TITLE: Does freeze-thaw action affect the extractability and bioavailability of Pb and As in contaminated soils?

RUNNING TITLE: Pb and As bioavailability changes in control and freeze-thaw soils

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Highlights

- Freeze-thaw increased the bioavailability of soil Pb and As;
- Changes were detectable under free-thaw treatments using a sequential extraction;
- In vitro simulation methods showed differences in freeze-thaw treated soils;
- Particle size changes after freeze-thaw were linked to Pb/As bioavailability change.

Abstract

As global warming intensifies, there will be increased uncertainty as to the environmental behavior and risks from heavy metals in industrial/legacy contaminated sites in permafrost regions. Bioavailability has been increasingly used for human health risk assessment of heavy metals in contaminated soils. Soil heavy metal bioavailability depends on soil physicochemical properties, and freeze-thaw affects soil physical, chemical and biological processes. However it is not clear whether freeze-thaw has an effect on the bioavailability of soil heavy metals. In this study, soils contaminated with Pb and As were collected from 10 industrial sites in northeast China, an area prone to freeze-thaw action. Extractability and bioavailability of soil As and Pb were determined by the Tessier sequential extraction method and four in vitro gastron-intestinal simulation methods under control and freeze-thaw treatments. The aims were: to compare the results of extraction and bioavailability from laboratory experiments which artificially simulate freeze-thaw conditions against control soils; to explore the correlation between bioavailability of Pb/As and soil properties. Freeze-thaw cycling significantly decreased soil pH, and increased the soil weight surface area. Results using the Tessier sequential extraction procedure showed that freeze-thaw decreased the percentage in the residual fraction, and increased the percentage of Pb and As in the exchangeable fraction, carbonate-bound fraction, Fe-Mn oxides-bound fraction and organic-bound fraction, relative to control soils. Results using four simulation methods showed that freeze-thaw significantly increased Pb and As bioavailability compared to the controls. Pb and As released in the gastric digestion phase of the four methods was significantly higher than that in the intestinal digestion phase. Further analysis of correlations between Pb and As bioavailability and soil properties indicated that total concentrations of Al, Fe and Mn, particle size, and weight surface area significantly correlated to Pb and As bioavailability. Overall, this study demonstrated that freeze-thaw did influence the bioavailability of soil heavy metals. It suggests the freeze-thaw action should be comprehensively considered in the human risk assessment of soil pollutants in permafrost regions.

Keywords: Heavy metals; Bioavailability; Tessier sequential extraction method; in vitro simulation methods; Seasonal permafrost regions; Industrial contaminated land

1. Introduction

Soils are subjected to freezing-thawing over large parts of the globe. Indeed, freezing-thawing is a key factor in soil formation in many parts of the world (Henry, 2007; Kim et al., 2012). Permanent and seasonal permafrost areas cover ~23% of the global land area (Zhou et al., 2018). Seasonal permafrost freezes for one month or more, at depths of a few tens of millimeters to a few meters; seasonal permafrost is cyclic, freezing in winter and thawing in spring and summer. It is estimated that the global area of stable permafrost will decrease from 1.07 × 10^5 km^2 in 2011 to 5.6 × 10^4 km^2 by 2110 (Wei et al., 2011). It has been hypothesized that pollutants accumulated in permafrost areas as a "sink" may then be re-mobilised and transformed during permafrost thawing, with unknown consequences (Wei et al., 2011). In China, soil heavy metal contamination is a key type of soil pollution, especially for As and Pb (Ministry of Environmental Protection of the People's Republic of China Ministry of Land and Resources of the People's Republic of China, 2014). As part of the increased development and re-development of urban areas, a large number of polluting enterprises in seasonal tundra areas have moved out from urban centers, leaving behind potentially contaminated sites, for example, old industrial bases in northeast China. Therefore, as global warming intensifies, there will be increased uncertainty as to the environmental behavior and risks from heavy metals in industrial/legacy contaminated sites in permafrost regions.

Understanding the fraction of contaminants that potentially enter human body is a key task of human health risk assessment. Often the focus for risk assessment of heavy metals in contaminated sites/soils is their potential to be absorbed by humans, through oral, dermal, and respiratory routes (Kelley et al., 2002). The use of bioavailability for human health risk assessment of soil heavy metals has been increasingly recognized by researchers and has resulted in a more mature human health risk system (USEPA, 2001; USEPA, 2017a; USEPA, 2017b). Many countries have also included bioavailability into the human health risk assessment of soil heavy metals (Ng et al., 2009; USEPA, 2007). The essence of freeze-thaw action is obviously the freezing and thawing of soil water, i.e., the repeated freezing and thawing of the soil surface layer and soil layers at certain depths when the temperature drops below 0 degrees and rises above 0 degrees. The physical effect produced by freezing and thawing will accelerate the fragmentation of large solid media, enhance water release and the water permeability coefficient of soil, and be accompanied by the leaching and migration of soluble components during the thawing process (Gao et al., 2016; Hou et al., 2021; Luo and Li, 2021). Freeze-thaw affects soil physical (e.g., particle size, soil porosity, and specific surface area), chemical (e.g., pH and organic matter), and biological processes (e.g., soil microbes and soil fauna) (Edwards, 2010; Lehrsch, 1998). Although it is not clear whether freeze-thaw has a particularly important effect on soil heavy metal bioavailability, recent studies have found that freeze-thaw can affect the transformation of heavy metal forms (Sun et al., 2016; Wang, 2017). Freeze-thaw cycles affected the mobilization and enrichment of heavy metals (Hou et al., 2020, 2022). The increase in pH under freeze-thaw can reduce metal bioavailability (Chen, 2021), while organic matter can complex and chelate with heavy metal ions, thus reducing the bioavailability and mobility of heavy metals (Li et al., 2013). Based on this, it is reasonable to assume that freeze-thawing may affect the release, forms and bioavailability of soil heavy metals, although very little research has systematically investigated these processes to date.

Chemical extraction methods (Sun et al., 2006), in vivo animal-based methods (Brattin & Casteel, 2013; Juhasz et al., 2010) and in vitro simulation methods (Li et al., 2016; Zhu et al., 2019) have been commonly used as the assessment methods for soil heavy metal bioavailability. Chemical extraction methods have been widely used to estimate the bioavailability of heavy metals; primary extraction methods (e.g. EDTA) and sequential extraction methods (e.g. the Tessier sequential extraction methodology) have been used. Although the evaluation of the mobilisation of heavy metals in soil by chemical extraction methods is relatively simple to perform, it obviously will not accurately mimic conditions in the gastro-intestinal tract, where absorption occurs. It has disadvantages and shortcomings, such as inaccurate characterization, poor comparability, and susceptibility to the influence of soil physical and chemical properties (Sun et al., 2006). The limitations of chemical extraction methods have become increasingly evident, with the increasing requirements of environmental health risk assessment. In vivo bioassays in different animal models have been used to assess the bioavailability of soil heavy metals, but due to ethical and cost issues, in vitro gastrointestinal simulation methods have been developed in recent years. Gastrointestinal simulation methods are intended to determine the dissolution of soil contaminants under conditions designed to simulate the human gastrointestinal tract, which is a key step determining bioavailability (i.e. absorption across the GI-tract lining). Examples of gastro-intestinal simulation methods include: the Physiologically Based Extraction Test (PBET), the Unified BARGE Method (UBM), the In Vitro Gastrointestinal Method (IVG) and the Soluble Bioavailability Research Consortium (SBRC) method. Around 10 different gastrointestinal simulation methods are available internationally now, but there is still a lack of carefully designed inter-comparison and calibration exercises, and there is not yet a recommended or preferred procedure, supported by the wider research community. The results from different gastrointestinal simulation methods for the same metals were found to vary widely in previous studies, for example (Li et al., 2020; Li et al., 2016). The SBRC has now been recommended by the USEPA as the standard method for predicting the biological effectiveness of soil Pb. Li et al. (2016) showed the ability of the IVG method to effectively predict the relative biological effectiveness of As, based on the in vivo - in vitro correlation (IVIVC) model. Zhu et al. (2019) confirmed that the UBM can be used as a valid method to predict the relative biological effectiveness of Pb, by constructing a mouse-UBM correlation experiment.

The objectives and design of this study were: to sample 10 soils from contaminated industrial legacy sites in northeast China, a major seasonal permafrost region; to compare the performance of established extraction (Tessier sequential extraction method) and in vitro simulation methods (PBET, IVG, SBRC and UBM) for Pb and As present in the soils from the legacy sites, in relation soil properties, ease of test performance etc.; to compare the extraction and bioavailability results from laboratory experiments which artificially simulate freeze-thaw conditions against soils maintained under control conditions. The results are discussed with respect to the test performances, influence of soil properties, the assays used and the potential importance of freeze-thaw actions on heavy metal behavior and bioavailability in soils.

2. Materials and methods

2.1. Soil sample collection and freeze-thaw experiment

Pb and As contaminated sites left by industries in cities in northeast China were selected for this study. A grid of 40 × 40 square meters was delineated at the geographic center of the sites. The diagonal method was used to collect samples from 5 points; the manual drilling method was used for sampling, to 0-20 cm depth. A total of 10 contaminated soil samples were collected from three types of industrial site: 4 steel plant sites, 5 non-ferrous metal smelting and processing plant sites, and 1 pharmaceutical factory site. The soil for testing and experiments was air-dried at room temperature, then debris such as gravel and plant residues were removed. The soils were then loosely disaggregated, sieved through 2 mm and 250 μm, then stored in sealed bags for chemical analysis and freeze-thaw treatment, respectively. The 250 μm soil samples were transferred into a sealed bag, and the relative field water holding capacity of the soil was adjusted to 60%. Soils were incubated at room temperature (10±5°C) for 15 days before freeze-thaw experiments (Zhang et al., 2013).

Using local meteorological data for northeast China, the freeze-thaw experiments used the following conditions: soil moisture content, 60%; soil freezing/thawing temperatures, -15°C and 6°C. With reference to "Standard Test Methods for Freezing and Thawing Compacted Soil-Cement Mixtures" (ASTMD560, 2016) issued by the American Society for Testing and Materials, the number of freeze-thaw cycles was set to 12. Incubated soils were placed in a low-temperature freezer at -15°C for 24 hours, then taken out and thawed in a refrigerator at 6°C for 24 hours. One freeze-thaw cycle was therefore 48 hours, and the process was repeated 12 times (Gu, 2020). At the same time, control soils were maintained at room temperature (10±5°C) with 0 cycles of freeze-thaw treatment.

2.2. Determination of soil properties and metal concentrations

The <2 mm sieved samples were used for determining the soil properties and metal concentration. These samples were used to determine soil pH (potentiometric method), cation exchange capacity (CEC) (barium chloride-sulfuric acid forced exchange method) and soil organic matter content (SOM) (potassium dichromate titration method). Soil particle surface area was determined by the BET specific surface area test and particle size distribution was determined using a TopSizer laser. Total metal concentrations were determined using the U.S. Environmental Protection Agency's digestion method (USEPA 3050B), with quantification by inductively coupled plasma mass spectrometry (ICP-MS).

2.3. Properties of the soils used in the study

Soil physicochemical properties and total metal concentrations are given in Table 1. In summary, values range as follows: soil pH, 7.20-7.59; SOM, 1.78-91.4 g/kg; CEC, 1.31-12.6 cmol/kg; mean particle size, 11.18-115 µm; weight specific surface area, 48.7-296 m^2/g; conductivity, 45.3-251 µs/cm. The key variables soil pH and SOM are broadly typical for soils from northeast China (Sun et al., 2020; Zhang, 2011). The total concentrations of the major elements Al, Fe and Mn, which can influence the chemistry of Pb, were ~18,000-55,000 mg/kg, ~37,000-130,000 mg/kg and ~690-4,000 mg/kg, respectively. Pb concentrations ranged between ~130-27,000 mg/kg, As between 77-930 mg/kg. Background (uncontaminated) soils are typically ~10-60 mg Pb/kg and ~2-35 mg As/kg (China National Environmental Monitoring Centre, 1990; Sun et al., 2019b), so these soils are clearly contaminated with Pb. The total concentrations of Pb, Al, Fe and Mn significantly correlated with each other (Figure S1).

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

Samples	Soil 01	Soil 02	Soil 03	Soil 04	Soil 05	Soil 06	Soil 07	Soil 08	Soil 09	Soil 10
pH	7.40	7.59	7.25	7.48	7.53	7.55	7.32	7.50	7.49	7.20
σ (µs/cm)	59.4	58.9	76.6	47.8	77.9	52.7	214.0	80.2	45.3	251.0
CEC (cmol/kg)	7.45	10.22	6.13	6.86	8.54	1.31	3.06	9.30	12.6	8.41
SOM (g/kg)	34.5	28.2	30.5	20.1	42.3	25.9	25.1	61.4	86.5	91.4
BET (m^2/kg)	93.2	76.2	194	82.0	261.1	51.8	48.7	89.6	295.7	223.1
PS (µm)	95.4	112.	106	109	69.0	11.8	11.2	115.0	62.7	47.5
Total Al (mg/kg)	24200	36800	24400	23500	29300	21400	1800	23000	28700	54600
Total As (mg/kg)	76.8	498	146	931	99	701	536	445	130	168
Total Fe (mg/kg)	38100	45400	36800	49200	42200	129000	94400	54400	38100	38800
Total Mn (mg/kg)	796	692	888	3900	713	3710	1800	1900	909	1340
Total Pb (mg/kg)	527	13600	4060	22000	243	27000	22700	12100	3420	137

Key: pH (soil pH), conductivity (σ), CEC, SOM, BET (Weight specific surface area), PS (Mean particle size), and total concentration of Fe (Total Fe), Al (Total Al), Mn (Total Mn), and Pb (Total Pb).

2.4. Determination of Pb and As extractability

The Tessier 5-step sequential extraction (Tessier method) (Tessier et al., 1979) was used to determine Pb and As extractability of the 10 soil samples (250 μm size fraction). The Tessier method determines five operationally defined fractions: exchangeable fraction (F1), carbonate bound fraction (F2), iron and manganese bound fraction (F3), organic bound fraction (F4) and residual fractions fraction (F5).

2.5. Heavy metal availability determined with simulated GI tract procedures

The soil fraction that passed through the 250 μm sieve was used for the experiments. Each soil sample was subjected to two freeze-thaw treatments (0 and 12 cycles). Four widely used gastrointestinal simulation methods (IVG, PBET, SBRC, UBM) were used to determine the bioavailability of Pb and As in the soils. All four methods generate gastric phase and intestinal phase extracts, but they differ in solid-fluid ratio, pH and extraction time. For example, pH of the gastric phase and intestinal phase of the UBM procedure are 1.2 and 6.3 respectively, and for PBET are 2.5 and 7.0, respectively. Details of the gastrointestinal fluid composition and extraction parameters for the four methods are provided in Table S1. The specific experimental components and experimental parameters refer to the research of Juhasz et al. (2009) and Denys et al. (2012). Three replicate analyses were performed for each method. Estimation of the bioavailable concentrations of Pb was as follows:

$$c_g = \frac{\rho(G_{Pb/As}) \times V_g}{m} \quad (1)$$

$$Bio_G = \frac{C_g}{C_s} \times 100\% \quad (2)$$

$$c_{gi} = \frac{\rho(GI_{Pb/As}) \times V_{gi}}{m} \quad (3)$$

$$Bio_{GI} = \frac{C_{gi}}{C_s} \times 100\% \quad (4)$$

where C_g is the available concentration of Pb/As in the stomach, mg/kg; C_{gi} is the available concentration of Pb/As in the intestine, mg/kg; $\rho(G_{Pb/As})$ is the concentration of Pb/As in simulated gastric juice, μg/L; $\rho(GI_{Pb/As})$ is the concentration of Pb/As in simulated intestinal fluid, μg/L; Vg is the volume of simulated gastric fluid, L; V_{gi} is the volume of simulated intestinal fluid, L; m is the mass of the soil sample during the test, g; Cs is the total concentration of Pb/As in soil samples, mg/kg; Bio_G is the human availability of Pb in the gastric stage; Bio_{GI} is the human availability of Pb/As in the intestinal stage.

2.6. Data analysis

Pb and As concentrations are presented as means, standard deviations determined on triplicate analyses. ANOVA was used to test the effect of freezing and thawing on Pb extractability/bioavailability versus the controls. Spearman correlation was used to test the correlation between soil Pb and soil factors (soil physicochemical properties and different occurrence forms). The R statistical package was used for all statistics and graphics.

3. Results

3.1. The influence of the freeze-thaw treatments on basic soil properties

The freeze-thaw treatment resulted in some changes to the soil properties, compared to the untreated control soils (see Table 2 & Table S2). Freeze-thaw treatment generally resulted in the following changes: slight reduction in soil pH (typically by 0.13-0.56 pH units); a decrease in SOM (by between 4-58 %), as determined by the potassium dichromate titration method; some differences in CEC, although this was not consistently up or down following treatment. However, the biggest change was in the surface area, as determined by the BET method. This likely reflects the effect of freeze-thaw action in disaggregating/fracturing soil particles. These results show freezing-thawing has important effects on the physical and chemical properties of soils.

Table 2. Differences in the soil properties between the freeze-thaw treatments and the controls. Values in bold show a significant difference between treatment and control soils. Values with minus and plus symbols show a reduction and increase in the treated soils versus the control soils, respectively.

Treatment vs. Control	Soil 01	Soil 02	Soil 03	Soil 04	Soil 05	Soil 06	Soil 07	Soil 08	Soil 09	Soil 10	*P*
pH	-0.27	-0.55	-0.52	0.00	0.00	-0.13	-0.56	-0.52	-0.30	-0.41	**0.0080**
σ (μs/cm)	79.80	16.80	-15.50	18.30	5.70	20.00	-21.80	28.80	9.90	-66.10	0.8055
CEC (cmol/kg)	-1.87	-6.09	-1.37	0.33	-3.56	-0.73	-0.34	-0.09	-4.08	1.41	0.1430
SOM (g/kg)	-2.85	-12.31	-7.18	-6.25	-3.00	-7.75	-3.76	-0.79	-7.71	-16.62	0.2568
BET (m^2/kg)	569.54	628.35	319.39	436.19	523.60	3.49	51.41	169.00	552.75	155.17	**0.0037**
PS (μm)	-72.93	-90.89	-77.87	-71.96	-53.66	195.74	127.24	-35.74	-50.63	-19.07	0.0601

Key: pH (Soil pH), σ (Conductivity), CEC, SOM, BET (Weight specific surface area), PS (Mean particle size).

To analyze the effect of freeze-thaw on Pb and As speciation fractions, the Tessier sequential extraction method was used on the control soils and the freeze-thaw treated soils. When comparing the percentage of five fractions between freeze-thaw treated soils and control soils, higher F1, F2 (P<0.05), F3 and F4 of Pb were found under the freeze-thaw treatment than under the control, while a greater percentage of Pb occurred in F5 Pb under the control than that under the freeze-thaw treatment; Higher F1 (P<0.05), F2 (P<0.05), F3 (P<0.05) and F4 As was found under the freeze-thaw treatment than under the control, while higher F5 (P<0.05) As was found under the control than that under the freeze-thaw treatment (Figure 1 & Table S3). Although the differences were small, they were statistically significant. These results for Pb and As indicated that freeze-thaw action enhanced movement from the residual fraction, organic bound fraction and Fe-Mn oxides bound fraction to the exchangeable and carbonate-bound states, (i.e. a move to the fractions typically associated with higher mobility and bioavailability).

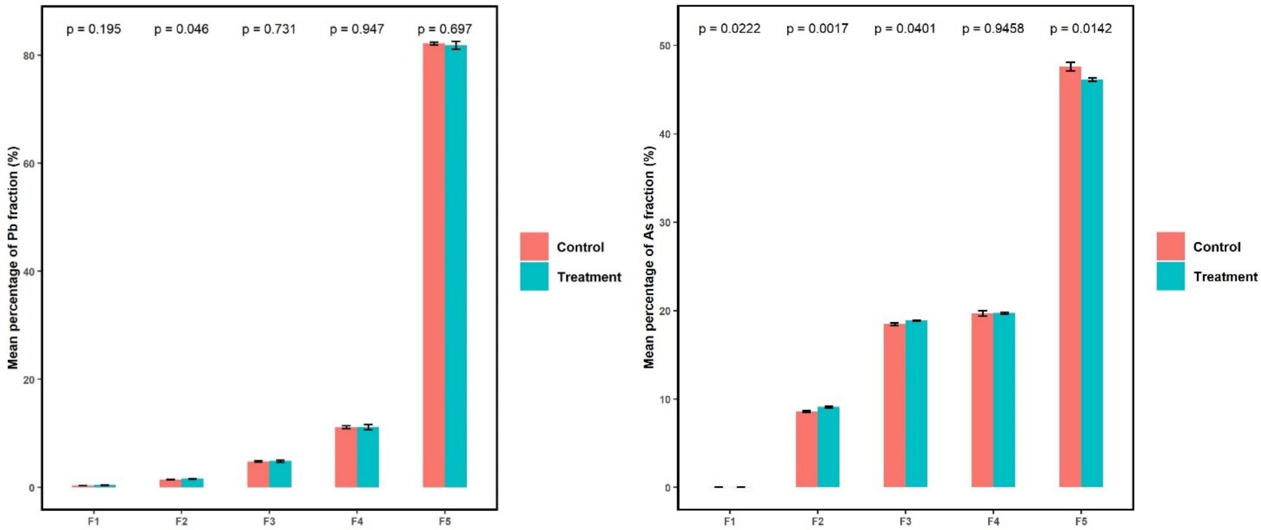

Figure 1. Mean percentage of the five Tessier extracts of Pb (left) and As (right) under freeze-thaw treatment and control averaged for the 10 soils. F1: Exchangeable fraction; F2: Carbonate-Bound fraction; F3: Fe-Mn Oxides-Bound fraction; F4: Organic-Bound fraction; F5: Residual fraction. P values showed the significant difference of each fraction between the freeze-thaw and control treatments.

3.3. Pb and As bioavailability in the control (untreated) soils determined using the 4 in vitro GI methods

Soil Pb and As bioavailability determined in the control soils using the 4 methods is shown in Figure 2 and Table S4. Mean values of Pb across the 10 soils range for each method as follows: IVG, 71.60±3.27-76.70±0.89 (gastric phase), 32.85±3.79-35.41±1.35 (intestinal phase); PBET, 38.68±1.79-46.00±0.70 (gastric phase), 22.96±1.48-31.09±0.60 (intestinal phase); SBRC, 49.79±1.09-58.98±0.56 (gastric phase), 16.04±1.52-20.67±0.59 (intestinal phase); UBM, 36.38±0.28-47.29±4.39 (gastric phase), 9.97±0.57-13.07±0.21 (intestinal phase). Mean values of As across the 10 soils range for each method as follows: IVG, 21.57±1.47-75.75±2.41 (gastric phase), 31.25±1.95-35.06±2.21 (intestinal phase); PBET, 36.46±3.44-44.94±4.30 (gastric phase), 8.03±0.72-31.31±6.14 (intestinal phase); SBRC, 46.88±3.44-57.53±2.21 (gastric phase), 15.62±2.26-19.03±1.89 (intestinal phase); UBM, 40.63±6.72-46.45±3.48 (gastric phase), 8.68±0.67-13.37±1.36 (intestinal phase). Bioavailability in the control soils in the gastric and phase of the four in vitro GI methods were in the sequence: IVG > SBRC > UBM > PBET for both Pb and As;

while in the intestinal phase: IVG>PBET>SBRC>UBM for Pb, IVG>SBRC>PBET>UBM for As. Both elements were lower in the intestinal phase than in the gastric phase for the control soils. From the 10 soils, Pb bioavailability followed the same order (IVG>SBRC>UBM>PBET) as the average trend in the gastric phase. The exceptions were Soils 02, 03 and 08 (IVG>SBRC>PBET>UBM). All 10 soils followed the same order (IVG>PBET>SBRC>UBM) in the intestinal phase. For As, bioavailability in 9 soils followed the same order (IVG>SBRC>UBM>PBET) in the gastric phase; the exception was Soil 08. In the intestinal phase, 9 soils also followed the same order, with the exception being Soil 05. For both Pb and As all 10 soils showed the closest agreement between the PBET and UBM GI methods (Figure S1).

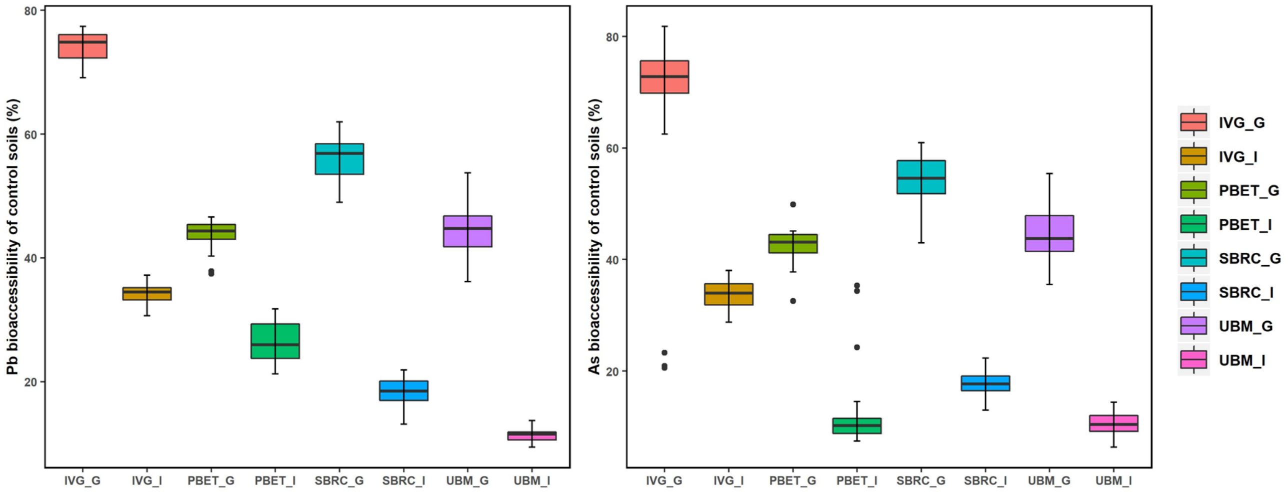

Figure 2. Mean Pb and As bioavailability in the control soils across 4 methods (n=10). G: Gastric phase, I: Intestinal phase.

3.4. Comparison of Pb bioavailability determinations between the control and freeze-thaw treated soils

Table 3 shows a comparison of Pb and As bioavailability derived using IVG, PBET, SBRC, and UBM between the control and the freeze-thaw treated soils. Significantly higher bioavailability of Pb and As determined by the gastric phase, the intestinal phase and the combined phases (total of G and I) of all four methods were found in freeze-thaw treated soils than in the control soils. The ratios of G:I in three of the methods (IVG, SBRC and UBM) were significantly lower in the freeze-thaw treated soils than in the control soils. Higher bioavailability of Pb and As occurred in the gastric phase, the intestinal phase and the combined phases using the IVG methodology, while the UBM and PBET methods gave the lowest values. Bioavailability of Pb and As determined by the gastric phase in the both of control soils and freeze-thaw treated soils was significant higher than that determined by the intestinal phases (Table 3). The distribution trend of As and Pb bioavailability in the ten control soils was the same as that in the ten freeze-thaw treated soils. For example, the lowest bioavailability was found in Soil 05 across all four GI methods and the highest was found in Soil 08 with all four GI methods (Figure S1).

Table 3. Comparison of Pb and As bioavailability of each method between treatments and phases averaged for the 10 soils. Mean value and standard deviation of Pb and As bioavailability were provided in the table. Uppercase letters represent for the significant difference in the Pb and As bioavailability between gastric phase and intestinal phase for each method; Lowercase letters represent for the significant difference in the Pb and As bioavailability between control and treatment for each method. G: Gastric phase; I: Intestinal phase.

Bioacce-ssibility (%)	Control				Freeze-thaw treatment			
	Gastric phase	Intestinal phase	Ratio of G:I	Total of G and I	Gastric phase	Intestinal phase	Ratio of G:I	Total of G and I
IVG_Pb	74.15±2.40A/a	34.21±1.59B/a	2.17±0.012a	108.35±2.75a	91.96±3.09A/b	45.89±1.86B/b	2.00±0.002b	137.85±3.38b
PBET_Pb	43.71±2.41A/a	26.44±3.06B/a	1.67±0.19a	70.15±4.29a	58.51±3.22A/b	28±3.28B/a	2.11±0.24b	86.51±5.11b
SBRC_Pb	56.02±3.46A/a	18.47±2.13B/a	3.05±0.29a	74.49±3.85a	59.4±3.40A/b	21.45±2.05B/b	2.79±0.26b	80.86±4.17b
UBM_Pb	44.40±4.87A/a	11.35±1.07B/a	3.93±0.38a	55.75±3.36a	54.21±3.84A/b	14.72±1.27B/b	3.70±0.36a	68.93±4.13b
IVG_As	68.01±16.25A/a	33.88±2.48B/a	2.01±0.49a	101.89±16.21a	90.90±4.79A/b	45.45±2.92B/b	2.00±0.02a	136.35±4.85b
PBET_As	42.43±3.13A/a	12.19±6.97B/a	4.06±1.25a	54.62±7.51a	57.01±4.05A/b	12.89±7.27B/a	5.17±1.60a	69.90±8.17b
SBRC_As	54.50±4.09A/a	17.51±2.21B/a	3.12±0.21a	72.01±3.99a	57.73±4.08A/b	20.42±2.45B/b	2.83±0.19b	78.12±4.33b
UBM_As	44.41±5.13A/a	10.70±2.09B/a	4.23±0.64a	55.12±2.18a	54.20±2.64A/b	13.86±1.93B/b	3.97±0.60a`	68.09±2.68b

3.5. Correlation of soil chemical properties and Pb and As bioavailability under the freeze-thaw treatments

Spearman correlation analysis was performed on the results from the 10 soils to further analyze whether any of the measured soil properties influenced Pb and As bioavailability. A significant correlation was found between the total concentration of Al, Fe and Mn with Pb and As bioavailability under both the control and freeze-thaw treatment (see Table 4). Particle size and BET had significant correlation with Pb and As bioavailability under the control treatment. Although most pH, all SOM, all CEC and all connectivity gave no significant correlation with Pb and As bioavailability (Table 4), they showed some trends with the change of Pb and As bioavailability from the control to the freeze-thaw treatment (Table 2).

Table 5. Correlation between Pb and As bioavailability and soil properties across different treatments. Values in the columns represent correlation coefficient between Pb and As bioavailability and soil properties. Values in bold red indicate P <0.05.

Treatment	Method	Total Al		Total Fe		Total Mn		BET		CEC		pH		PS		SOM		σ	
		Pb	As	Pb	As	Pb	As	Pb	As	Pb	As	Pb	As	Pb	As	Pb	As	Pb	As
Control	IVG_G	-0.5	-0.31	0.28	0.5	**0.66**	0.43	-0.16	-0.48	0.1	-0.41	0.04	0.18	0.21	0.36	0.03	-0.56	-0.33	-0.37
Control	IVG_I	-0.59	-0.56	0.28	**0.72**	0.53	**0.73**	-0.22	-0.41	0.12	-0.07	0.25	0.26	0.36	0.54	-0.15	-0.09	-0.49	-0.01

Control	PBET_G	-0.42	-0.53	**0.66**	**0.76**	0.42	0.41	**-0.75**	**-0.68**	-0.07	-0.16	0.35	0.55	**0.7**	**0.84**	-0.39	-0.45	-0.04	-0.03
Control	PBET_I	-0.18	0.14	0.39	-0.16	0.15	0.2	-0.58	0.49	-0.13	-0.09	0.28	-0.02	0.52	-0.48	-0.52	-0.05	-0.31	-0.35
Control	SBRC_G	-0.47	-0.54	**0.62**	**0.72**	0.39	0.44	**-0.76**	**-0.67**	-0.1	-0.13	0.36	0.53	**0.75**	**0.83**	-0.45	-0.43	-0.09	-0.1
Control	SBRC_I	**-0.7**	-0.3	0.5	0.59	**0.62**	0.58	-0.33	-0.45	-0.13	-0.01	-0.04	-0.08	0.39	0.41	-0.21	-0.26	-0.08	0.22
Control	UBM_G	-0.31	-0.41	0.5	0.53	0.09	0.22	-0.42	-0.32	-0.14	0.26	0.42	0.47	0.38	0.56	-0.42	-0.08	-0.31	-0.02
Control	UBM_I	0.09	0.05	0.18	0.03	0.04	0.43	-0.28	0.31	0.14	-0.12	0.24	0.16	0.19	-0.3	-0.33	-0.02	-0.41	-0.47
Freeze-thaw	IVG_G	-0.54	-0.61	0.37	**0.81**	**0.73**	**0.73**	-0.25	-0.55	0.36	-0.08	0.19	0.07	0.39	**0.65**	0.13	-0.16	-0.17	-0.01
Freeze-thaw	IVG_I	-0.58	**-0.64**	0.31	**0.82**	**0.77**	**0.68**	-0.28	-0.49	0.37	-0.14	0.14	0.15	0.42	0.6	0.16	-0.19	-0.2	-0.05
Freeze-thaw	PBET_G	-0.47	-0.54	**0.62**	**0.72**	0.39	0.44	-0.45	-0.52	-0.16	-0.37	-0.13	-0.09	0.56	0.58	-0.39	-0.43	0.07	-0.13
Freeze-thaw	PBET_I	-0.21	0.14	0.37	-0.16	0.16	0.2	-0.21	0.31	-0.54	0.15	-0.2	**0.68**	0.25	-0.22	-0.58	0.13	-0.21	-0.44
Freeze-thaw	SBRC_G	-0.42	-0.55	**0.66**	**0.75**	0.42	0.45	-0.47	-0.54	-0.09	-0.47	-0.1	-0.04	0.58	0.6	-0.36	-0.49	0.15	-0.17
Freeze-thaw	SBRC_I	**-0.7**	-0.21	0.5	0.54	**0.62**	0.55	-0.22	-0.36	0.12	0.18	0.19	-0.18	0.39	0.45	0.02	-0.18	0.18	0.28
Freeze-thaw	UBM_G	-0.31	-0.38	0.5	0.45	0.09	0.15	-0.03	-0.18	-0.58	-0.19	0.33	-0.15	0.1	0.18	-0.36	0.09	0.01	-0.02
Freeze-thaw	UBM_I	0.04	0.05	0.14	0.03	0.02	0.43	0.15	0.1	-0.37	0.18	-0.02	0.7	-0.09	0.01	-0.49	-0.03	-0.41	-0.58

Key: pH (Soil pH), σ (conductivity), CEC, SOM, BET (Weight specific surface area), PS (Mean particle size), total concentration of Fe (Total Fe), Al (Total Al), and Mn (Total Mn).

4. Discussion

4.1. Effect of freeze-thaw on Pb and As bioavailability

Our results indicated that the effect of freeze-thaw increased Pb and As bioavailability with all four in vitro methods. Although previous studies (Gao et al., 2016; Sun et al., 2016; Wang, 2017) did not show the direct effect of freeze-thaw on the bioavailability of heavy metals, authors have speculated that freeze-thaw will change soil physicochemical properties, thereby directly affecting metal speciation and mobility, and then indirectly affecting the bioavailability of soil heavy metals (Guo et al., 2012; Luo and Li, 2021; Wang et al., 2013). Metal-soil adsorption and desorption will presumably be affected by freeze-thaw water content and freeze-thaw temperature (Meeravali et al., 2020; Sun et al., 2019a; Yang et al., 2017). Guo et al. (2012) analyzed black soil and brown loam soil from agricultural land under freeze-thaw treatments and found that the amount of Pb immobilized and adsorbed in the freeze-thaw soil was lower than that in non-freeze-thaw soils. The soil samples in this study were all black soil and brown loam, and the results were consistent with this study. The adsorption capacity of soil for heavy metals decreased with decreasing freeze-thaw temperature, decreased with increasing freeze-thaw cycles, and first increased, then decreased and then stabilized with the increase of freeze-thaw water content (Wang et al., 2011), indicating that freeze-thaw may not increase Pb bioavailability in all studies/scenarios. Although only 12 freeze-thaw cycles with fixed temperature and water content parameters were used for the freeze-thaw treatments in this study, the 12 freeze-thaw cycles have been shown previously to be a threshold value of freeze-thaw cycles (ASTMD560, 2016).

The proportions of bioavailable Pb and As varied with the four methods and the two phases (gastric phase >

intestinal phase; P<0.05, Table 3) in this study. This is consistent with other studies, which have found that more Pb is solubilized in the first gastric) phase (Chen, 2021) and that there are also methodological differences (Aurélie et al., 2010; Li et al., 2020). Previous studies have shown that increasing soil pH can increase the adsorption of heavy metal ions in soils (Harter, 1983). The availability of heavy metals in soil is significantly affected by pH, but the relationship between bioavailability and pH remains complex. Yang et al. (2003) found that with the increase of soil pH, the bioavailability of Pb decreased significantly. However, in this study, we found that there was no significant relationship between Pb bioavailability and pH in the control soils (Table 4). This difference between our study and previous studies may be due to the relatively small pH variations for the soils studied here (7.20-7.59), so that the effect of pH on the bioavailability of heavy metals in the soil could not be detected.

In the intestinal phase under the control treatment, the highest values of Pb bioavailability were found in IVG, followed by PBET, SBRC and UBM. IVG has the highest Pb bioavailability in gastric phase, PBET has the lowest pH in gastric phase (1.2 versus 1.5, 1.8, 2.5) of these four methods (Table S1). This indicated that pH is the factor controlling bioavailability in the gastric stage. Previous studies have indicated that the bioavailability of soil heavy metals in the gastric stage of in vitro simulation methods is affected by many factors, with the pH of gastric juice an important factor. For example, Oomen et al. (2002) compared the results of five in vitro digestion methods and concluded that gastric pH was the most significant factor affecting the bioavailability of heavy metals in soils. In addition, some studies have reported that the solid-liquid ratio is also an important influencing factor. Among the four methods used in this paper, the IVG method has the highest soil-liquid ratio (1:150) in the gastric stage, and related studies show that when the soil-liquid ratio increases from 1:25 to 1:1000, the bioavailability of heavy metals increased.

4.2. General consideration of soil changes and factors affecting Pb and As bioavailability under freeze-thaw conditions

The results showed soil particle size, BET weight surface area and metal concentrations (As, Pb, Al, Fe and Mn) were correlated under both the freeze-thaw and control treatments, suggesting they had similar sources/behaviours. The traditional soil extraction methods were not sensitive/responsive enough to show major differences in Pb behaviour in the freeze-thaw experiments. There was no direct significant relationship between Pb bioavailability and most soil properties (pH, SOM, and CEC), even though these variables are known to be important in affecting heavy metal fate and behaviour. This may be because: (1) the limited amount of samples collected in this study; (2)

the pH variation between the different soils studied and with the treatments was relatively small, so that an effect of pH on Pb and As bioavailability could not be detected. However, the results still indicated changes of these properties under the freeze-thaw treatment (Table 2). Changes in soil physicochemical properties are important factors affecting the environmental behavior of soil heavy metals, among which soil porosity, aggregate stability, pH and organic matter are all important factors affecting soil bioavailability of heavy metals (Liu et al., 2017a; Zhao et al., 2010).

The Our results showed that soil particle size and BET weight surface area significantly correlated to the bioavailability of Pb and As. Although there are no direct previous studies on freeze-thaw soils, other studies have shown soil particle size is a key factor affecting the bioavailability of Pb in soil (Anthony et al., 2007). For example, Ljung et al. (2007) showed that when the soil particle size was 0-50 μm, the bioavailability of heavy metals in simulated gastric phase had a significant correlation with the pH of gastric phase, but when the particle size increased, the correlation was not significant. In addition, our results found that lower values of soil particle size and higher values of BET weight surface area were in soils under the freeze-thaw treatments than in the non-freeze-thaw treatments. The physical action produced by freeze-thaw (Liu et al., 2017b; Grogan et al., 2004; Oztas and Fayetorbay, 2003; Zhao et al., 2004) will accelerate the fragmentation of large-particle solid media, so soil pore particle size will decrease and BET weight surface area in soils will increase.

A total of 10 soils contaminated with Pb and As from Northeast of China were used in this study. Soils in the north area of China are generally alkaline (Sun et al., 2020), which is consistent with the results of soil pH (7.20-7.59) under the non-freeze-thaw treatments across all study sites. However, soil pH under the freeze-thaw treatments generally decreased slightly, except for two samples). A decreasing trend was also found in previous studies (Gao et al., 2016; Sun et al., 2016); this may be because freeze-thaw could promote soil nitrification and release of dissolved organic acids, thereby increasing the concentration of H ions in soil and reducing soil pH (Freppaz et al., 2007; Wang et al., 2007; Zhou et al., 2011). The decrease of soil solution pH reduces the negative charges carried on the soil colloid surface, resulting in the decrease of soil CEC, which is basically consistent with our results of soil CEC under the freeze-thaw treatments (see Table 2). In addition, some studies have reported that freeze-thaw can release easily decomposed organic matter by destroying soil aggregates, but our results showed SOM decreased under the freeze-thaw treatment compared to the controls. This might be because freeze-thaw can promote the mineralization of organic matter by microorganisms, resulting in decreasing SOM with the freeze-thaw cycles (Wang et al., 2007).

To sum up, freeze-thaw action can have a comprehensive effect of soil properties which effect the adsorption and desorption of heavy metals, not just one aspect of it. This study to analyze the effect of freeze-thaw action on Pb and

As has some limitations. First, because of the close and small size (n=10) of sampling sites, the pH, SOM and CEC results may not have ranged widely enough to show a significant correlation to Pb and As bioavailability. Second, different times, temperatures and water contents could have been investigated in freeze-thaw treatments in this study. Larger sampling size and design of freeze-thaw experiments, perhaps also combined with in vivo experiments could be conducted in future.

5. Conclusions

We investigated the effect of freeze-thaw action on the extractability and bioavailability of Pb and As in contaminated soils, based on the Tessier sequential extraction method and four G-I tract simulation methods (IVG, PBET, SBRC and UBM). Our results indicated that (1) the freeze-thaw action significantly increased the bioavailability of Pb and As in soil; (2) the freeze-thaw action decreased the percentage of residual fraction (F5), and increased the percentage of exchangeable fraction (F1), carbonate-bound fraction (F2), Fe-Mn oxides-bound fraction (F3) and organic-bound fraction (F4) of Pb and As in soil; (3) Total concentrations of Al, Fe and Mn, particle size, and weight surface area were the main factors correlated with soil Pb and As bioavailability. Our findings suggest that the assessment of soil pollutants in frozen regions needs to comprehensively consider the effect of freeze-thaw on soil properties and pollutant behaviour. In addition, due to the limitation of sampling size and design of freeze-thaw treatments, further studies related to the larger sampling size and more deep design of freeze-thaw experiments (e.g., temperature and water content) for in vivo experiments are required to confirm our findings and explore how the freeze-thaw action affecting on the bioavailability.

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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.

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