycles was not explored in this study, but we will continue to work on this issue in the future. The environmental risk of arsenic is closely related to its bioavailability, so mastering the threshold value of arsenic under special environmental conditions is a key issue in solving As risk assessment and environmental remediation. Due to the limitations of objective factors, such as human and physical, the threshold of arsenic bioavailability under freeze-thaw cycles was not explored in this study, but we will continue to work on this issue in the future.

Many studies have shown that soil pH is one of the crucial factors determining the transformation of As species, thereby affecting the bioavailability of As in the soil. Different pH values lead to different As species. The higher the pH, the poorer the adsorption of As by the soil, leading to a higher content of available As in the soil and, consequently, higher bioavailability. Changes in soil pH can cause mutual transformations of various As species in the soil, affecting the bioavailability and environmental risk of soil As. Soil As mainly exists in anionic forms. At lower pH, $H_2AsO_4^-$ and $HAsO_4^{2-}$ can be rapidly adsorbed by positively charged iron oxides. With increasing pH, the negative charge on the surface of adsorbents such as iron oxides increases, weakening the adsorption force and causing the desorption of As anions into the soil solution, thereby increasing the availability of As [56-58]. In this study, pH showed a significant effect on As bioavailability despite the small change in pH (~0.5 pH) between control and freeze-thaw soil. This is mainly because, although the pH change was small, the soil pH decreased to nearly acidic or acidic under freeze-thaw soils (6.73-7.48). Under acidic conditions, (1) the solubility of As typically increases and As is more readily available in soluble form in soil solutions, thereby increasing biological uptake of As; and (2) the density of negative charges on the surface of soil particles decreases, which will reduce the adsorption of As to soil particles, thereby increasing biological exposure to and uptake of As. Organic matter is an essential component of the soil and a significant factor affecting the transformation and migration of heavy metals [59-61]. Previous studies have shown that the organic compound-bound fraction of heavy metals is mainly related to the content of soil organic matter. As the content of soil organic matter increases, the speciation of heavy metals in the soil will change from carbonate-bound to organic-bound forms. Soil organic matter has a strong ability to adsorb and complex heavy metal ions, affecting the transformation and migration of heavy metal chemical species by complexing with them [62-64]. Soil variable charge mainly originates from functional groups in soil organic matter, such as carboxyl groups (R-OOH). Studies have found that organic matter promotes the adsorption of As [65], polar groups in organic matter can chelate As, forming structurally complex chelates with As, thus increasing its bioavailability.

In addition to pH and SOM, total soil arsenic concentration is also an important factor affecting arsenic bioavailability. However, in this study, we did not find that the changes in total soil concentration and bioavailability before and after freeze-thaw were not always consistent and the correlation between them was not significant. This is consistent with previous studies and may be related to the As speciation, especially for labile species of arsenic [66, 67]. Besides of labile species of arsenic, due to the toxicity of As is closely related to its different forms in the soil, the toxicity of inorganic As is generally greater than that of methylarsonic acid (MMA) and dimethylarsinic acid (DMA); trivalent As (AsIII) is more toxic than pentavalent As (AsV). The vast majority of As oxides (such as arsenic trioxide) and its related salts have highly toxic characteristics. Therefore, in future research, we need to specifically investigate the changes in the content of different valence states of As due to freeze-thaw action, especially trivalent and pentavalent As, to provide a theoretical basis for further refining As human health risk assessment. Additionally, the results of the significance analysis were inconsistent, which is preliminarily attributed to the limitation of the number of research sites. Therefore, in future studies, we need to expand the sampling scope and increase the number of samples to verify the correlations between these factors and As form and bioavailability.

4 Conclusion

This study investigated the effect of freeze-thaw action on the bioavailability of soil arsenic, using in vivo and in vitro bioavailability measurement methods under the freeze-thaw control condition. We found that freeze-thaw action reduced soil pH (P<0.05), CEC, SOM, and particle size, while increased BET; Freeze-thaw action increased the proportions of exchangeable (P<0.05), carbonate-bound, and iron-manganese oxide-bound As, while reduced the proportions of organic-bound and residual As. Both in vivo and in vitro results under control and freeze-thaw soils consistently confirmed that freeze-thaw action increased the bioavailability of As in contaminated soils. The findings suggest that freeze-thaw action increase the environmental risk of soil arsenic. By further exploring the relationships between soil physicochemical properties, As speciation, and differences in As bioavailability, we found soil pH, SOM, BET and EC are main factors contributing to the increased bioavailability of As due to freeze-thaw processes. The findings verified that freeze-thaw action indirectly increased As bioavailability by direct affecting soil physicochemical properties.. Clearly, freeze-thaw cycles, temperature and humidity also might be key indicators effecting on soil arsenic bioavailability, which should be paid attention when

Acknowledgements

This work was supported by National Key R&D Program of China (2019YFC1803800) and Budget Surplus of Central Financial Science and Technology Plan (2021-JY-36). Yiming Sun and Qingbao Gu have received research support from Ministry of Science and Technology of the People's Republic of China (2019YFC1803800) and Chinese Research Academy of Environmental Sciences (2021-JY-36), respectively.

Conflicts of Interest Statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethics approval

This work has received approval for research ethics from Sinoresearch (Beijing) Biotechnology Co., Ltd. and a proof/certificate of approval is available in the uploaded file.

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