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In vitro estimates of bioaccessible nickel in field-contaminated soils, and comparison with *in vivo* measurement of bioavailability and identification of mineralogy

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ABSTRACT

This study determined nickel (Ni) bioaccessibility in weathered smelter-contaminated soils, separately for particle-sized fractions using two *in vitro* methods: simulated gastrointestinal digestion (PBET) and PBET followed by absorption by Caco-2 cells. Relative bioavailability of Ni in soils was determined *in vivo* using rats, validating *in vitro* estimates; a mineralogical basis of variation in bioavailability/bioaccessibility among soils was explored. *In vitro* assays identified the same difference in bioaccessibility for Ni among particle size fractions. PBET estimates were more precise, thus likely to be more useful in discerning differences among soils. *In vivo* bioavailability for Ni was below limit of detection for the small soil particles, and 31% and 56% for the larger particles. The relative bioavailability calculated from this work suggests that risk from ingesting Ni-contaminated soils could be overestimated by between 2- and 50-fold if the estimates of exposure are not adjusted for the lower bioavailability of weathered Ni originating from smelter emissions. The overestimation that would occur by using total Ni is greatest for the particle size that is most likely to adhere to the hands of children, demonstrating the importance of particle-size separation of soils for bioavailability determination and risk assessment.

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1. Introduction

Frameworks for human health risk assessment (HHRA) of contaminated soil typically include the option to consider site-specific bioavailability of trace elements that are ingested with soil, in the estimate of exposure for calculating risk (CCME, 2006). As the site-specific bioavailability for a trace element may be quite different than in the studies which formed the basis for its oral reference dose (RfD), adjusting exposure estimates for site-specific bioavailability is appropriate and could significantly reduce the identified risk, and thus the scale of remediation. The adjustment factor, "Relative Bioavailability" (RBA) is the ratio of absolute bioavailability (ABA_{test}) of the test material to absolute bioavailability of the reference ($ABA_{reference}$) dose material. Site-specific bioavailability (ABA_{test}) is typically determined by *in vivo* feeding studies using the contaminated soils, although validated *in vitro* assays can be used as a surrogate for estimating *in vivo* bioavailability. The U.S. EPA (2007) developed guidance for determining site-specific adjustment factors: bioaccessibility and bioavailability are trace element specific, should be determined for a number of field-contaminated soils, and provided that the correlation between these estimates meets statistical criteria, bioaccessibility assay data can be used to determine site-specific RBA values for exposure assessment. Casteel et al. (2006) described a validated *in vitro* assay for lead (Pb) oral availability, using 19 soils or soil-like materials, measuring bioaccessibility with a physiologically-based *in vitro* extraction technique (PBET) extraction, and measuring bioavailability *in vivo* using juvenile swine. The RBA for the individual soils varied from 5 to 105% relative to lead acetate, this wide range demonstrating the potential importance of site-specific RBA. Mineralogical characterization of the soils supported the range of RBA: soil-Pb in the form of galena or anglesite ($PbSO_4$) had an RBA of less than 10%, whereas Pb associated with manganese oxide or Cerussite ($PbCO_3$) had an RBA close to 100%, demonstrating the contribution of mineralogy to the control of RBA.

The conditions under which both the *in vivo* and *in vitro* assays are conducted can vary broadly, and influence the outcome, with the variations typically arising in order to make the assay more comparative to juvenile human ingestion. *In vitro* assays simulate the gut chemistry: some extractions are simply buffered acid at stomach pH (Drexler and Brattin, 2007), while others simulate to some degree the biochemistry of the gastric and intestinal phases of digestion (Oomen et al., 2001). These methods measure the liberation of a trace element from the soil matrix (i.e. "bioaccessible"). Free-metal may be sorbed during the *in vitro* assay by addition of iron oxide gel (Rodriguez and Basta., 1999) or after the assay using cultured intestinal epithelial cells (Oomen et al., 2001). *In vitro* assays using Caco-2 intestinal cells in culture have been applied to evaluating the absorption of drugs, and to the study of toxicity and

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metabolism of xenobiotics (Duizer et al., 1999; Vasiluk et al., 2007; Vasiluk et al., 2008). They have also been used to study bioavailability of iron from foods (He et al., 2008; Zhu et al., 2006) through measurement of ferritin induction, so may be well-suited to studying bioaccessibility of trace metals in soils. After three weeks in culture, Caco-2 (derived from a colonic adenocarcinoma (Artursson and Borchardt, 1997)) cells undergo spontaneous differentiation forming a monolayer of highly polarized cells, joined by functional tight junctions, with well-developed and organized microvilli on the apical membrane (Hidalgo et al., 1989). Caco-2 cell monolayers resemble mature intestinal enterocytes both morphologically and biochemically, and express many brush border enzymes.

Soil consists of a wide range of particle sizes; for estimating risk to children, the US EPA (U.S. EPA., 2008) assumes that most children ingest relatively small quantities of soil, e.g., <100 mg/day. The soil ingested involuntarily by children is usually of smaller particle size (Calabrese et al., 1997). Sheppard and Evenden (1994) found that soil particles less than 50 μm in diameter tended to preferentially adhere to dry skin, regardless of soil type. It was also found that concentrations of soil-bound contaminants, such as lead, mercury, uranium and hexachlorobenzene were up to 10-fold higher in fine adhering fraction of the soil (Sheppard and Evenden, 1994). Children may also deliberately ingest larger quantities of soil (on the order of 1000–5000 mg/day or more), which can be either occasional or pica behavior. The soil in this scenario is presumed to consist of both large and small particles (Ljung et al., 2007). Most studies of oral bioaccessibility/bioavailability for metallic elements have used bulk soil with particles <250 μm, characterized by the USEPA (cited in Richardson et al., 2006) as a reasonable upper bound (U.S. EPA., 2008). This debate about relevant particle size is only important if total metal concentration, and bioavailability of the metal, vary with particle size. There is a growing body of evidence that metal concentration and bioavailability do vary considerably with particle size fraction (Edwards and Lioy, 1999; Kissel et al., 1996; Ljung et al., 2006; Madrid et al., 2008; Sheppard and Evenden, 1994), which could be anticipated as larger soil particles are those that resist weathering, so typically with quartz as the parent material which has a limited capacity for binding of cationic metals.

The mineralogy of the trace element in the soil is a well-known influence on bioaccessibility/bioavailability — demonstrated for arsenic (As) (Meunier et al., 2010), for Pb (Casteel et al., 2006) and for nickel (Ni) in simulated lung fluid (Oller et al., 2009). Mineralogy can be inferred from sequential extraction-type assays (e.g. CISED, (Cave et al., 2004)) or SEM–EDS (Scanning Electron Microscope equipped with an Energy Dispersive X-ray Spectrometer) (SGS, 2009); once the predominant minerals in the soil associated with the trace element are identified, their relative chemical solubilities can be compared to the outcomes of the *in vivo* and *in vitro* assays.

Currently, the favored *in vivo* model animal for bioavailability studies is the juvenile swine (Casteel et al., 2006) because of the purported similarity in the physiology of their digestive tract to that of children. In some cases, rodents might be more relevant particularly for trace elements for which the RfD was determined from toxicity studies using rodents.

Published studies to date have primarily focused on As and Pb due to their prevalence at contaminated sites and their toxicity to humans. There is a need to extend the principles of bioaccessibility/bioavailability to other trace elements to address current and legacy contamination from Ni smelting and refinery sites, some of which have operated for a century, such as the Vale complex in Sudbury, ON. The Ni species found nearby Vale complexes are common to all sulfidic nickel operations, and some species (oxide and metal) are common to many types of Ni refineries. Also, oxide and sulfate are common species in ambient air as a result of fuel combustion. Ni oxides are common species found around stainless steel manufacturing too, making this study of broad interest. Ni bioaccessibility has not commonly been assessed (Hamel et al., 1998; MOE., 2002; Oller et al., 2009). The first objective of this study was to evaluate the differential bioaccessibility of Ni in particle-sized fractions of soils containing weathered smelter emissions using two methods: *in vitro* simulated gastric or gastrointestinal digestion, alone, as well as followed by absorption by Caco-2 cells. The mineralogy of the soil-Ni in the particle size fractions was characterized using SEM–EDS, with the goal of explaining variation in bioavailability/bioaccessibility among soils. The second objective of this study was to determine ABA of these soils *in vivo*, and calculate RBA relative to the reference material in the present study and to the material used to derive the RfD (nickel sulfate hexahydrate).

2. Materials and methods

2.1. Soil characteristics

Two naturally weathered soils with elevated metal concentrations from historical Ni refinery emissions were used in this study. A sandy soil was collected from the 0 to 15 cm layer of a sparsely wooded area located just outside Sudbury, ON, within 10 km of two major Ni–Cu smelters, where total soil-Ni concentrations were four-fold higher than the Soil Quality Guideline for industrial soils from Canadian Council of Ministers of the Environment (CCME) (50 mg/kg). The top 30 cm of a heavy clay soil was collected from an agricultural field located north of Lake Erie, in Port Colborne, ON which had received emissions of Ni as well as Cu, Co and As from the Inco Ni refinery, which operated from 1918 to 1984 (Everhart et al., 2006). Soil concentrations of Ni in this field approached 40-times the CCME Soil Quality Guideline.

The soils were air-dried, dis-aggregated in a plate grinder with a gap of 3 mm, then screened (2 mm) to remove large pieces, and then screened to exclude particles greater than 250 μm. The "bulk" soil (<250 μm) was sieved using fluorocarbon screens into four fractions of particle size: 150–250 μm, 105–150 μm, 70–105 μm, and <70 μm, although not all soils had particles in each of these ranges. NIST standard reference material 2710 (U.S. Department of Commerce, National Institute of Standards and Technology, Gaithersburg, MD) was included as a third soil and a basis of comparison with previously published bioaccessibility studies. Ni concentration in soils was determined by hot-acid digestion, as described later in this section.

2.2. In vitro PBET digestion

The PBET digestion process used physiological constituents and transit times for fasting conditions in children, beginning with stomach digestion. The composition of synthetic gastric and intestinal fluids were as described in Minhas et al. (2006), which was a modification of Ruby et al. (1993). "Gastric" bioaccessibility used a 1:100 ratio, with 10 mg of soil added to 1 ml of gastric fluid, followed by incubation at 37 °C for 2 h. For "intestinal" bioaccessibility, the leached gastric solution was further incubated with bile salts, pancreatin, and bovine serum albumin (Minhas et al., 2006). Solutions resulting from gastric and gastric + intestinal digestions were analyzed for Ni by GF-AAS following filtration using Whatman® paper filter Grade 42 (particle retention size >2.5 μm) (Whatman Inc., NJ, USA). The mass of Ni present in the total volume of the supernatant expressed as a fraction of the total mass of Ni in the soil before digestion was "ABA_{PBET}".

2.3. In vitro PBET plus Caco-2 cells

The human colorectal carcinoma cell line (Caco-2) was obtained from the American Type Culture Collection (ATCC catalog No. HTB-37). Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Gibco BRL, Burlington, ON) with high glucose (4.5 g/l) supplemented with 1% non-essential amino acids, penicillin (25 IU/ml) and gentamicin (0.5%, v/v), with 10% Bovine Growth Serum, at 37 °C in an atmosphere of

5% CO_2 and 90% relative humidity. Caco-2 cells between passages 30 and 40 were seeded ($1–2\times10^5$ cells/insert) on 12-well Transwell polycarbonate cell culture inserts (Becton Dickinson, Franklin Lakes, NJ, USA) with high pore density (0.4 μm pore size). Cells were incubated for three weeks and used for experiments between 21 and 28 days after seeding. The formation of a sealed monolayer was confirmed by the measurement of transepithelial electrical resistance (TEER) before and after exposure to the supernatant (Vasiluk et al., 2005). Decreases in the TEER values are indicative of increased paracellular permeability and correlate with increased permeability to low molecular weight chemicals such as mannitol (Konsoula and Barile, 2005). Therefore, any cell insert with a TEER value <200 Ω/cm^2 was rejected.

Cells were washed with a buffered solution (pH 7.4) to remove culture medium, and neutralized intestinal fluid from PBET digestion (pH adjusted to 7 using granular $NaHNO_3$) was added to the apical chamber. Cells were incubated at 37 °C with gentle shaking and the basal medium was changed after 1 h to mimic blood flow. At the end of the second hour of incubation, cells were washed again with buffered solution, trypsinized and scraped off the inserts for hot acid digestion and analysis for metal content. The $ABA_{PBET+\ Caco2}$ for Ni was calculated by dividing Caco-2-sorbed Ni mass by the pseudo-total Ni mass of the soil sample obtained by hot nitric acid digestion.

2.4. Sample digestion and measurement of metals

Nickel concentration in soil and cell matrices was determined by the modified hot nitric acid Teflon-vessel digestion procedure (Topper and Kotubyamacher, 1990). Briefly, approximately 10 mg of soil or trypsinized and scraped Caco-2 cells were placed in a 60-ml Teflon digestion vessel with trace metal grade HNO_3 (1 ml for ~10 mg of soil sample or cell tissue), sealed and oven-heated at 110 °C overnight (pre-digestion was not required). At the end of the digestion, 18 MΩ water was added for a total volume of 5 ml. NIST SRM 2710 was digested with each batch of soil samples, and all were analyzed using graphite furnace atomic absorption spectrometry (GF-AAS) with Zeeman background correction. Recovery values for SRM 2710 were 11.8 ± 4.2 mg/kg of Ni (leachable value 10.1 mg/kg). Acid blanks of PBET solution were also digested and analyzed with each batch of samples. Blank samples were found to be "clean". Soil samples were diluted or analyzed using Flame AAS if the concentration of the metal exceeded the upper boundary of the optimal working range (100 μg/kg) for the GF-AAS. A blank and an internal quality control spiked sample were run every 10 samples, and the instrument was recalibrated every 30th sample. If the relative standard deviation (RSD) of absorbance was greater than 10%, then the instrument was recalibrated.

2.5. Mineralogy (SEM–EDS)

The mineralogy and size distribution of the particles within each size-class for the soils was explored using scanning electron microscopy equipped with an energy dispersive spectrometer (SEM–EDS) (SGS, 2009). One 30 mm polished section was made from a representative portion of each sample as received, including single point analysis on about 30 individual grains. The longest length and width of each particle were measured.

2.6. In vivo experiments

Male Sprague–Dawley rats, weighing between 126 and 150 g (38–42 days old), (Charles River Laboratories, Montreal, QC), were chosen for this study for comparability with the earlier *in vivo* studies of Ni fate and toxicity (Ambrose et al., 1976; Ishimatsu et al., 1995) upon which the RfD for Ni is based (IRIS, 1996).The animals were individually housed for the first week in regular cages for acclimation to reversed light cycle, and then were randomly assigned to one of the treatment groups. In order to adapt the rats to metabolic cages for the subsequent feeding and mass balance study, they were acclimated to these cages for ~2 h each day for three days preceding the study. During this period, the rats were introduced to the experimental diet (Teklad Global 18% Protein Rodent Diet, formula 2018, Harlan Teklad Certified Diets, Madison, WI, USA) in ground form to prevent animals from taking the large food pellets into the cages and contaminating urine and feces. During the whole period, the animals were given 18 MΩ water to drink and the rodent food *ad libitum*. Adverse effects from Ni exposure were not detected based on daily health evaluations, food consumption, and weight gain of the animals.

At time 0, the rats were given a single dose of either Ni-contaminated soil, or $NiSO_4\cdot6H_2O$, administered by gavage and using 0.5% methylcellulose as a carrier. Negative control animals received only 0.5% methylcellulose. Each gavage dose for each animal was prepared individually, to ensure similar doses: 0.3 g of soil per rat was brought up to 1.5 ml total volume with methylcellulose. A non-treated group served as a control for determining background Ni exposure from diet and drinking water. Immediately after the dosing, the animals were placed in the metabolic cages for the remainder of the experiment. Urine and feces were collected every 24 h for 72 h, as a preliminary study indicated that this was sufficient to clear the gut. At the end of 72 h, the rats were weighed and euthanized using CO_2. Liver, kidney and blood serum samples were collected, and stored at −20 °C. Collected feces were dried at 60 °C, weighed for determination of total dry mass eliminated and then processed in a small grinding mill to fine particles in order to homogenize the material for subsampling. A soil splitter was used to accurately divide the collected mass into two representative samples of the fecal output of each animal, at each time point. One half was archived, and the other half of the fecal sample was weighed into a flat bottom 250 ml boiling flask. Each weigh boat was rinsed with 18 MΩ water to ensure that the entire sample was in the flask. Twenty milliliters of trace metal grade nitric acid was added to each flask, swirled and left open in the fume hood for overnight pre-digestion. Twelve hours later, 5 ml of HCl (12 M) was added to each flask before placing on a hot plate (~90 °C). When boiling flasks were no longer emitting brown smoke, indicating complete oxidation of the sample's organic matter, the flasks were removed and cooled slightly and another 5 ml of HNO_3 was added to each flask, which was then returned to hot plate. After approximately 8 h of digestion, the hot plate was turned off and flasks were left to cool down gradually on the plate. The entire solution was filtered as previously described the next day into a 50 ml volumetric flask, rinsed and then brought up to 50 ml volume. All the samples were analyzed on the FAAS instrument.

3. Results

3.1. Study soils

Only two of the possible four particle-size fractions, the biggest (150–250 μm) and the smallest (<70 μm), were used for this study. The smaller particles are more likely to adhere to surfaces for transport or/and ingestion and also have a larger surface area–volume ratio, leading to greater contact with gastrointestinal fluids. The smaller particle-size fraction of Sudbury soil (S<70 μm) had an almost two-fold greater concentration of Ni compared to the bulk soil and almost eight-fold greater concentration of Ni than the larger particle size fraction of the same soil (S150–250) (Table 1). The Sudbury soil was sandy, as indicated by the percent of total mass represented by the larger particles. Sandy soils typically have lower cation exchange capacity and greater bioaccessibility of metals. In contrast, more of the particles from the Port Colborne (PC) soil were <70 μm (42.43% from the bulk), and the soil would be classified as a heavy clay. Total Ni concentrations were not different between the two particle sizes of PC soils, but were much higher than the soils from Sudbury (Table 1). All soils had relatively low total organic carbon content, although the Port

Table 1
Characteristics of bulk soils and the particle-sized fractions. Each value is a mean of three independent experiments with three replications, and the SEM (standard error of mean).

Soils	Fraction, % from bulk (weight)	pH	TOC%	Ni mg/kg
CCME (residential)				50
NIST 2710 (<74 μm)	N/A	5.81[a]	3[a]	14.3 ± 1.0
S bulk <250 μm	N/A	3.7	5.00	192 ± 6.4
S150–250 μm	34.31	3.3	3.17	46.5 ± 3.1
S<70 μm	36.38	3.6	6.24	361 ± 14.1
PC bulk <250 μm	N/A	5.1	5.08	1920 ± 62.0
PC150–250 μm	27.61	5.1	5.60	1720 ± 53.0
PC<70 μm	42.43	5.0	4.58	1990 ± 52.4

[a] Data from (Ahnstrom and Parker., 1999).

Colborne soils had twice that of the Sudbury soils (Table 1). All soils were acidic: the soil fractions from Sudbury had a pH between 3.3 and 3.7, and the soil fractions from Port Colborne had a pH of 5 or 5.1 (Table 1).

3.2. Bioaccessibility

After gastrointestinal digestion, the average bioaccessibility of Ni in S<70 was 3.59% whereas the average bioaccessibility of Ni in S150–250 was 18.7% (Table 2A). The bioaccessibility of Ni in both particle size fractions of Port Colborne soil was about 11%. When bioaccessibility is taken into account, the eight-fold greater Ni concentrations in the <70 μm fraction of the Sudbury soil translates into only a four-fold greater release of Ni relative to the 150–250 μm fraction. The fraction of total soil Ni mobilized by PBET was not related to total soil Ni concentration among the four soil fractions. The bioaccessibility of Ni in SRM2710 was approximately 15% (data not shown), which compares with 12% bioaccessible Ni in SRM 2710 after the gastric phase reported by Hamel et al. (1998) using 100:1 fluid to solid ratio. For the soils used in this study, gastric bioaccessibility was slightly higher than intestinal bioaccessibility only for Port Colborne soils (Table 2A).

Simulating absorption of the solubilized soil Ni using Caco-2 cells ("pseudo-bioavailable") resulted in much lower estimates of orally bioaccessible Ni than PBET alone — it was approximately seven-fold lower than that of PBET (S<70 μm) and almost negligible for the Port Colborne soil fractions (Table 2A). However, the ranking of pseudo-bioavailability among soils was similar to that resulting from PBET (Table 2A). Pseudo-bioavailable metal concentrations were also more variable for soils with lower total metal concentrations (S150–250 and SRM 2710, Table 2) having values very close to LOD, suggesting a practical lower limit on the usefulness of Caco-2 cells.

The soils, and particle size fractions within the soil, varied in their influence on pH during the gastric and intestinal phases (Table 2B): the smaller particle size fraction elevated pH during gastric phase more (0.71 and 0.17, Port Colborne and Sudbury, respectively) than the larger particles (0.48 and 0.034, Port Colborne and Sudbury, respectively). All four soils raised pH during the intestinal phase: for the Port Colborne soils, the change was smaller (0.30 and 0.25, smaller and larger particles, respectively) relative to those for the gastric phase. For Sudbury soils, the changes were larger (0.35 and 0.54, smaller and larger particles, respectively) than those observed for the gastric phase (Table 2B).

Table 2
Oral bioaccessibility of Ni in soils following gastric and gastric/intestinal simulated digestions; vpH drift during gastric and intestinal digestion. Each value is a mean ± SEM (standard error of the mean was calculated using following formula $\left(\frac{Error\ \ Total\ \ Ni}{Mean\ \ Total\ \ Ni}\right)^2 + \left(\frac{Error\ \ PBET}{Mean\ \ PBET}\right)^2 = \left(\frac{Error\ \ BA}{Mean\ \ BA}\right)^2$). Coefficient of variation (CV in parenthesis) values used to describe variability in measurements.

A.

Soil	% BA (gastric)	%BA (gastric + intestinal)	%BA (gastric + intestinal + Caco-2 cells)
SRM 2710	N/D	16.6 ± 1.5 (0.09)	<LOD
S150–250	17.2 ± 4.8 (0.28)	18.7 ± 4.9 (0.26)	30.7 ± 17.5 (0.57)
S<70	3.88 ± 0.9 (0.23)	3.59 ± 1.1 (0.30)	1.44 ± 0.5 (0.35)
PC150–250	13.7 ± 3.0 (0.22)	11.4 ± 2.0 (0.17)	0.18 ± 0.04 (0.22)
PC<70	13.5 ± 3.8 (0.28)	10.8 ± 3.0 (0.28)	0.52 ± 0.2 (0.38)

B.

Soil	pH drift (gastric)	pH drift (intestinal)
S150–250	+0.034	+0.54
S<70	+0.17	+0.35
PC150–250	+0.48	+0.25
PC<70	+0.71	+0.30

N/D = not determined.

3.3. Estimating mineralogy

SEM–EDS analysis of Sudbury soils yielded mainly high concentrations of Fe and O, containing different amounts of Ni and Cu or both (Table 3A). Semi-quantitative analysis of the various minerals was performed for 33 grains of S<70: 27 grains out of 30 were Ni particles. The majority of Ni grains also included Fe oxide (15 grains) or Fe-oxide-Cu (10 grains); only two grains were found as Ni-oxide. Ni and Cu alloys were not observed in Sudbury soils. Almost all of the Ni-containing grains were liberated, spherical or anhedral in shape and were commonly intergrown with carbonaceous material. Twenty four grains were analyzed for S150–250, of which 13 grains contained Ni — seven of the thirteen grains also contained Fe oxide and six of the thirteen grains contained Fe-oxide-Cu (Table 3A). The mean values of the two longest dimensions ("length" and "width") of Ni-bearing grains were the same for both particle-size classes of Sudbury soils, and all four means had very high variability (Table 3B). However, only S<70 had Ni-bearing grains for which both dimensions were less than 2.5 μm, and they represented 22% of the total number of Ni-bearing grains (Table 3B).

SEM–EDS analysis of Port Colborne soils also produced mainly Ni and Cu dominated particles. Semi-quantitative analysis of PC<70 was performed on 31 grains, of which 23 had Ni (Table 3A), with NiO in 16 of the 23 grains. Three of the 23 grains were Ni-Cu-oxide and one was Ni alloy. Only two of the 23 grains were Ni associated with Fe-oxide, and one was Ni associated with Fe-oxide-Cu. Thirty-two grains of PC150–250 were interrogated, 31 of which contained Ni; 26 were Ni-oxide, three were a Ni alloy and two were a Ni–Cu alloy (Table 3A). The metals were all liberated (Ni particle did not have attachments of other minerals); mainly associated with particles with irregular surface and only few grains had a spherical shape. The mean values of the two longest

Table 3
Frequency (A), mean width and length (B) (±SEM), and% of Ni-containing grains in soils with both dimensions <2.5 μm determined using SEM–EDS.

A.

	S<70	S150–250	PC<70	PC150–250
Ni oxide	2	0	16	26
Ni alloy	0	0	1	3
Ni–Cu alloy	0	0	0	1
Ni–Cu oxide	0	1	3	1
FeO–Ni	15	7	2	0
FeO–Ni–Cu	10	6	1	0
Ni-particles/total particles	27/33 (82%)	14/24 (58%)	23/31 (74%)	31/32 (97%)

B.

Soil	Width, μm	Length, μm	Ni-bearing grains with both dimensions <2.5 μm (%)
S<70	23 ± 4	21 ± 4	22
S150–250	19 ± 5	20 ± 5	0
PC<70	13 ± 3	10 ± 3	26
PC150–250	26 ± 5	24 ± 6	6

dimensions ("length" and "width") of Ni-bearing grains for PC<70 were arithmetically smaller than for PC150–250, but were statistically similar because of the high variability associated with these means (Table 3B). Both size classes had Ni-bearing grains for which both dimensions were less than 2.5 μm, but for PC<70 they represented 26% of the total number of Ni-bearing grains, vs. 6% for PC150–250 (Table 3B).

3.4. In vivo studies

Ishimatsu et al. (1995) reported that soluble Ni compounds orally administered to rats have half-lives of about 5 to 6 h. Based on this estimate, over 90% of dosed Ni was expected to be excreted within 24 h; 35 h (five times the estimated half-life) would allow for almost complete clearance. A preliminary study demonstrated that by the end of 72 h, all of the administered Ni had been cleared (Fig. 1). After 24 h, approximately 39% of dosed $NiSO_4 6H_2O$ was absorbed by the animals (Table 4). In contrast, none of the soil Ni was absorbed from the smaller particles, and approximately 12% of the Ni associated with the bigger particles was absorbed by the animals (Table 4). As for Sudbury soils, Ni bioavailability for the smaller Port Colborne particles was negligible (<LOD), while the Ni in the larger soil particles was about 22% bioavailable (Table 4). These results differed from the mean values for Ni bioaccessibility in Port Colborne soils, for which soil-Ni in both particle-size fractions was about 11% (Table 2A). Relative bioavailability (RBA) for Ni in soils was nil for the small particles, and 31% and 56% for the larger particles of Sudbury and Port Colborne, respectively (Table 4).

4. Discussion

It is quite typical that total metal concentrations are higher in smaller soil particles than larger, as clay has a greater capacity for surface complexation of cations (Adhami et al., 2008; Bolton and Evans, 1996). Total As concentration was observed to be larger or similar in small particles relative to bulk (Girouard and Zagury, 2009). Ljung et al. (2006) found that As concentrations in the <50 μm fraction of playground soils were higher than those found in the <4 mm fraction. This was also observed by Sheppard and Evenden (1994) for Mn, where they found higher concentration in the smaller particle size fraction compared to the total fraction. Siciliano et al. (2009) observed that metal concentrations were significantly enriched in particles <150 μm relative to the bulk soil: 140% for Cr, 140% for Cu, 130% for Ni, 110% for Pb, and very significantly enriched for silver (Ag) (810%), selenium (Se) (500%) and As (420%). The present study was only partially consistent with these observations: the Port Colborne soil had similar Ni concentrations in the two particle size fractions, whereas the Sudbury soils had greater total concentration of Ni in the smaller particles. Generalizing a relationship between particle size, metal concentration, and bioavailability is complicated by assumptions of size cut-off for the "adhering fraction". Particles <70 μm may also include some sand as well as clay and silt (50 μm–2 mm) (Shedrick and Wang, 1993), whereas a cut-off of 45 μm (based on median size of adhered particles of 36 μm (Siciliano et al., 2009) or 39 ± 26 μm (Yamamoto et al., 2006)), include only clay (<2 μm) and silt (2–50 μm). Among soils sieved to the same particle size cut-off, there could be great variance in total capacity for surface complexation. These findings highlight the importance of particle size separation for assessing the risk to human health from accidental ingestion of contaminated soils, even if bioaccessibility of the contaminant is not taken into account.

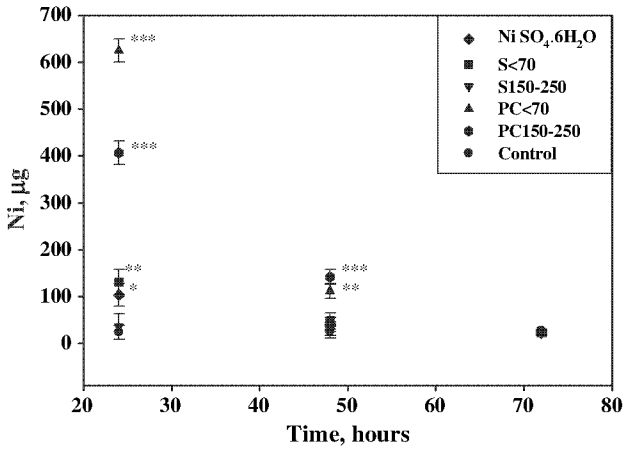


Fig. 1. Mass of Ni eliminated within 72 h after one-time orally administered dose by gavage. Eliminated Ni sulfate hexahydrate (closed diamonds), S<70 μm (closed squares), S150–250 μm (closed triangles down), PC<70 μm (closed triangles up), PC 250–150 (closed hexagons) and negative control (closed circles). Each datum is the mean ± standard error of mean (SEM) of two repeats, with three independent animals in each repeat. Mass of Ni excreted by experimental animals was greater than by the negative control group at P<0.01 (*), P<0.005 (**) and P<0.001(***).

Table 4
Absolute bioavailability of ingested Ni 24 h after dosing (least squares mean ± standard error). Relative bioavailability of ingested Ni ($ABA_{test}/ABA_{reference}$) where $ABA_{reference}$ is for $NiSO_4 6H_2O$ as determined from this study.

	Reference	Test materials			
	$NiSO_4 6H_2O$	S<70	S150–250	PC<70	PC150–250
ABA (%)	39 ± 12	0.2* ± 13	12 ± 13	−4* ± 12	22 ± 12
RBA (%)	100	0.5	31	−10	56

* Mean value smaller than mean value for Reference (P<0.05).

Ruby has shown that gastrointestinal bioaccessibility of contaminants is greater in smaller particle size fractions because of their greater ratio of surface area to volume (Ruby, 2004). This generalization depends on the reactive capacity of the extractant not being exhausted by the surface area, which depends on the aggregate surface area of the particles in the fraction, as well as the acid neutralization capacity of the soil. Girouard and Zagury (2009) observed greater bioaccessibility of As (mg/kg of soil) in fine particles (<90 μm) of two soils that had the same total As concentration as bulk (<250 μm) soil, likely because more total surface area of the small particles was exposed to extractant. However, this mechanism is likely pH dependent: the bioaccessibility of Ni in playground soils was greater for particles <4 mm than for particles <50 μm (Ljung et al., 2007), and the smaller particles had a slightly higher pH in both gastric and intestinal phases. The authors attribute this to the greater reactive surface area exhausting the supply of protons in the solution, resulting in less liberation of metal from the smaller soil particles relative to their mass. Similarly, in the present study, pH was higher after the gastric phase for both smaller particle size fractions, with PC<70 increasing pH the most.

This generalization about the relationship between particle size and trace element bioaccessibility also requires similarity of mineralogy among particle sizes, as the mineral form of the contaminant is a critical determinant of bioaccessibility/bioavailability. Meunier et al. (2010) demonstrated that arsenic sulfides and iron arsenates were much less bioaccessible mineral forms of As than were arsenic sulfates and calcium-iron arsenates. Casteel et al. (2006) demonstrated that for Pb, certain types of minerals (such as cerussite, Mn/Pb oxide) were well absorbed whereas other minerals (for example, galena and anglesite) were not. However, these authors concluded that they did not have enough data to robustly predict RBA from mineralogy alone. Oller et al. (2009) demonstrated that Ni was almost completely released from the salts nickel sulfate and nickel carbonate into ammonium citrate, but that the release of Ni from Ni metal powder, Ni_3S_2 and green NiO was sparing. In the present study, the mineralogy of the soil fractions from the same site was similar, but differed between the two sites: Port Colborne soils were dominated by NiO whereas almost all of the Ni-

bearing particles in the Sudbury soils were FeO–Ni. Neither of these mineral forms of Ni is very soluble, even in acid: Girouard and Zagury (2009) reported that Fe and Al oxides did not dissolve during the simulated gastrointestinal digestion, and NiO is poorly soluble even in acid at 20 °C (Rhoda et al., 1981). The disjunct in the relationship between bioaccessibility and bioavailability for the Ni in the Port Colborne soils may be because some Ni-bearing grains in the PBET solutions from the smaller particle-size fraction were not captured by filtration after PBET. This possibility is supported by the grain-size measurements (Table 3B): the average length and width of Ni-bearing particles in PC<70 were the smallest of the four soils. Smaller particles can have lower bioaccessibility than the same mass of larger particle sizes if dissolution is limited by surface area:proton concentration during the gastric or intestinal phase. All four of the soils elevated the pH of the digestion (suggesting greater acid neutralization capacity), but Port Colborne soils raised the pH more than Sudbury, and the smaller particles raised pH more than the larger particles.

In vitro estimates of bioavailability (i.e. bioaccessibility) are of value to risk assessment when they are validated by correlation with *in vivo* bioavailability estimates for the same soil. While some feel that the correlation needs to be 1:1, others are of the view that a statistically significant correlation is sufficient for "validation". Bioaccessibility was highly correlated with bioavailability estimated using weanling rats for Pb in a variety of contaminated soils, with bioaccessibility of Pb being about 70% of the bioavailability in the same soil (Ruby et al., 1996). A similar correlation for Pb in soils was shown between the gastric phase of PBET and juvenile swine (Kelly et al., 2002), leading the US EPA to develop guidance as to the methods for estimating site-specific bioavailability of Pb in soils that can be used to develop Pb relative bioavailability values for use in HHRA. The correlations between *in vitro* and *in vivo* data with As have been less comprehensive (Ruby, 2004). In the present study, PBET and Caco-2 assays returned similar ranking of estimates of bioaccessible soil–metals, and identified the same difference in bioaccessibility for Ni among particle size fractions; however PBET estimates for Ni were more precise than Caco-2 estimates, thus likely to be more useful in discerning differences among soils. In addition, Caco-2 estimates of bioaccessibility underestimated bioavailability, as determined *in vivo*. The relationship between the bioavailability and bioaccessibility (PBET) of soils in the present study was not obvious, although the number of data points was too small to calculate correlation (Fig. 2). For one of the soil fractions (PC150–250) the bioaccessibility of Ni was lower than its bioavailability; for the other three soil fractions, bioavailability was lower than bioaccessibility. This is typically interpreted to be due to the fact that bioaccessibility does not account for absorption. Since *in vitro* dissolution cannot estimate the rate of absorption from the GI tract, it will be predictive of bioavailability to the degree that the mobilization from the soil matrix in the gastrointestinal solution limits oral bioavailability. For As (Rodriguez et al., 1999), the slope between bioaccessibility (IVG or PBET) and bioavailability was always less than one (i.e. bioaccessibility returned a lower value than bioavailability). The linear regression slope determined for Pb by Drexler and Brattin (2007) using swine was 0.88, and they reported that the slope of the linear correlation derived by Medlin (1997) for swine was 0.90. The linear regression slope derived by Schroder et al. (2004) for swine was 0.39 (gastric IVG without dough) or 0.65 (gastric IVG with dough). Using rats for the bioavailability testing in this study allowed the calculation of an RBA that is directly useful to risk assessment, as the original study from which the RfD was derived (Ambrose et al., 1976) was also conducted using Sprague–Dawley rats dosed with $NiSO_4 \cdot 6H_2O$ in food. The RBA's calculated from this work suggest that risk from ingesting Ni-contaminated soils could be overestimated by between 2- and 50-fold if the estimates of exposure are not adjusted for the lower bioavailability of Ni originating from smelter emissions. It should be emphasized that the overestimation of risk by using total Ni concentration in the soils rather than the bioavailable Ni concentration, is greatest for the particle size that is most likely to adhere to the hands of children. This supports the importance of soil particle size separation and bioavailability evaluation for risk assessment. The RBA estimates are uncertain due to variability among animals in measured bioavailability of the same soil, as noted by Casteel et al. (2006). However, even with this uncertainty (which could certainly be reduced by using more animals per dose group), the reduction in ABA relative to the reference material is substantial.

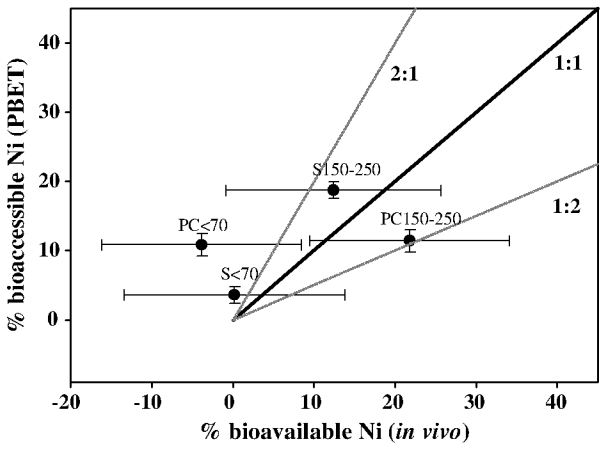


Fig. 2. Correlation between Ni bioaccessibility measured using PBET and Ni bioavailability after 24 h measured *in vivo*. Each value is the least-square mean ± SEM.

5. Conclusions

The mechanistic bases of differences between *in vivo* estimates of bioavailable Ni and *in vitro* estimates of bioaccessibility are not fully understood, but distributions of metals among size fractions, particle size/surface area ratio, mineralogy, buffering capacity of soil, absorption processes, and the interactions among these factors all likely play a role. A much larger suite of field-contaminated soils would be needed to define these relationships better, although it is notable that other studies with 20 or so soils contaminated with Pb or As have not been able to define predictive relationships between soil characteristics and bioaccessibility/bioavailability. Despite the limited number of soils in the present study, the influence of particle size on estimating exposure was clearly demonstrated for Ni in smelter-impacted soils. Risk estimates from incidental ingestion of soil particles contaminated with smelter-emitted Ni is at least less than half of what would be predicted using the current RfD (IRIS, 1996) and if estimated for the size of particles that adhere to hands, much less than half. The recently completed Sudbury Soils Study estimated risks due to soil ingestion, and bioaccessibility testing was used to estimate that on average only 44% of the total Ni in soil was bioavailable (SARA, 2008). In the present study, bioaccessibility with and without Caco-2 cells and gavage dosing of rats with two Ni-contaminated soils from Sudbury and Port Colborne resulted in bioavailability/bioaccessibility estimates that did not exceed 30%, with the finer soil fractions having lower bioavailability/bioaccessibility. Current bioaccessibility methods cannot mimic the absorptive aspects of bioavailability. As a result, bioaccessibility generally over-predicts the true bioavailability/exposure, enabling more accurate estimates of risk relative to total metal concentrations in soil, but with an inherent margin of safety.

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