EVALUATING METAL BIOACCESSIBILITY OF SOILS AND FOODS USING THE SHIME

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By

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ABSTRACT

Ingestion exposure estimates typically use a default bioavailability of 100%, thereby assuming that the entirety of an ingested dose is absorbed into systemic circulation. However, the actual bioavailability of ingested contaminants is oftentimes lower than 100%. The research described herein investigates the use of the Simulator of the Human Intestinal Microbial Ecosystem (SHIME) for the calculation of *in vitro* bioaccessibility (IVBA), a conservative predictor of bioavailability, of mercury (Hg) from traditional northern foods and arsenic (As) from soils. The primary objective of the research described herein is to address data-gaps which have hindered attempts to incorporate IVBA into risk assessment on more than a case-by-case basis. The hypotheses of this thesis are that (1) the bioaccessibility of contaminants is dependent upon concentration due to kinetic limitations on dissolution, (2) gastrointestinal (GI) microbes in the ileum and colon alter contaminant bioaccessibility and/or speciation, (3) the GI microbial effect on bioaccessibility is toxicologically relevant, and (4) metal bioaccessibility is predictable according to dissolution kinetics.

Mercury bioaccessibility from country food samples was independent of total Hg concentration ($F=0.5726$, $P=0.578$) whereas As bioaccessibility was inversely related to total As concentration for Nova Scotia mine tailings, synthesized ferrihydrite with adsorbed As^{V}, and synthesized amorphous scorodite ($P=2 \times 10^{-10}$). Isotherm analysis indicated that, at high soil As concentrations, saturation of simulated GI fluids limited As bioaccessibility under gastric conditions whereas kinetic limitations constrained As bioaccessibility under intestinal conditions. Additionally, we demonstrated that GI microbes may affect Hg bioaccessibility, either increasing or decreasing bioaccessibility depending upon the type of food. For example, the bioaccessibility of HgT decreased in the presence of GI microbial activity for caribou kidney, caribou tongue, seal blood, seal brain, seal liver, and walrus flesh. In contrast, HgT

bioaccessibility from Arctic char and seal intestine increased in the presence of GI microbial activity. Similarly, colon microbial activity increased (Fishers Protected LSD, $P<0.05$) As bioaccessibility from synthesized amorphous scorodite (56 – 110%), Nova Scotia mine tailings (140 – 300%), an agricultural soil (53%) and an ironstone soil (350%) containing elevated arsenic concentrations. However, under small intestinal conditions, this microbial effect was transient and demonstrated a small effect size.

The toxicological relevance of microbial effects upon As bioaccessibility was assessed using a juvenile swine model with co-administration of oral antibiotics (neomycin and metronidazole). This study research indicated that microbial effects on As bioaccessibility are not reflected in the juvenile swine model. For example, the microbial communities present in the pig's proximal colon clustered according to antibiotic treatment (e.g. microbial communities of antibiotic treated pigs differed from non-treated pigs). Despite this, the urinary arsenic excretion (and hence arsenic bioavailability) of antibiotic-treated juvenile swine orally exposed to soil-borne arsenic was equivalent (Holm-Sidak, $P=0.930$) to the urinary arsenic excretion of juvenile swine not treated with antibiotics. Therefore, *in vitro* GI models may not need to include a microbially active intestinal stage when measuring As IVBA.

Metal bioaccessibility from soils appears predictable according to fundamental chemical properties of the metal-of-concern. Specifically, metal bioaccessibility of 7 of the 13 metals (V, Ni, Zn, Cu, U, Cd, & Ba but not Tl, Pb, As, Se, Cr, and Hg) regulated according to Canadian Council of Ministers of the Environment Soil Quality Guidelines (CCME SQG) were strongly dependent ($R^2 = 0.7$) on water exchange rate constants of metal cations (k_{H20}) indicating that desorption kinetics may serve as the foundation of a predictive model of metal bioaccessibility.

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LIST OF ABBREVIATIONS

AF_{GIT}	Absorption Factor (gastrointestinal tract)
ANOVA	Analysis of Variance
As^{V}	Inorganic arsenate
As^{III}	Inorganic arsenite
BGS	British Geological Survey
BW	Body weight
CCME	Canadian Council of Ministers of the Environment
CV	Coefficient of Variation
DARP	Dissimilatory Arsenic Reducing Prokaryote
DIRP	Dissimilatory Iron Reducing Prokaryote
DMA^{V}	Dimethylarsinic acid
GI	Gastrointestinal
H5	Hoboken Soil #5
H18	Hoboken Soil #18
H50	Hoboken Soil #50
HGAFS	Hydride Generation Atomic Fluorescence Spectroscopy
Hg^{II}	Mercuric mercury (Inorganic)
HgT	Total mercury
$[HgT]_{filtrate}$	Total mercury concentration in <10 kDa filtrate
$[HgT]_{food}$	Total mercury concentration in food items
HgTu/LC-CVAFS	Hg-Thiourea complex Liquid Chromatography – Cold Vapour Atomic Fluorescence Spectrometry
HHRA	Human Health Risk Assessment

ICP-MS	Inductively Coupled Plasma Mass Spectrometry
I-PDVB	Polydivinylbenzene
IVBA	*In vitro* bioaccessibility
LE	Life expectancy
LOAEL	Lowest Observed Adverse Effect Level
LOD_{soil}	Soil Limit of Detection
LSD	Least Significant Difference
LSR	Liquid:Soil Ratio
MAD	Median Absolute Deviation
MDL	Methodological Detection Limit
MeHg	Methylmercury
$[MeHg]_{10kDa,Food}$	Bioaccessible (<10 kDa) MeHg concentration in food item
$[MeHg]_{filtrate}$	MeHg concentration in <10 kDa filtrate
MMA^V	Monomethylarsonic acid
MPHW	Mass Per Handwashing
NIST	National Institute of Sciences and Technology
NLS	Nonlinear Regression
PSCI	Prairie Swine Center
PQRA	Preliminary Quantitative Risk Assessment
RAF_{GIT}	Relative Absorption Factor (gastrointestinal tract)
RBA	Relative Bioavailability
SHIME	Simulator of the Human Intestinal Microbial Ecosystem
SSRA	Site Specific Risk Assessment

TRV	Toxicological Reference Value
TM1	Test Material #1 (Overburden from Mount Nanzen mine)
TMAO	Trimethylarsine oxide
TU	Thiourea
UEF	Urinary Excretion Fraction
XANES	X-Ray Absorption Near Edge Spectroscopy

1 INTRODUCTION

Risk assessors use gastrointestinal (GI) models for the calculation of *in vitro* bioaccessibility (IVBA) to estimate the percentage of ingested arsenic (As), mercury (Hg), and other contaminants likely to be solubilized into GI fluids. In contrast to bioaccessibility, oral bioavailability, which is calculated using animal testing, refers to the percentage of an ingested contaminant that is absorbed across the GI epithelium and reaches systemic circulation. Since dissolution into GI fluids is a requisite precursor for absorption, bioaccessibility is often a conservative predictor of oral bioavailability. Thus, incorporating bioaccessibility and bioavailability into exposure assessments enables risk assessors to account for limitations on the release and subsequent absorption of As and Hg after the ingestion of contaminated soils or foods. However, numerous data gaps have impeded regulators attempts to incorporate IVBA into risk assessment for more than a case-by-case basis. One such data-gap is whether or not it is necessary to include a microbially-active colon stage for evaluations of metal bioaccessibility. A second data-gap that has impeded the widespread use of bioaccessibility in risk assessment is the lack of knowledge pertaining to the factors that determine bioaccessibility of metals other than As and lead (Pb). The PhD thesis research described herein addresses both of these data gaps.

1.1 Objectives and Hypotheses

The global hypothesis of my PhD research is that the default assumption of 100% contaminant bioaccessibility overestimates human exposure to As from contaminated soils and to Hg from northern country foods. During the investigation of this global hypothesis, four sub-hypotheses are evaluated: (1) bioaccessibility of contaminants is dependent upon concentration due to kinetic limitations on dissolution, (2) GI microbes in the ileum and colon alter contaminant bioaccessibility and/or speciation, (3) the GI microbial effect on bioaccessibility is

toxicologically relevant, (4) metal bioaccessibility can be predicted according to dissolution kinetics.

For the first sub-hypothesis, the relationship between Hg IVBA vs. concentration is plotted for northern country foods and the relationship between As IVBA vs. concentration is plotted for contaminated soils. For the second sub-hypothesis, the effect of GI microbes on Hg and As IVBA was evaluated for both northern country foods and contaminated soils. The results for this research are presented in Chapter 3 (Bioaccessibility of Mercury from Traditional Northern Country Foods Measured Using an *In Vitro* Gastrointestinal Model is Independent of Mercury Concentration) and Chapter 4 (Gastrointestinal Microbes Increase Arsenic Bioaccessibility of Ingested Mine Tailings Using the SHIME). The mechanism by which total concentration limits IVBA is discussed in Chapter 5 (The Effect of Residence Time and LS Ratio on *In Vitro* Arsenic Bioaccessibility from Poorly Crystalline Scorodite). For the third sub-hypothesis, *in vitro* and *in vivo* research is presented in Chapter 6 (Increased Arsenic Bioaccessibility Due to Microbial Activity Does Not Affect Arsenic Bioavailability) to determine whether increased bioaccessibility is likely to result in increased bioavailability. The fourth sub-hypothesis is evaluated in Chapter 7 (Predicting Metal Bioaccessibility According to Lability via Water Exchange Rate Constants).

Some degree of redundancy in the presentation of this PhD thesis was unavoidable since each research chapter was written as an independent manuscript for publication in peer-reviewed journals. At the time of submission of this PhD thesis, Chapters 3, 4, and 5 were previously published in the academic journals Science of the Total Environment, Environmental Science and Technology, and Journal of Environmental Science and Health: Part A

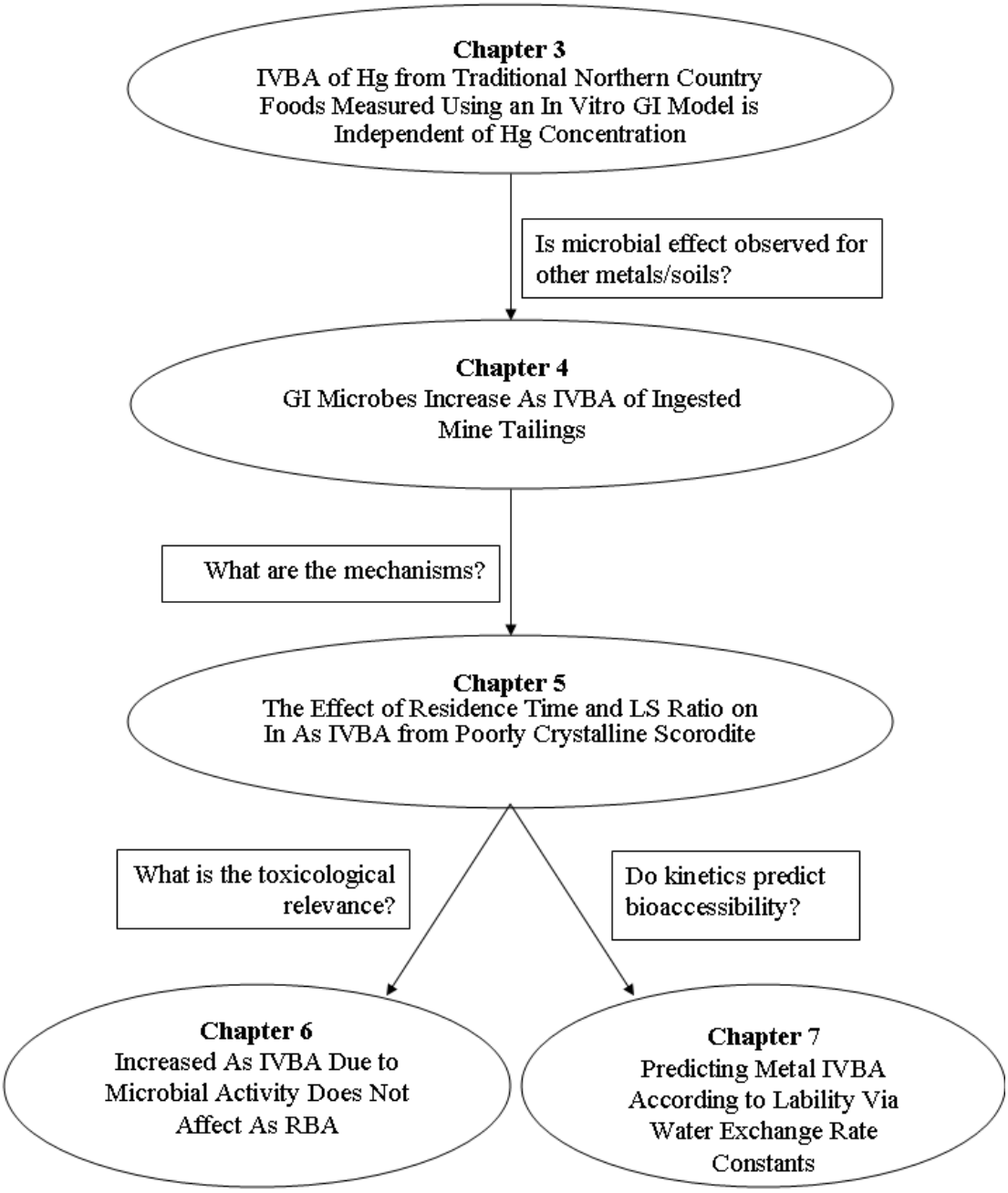

Figure 1.1 Chapter overview of BD Laird's Ph.D. thesis "Evaluating Metal Bioaccessibility of Soils and Foods using the Simulator of the Human Intestinal Microbial Ecosystem".

2 LITERATURE REVIEW

2.1 Human Health Risk Assessment

Human health risk assessments (HHRA) are performed according to standardized guidelines (Health Canada 1994, 2004a) designed to ensure that consistent approaches are adopted in the remediation and creation of public policy regarding environmental contamination in Canada. These Health Canada guidelines encourage the use of a tiered framework to maximize the efficiency of HHRA so that the attention of regulators is not monopolized by substances for which negligible risks exist. Early stages of tiered HHRA, also known as Preliminary Quantitative Risk Assessment or PQRA), require ultra-conservative assumptions to account for a lack of knowledge pertaining to the toxicity and exposure at a contaminated site. If the PQRA indicates that the environmental contamination doesn't present human health risks even with the most conservative of assumptions, than it is unlikely that interventions or intensive remediation will be required. However, if the PQRA indicates that the site may present health risk then further work is necessary to replace the ultra-conservative assumptions with realistic data. This research-intensive stage in tiered risk assessment is known as Site Specific Risk Assessment or SSRA. Both PQRA and SSRA are similar in that they characterize the effects (i.e., toxicity) and exposure (e.g., magnitude and probability) and define health risk according to the overlap of these point estimates or distributions (**Figure 2.1**).

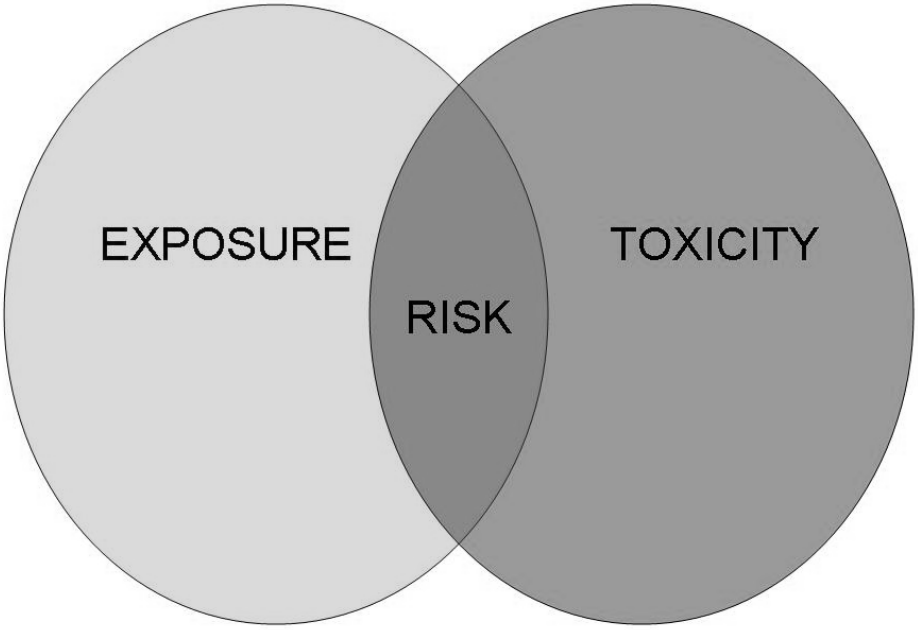

Figure 2.1 Venn diagram illustrating human health risk as a function of the exposure and toxicity of a chemical stressor at a contaminated site.

2.2 Effects Characterizations of Arsenic and Mercury

Comprehensive reviews of the human health hazards posed by arsenic (ATSDR 2007; IPCS 2001; Ratnaike 2003) and mercury (ATSDR 1999; Clarkson 2006; IPCS 1976; NRC 2000) are presented elsewhere. The purpose of this chapter is not to thoroughly survey decades of research investigating the toxicodynamics (i.e., mechanism(s) of action) and health effects of arsenic and mercury. Instead, the most critical aspects of arsenic and mercury toxicity (e.g., sensitive organs systems, effect measures, and the role of speciation) are introduced, thus providing the toxicological context for the exposure analysis described in subsequent research chapters.

2.2.1 Arsenic health effects

Arsenic is acutely toxic to humans and lab animals, with a LD_{50} as low as 4 mg kg^{-1} following a single intraperitoneal injection of sodium arsenite (IPCS 2001; Ratnaike 2003). However, a large variation in toxicity is observed between chemical forms of arsenic (ATSDR 2007). For

example, the juvenile mouse LD_{50} for sodium arsenate is 21 mg kg^{-1} but is 8 mg kg^{-1} for sodium arsenite. An even larger variation in acute toxicity is observed when comparing organoarsenicals to inorganic arsenic species. For example, the Lowest Observed Adverse Effects Level or LOAEL causing death for Sprague-Dawley rats exposed to monomethylarsonic acid (MMA^V) ranged between 2449 and 3184 mg $kg^{-1}day^{-1}$ (IPCS 2001). Furthermore, the organoarsenicals arsenobetaine and arsenocholine, which are naturally present in fish and shellfish, are thought to be essentially non-toxic (ATSDR 2007). Both MMA^V and its dimethylated counterpart dimethylarsinic acid (DMA^V) also demonstrate rapid excretion with little further metabolism and low acute toxicity (ATSDR 2007; Cohen 2006), thereby resulting in the misconception that all organoarsenicals are generally non-toxic. In fact, MMA^V causes gastrointestinal (GI) effects (e.g., diarrhea, tissue damage) at concentrations as low as 1.8 mg As kg^{-1} day^{-1} whereas DMA^V causes bladder and kidney cytotoxicity at concentrations as low as 1.7 mg As kg^{-1} day (ATSDR 2007). In addition to these very sensitive endpoints, MMA^V exposure in laboratory animals results in reproductive (e.g., decreased pregnancy rate and male fertility) and teratogenic effects (e.g., decreased fetal growth and minor skeletal defects) (ATSDR 2007; IPCS 2001). Additionally, DMA^V exposure results in thyroid hypertrophy and GI effects in laboratory animals. Rats further metabolize ingested DMA^V to trimethylarsine oxide (TMAO) resulting in the formation of DMA^{III} as a reactive intermediate (Cohen 2006). This metabolism of ingested DMA, which is unique to rats, is believed to be responsible for the carcinogenicity (neoplastic bladder tumors) of DMA^V to rats (ATSDR 2007). DMA^V has also been shown to act as a tumor promoter, increasing the incidence of bladder, kidney, liver, and thyroid cancers to rats co-administered DNA-reactive carcinogens (Cohen 2006).

In addition to these effects, long-term arsenic exposure can result in a multitude of serious adverse effects, from cardiovascular to carcinogenic (Ratnaike 2003). Initial clinical diagnosis of chronic arsenic exposure is often based upon dermatological effects, including hyperpigmentation (dark brown spots on skin) and hyperkeratosis (either uniform thickening or distinct nodules) (Ratnaike 2003). Occupational arsenic exposure has been linked with increased rates of cardiovascular disease (Ratnaike 2003), likely due to increased rates of hypertension. Neurological effects, including memory loss, behavior changes, and confusion, have been observed following arsenic exposure. Evidence for causality for neurological toxicity is strengthen by a case-study where the mental function of two adversely affected workers returned to normal after withdrawal from arsenic exposure (Ratnaike 2003). Arsenic is a known human carcinogen (ATSDR 2007; IPCS 2001), resulting in malignancies in virtually all organs of the body (Ratnaike 2003). Interspecies variation in the metabolism, distribution, and excretion of arsenic has, however, prevented laboratory animal experiments from modeling the carcinogenic effects of arsenic observed in humans (Cohen 2006). Numerous epidemiological studies investigating drinking water contamination in Bangladesh, India, and Taiwan have shown that inorganic arsenic oral exposure can result in malignancies of the skin, lung, kidney, bladder, bone, liver, larynx, lymphatic system, and colon (Guo 2004; Ratnaike 2003; Tchounwou 2004; Tsuda 1992). However, it is notable that early epidemiological studies of exposed populations in the United States did not demonstrate a strong correlation between arsenic exposure and skin cancer risk. It is unknown at this time whether this is a function of limited exposed population sizes or upon an increased sensitivity of south Asian populations due to nutritional differences and/or deficiencies (ATSDR 2007; Ratnaike 2003).

2.2.2 Mercury health effects

As observed for arsenic, there are considerable differences in the oral toxicity of mercury between chemical species. For example, one may ingest several grams of elemental mercury without appreciable adverse effects, while a lethal dose of inorganic mercury can be as low as 50 mg kg^{-1}, whereas the lethal dose of methylmercury can be as low as 16 mg kg^{-1} (ATSDR 1999). However, it is inaccurate to assert that methylmercury is more toxic than inorganic mercury since, before a toxic effect is observed, methylmercury is first intracellularly converted to inorganic mercury. It is the high bioavailability of methylmercury (i.e., the percent absorbed across the GI tract epithelium into systemic circulation) that results in methylmercury's higher hazard relative to inorganic mercury (Clarkson 2007).

Effects upon the central nervous system and kidneys most commonly drive the HHRA of mercury (ATSDR 1999; Clarkson 2007). However, oral exposure to mercury may also result in serious GI effects. GI symptoms of acute mercury exposure include gastric epithelial inflammation, ulceration, and necrosis coupled with nausea, vomiting, abdominal cramps, and diarrhea (ATSDR 1999). If exposure is prolonged, inflammation of the distal intestine may occur in addition to the formation of forestomach squamous cell papillomas (ATSDR 1999). The International Agency for Research on Cancer (IARC) lists methylmercury as a possible human carcinogen based upon a small number of epidemiological studies that demonstrated a correlation between methylmercury exposure and acute leukemia. Additionally, laboratory animal experiments indicate that chronic exposure to methylmercury can result in the formation of kidney tumors (NRC 2000). In addition to these tumorigenic effects, mercury exposure potentially affects the immune system via lymphocyte proliferation; however, once again the effect is dependent upon the mercury chemical species (NRC 2000).

2.3 Exposure Characterization

2.3.1 Exposure pathways

Human exposure to environmental contaminants can occur through several pathways: dietary consumption of contaminated foodstuffs, consumption of contaminated drinking water, inhalation of contaminated particles, and the accidental ingestion of contaminated particles (Health Canada 2004a; Paustenbach 2002). The consumption of contaminated foodstuffs and drinking water are generally the most important routes of exposure for environmental contaminants, with numerous case studies demonstrating the human cost of pollution. For example, the contamination of deep-water wells in Taiwan, Bangladesh, and India is associated with increased cancer rates and cardiovascular effects, thereby affecting millions of lives (Ratnaike 2003). Mass poisonings due to dietary mercury exposure occurred in Minamata, Japan in the 1950s and in Iraq in the early 1970s. Although the mercury source varied between these case studies, both incidents resulted in numerous developmental and neurological effects in exposed individuals (IPCS 1976). These adverse effects included sensory impairment, seizures, cerebral palsy, and permanent mental retardation of children exposed during childhood or *in utero* (NRC 2000).

For sites possessing contaminated soil, the incidental ingestion of soil is often the primary route of exposure. For example, in a HHRA of dioxin contaminated soil, 95% of exposure was via the ingestion of soil with approximately 78% of an individual's lifetime exposure occurring prior to the age of 5 (Paustenbach 1989). Consequentially, human health risks predicted to occur due to the ingestion of contaminated soil drive the remediation strategies of hazardous waste sites in the United States (Paustenbach 2002). Health risks due to the incidental ingestion of soil are thought to be greatest for children due in-part to the prevalence of hand-to-mouth behavior

by toddlers, resulting in soil ingestion rates between 2 and 10-fold greater than those observed in adults (Health Canada 2004a; NCEA 1997; Paustenbach 1989, 2002).

2.3.2 Quantifying ingestion exposure

Exposure to ingested contaminants is quantified for dietary consumption of foodstuffs (2.1), drinking water consumption (2.2), and the accidental ingestion of contaminated soils and soil-like particles (2.3) (Health Canada 2004a):

$$Dose\,(mg\ kg^{-1}d^{-1}) = \frac{\left[\sum\left[C_{Food} \times IR_{Food} \times RAF_{GIT} \times D_1\right]\right] \times D_2}{BW \times 365 \times LE} \quad \text{(Eqn 2.1)}$$

$$Dose\,(mg\ kg^{-1}d^{-1}) = \frac{C_W \times IR_W \times AF_{GIT} \times D_1 \times D_2 \times D_3}{BW \times LE} \quad \text{(Eqn 2.2)}$$

$$Dose\,(mg\ kg^{-1}d^{-1}) = \frac{C_S \times IR_S \times AF_{GIT} \times D_1 \times D \times D_3}{BW \times LE} \quad \text{(Eqn 2.3)}$$

Where C_S (mg kg^{-1}) represents the contaminant concentration in ingested soil, C_W (mg L^{-1}) represents the contaminant concentration drinking water, and C_{Food} (mg kg^{-1}) represents the contaminant concentration in food items. Similarly, IR_S (kg d^{-1}), IR_W (L d^{-1}), and IR_{Food} (kg d^{-1}) represent the receptor (e.g., infant, toddler, teenager, or adult) intake rates for ingested soil, drinking water, and food items, respectively. Both the AF_{GIT} and RAF_{GIT} (unitless) refer to the relative bioavailability (RBA) of the ingested contaminant, which reflects the percent of a contaminant that is absorbed into systemic circulation relative to that of a reference dose. D_1, D_2, and D_3 are exposure duration modifiers representing the fraction of days per year and number of years per lifetime to which the receptor is exposed to the contaminant. Body weight (BW) represents the body weight of the receptor and LE represents the life expectancy of the receptor. Notably, the number of years to which people are exposed to the contaminant and the LE of the receptor are only included in the exposure estimates for carcinogens (Health Canada 2004a).

2.3.3 Estimating gastrointestinal absorption

Risk assessors performing PQRA in Canada are typically required to assume that 100% of an ingested contaminant is absorbed into systemic circulation (i.e., $AF_{GIT} = 1.0$) (Health Canada 2004a). However, if the PQRA indicates that a higher-tier (i.e., more realistic albeit less protective) SSRA is necessary, then limitations on GI absorption may be accounted for by setting the AF_{GIT} to a value less than 1.0 (US EPA Region 8 2005). The relative bioavailability (RBA) of ingested contaminants following oral exposure is traditionally calculated using *in vivo* animal experiments that monitor the percent of an ingested dose to be absorbed into the animals' bloodstream (Diamond 1998). The RBA can be calculated using any one of a number of model organisms; however, bioavailability researchers have of late emphasized the importance of selecting test organisms (e.g., cynomolgus monkeys, juvenile swine) that possess GI tracts similar to humans (Roberts 2006). Additionally, the approach used to estimate contaminant RBA varies with the toxicokinetics of the contaminant of concern (Diamond 1998). For example, arsenic RBA is measured using arsenic urinary excretion since the majority of an ingested arsenic dose is metabolized and then excreted in urine within several days (Casteel 2001; Rodriguez 1999). In contrast, mercury is slowly eliminated via the bile duct, thereby under going enterohepatic circulation prior to fecal excretion (ATSDR 1999). Consequentially, researchers have utilized gamma-emitting Hg isotopes to evaluate the retention and bioavailability of ingested Hg (Nielsen 1992). A similar body-retention approach is used for PAHs and other lipophilic contaminants (Budinsky 2008); however, blood, liver, and/or adipose tissue concentrations are typically employed rather than radio-labeling. Regardless of the approach used to estimate contaminant oral bioavailability (e.g., urinary excretion, radio-labeled isotopes, or blood concentrations), the exorbitant time and financial investments required make such tests

inefficient for SSRAs. For example, researchers may spend up to 4 weeks and $10,000, requiring the euthanasia of at least 12 animals, to calculate the RBA of As from a single soil sample.

Since the costs of such *in vivo* animal experiments are often prohibitively expensive, not to mention ethically troubling, researchers have long sought *in vitro* alternatives for calculating AF_{GIT} (Ruby 1999). The Physiologically Based Extraction Test (PBET), an *in vitro* gastrointestinal (GI) model that simulates the physical, chemical, and enzymatic conditions of the human GI tract, was first developed for the calculation of lead bioaccessibility from contaminated soils (Ruby 1993). Contaminant bioaccessibility, in contrast to bioavailability, refers to the fraction/percent of an ingested contaminant that is released into simulated GI fluids (Rodriguez 1999). Since contaminant dissolution is typically required prior to the absorption of the contaminant across the GI epithelium, bioaccessibility is considered to be a conservative predictor and *in vitro* surrogate for *in vivo* bioavailability (Oomen 2003). Numerous other *in vitro* GI models (e.g., Simple Bioaccessibility Extraction Test or SBET; Ohio-State *In Vitro* Gastrointestinal Tract or OSU-IVG; Simulator of the Human Intestinal Microbial Ecosystem or SHIME; Unified BARGE Model or UBM, among others) have since been developed and refined for the calculation of metals and metalloids (e.g., arsenic, mercury, chromium, copper, zinc, nickel, uranium) and organic contaminants (e.g., PAHs and PCBs) (Denys 2008; Fendorf 2004; Frelon 2007; Hack 1996; Meunier 2010; Rasmussen 2008; Rodriguez 1999; Van de Wiele 2004; Welfringer 2009).

2.3.4 Validating *in vitro* bioaccessibility

Before measures of *in vitro* bioaccessibility may be accepted for SSRA, the *in vitro* GI model must be validated and proven to reflect bioavailability data collected using one of the standard *in vivo* techniques described above (Basta 2007; Rodriguez 1999; US EPA Region 8 2005). Extensive work is underway to validate *in vitro* GI models for the measurement of lead, arsenic,

and more recently cadmium and antimony in contaminated soils (Basta 2007; Caboche 2009; Casteel 2001; Roberts 2006; Rodriguez 1999; Schroder 2004). No such validation procedures have yet been performed for contaminated foodstuffs. To perform these validation studies, the soil contaminant concentration is measured in numerous test materials from a contaminated site. Subsequently, the contaminant percent bioaccessibility under simulated gastric and/or intestinal conditions are quantified using an *in vitro* GI model. Thereafter, the RBA of the contaminant is measured using an *in vivo* animal model. Finally, the correlation between percent bioaccessibility and contaminant RBA is assessed and the model declared valid according to the model fit, and occasionally slope, of the regression equation (US EPA Region 8 2005).

This approach to GI model validation is, however, not without its limitations. For example, *in vivo* bioavailability trials are typically performed for test materials with high contaminant concentrations (e.g., >500 mg kg^{-1} As) due to detectability constraints. For example, the measurement of the RBA of As (Casteel 2001) in a moderately contaminated soil (e.g., 250 mg As $kg\ soil^{-1}$) requires each animal in the highest dosing group to ingest 10 g of soil per day. Such large soil ingestion rates are impractical due to difficulties ensuring complete dose ingestion. In contrast, IVBA is most useful for soils containing lower contaminant concentrations as these are the soils for which bioaccessibility is most likely to effect decision making. For example, an arsenic contaminated soil containing a concentration 2000 mg kg^{-1} greater than background would require a bioavailability of 0.6% to be equivalent to the CCME SQG of 12 mg kg^{-1} (i.e., [12 mg kg^{-1}/2000 mg kg^{-1}] x100% = 0.6%). However, neither *in vitro* nor *in vivo* techniques offer sufficient precision to confidently and reliability predict bioaccessibilities or bioavailabilities below 1%. Therefore, remediation options (e.g., excavation, phytoremediation, etc) are almost certain to proceed for such highly contaminated sites regardless of bioavailability.

Consequentially, current procedures validating the use of *in vitro* GI models may be predicated on the assumption that the correlation between bioaccessibility and bioavailability is dose independent. In addition, current validation procedures suggest *in vitro* bioaccessibility under simulated gastric conditions predicts *in vivo* bioavailability just as well as under simulated intestinal conditions (Juhasz 2008). This observation is contrary to the vast majority of physiological knowledge that places the majority of GI uptake in human intestine (Gad 2007).

2.3.5 Uncertainty and data gaps

Several data gaps have impeded the widespread utilization of *in vitro* GI models for HHRA. One of these data gaps is whether or not bioaccessibility is relevant to the estimation of mercury exposure from foodstuffs. Adult exposure to environmental contaminants such as mercury is rarely driven by accidental ingestion of soils (Paustenbach 1989) and may, instead, be of dietary origin (BCS 2007). However, the vast majority of bioaccessibility data for metal contaminants has focused upon contaminated soils and soil-like materials (Ruby 1996). Consequentially, it is as of yet unknown whether contaminant solubilization from ingested foods limits GI absorption and, thus, oral exposure to dietary contaminants.

Despite decades of research evaluating the use of *in vitro* GI models for measuring soil contaminant bioaccessibility, a number of data gaps persist (Ruby 1993). For example, *in vitro* bioaccessibility is occasionally dependent upon concentration (Beak 2006b); however, the factors that result in this relationship being only occasionally observed are largely unknown. An inverse relationship between bioaccessibility and concentration is of particular concern since this may result in the underestimation of bioaccessibility if not also observed *in vivo*. For example, the Liquid to Soil ratio (LSR), representing the volume of simulated GI fluid per gram of soil, of *in vitro* models is typically far lower than observed *in vivo* (Richardson 2006). Consequentially,

solubility limits may be reached in GI models, thereby artificially restraining contaminant dissolution and *in vitro* bioaccessibility.

Another data gap impeding the widespread application of *in vitro* GI models is whether it is necessary to measure bioaccessibility under simulated gastric conditions only or if simulated intestinal conditions must also be included. Although the majority of *in vitro* GI models published in peer-reviewed literature include an intestinal phase (Van de Wiele 2007), validation studies indicate that gastric bioaccessibility offers superior correlation with *in vivo* bioavailability results. Additionally, most *in vitro* GI models that include a simulated intestinal stage do not incorporate microbial activity (Van de Wiele 2007) even though microbes are known to modify the speciation (Kuroda 2001) and biological activity of ingested contaminants (Van de Wiele 2005). However, the relevance of microbial effects upon contaminant bioaccessibility to HHRA has yet to be established.

It is likely, though, that the largest impediment to the widespread use of *in vitro* bioaccessibility is that the development of GI models has largely proceeded on an *ad hoc* basis. Consequentially, the majority of bioaccessibility results obtained to date focus upon arsenic, and to a lesser extent lead, with far less information available for other metals and organic contaminants. Although numerous articles have assessed the role of soil properties (Juhasz 2007; Roberts 2006; Yang 2002; Yang 2005), constituents (Wragg 2007a), and mineralogy (Meunier 2010; US EPA Region 8 2005) on soil contaminant bioaccessibility, these studies have focused exclusively on arsenic bioaccessibility. Therefore, it remains unknown whether the soil properties that determine arsenic bioaccessibility (e.g., pH, Fe_{total}, $FeSO_4$, clay, fine-grained silicate, etc) also influence the bioaccessibility of other metal contaminants. As such, utilizing multi-metal approaches to identify fundamental metal properties controlling metal

bioaccessibility addresses a critical data-gap preventing the widespread use of metal bioaccessibility in HHRA.

3 BIOACCESSIBILITY OF MERCURY FROM TRADITIONAL NORTHERN COUNTRY FOODS MEASURED USING AN *IN VITRO* GASTROINTESITNAL MODEL IS INDEPENDENT OF MERCURY CONCENTRATION

3.1 Introduction

Traditional country foods offer tremendous social, health, and economic benefits to Inuit in northern Canada (NCP 2003); however, the presence of environmental contaminants such as mercury (Hg) in country foods may also present health risks. The risk assessment of ingested Hg to the Canadian public is calculated under the assumption that 100% of the Hg in fish flesh is in the form of methylmercury (MeHg) (BCS 2007). The hazard of MeHg makes this assumption both conservative and protective (BCS 2007). The assumption that dietary mercury is 100% MeHg is realistic for southern Canadians (i.e., those consuming typical western diets of cereals, legumes, dairy products, fruits, vegetables, and meat) since fish is their primary source of Hg exposure (BCS 2007) and the majority of Hg in fish is MeHg (ATSDR 1999; BCS 2007; Wagemann 1997). Although fish consumption rates of Inuit can be substantially higher relative to southern Canadians (BCS 2007), fish consumption constitutes a small proportion of the Inuit Hg exposure (NCP 2003). The dietary Hg risk assessment of Inuit could be improved by systemically evaluating the concentration, speciation, and bioavailability of Hg from a variety of traditional country foods.

Although both Hg^{II} and MeHg are primarily absorbed in the small intestine, toxicokinetic differences exist between Hg^{II} and MeHg (ATSDR 1999; Clarkson 2007; Nielsen 1992). For example, only a fraction of ingested Hg^{II} is absorbed across the gastrointestinal (GI) tract (Kamijo 2004; Nielsen 1992). In contrast, both human and animal studies demonstrated nearly complete absorption of Hg when ingested as MeHg contaminated fish, or as MeHg spiked into water, oils, or fish muscle homogenates (ATSDR 1999; Clarkson 2007). The absorption of MeHg across the intestinal epithelium is mediated by brush border dipeptide transporters due to

the structural homology of L-cysteine-MeHg complexes and methionine (Clarkson 2007). In contrast, the mechanisms of Hg^{II} absorption are not yet completely understood (Hoyle 2005).

In vitro GI models are useful tools in the measurement of Hg bioaccessibility, i.e., the fraction of ingested Hg that is solubilized into GI fluids (Ruby 1999). In contrast, Hg bioavailability refers to the fraction of ingested Hg that is absorbed across the intestinal epithelium into systemic circulation (Ruby 1999). Oral Hg bioavailability may be reduced due to complexation with fiber and phytates (ATSDR 1999); therefore, assuming Hg from foods to be completely absorbed may overestimate the health risk of consuming traditional country foods. The bioaccessible fraction of Hg, that which is solubilized in the human GI tract, may be a better indicator of exposure for use in risk assessment. This principle, in conjunction with the high cost of *in vivo* oral bioavailability assessments, has led to the development of *in vitro* GI models for the evaluation of contaminant bioaccessibility from soils, slags, and mine tailings (Ruby 1999). Similarly, *in vitro* models were used to predict GI Hg absorption in channel catfish, *Ictalurus punctatus* (Leaner 2002), and humans (Cabanero 2004; Cabanero 2007; Shim 2009). The Simulator of the Human Intestinal Microbial Ecosystem (SHIME) is an *in vitro* GI model that is unique in comparison with other *in vitro* models because it incorporates the activity of the human GI microbiota (De Boever 2000; Van de Wiele 2007). The SHIME mimics the physiological conditions (e.g., pH, residence time, enzymes, etc) of the stomach, small intestine, and colon to monitor the solubilization of ingested contaminants in the GI tract. Microorganisms (e.g., sulfate reducing bacteria) are capable of reducing mercury to form unabsorbable sulfide complexes (ATSDR 1999), methylating Hg^{II} to form MeHg or demethylating MeHg to form Hg^{II} (Craig 2004). Antibiotic treatment of rats decreased Hg elimination following oral exposure to methylmercuric chloride, indicating that the rat GI microflora metabolized MeHg (Rowland

1980) via the production of elemental mercury and a volatile sulfur derivative of methylmercury (Rowland 1978). Furthermore, the microbial community of the rat jejunum first methylated $HgCl_2$, which was subsequently reduced to elemental Hg via microbial metabolism (Ludwicki 1990). However, the methylation of ingested inorganic Hg following human oral exposure has yet to be conclusively demonstrated *in vivo* (Barregard 1994).

Metal(loid) concentration of ingested matrices sometimes limits bioaccessibility and bioavailability due to constraints upon contaminant dissolution and/or absorption. Although MeHg bioavailability was independent of MeHg concentration in an *in vivo* mouse model (Nielsen 1992), it is unknown if this is also true for *in vitro* bioaccessibility. The objective of this research is to assess the relationship between Hg bioaccessibility and concentration in country foods. In addition, we will evaluate for the presence of Hg biotransformation (e.g., Hg species demethylation or methylation) from several country foods. As such, we hypothesize that Hg bioaccessibility is inversely related to the Hg concentration in the food and that GI microorganisms alter the speciation of the ingested Hg via methylation or demethylation of Hg species in the GI lumen. The evaluation of these hypotheses are limited by the fact that the SHIME, like other *in vitro* GI models, are not yet validated for the measurement of Hg bioaccessibility relative to *in vivo* data.

3.2 Materials and Methods

3.2.1 Inuit food samples

Food samples collected between 1998 and 2006 (n=16), were acquired from sample archives maintained by Dr Hing Man Chan (University of Northern British Columbia, Canada) and Dr Derek Muir (Environment Canada, Burlington, Ontario, Canada) as part of their on-going monitoring studies. The food samples, which were stored at -20°C, included the kidney and tongue of caribou (*Rangifer tarandus* L.), the intestine of bearded seal (*Erignathus barbartus* E.),

the blood, brain, and liver of ringed seal (*Phoca hispida* S.), the flesh of walrus (*Odobenus rosmarus* L.), the skin of narwhal (*Monodon monoceros* L.) and beluga (*Delphinapterus leucas* P.), and the flesh of Arctic char (*Salvelinus alpinus* L.). The majority of the foods (14 of 16) were uncooked; however the bearded seal intestine was boiled and one of two walrus flesh samples were aged. The total Hg (HgT) concentration (i.e., [HgT]) in the various foods ranged between 0.01 mg kg^{-1} and 3 mg kg^{-1} (wet weight).

3.2.2 Dynamic Simulator of the Human Intestinal Microbial Ecosystem

A three compartment dynamic SHIME model, constructed at the University of Saskatchewan (Saskatoon, Saskatchewan, Canada) according to previously published specifications (De Boever 2000; Laird 2007), maintained a microbial community representative of that found in the distal human intestine. The SHIME is operated under anaerobic conditions with human GI microorganisms in the colon stage since GI microbes may influence Hg bioaccessibility and speciation. In brief, a feces: thioglycolate buffer mixture was centrifuged and the supernatant was added to a carbohydrate-based nutrition solution (previously autoclaved) in a double-jacketed reactor flask. The carbohydrate nutrition solution contained 5.01 g $KHCO_3$ (MP Biomedicals), 2.92 g NaCl (EMD), 0.5 g arabinogalactan (Fluka), 1.0 g pectin (ICN Biomedicals), 0.5 g xylan (Sigma), 2.1 g potato starch (EMD), 0.2 g glucose (EMD), 1.5 g yeast extract (BD Bacto™), 0.5 g peptone (BD Bacto™), 2.0 g mucin (Sigma), 0.25 g L-cysteine (EM Science) per liter of Milli-Q™ water. The L-cysteine concentration in the dynamic SHIME colon suspension is equivalent to the maximum dietary cysteine used in a recent publication investigating the role of dietary cysteine in the synthesis of erythrocyte glutathione (Courtney-Martin 2008). Nutrition solution (100 mL) was acidified to pH 2 via the addition 12 M HCl (EMD OmniTrace™) and was added to the stomach every 8 hours. Following a 2 hour residence

time in the stomach, the nutrition solution was pumped into the small intestine and mixed with small intestinal solution (50 mL) containing 12.5 g L^{-1} $NaHCO_3$ (EMD), 6.0 g L^{-1} Oxgall (BD Difco^{TM}), 0.9 g L^{-1} pancreatin (Sigma). Following two hours in the small intestine, the nutrition/small intestine mixture was pumped into the colon stage containing the large intestinal microbial community. The SHIME, maintained at 37°C under nitrogen atmosphere, containing 10^8 total aerobes CFU mL^{-1} and 10^8 total anaerobes CFU mL^{-1}, included Fecal Coliforms, Clostridia, Enterococci, Staphylococci, Bifidobacteria, and Fungi. Aliquots of the colon microbial cultured in the dynamic SHIME were used for static batch colon digests of country foods.

3.2.3 Static Simulator of the Human Intestinal Microbial Ecosystem

Mercury bioaccessibility from country foods was measured from the stomach, small intestine, and colon stage of the SHIME using a static batch design (Laird 2007) making sample digestion order irrelevant to the calculation of HgT bioaccessibility. In brief, 0.2 g of country food was added to 50 mL polypropylene conical centrifuge tubes and then 8 mL of pH 1.5 diluted HCl (EMD OmniTrace^{TM}) was added. The pH of the food/gastric solution was corrected to 1.5 ± 0.1 and 6.8 μL of concentrated porcine pepsin (Calbiochem) was added to the mixture to facilitate protein digestion. The country food stomach digests were horizontally shaken at 70 rpm in an incubator shaker heated to 37°C for 2 hours. Following the stomach residence time, 4 mL of small intestinal solution (as described above) was added to the centrifuge tube. Sample pH was corrected to 6.5 ± 0.1 and the headspace was replaced with a nitrogen atmosphere using an anaerobic glove box. Subsequently, the small intestine digests were returned to the incubator shaker and incubated with shaking for an additional 2 hours. Following the small intestinal residence time, 8 mL of colon SHIME solution from the dynamic SHIME colon was added to the intestinal country food digests. The pH was corrected to 6.3 ± 0.1 and the headspace was

replaced with a nitrogen atmosphere, as previously described. Subsequently, the colon digests were returned to the incubator shaker and incubated with shaking for an additional 18 hours. Additionally, the colon SHIME digests of the country foods were repeated using previously sterilized (i.e., autoclaved) and cooled dynamic SHIME colon suspension.

The separation of bioaccessible contaminants from *in vitro* GI fluids often uses centrifugation, microfiltration (0.45 μm) or ultrafiltration for the assessment of contaminant bioaccessibility. It is not clear what method best estimates Hg bioaccessibility; however, ultrafiltration may be most appropriate because it represents the size fraction thought to cross the intestinal lumen (Van de Wiele 2007). Following the stomach, small intestinal, and colon stages, the SHIME fluid/country food mixtures were first syringe filtered (Whatman 0.45 μm, Glass Micro-Fiber) and then 3.5 mL of the filtered solution was passed through the 10 kDa membrane of an ultrafiltration centrifuge cartridge (Pall, Microsep, 10 kDa). The 10 kDa filtrate was transferred from the ultrafiltration centrifuge cartridge, preserved in 5% HCl (EMD OmniTrace^{TM}), and frozen prior to transportation to Quicksilver Scientific (Denver, CO, USA) for chemical analysis.

Mercury in the 10 kDa filtrate was defined as bioaccessible and percent HgT bioaccessibility was calculated according to:

$$HgT\ Bioaccessibility = \left([HgT]_{filtrate} \times Fluid\ Volume\right) / \left([HgT]_{food} \times Food\ Mass\right) \times 100\% \qquad \text{(Eqn 3.1)}$$

Where $[HgT]_{filtrate}$ is the total Hg concentration (μg mL^{-1}) in the 10 kDa filtrate, Fluid Volume is the total volume of the GI fluid (mL), $[HgT]_{food}$ is the total Hg concentration in the food item (μg g^{-1}), and Food Mass is the total mass (g) of the food digested through the SHIME. Since the concentration of MeHg and Hg^{II} in the undigested country foods was not evaluated, the IVBA of MeHg and IVBA of Hg^{II} cannot be calculated. Instead, the bioaccessible MeHg concentration of

the country foods (i.e., $[MeHg]_{10\ kDa,Food}$) was calculated to evaluate the role of GI microbes of the speciation of bioaccessible Hg:

$$[MeHg]_{10kDa,\,Food} = \left([MeHg]_{\,filtrate} \times Fluid\ Volume\right) / Food\ Mass \quad \text{(Eqn 3.2)}$$

Where $[MeHg]_{filtrate}$ is the MeHg concentration ($\mu g\ mL^{-1}$) in the 10 kDa filtrate, Fluid Volume is the total volume of the gastrointestinal fluid (mL), and Food Mass is the total mass (g) of the food digested through the SHIME. $[MeHg]_{filtrate}$ was adjusted for background MeHg in the SHIME digest (see Chemical Analysis); however, the calculation of $[MeHg]_{filtrate}$ assumes negligible loss (e.g. adsorption) of MeHg in the ultrafiltration cartridge. This assumption has yet to be verified. The GI microbial effect size on HgT bioaccessibility, $[MeHg]_{10\ kDa,Food}$, and the proportion of bioaccessible HgT as MeHg was calculated according to:

$$Effect\ \ Size = \left(\left(Active - Sterile\right) / Sterile\right) \times 100\% \quad \text{(Eqn 3.3)}$$

Where Active denotes the HgT bioaccessibility, $[MeHg]_{10\ kDa,Food}$, or proportion of bioaccessible HgT as MeHg in the presence of GI microbial activity. Sterile denotes the HgT bioaccessibility, $[MeHg]_{10\ kDa,Food}$, or proportion of bioaccessible HgT as MeHg, without GI microbial activity. The calculation of Effect Size (Equation 3.3) assumes that the microbial activity is the dominant source of bacterial metabolism in the SHIME and was not affected by microorganisms originally present within the country foods. The presence of cysteine and thioglycolate in the SHIME colon suspension may have facilitated the release of Hg from the foods into solution. However, the calculation of the microbial effect size controls for this factor since the active and sterile SHIME colon contained the same concentrations of cysteine and thioglycolate.

3.2.4 Chemical analysis

Determination of total $[HgT]_{food}$ in the majority of the food samples was accomplished through the use of a Hg analyzer (MA-2000, Nippon Instruments Corporation) that uses sample combustion to, gold amalgamation, and Hg detection by atomic absorption spectrometry (C-GA-AAS). Approximately 5-10 mg of food samples were used in each measurement from three subsamples of each food. The sample was placed in ceramic boats containing additives (1:1 calcium hydroxide: sodium carbonate and activated alumina). The purpose of the additive is to absorb substances such as sulfates or halogens that could potentially be released during sample combustion, which could interfere with the absorbance measurement. The samples were combusted at 850°C. The Hg vapor that is released is trapped by gold-coated diatomite particles and is subsequently released into an absorption cell. The Hg concentration is then determined using atomic absorption spectrometry at a wavelength of 253.7 nm. The detection limit for the analysis of HgT content of the foods was 1 ng g^{-1}. The ensure accuracy, blanks and standard reference material check were run periodically. Blanks, which are ceramic boats containing only additive, gave values below the detection limit. Moreover the standard reference materials Dogfish Muscle Certified Reference Material for Trace Metals DORM-2 (NRC, Ottawa) consistently gave recoveries above 85%. The mean (n=3) of the HgT was calculated and the coefficient of variation for the HgT concentrations was consistently less than 10%.

The $[HgT]_{food}$ of the Arctic char was measured in triplicate at the National Laboratory for Environmental Testing at the Canada Centre for Inland Waters, as previously described (Gantner 2009). In brief, Arctic char flesh was acid-digested in a closed-vessel high-pressure microwave oven and total Hg was measured using cold-vapor atomic absorption spectrophotometry. Average Hg recovery, measured from certified reference materials (DOLT-2, DORM-2, TORT-2; National Research Council of Canada, Ottawa, ON) for each batch of 20 samples, was 129%.

$[HgT]_{food}$ of the Arctic char samples were only accepted when the coefficient of variation of the mean of the triplicate samples was less than 10%.

The $[Hg^{II}]$ and [MeHg] in the 10 kDa filtrate (5 mL) were measured using Hg-Thiourea complex Liquid Chromatography – Cold Vapour Atomic Fluoresence Spectrometry (HgTu/LC-CVAFS) according to a previously published technique utilizing the complexation affinity of thiourea with Hg (Shade 2008). In brief, a thiourea (TU) and hydrochloric acid leaching solution was added to each of the acid-preserved 10 kDa filtrate samples, forming hydrophilic cationic $Hg(Tu)_2^{2+}$and CH_3HgTu^+ complexes with both Hg^{II} and MeHg. This step released any Hg species potentially bound to the polypropylene container walls. To evaluate the efficacy of TU in mobilizing Hg from the polypropylene tubes we performed a TU leaching test on the extracted tubes. This TU leaching test indicated that the average Hg recovery was 89.6% (n=4). The samples were then transferred to acid-cleaned borosilicate glass vials and potassium iodide added to create neutral CH_3HgI and HgI_2 complexes that were then pre-concentrated on a polydivinylbenzene (I-PDVB). Excess iodide was then washed from the column prior to elution with acidic thiourea and sequential on-line separation by ion-pairing reversed-phase liquid chromatography, cold vapor generation, gas-liquid separation, and detection of formed Hg^0 by atomic fluorescence. The absolute detection limits of the analysis are 0.4 pg for MeHg and 0.7 pg for Hg^{II} (Shade 2008), relative detection limits depend on the amount of sample prepared and the volume of the prepared solution pre-concentrated. Certified Reference Material (BCR-463, DOLT-3, Mussel SRM 2796) recovery ranged between 95 and 106% for MeHg (n=5) and 98 and 105% for Hg^{II}(n=5). Matrix spike recovery ranged between 92 and 98% for MeHg and 92 and 101% of Hg^{II} and the average percent deviation of MeHg and Hg^{II} was 9% and 14%,

respectively. The mean (n=6) procedural SHIME blanks concentration was $1.5x10^{-4}$ ng mL^{-1} MeHg and $6.5x10^{-3}$ ng mL^{-1} Hg^{II}.

3.2.5 Statistical analysis

Fisher-Protected Least Significant Difference (LSD) (α=0.05, n=15) tested the hypothesis that the concentration of bioaccessible (i.e., that which passes the 10 kDa filter) MeHg in the colon is affected by the activity of GI microorganisms. A lack of replication within different types of food necessitated the pooling across food type for the comparison of mean $[MeHg]_{10kDa,Food}$ for the two microbial treatments (i.e., active versus sterile colon). Therefore, the mean $[MeHg]_{10\ kDa,\ Food}$ of the 15 foods was compared with or without the activity of GI microbes.

To evaluate if HgT IVBA from foods was inversely related to $[HgT]_{food}$, ANOVA nonlinear regression analysis was performed. A null (i.e., intercept-only) model (HgT ABA ~ a) was compared (F-test, α=0.05) to a 2-parameter reciprocal (HgT ABA ~ $a - b^{-c*HgTppm}$) model where HgT ABA is the total Hg bioaccessibility and HgT ppm is the total Hg concentration in the food. To evaluate if HgT bioaccessibility of mammal organs differed from fish flesh, small intestinal HgT IVBA of foods from mammal organs (terrestrial and marine, n=5) was compared with that of fish (i.e., Arctic char, n=6) using a Fisher Protected LSD to test the hypothesis that type of food influences (α=0.05) HgT IVBA in the SHIME small intestine. Similarly, the GI microbial effect size on $[MeHg]_{10\ kDa\ Food}$ from mammals was compared with that of fish using a Fisher's Protected LSD, thereby testing the hypotheses that type of food influences the GI microorganism's affect upon $[MeHg]_{10\ kDa,Food}$. The small number of samples limits the interpretation of the data to this set of samples; therefore, it would be inappropriate to extrapolate the results of this comparison to Inuit country foods in general.

3.3 Results

Small intestinal HgT IVBA for these 16 country food samples ranged between 1 and 93% and was independent of $[HgT]_{food}$ (**Table 3.1**). The nonlinear (reciprocal) model of $[HgT]_{food}$ and HgT bioaccessibility did not significantly improve the regression when compared to the intercept-only model ($F= 0.5726$, $p=0.578$). The CV of small intestinal HgT IVBA was greater from duplicated (n=2) digests of Beluga skin than from Arctic char, indicating that future studies should digest samples in triplicate to reduce measurement uncertainty. Mean small intestinal HgT IVBA, pooled between foods, was 35 % (n = 16, CV = 79%), which is higher than the HgT IVBA previously reported from other fish species (Tuna 9%; Sardine 13%; Swordfish 17%) (Cabanero 2004; Cabanero 2007). The higher HgT IVBA in this study could be due to differences in either sample composition or *in vitro* model operational parameters. Since Hg in the fish flesh is predominantly MeHg (BCS 2007; Wagemann 1997), Hg ingested via fish consumption is thought to be completely absorbed across the GI tract. In contrast, mean small intestinal Arctic char HgT bioaccessibility was 56% (n =6, CV = 38%) in the SHIME and Cabanero *et al.* (2004, 2007) reported fish HgT bioaccessibility below 20%. Therefore, *in vitro* Hg bioaccessibility may systematically underestimate Hg bioavailability from foods, perhaps due to a lack of brush border enzymes and/or Hg absorption mechanisms. Consequentially, it is essential that *in vitro* Hg bioaccessibility be calibrated (i.e., validated) according to *in vivo* oral bioavailability prior to use in human health risk assessment to ensure the accuracy of Hg bioaccessibility data.

Table 3.1 Food total mercury (HgT) concentrations ($[HgT]_{food}$), small intestinal HgT bioaccessibility, and bioaccessible methylmercury (MeHg) concentrations ($[MeHg]_{10\ kDa\ food}$) digested through the Simulator of the Human Intestinal Microbial Ecosystem. Concentrations were expressed by a wet weight basis.

Species (*Scientific name*)	Food Tissue	$[HgT]_{food}$ (mg kg^{-1})	% HgT Bioaccessibility (Std Dev)	$[MeHg]_{10\ kDa,Food}$ (mg kg^{-1})
Caribou (*R. tarandus)*	Tongue	0.02	25.0	0.0058
Walrus *(O. rosmarus)*	Flesh	0.07	15.5	0.011
Ringed seal *(P.hispida)*	Blood	0.11	66.1	0.082
Walrus *(O. rosmarus)*	Flesh	0.12	47.7	0.063
Bearded seal *(E. barbartus)*	Intestine	0.14	4.6	0.0068
Arctic char *(S. alpinus)*	Flesh-I	0.15	52.3 (1.6)	0.091 (0.003)
Ringed seal *(P. hispida)*	Brain	0.16	0.7	0.0058
Caribou (*R. tarandus)*	Kidney	0.19	0.7	< Detection Limit[a]
Arctic char *(S. alpinus)*	Flesh-II	0.26	41.1	0.12
Narwhal *(M. monoceros)*	Skin	0.34	0.5	0.0022
Arctic char *(S. alpinus)*	Flesh-III	0.34	93.9	0.36
Beluga *(D. leucas)*	Skin	0.37	41.4 (7.8)	0.17 (0.03)
Ringed seal *(P. hispida)*	Liver	0.45	18.9	0.068
Arctic char *(S. alpinus)*	Flesh-IV	1.29	55.5	0.81
Arctic char *(S. alpinus)*	Flesh-V	2.09	33.2	1.51
Arctic char *(S. alpinus)*	Flesh-VI	3.40	60.2	2.32

[a] Detection Limit = 0.4 pg

The thioglycolate and L-cysteine in the dynamic SHIME may have encouraged the release of Hg into the SHIME fluid due to their complexation affinity for Hg. Since the

thioglycolate and L-cysteine were present in the static SHIME colon but not in the SHIME small intestine, comparing the HgT bioaccessibility between these SHIME stages provides indirect evidence that the thioglycolate and L-cysteine did not bias the HgT bioaccessibility results. The addition of the colon SHIME suspension consistently decreased HgT bioaccessibility in the SHIME colon (with thioglycolate; HgT bioaccessibility: 0.2 – 42%) relative to the SHIME small intestine (without thioglycolate; HgT bioaccessibility 0.5 – 93%). These results suggest that the presence of thioglycolate and L-cysteine did not increase HgT bioaccessibility from country foods in the SHIME.

3.4 Discussion

No differences were observed between either $[MeHg]_{10\ kDa,\ Food}$ ($F_{1,28} = 1.75$, $p=0.197$) or HgT bioaccessibility ($F_{1,28} = 2.32$, $P=0.139$) of country foods in the presence or absence of GI microbial activity. However, our ability to detect differences between the active and sterile colon was confounded by pooling differing types of foods. For example, GI microorganism activity decreased $[MeHg]_{10\ kDa,\ Food}$ between 13 and 100% relative to the sterile control from the majority (7 out of 8) of uncooked mammalian country foods (**Figure 3.1**).

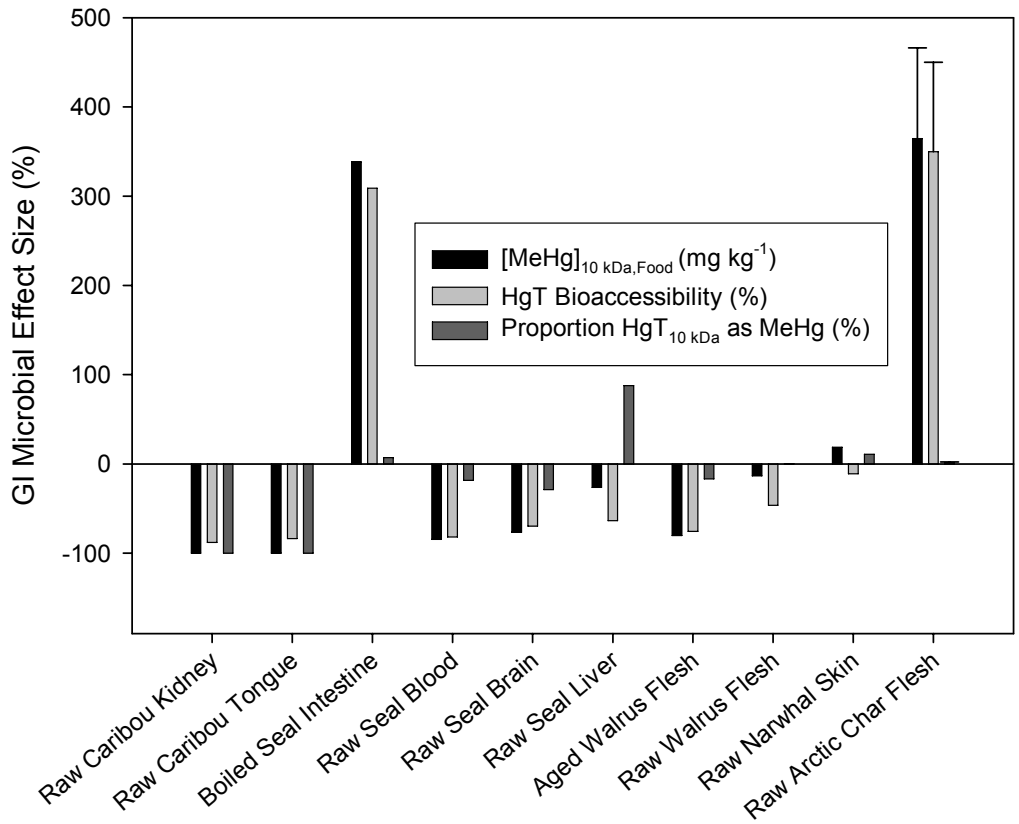

Figure 3.1 Gastrointestinal microbial effect size on 10 kDa MeHg concentration, total mercury (HgT) bioaccessibility, and proportion of bioaccessible mercury (Hg) as methylmercury (MeHg) from country foods digested through the Simulator of the Human Intestinal Microbial Ecosystem. Of the 10 food types, only Arctic char flesh was replicated (n=6) via pooling results from the 6 Arctic char samples. Error bars represent standard error of the mean.

In contrast, GI microbial activity increased $[MeHg]_{10\ kDa,Food}$ for uncooked narwhal skin (19%), boiled seal intestine (340%), and uncooked Arctic char flesh (n=6, 67 – 760%) (**Figure 3.1**). A similar pattern was observed for HgT bioaccessibility, thereby demonstrating a correlation between HgT bioaccessibility and $[MeHg]_{10\ kDa}$ as HgT concentrations in the GI fluids were driven by MeHg. The proportion of bioaccessible HgT as MeHg decreased up to 100% in the presence of GI microorganisms relative to sterile control samples (**Figure 3.1**). The decrease in $[MeHg]_{10\ kDa,Food}$ from uncooked mammalian foods in the presence of GI microbes

may be due to microbial demethylation of MeHg present in the country foods. Alternatively, variations in $[MeHg]_{10\ kDa,Food}$ and the proportion of bioaccessible HgT as MeHg observed from some foods (**Figure 3.1**) may be due to preferential coordination of MeHg to ligands greater than 10 kDa (i.e., not bioaccessible) in the active versus sterile colon. However, microbial demethylation is the more plausible mechanism explaining decreases in $[MeHg]_{10kDa,Food}$ since the matrix composition of the active and sterile colon suspension is likely equivalent. Demethylation of ingested MeHg was previously observed *in vivo* (Seko 2001) and *in vitro* in the presence of *Escherichia coli* (Oremland 1991). Increases in $[MeHg]_{10kDa,Food}$ from fish and boiled seal intestine did not correspond to increases in the proportion of bioaccessible HgT as MeHg (**Figure 3.1**). Similarly, the proportion of bioaccessible HgT as MeHg from raw seal liver increased 88% in the active colon relative to the sterile control whereas the bioaccessible MeHg concentration in the raw seal liver decreased 26% (**Figure 3.1**). Therefore, our results suggest that although GI microorganisms may methylate Hg locally in the GI tract, overall the demethylation of MeHg in the GI tract may limit the production of MeHg.

Food type may influence percent HgT bioaccessibility; however, the experimental design of this study (i.e., lack of replication within most food types) does not facilitate conclusive interpretations regarding the effect of food type on Hg bioaccessibility. However, selecting two groups (mammal organs or Arctic char flesh) allowed us to develop hypotheses regarding food type effects which will be investigated in the future. Our data suggests that the small intestinal HgT bioaccessibility from Arctic char flesh is 6-fold greater ($F_{1,9} = 19.17$, $P = 0.002$) than observed from mammal organs (**Figure 3.2**). The low variability (CV 3.1%) of duplicated digests of the flesh from one Arctic char sample suggests that the differences in HgT bioaccessibility observed between Arctic char samples are likely significant. Furthermore, our

results suggest that the GI microbial effect size on the $[MeHg]_{10kDa,Food}$ is 50-fold lower ($F_{1,9}$ = 6.94, P = 0.027) from mammal organs than from Arctic char flesh (**Figure 3.2**). Consequentially, the GI absorption of Hg from mammal organs is potentially lower than that of fish. However, the conclusive interpretation of the role of food type on Hg bioaccessibility will require a much larger and comprehensive set of samples than available in this study.

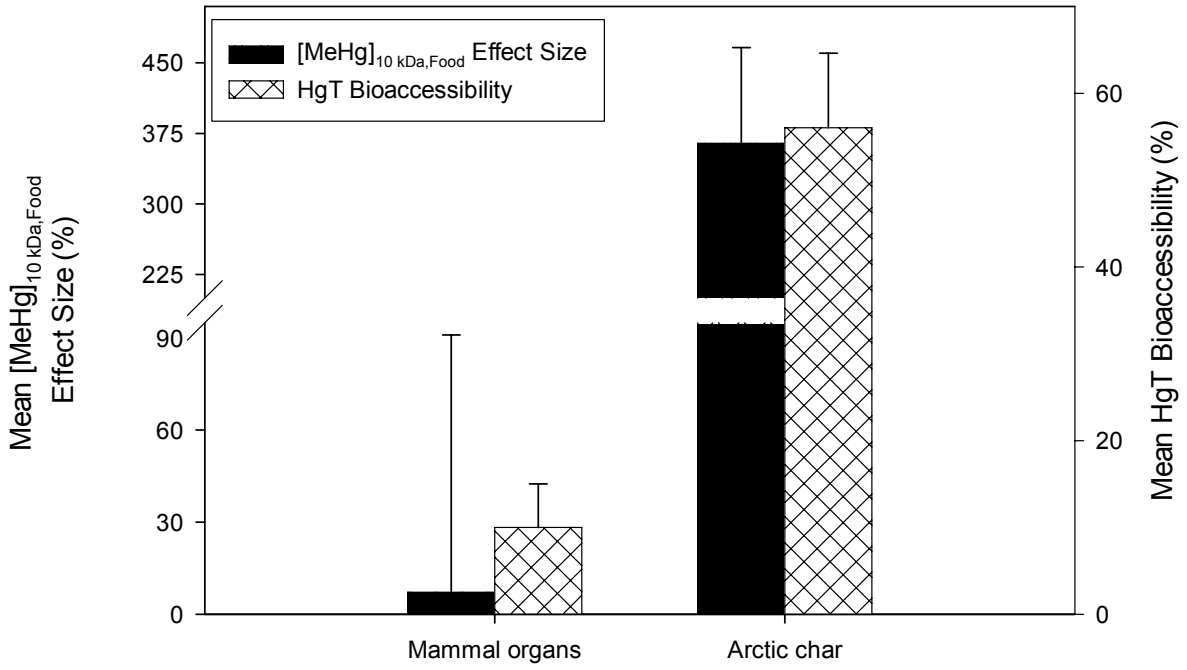

Figure 3.2 Gastrointestinal microbial effect size on small intestinal total mercury (HgT) bioaccessibility and bioaccessible methylmercury concentration ($[MeHg]_{10kDa,Food}$) for Arctic char and mammal organs. Error bars denote standard error of the mean.

We demonstrated that HgT bioaccessibility is independent of $[HgT]_{food}$, for these 16 country food samples, thereby suggesting that the same proportion of dietary Hg is potentially available for absorption regardless of the Hg concentration. Furthermore, our results indicate that GI microorganisms may influence the speciation of dietary Hg in the intestinal lumen via the conversion of MeHg to Hg^{II}, although this effect appears dependent upon the type of food consumed. The interpretation of the speciation results were complicated by the possibility of

dynamic Hg transfer between the bioaccessible (i.e., <10 kDa fraction) and non-bioaccessible (>10 kDa fraction). Finally, our results indicate that the bioaccessibility of HgT from mammal organs is lower than that of Arctic char flesh. It should be emphasized that the results of this study are limited by the small number of samples investigated and should not be extrapolated to Inuit country foods as a whole. Furthermore, it is as of yet unknown if the activity of the GI microbial of the SHIME is representative of Inuit GI microbial ecology. It is necessary for *in vitro* Hg bioaccessibility measured using the SHIME be validated according to *in vivo* Hg bioavailability before the results of this research be used in human health risk assessment.

4 GASTROINTESTINAL MICROBES INCREASE ARSENIC BIOACCESSIBILITY OF INGESTED MINE TAILINGS USING THE SIMULATOR OF THE HUMAN INTESTINAL MICROBIAL ECOSYSTEM

4.1 Introduction

Three million tonnes of mine tailings were generated within 64 gold-mining districts in Nova Scotia through mercury-amalgamation and cyanidation techniques between 1861 and the mid-1940s (Bates 1987; Parsons 2004). Exposure to arsenic (As) through the ingestion of mine tailings, crushed rock waste produced during the extraction of gold from ore, poses a potential human health risk to communities in Nova Scotia, Canada. Inorganic As consumption is linked to blackfoot disease (Tseng 2005), neonatal death and spontaneous abortions (Milton 2005) as well as skin, bladder, and colon cancer (Guo 2004; Tchounwou 2004). The potential for As exposure is exacerbated by the small particle size of tailings since the probability of ingestion often increases with decreasing particle size (Bright 2006). Current practice is to assume that the bioaccessible fraction, the fraction of an ingested contaminant solubilized in the gastrointestinal (GI) tract, is close to one hundred percent, or at least equal to the material used to derive the Toxicological Reference Value (TRV) (Ruby 1996; Yang 2003). Since metal bioaccessibility varies between matrices (i.e., mine tailings, soils, etc.) there is a need to develop bioaccessibility estimates for use in site-specific risk assessments (Hund-Rinke 2003; Ruby 1996).

Bioaccessibility of As has been measured using several *in vitro* gastrointestinal models to estimate human health risk from the ingestion of As-contaminated soils and mine tailings (Ellickson 2001; Pouschat 2006; Rodriguez 2003; Ruby 1996; Yang 2003). The bioavailable fraction, that which is absorbed across the intestinal epithelium into systemic circulation, of metal(loid) contaminants may decrease with increasing exposure due to saturation of absorption mechanisms (Ellickson 2001; Ruby 1996). However, the role of As concentration in determining As bioaccessibility using *in vitro* models has been less conclusive. Two studies utilizing

different *in vitro* GI models demonstrated that As bioaccessibility is independent of As concentration (Pouschat 2006; Ruby 1996). However, when bioaccessibility was greater than the detection limit, decreasing bioaccessibility was observed for increasing concentrations of arsenate (As^V) adsorbed to the iron-oxide mineral ferrihydrite in the stomach stage of an *in vitro* model (Beak 2006b). In contrast, As bioaccessibility in soils increased with increasing concentrations using a different *in vitro* model (Yang 2003). These conflicting results may be due to *in vitro* model differences which may have facilitated or limited As dissolution. For example, the pH, residence times, LSRs, and bioaccessible fraction separation procedure vary with *in vitro* models and may influence the dissolution of contaminants from soils (Oomen 2003; Van de Wiele 2007). Alternatively, varying soil properties (i.e, pH and iron concentration) and mineralogy influence As release from soil particles (Yang 2005) as demonstrated by several selective sequential extraction studies (Corriveau 2006; Frau 2004).

The chemical dissolution of As occurs as a result of desorption of surficially-adsorbed As and the solubilization of discrete As-bearing minerals (Rodriguez 2003). In the GI tract, chemical dissolution may be limited by the desorption of surficially-adsorbed As, rather than solubilization of discrete As minerals (Davis 1996; Ruby 1999). However, the low pH observed in the stomach may result in the dissolution of minerals resulting in the release of surficially-bound As (Beak 2006b). Bioavailable As is likely composed of surficially-adsorbed As and a portion of the As incorporated in discrete iron-oxide As minerals (Rodriguez 2003) rather than from As incorporated into sulfide containing minerals (i.e., arsenopyrite) (Davis 1996; Ruby 1999). However, not all surficially-bound As is bioaccessible since As may be strongly adsorbed to iron-oxide minerals (Fendorf 2004) or iron sulfide minerals (Bostick 2003). Microorganisms can directly solubilize arsenic by two different mechanisms involving anaerobic respiration

(Oremland 2003). Firstly, dissimilatory As reducing prokaryotes (DARPs) can reduce As^{V}, adsorbed to or complexed within soil minerals, thereby releasing arsenite (As^{III}) into solution (Ahmann 1997; Zobrist 2000). Alternatively, dissimilatory iron reducing prokaryotes (DIRPs) may reduce iron, thus releasing As contained in the iron mineral (Cummings 1999). DARPs, such as the Bacillus sp. identified in the GI tracts of mammals (Herbel 2002) may also utilize iron as an electron acceptor (Oremland 2003).

The Simulator of the Human Intestinal Microbial Ecosystem (SHIME) is unique from other *in vitro* GI models by incorporating the microbial community present in the human colon (Van de Wiele 2004). The conditions of the SHIME were selected from the range of physiological pH's and residence times of the human GI tract likely to facilitate the dissolution of ingested contaminants. The small intestine stage of the SHIME includes the addition of bile salts, which may impact metal bioaccessibility through alterations of surface tension and the formation of labile bile complexes (Oomen 2004). The SHIME is operated under anaerobic conditions with human GI microorganisms in the colon stage, thereby facilitating the potential role of DARPs and/or DIRPs in contributing to As release. The assessment of metal bioaccessibility from *in vitro* GI fluids often uses centrifugation, microfiltration (0.45 μm) or ultrafiltration for the separation of the bioaccessible fraction (Beak 2006b; Rodriguez 1999; Ruby 1996; Van de Wiele 2007). It is not clear what method best estimates bioaccessibility but ultrafiltration may be the most appropriate because it represents the defined size fraction known to cross the intestinal lumen (Van de Wiele 2007).

For this study, we tested the hypothesis that the percent of As that is bioaccessible to humans from mine tailings depends on the total concentration of arsenic and the action of GI microorganisms. The As bioaccessibility of two size fractions (bulk and < 38 μm) of mine

tailings from three gold districts in Nova Scotia (Goldenville, Lower Seal Harbour, and Montague) was evaluated in the small intestine and colon stages of the SHIME. Additionally, the analyses were repeated using autoclaved colon SHIME suspension to assess the influence of GI microbes on As bioaccessibility.

4.2 Materials and Methods

4.2.1 Sample description

Mine tailings were collected from Goldenville, Lower Seal Harbour, and Montague, all historic gold mine sites near rural or suburban residential communities in Nova Scotia, Canada. This sampling was part of a large multidisciplinary study of Nova Scotia gold districts that has been ongoing since 2003 (Corriveau 2006; Parsons 2004). A bulk sample of near-surface tailings (0-5 cm) was collected from each of the three mine tailings locations and a portion dry-sieved to <38 microns. The <38 μm fraction was selected since small particle sizes are more likely to adhere to hands and be accidentally ingested (Richardson 2006) and <38 μm was the finest particle size that could be obtained by dry-sieving. Wet sieving was avoided since it may dissolve soluble phases and/or cause cementation. In addition to these two size fractions, a cemented material (hardpan) formed by the precipitation of minerals from groundwater was collected from Montague.

The mineral hosts for As in these tailings samples have been characterized using a variety of techniques including optical mineralogy, electron microprobe, traditional X-ray diffraction (XRD), sequential leach extractions, and synchrotron-based micro-XRD and micro-X-ray absorption near edge structure. Detailed results of these mineralogical investigations are reported elsewhere (Meunier 2010; Walker 2009). Arsenic occurs naturally in the Nova Scotia gold deposits as arsenopyrite (FeAsS), but extensive near-surface weathering of these mine tailings has oxidized almost all of the sulfide to secondary phases. Minor amounts (< 2 vol.%) of

sulfide minerals were observed in the samples from Goldenville and Lower Seal Harbour, including arsenopyrite, pyrite, pyrrhotite, and chalcopyrite. No sulfides were identified in the Montague sample. The Montague tailings contain abundant hardpan material that has been variably crushed by off-road vehicles. The hardpan cement consists primarily of crystalline scorodite ($FeAsO_4$ $2H_2O$) and amorphous Fe arsenate. Mineralogical analyses of the Goldenville and Lower Seal Harbour tailings samples have identified a more diverse range of secondary arsenic minerals, including Ca-Fe arsenates, and Fe oxyhydroxides containing up to 30 wt % As_2O_5. These phases occur as discrete grains, reaction rims on oxidized arsenopyrite, and as grain coatings on silicate minerals (Walker 2009). In summary, the three samples of historical gold mine tailings evaluated in the present study contain a variety of arsenic-bearing minerals to which individuals may be exposed. The relative abundance of these phases at Goldenville, Lower Seal Harbour, and Montague varies laterally and vertically in the tailings deposits, and is not fully represented by the bulk samples collected for this study.

4.2.2 Dynamic Simulator of the Human Intestinal Microbial Ecosystem

As described in **Section 3.2.2**, a three-stage dynamic SHIME model was used to create the colon community used in the batch SHIME colon digests according to previously published procedures (Van de Wiele 2007). Stomach nutrition contained per liter: 5 g $KHCO_3$ (Alfa Aesar), 3 g NaCl (EM Sci), 0.5 g arabinogalactan (Sigma), 1 g pectin (Sigma), 0.5 g xylan (Sigma), 2 g potato starch (Nutricia), 0.2 g glucose (Sigma), 1.5 g yeast extract (Difco), 0.5 g pepton (Difco), 2 g mucin (Sigma) and 0.25 g cysteine (Sigma). The nutrition solution was acidified to pH 2 by the addition of hydrochloric acid and was then pumped into the stomach compartment and mixed constantly at 37ºC for two hours. Following the stomach incubation, a pancreatic solution containing 12.5 g L^{-1} $NaHCO_3$, 6 g L^{-1} Oxgall (Difco) and 0.9 g L^{-1} porcine pancreatin powder (Sigma, Belgium) was pumped into the small intestine vessel in a 2:1 (stomach: pancreatic

solution) ratio, thereby creating a mixture of pH 6.5. Following a 5 hour retention time, the small intestine mixture was pumped into the colon compartment, which had been previously inoculated with a fecal culture from a healthy human adult. The fecal culture was generated according to previously published procedures (De Boever 2000). In brief, freshly excreted feces from a healthy adult male (10 g) was diluted and homogenized with sterile phosphate buffer (0.1 M, pH 7.0, 100 mL), to which 100 mg of sodium thioglycolate was added. Particulate matter was removed by centrifugation (500g, 1 min) (De Boever 2000). This fecal culture was stored in 20% glycerol at -80°C until the time of the experiment. Following the 3 week stabilization period (De Boever 2000), the total aerobe and anaerobe concentrations of the colon stage were 9.9 and 10.9 log CFU mL^{-1} and included microbiota such as Lactobacilli, Bifidobacteria, Enterococci, Fungi, Staphylococci and Clostridia. This colon digest was maintained between pH 5.6 and 5.9, as observed in the human ascending colon, incubated at 37°C and stirred at 150 rpm for 18 hours. Regular flushing of the compartments with nitrogen gas maintained anaerobic conditions. The redox potential of the three SHIME stages were not monitored; however, previous experiments have demonstrated Eh's of -70 mV in the stomach and small intestinal compartments and -160 mV in the colon stage. Nutrient and pancreatic solutions were maintained at 4ºC and replaced every 3 days. Aliquots of the suspension from the colon compartment of the dynamic SHIME provided the solutions used in static batch experiments to measure arsenic bioaccessibility.

4.2.3 Static Simulator of the Human Intestinal Microbial Ecosystem

As described in **Section 3.2.3,** mine tailings sample (150 mg each) was added to two 30-mL glass vessels for the quantification of As bioaccessibility in the small intestine and colon (Van de Wiele 2007). Fasted stomach solution (6 mL) was added to each vessel. The fasted stomach solution is as described above except that no nutrition was added. The pH was adjusted to 1.5 ± 0.05 with 0.5 M HCl (EMD OmniTrace™). Anaerobic conditions were produced in the static

SHIME by flushing with nitrogen gas by applying consecutive cycles of overpressure (1 bar N_2-gas; 2 min) and underpressure (1 bar vacuum; 2 min) for 30 min (Van de Wiele 2010). The vessels were capped with teflon-coated lids and flushed with nitrogen gas prior to stomach incubation at 37ºC for 2.5 hours with rotary-mixing (10 rpm). Following stomach incubation, 3 mL of pancreatic solution was added to each vessel and the pH corrected to 6.2±0.05 with 0.5 M HCl. The vessels were then flushed with nitrogen gas and incubated for an additional 3 hours. Following incubation, the small intestine vessels were removed for separation of the bioaccessible arsenic fraction. Colon SHIME suspension (6 mL) from the Dynamic SHIME was added to each active colon vessel while 6 mL of previously autoclaved and cooled colon SHIME suspension was added to each sterile colon vessel. The pH of the colon vessels were adjusted to 5.9 ± 0.05 with 0.5 M HCl. The vessels were then flushed with nitrogen gas for 30 minutes and incubated at 37ºC for 18 hours.

4.2.4 Separation of bioaccessible fraction

The contents of each SHIME vessel were centrifuged at 12,000g for 20 minutes, thereby accelerating the subsequent ultrafiltration of the SHIME solution. Less rigorous centrifugation is typically used in the separation of the bioaccessible fraction (Hamel 1998; Ruby 1996); however, this strong centrifugation was required to pellet a precipitate formed in the colon stage which, if present, prevented ultrafiltration. The supernatant was decanted into a test tube and weighed. Aliquots of the small intestine supernatant (6 mL) and colon supernatant (9 mL) were weighed and transferred into ultrafiltration devices (10K Microsep; Pall) which were subsequently centrifuged (3000 rpm). The mass of the filtrate of each sample were recorded for the As bioaccessibility calculation. The As contained in the filtrate was defined as the bioaccessible As fraction, containing free As oxyanions and small arsenic-containing complexes. The As bioaccessibility (%) was calculated by:

$$\text{Bioaccessibility}\,(\%) = \frac{[\text{Filtrate As}] \times \dfrac{\text{Filtrate Mass} \times \text{Total Supernatant Mass}}{\text{Total Ultrafiltration Mass}}}{\text{Total As Concentration} \times \text{Sample Mass}} \times 100\% \qquad \text{(Eqn 4.1)}$$

Where [Filtrate As] is the As concentration (μg mL^{-1}) in SHIME filtrate, Total As Concentration refers to the As concentration (mg kg^{-1}) in the mine tailing, and the Sample Mass is the amount of mine tailing extracted in the Static SHIME. The Filtrate, Total Supernatant, and Total Ultrafiltration Mass is the mass (g) of each solution fraction assuming solution densities of 1 g mL^{-1}.

4.2.5 Measurement of arsenic via Hydride Generation Atomic Fluorescence Spectroscopy

Digestion of the bioaccessible fraction was performed using a CEM-2000 microwave according to a previously published procedure, with modifications (Rahman 2000). In brief, 0.5 mL of SHIME suspension was added to 12 M HCl (5 mL) and the mixture digested at 40% power for 15 minutes. Subsequently, hydrogen peroxide (EMD, 1.0 mL, 30%) was added and the mixture digested at 40% power for an additional 15 minutes. A further 1.0 mL of hydrogen peroxide was added to the mixture, which was digested for another 15 minutes. The digested SHIME solution was diluted to 30 mL with MQ water and stored at 4°C until As analysis.

Arsenic was measured using hydride generation atomic fluorescence spectroscopy (HGAFS) (Millenium Excalibur, PS Analytical) (Rahman 2000). Six mL of pure OmniTrace® HCl (12N, 37%), 4.5 mL Milli-Q® ultrapure water, and 0.4 mL 3.01 M potassium iodide (KI, EMD) and 0.57 M ascorbic acid ($C_6H_8O_6$, JT Baker) solution were added to 9 mL aliquots of each digested SHIME solution. Potassium iodide served to reduce any As^V in solution to As^{III}, which forms the hydride necessary for spectrometric analysis. Fresh $NaBH_4$ (1.4%, Sigma) and reagent blank solutions were prepared daily. Standard solutions were prepared by dilution of stock 1000 mgL^{-1} As standard (EMD Chemicals Inc., Darmstadt, Germany). All glassware was soaked in 10%

Omnitrace® nitric acid (EMD Chemicals Inc., Darmstadt, Germany) prior to use and then rinsed five times with distilled water.

4.2.6 Quality Assurance - Quality Control

Blank SHIME suspension (without mine tailings) was microwave digested and measured for As according to the procedures described above and returned an average concentration of 0.27 $\mu g\ L^{-1}$ (SE = 0.008). Duplicate SHIME subsamples were digested and measured for As for every ten samples processed. Additionally, a calibration blank, standard, and an analytical duplicate were measured for every ten samples analyzed to ensure instrumental accuracy and precision. The US EPA defines the methodological detection limit (MDL) as the minimum concentration that can be determined with 99% confidence that the true concentration is greater than zero (US EPA 1998). The methodological detection limit (MDL) for the measurement of arsenic in microwave digested SHIME solution is calculated as follows:

$$MDL = (t_{n-1,\ 0.01}) \bullet sd \qquad \text{(Eqn 4.2)}$$

Where the Student's t-value at the 0.01 level of probability for 7 samples is 3.143, and 'sd' is the standard deviation of 7 microwave digested blank SHIME solutions. The standard deviation was 21.5 ng L^{-1} for a methodological detection limit of 68 ng As L^{-1}.

4.2.7 Statistical analysis

Nested factorial Analysis of Variance (ANOVA) was used to compare mean As bioaccessibility from the bulk and <38 μm mine tailings fractions from Goldenville, Lower Seal Harbour, and Montague in the small intestine and microbially active and sterile colon. ANOVA assumptions of normality and homogenous variance were verified using a Type I error rate of 0.05. Fisher's protected Least-Significant Difference (LSD) (GLM, α=0.05) was used to test for significant differences.

4.3 Results

Percent As bioaccessibility was inversely related to sample As mass despite increasing As release (**Figure 4.1**). Bioaccessibility ranged between 2.5 and 20% in the small intestine compared to 4 and 70% in the colon. Bioaccessibility of As was log-normally distributed (W-Test, P>0.05) with homogenous variance (Levene's Test, P>0.05). In the small intestine, there was little difference between the mass of bioaccessible As in the 5000 and 17000 µg As samples suggesting that As release had peaked by 5000 µg. In contrast, the mass of bioaccessible As continued to increase with increasing total As in the colon.

Arsenic bioaccessibility in bulk tailings was greater (Fisher protected LSD, P=0.001) than in the <38 µm fraction in both the small intestine and colon (**Figure 4.2**). No significant interaction in As bioaccessibility existed between mine tailings location and size fraction (GLM, P=0.814). Thus, the As bioaccessibility of the mine tailings locations were pooled for both the small intestine (n=9) and colon (n=9) stages of the SHIME. Total As mass was consistently less in the bulk than in the <38 µm fraction of all mine tailings (Goldenville: Bulk = 62 µg As, < 38 µm = 455 µg As; Montague: Bulk = 4608 µg As, < 8 µm = 16764 µg As; Lower Seal Harbour: Bulk = 97 µg As, < 38 µm = 791 µg As). The bioaccessible arsenic concentration in the SHIME fluid ranged between 1.3 and 16.2 mg L^{-1} in the small intestine and 2.9 and 27.3 mg L^{-1} in the colon.

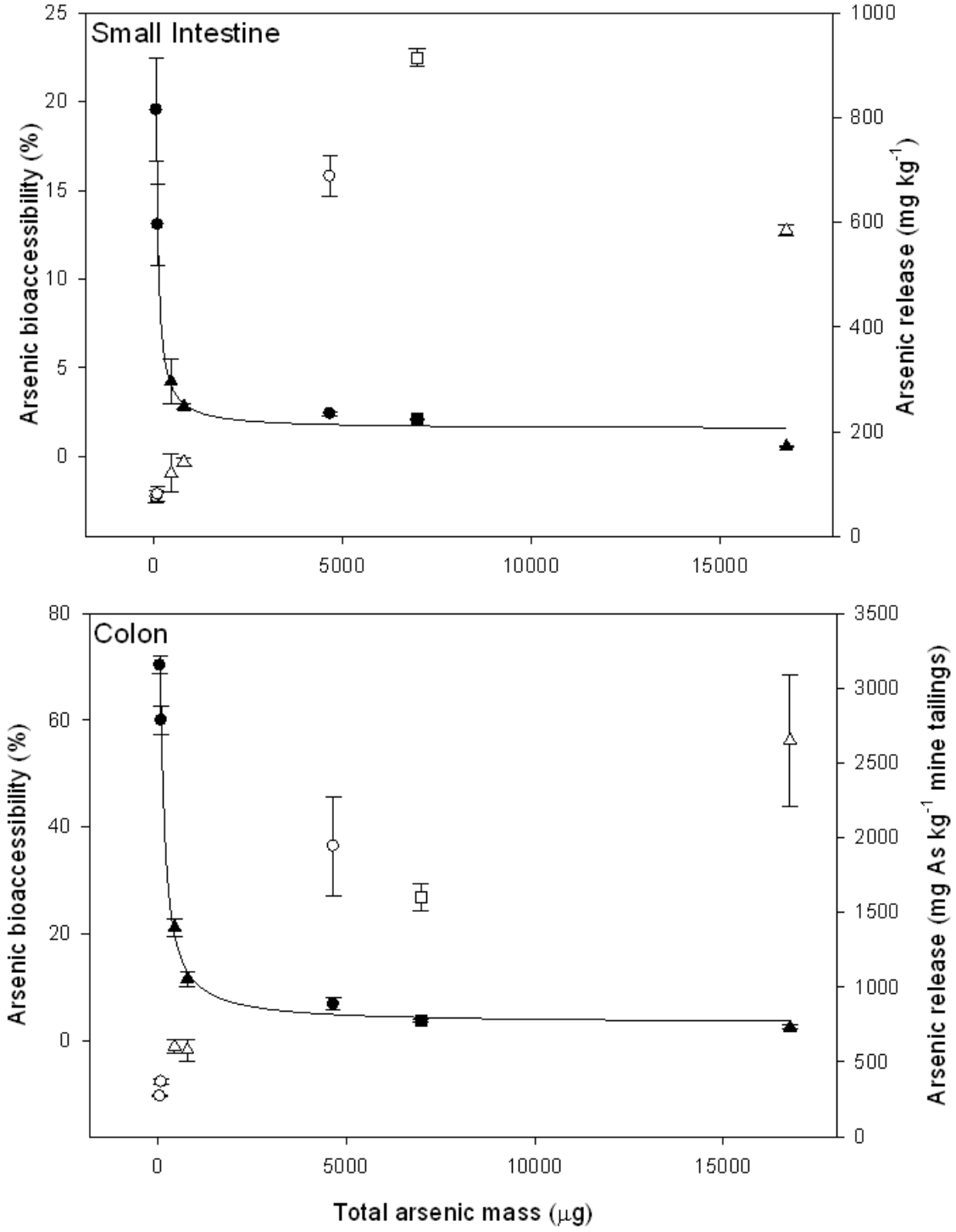

Figure 4.1 Relationship between As bioaccessibility (left Y-axis, closed symbols) and As release (right Y-axis, open symbols) and total As mass in the Small Intestine (Inverse First Order, R^2=0.994) and Colon (Inverse Second Order, R^2=0.998) stages of the SHIME for bulk (circle), <38 μm (triangle), and hardpan (square) fractions of mine tailings. Error bars represent standard error of the mean.

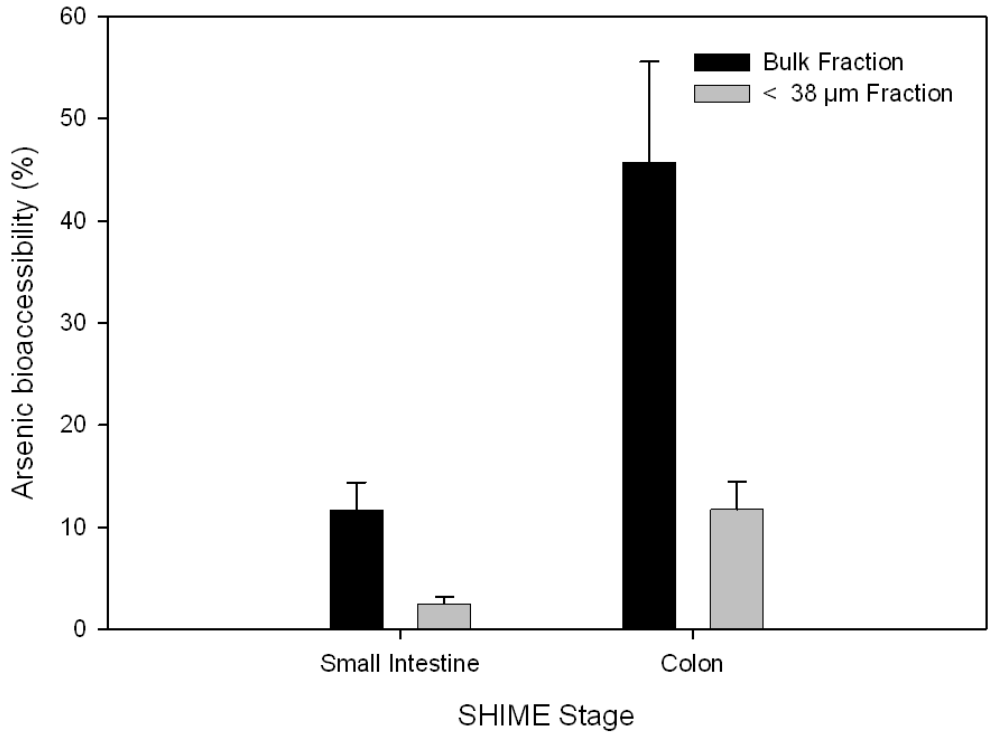

Figure 4.2 Arsenic bioaccessibility from bulk mine tailings was significantly greater (Fisher's Protected LSD, P<0.001) than that of the <38 µm of the same tailings using the Simulator of the Human Intestinal Microbial Ecosystem. Error bars denote standard error of the mean.

Small intestine As bioaccessibility of the bulk and < 38 µm mine tailings fractions was greater from Goldenville than Lower Seal Harbour which was greater than Montague (Fisher protected LSD, P<0.001) (**Figure 4.3**). The same order of As bioaccessibility was observed in the colon stage; however, the colon As bioaccessibility of the bulk fraction of tailings from Goldenville and Lower Seal Harbour were both greater (Fisher protected LSD, P<0.001) than that of tailings from Montague. No difference (Fisher protected LSD, P>0.05) was observed between the colon As bioaccessibilities of the bulk fraction of tailings from Goldenville and Lower Seal Harbour. In contrast, all pairwise comparisons of colon As bioaccessibility from the < 38 µm from the three locations were significant. The As bioaccessibility of the active colon stage of the SHIME

was greater than the small intestine (**Figure 4.4**) for all three locations (Fisher-Protected LSD, P<0.001). Sterilization of the colon SHIME suspension prior to incubation decreased As bioaccessibility to levels similar to the small intestine. A black precipitate was formed following the active colon incubation of mine tailings, but not in the absence of mine tailings. In contrast, no precipitate was formed when the colon suspension was autoclaved prior to colon incubation with mine tailings.

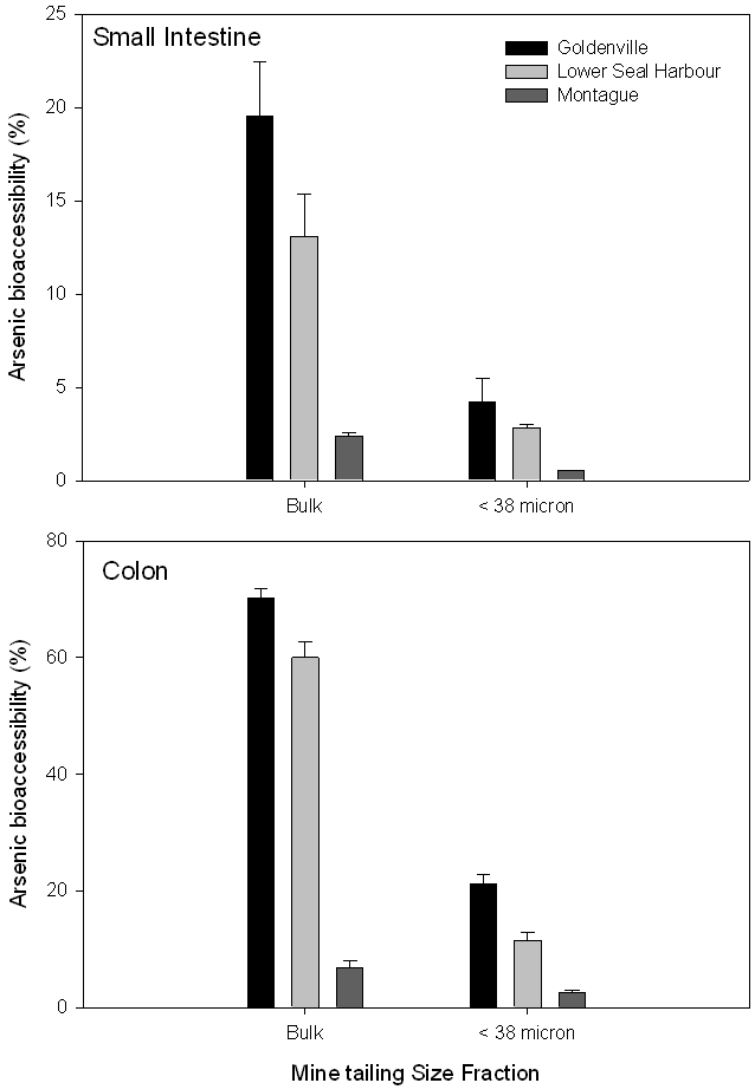

Figure 4.3 Mean As bioaccessibility (± standard error) for two size fractions (Bulk, < 38 µm) of mine tailings in the small intestine and colon stages the Simulator of the Human Intestinal Microbial Ecosystem.

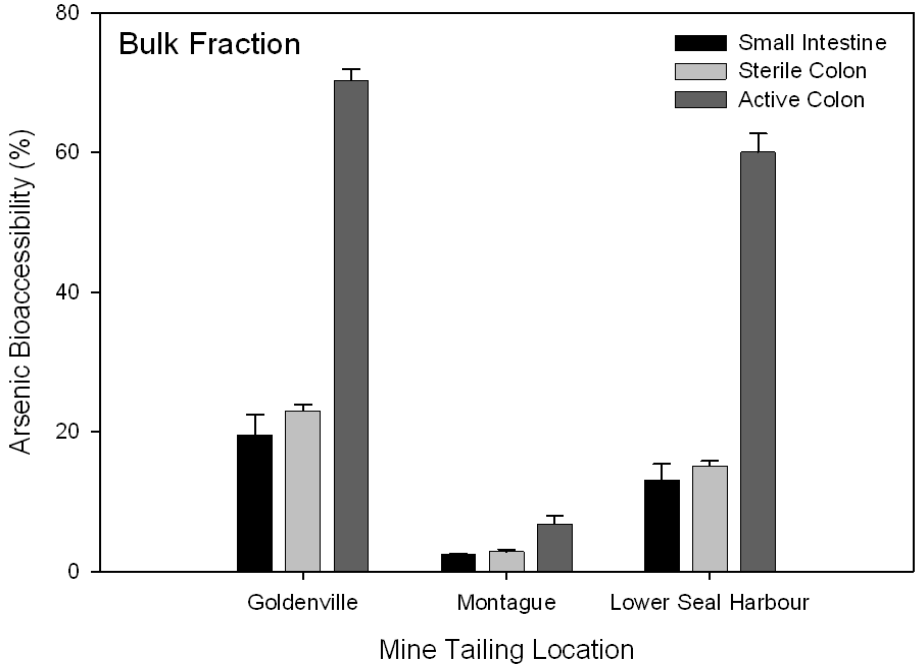

Figure 4.4 Mean As bioaccessibility was greater (Fisher's Protected LSD, $P<0.001$) in the Active Colon (i.e., with gastrointestinal microbial activity) than Small Intestine and Sterile Colon stages (i.e., without gastrointestinal microbial activity) of the Simulator of the Human Intestinal Microbial Ecosystem.

4.4 Discussion

As Beak *et al.* (Beak 2006b) observed in the gastric phase of an *in vitro* GI model, increasing As concentration decreased the percent As bioaccessibility in the small intestine of the SHIME. This same relationship was also observed in the colon stage of the SHIME despite increased solubilization of arsenic due to the activity of colon microbiota. An investigation of the mechanism of the relationship between As bioaccessibility and concentration will be necessary before conclusions may be made regarding why this inverse relationship was observed with the mine tailings in the SHIME but not in studies of other As-contaminated soils (Pouschat 2006; Ruby 1996; Yang 2003). The inverse relationship between As concentration and bioaccessibility in the SHIME may have arisen due to the saturation of the GI fluid or kinetic limitations of As

release. Our study used mine tailings from different sites of varying particle sizes and mineralogy (Corriveau 2006) and thus we are unable to construct an isotherm to assess the K_d of arsenic in GI fluid.

The bioaccessibility of ingested contaminants is typically greater from small particle sizes of soils (Ruby 2004). However, the As bioaccessibility from mine tailings in the SHIME was greater in the bulk than in the <38 μm fraction. Although this discrepancy may be due to differences in the mineralogy of the particle sizes, it may also be due to the high As concentration present in the <38 μm fraction relative to the bulk fraction. Thus, the lower percent bioaccessibility in small size fractions of the mine tailings may arise from the greater As concentrations, thereby confounding the size/bioaccessibility relationship often observed. Since contaminant distribution among size fractions varies between soils and size fraction affects probability of exposure, this result emphasizes the need to assess both the concentration and bioaccessible fraction distribution among various size fractions for use in human health risk assessment (Bright 2006; Richardson 2006).

Inclusion of the colon stage for *in vitro* As bioaccessibility analyses significantly increases the mobilization of As from mine tailings. However, the toxicological importance of this result remains to be demonstrated since the principal site of As absorption is likely in the small intestine (Kayne 1993). Although the rate of uptake of As in the colon will be markedly reduced relative to the small intestine, the much longer residence time in conjunction with increased arsenic bioaccessibility in the colon may result in appreciable As absorption. Additionally, genotoxic arsenic metabolites have been identified in the colon of mammals (Kuroda 2001; Kuroda 2004) and higher mortality rates due to colon cancer have been observed in populations that ingest high concentrations of As (Tchounwou 2004). Previous studies have attempted to

validate As bioaccessibility estimates generated with *in vitro* GI models with the oral bioavailability of ingested As from animal-dosing experiments (Ellickson 2001; Rodriguez 1999; Ruby 1996). The implicit assumption of this comparison for use in human health risk assessment is that the GI microbiota of the animal model is functionally similar to that of humans. Studies investigating the role of DIRPs and DARPs in the toxicokinetics of As ingestion will be necessary before conclusions regarding the validity of bioaccessibility estimates generated with or without the colon stage will be possible.

The use of total As concentration from ingested soils and mine tailings results in the over-estimation of human health risk due to the limited dissolution of contaminants from ingested soil particles relative to that of the soluble salts administered in toxicity studies (Hund-Rinke 2003; Ruby 1999). *In vitro* GI models such as the SHIME have gained acceptance as a valuable tool for estimating the relative bioavailability of As contaminated soils (Hund-Rinke 2003; Oomen 2003; Ruby 1999). The experiments detailed in this paper demonstrate that assumptions of constant bioaccessibility from varying As concentrations and size fractions could lead to inaccurate exposure estimates for use in risk assessments, thereby agreeing with recent recommendations for the incorporation of bioaccessibility estimates into human health risk assessment (Richardson 2006). Furthermore, neglecting to include the colon in previous assessments of As bioaccessibility may have resulted in an underestimation of the relative bioavailability and risk due to ingestion of As-contaminated soils.

5 THE EFFECT OF RESIDENCE TIME AND LIQUID:SOIL RATIO ON *IN VITRO* ARSENIC BIOACCESSIBILITY FROM POORLY CRYSTALLINE SCORODITE

5.1 Introduction

The use of the total As concentration from contaminated particles for human health risk assessments potentially overestimates ingestion As exposure because only a fraction of arsenic present in the particle is likely to be absorbed into systemic circulation (Davis 1996; Ruby 1996). The fraction of a soil-borne contaminant that is absorbed into systemic circulation is defined as the oral bioavailability and is routinely measured using *in vivo* models (Davis 1996; Rodriguez 1999). *In vitro* gastrointestinal (GI) models offer a means of predicting oral bioavailability via a surrogate measure termed bioaccessibility, which represents the fraction of a soil-borne contaminant released into GI fluids (Basta 2007; Jamieson 2006; Oomen 2004; Ruby 1993; Van de Wiele 2007). *In vitro* GI models offer risk assessors a means of reproducibly measuring bioaccessibility (and thus predicting bioavailability) without relying upon expensive and time-consuming animal models.

Researchers have extensively investigated factors that influence As bioaccessibility with the ultimate goal being the development of a predictive model of As bioaccessibility and bioavailability. Arsenic bioaccessibility from spiked soils is primarily determined by soil iron content and pH (Yang 2002; Yang 2005). Additionally, bioaccessible As from UK soils was successfully predicted according to several intrinsic soil components including amended soil, iron oxide, clay, fine grained silicate, and Fe/Al association constituents (Wragg 2007b). Investigations are also currently underway evaluating the role of mineralogy on As bioaccessibility using synchrotron-based X-Ray Absorption Near Edge Spectroscopy (XANES) techniques (Jamieson 2006). It is commonly presumed that particle size is inversely proportional to As bioaccessibility (Richardson 2006; Ruby 1999) since larger surface area facilitates

dissolution reactions at the particle surface. However, increasing As concentration with decreased particle size potentially confounds, and may even reverse, the usual trend of increased bioaccessibility from smaller particles (Laird 2007).

Arsenic concentration potentially affects As bioaccessibility of contaminated particles; however, trends from previous studies are at times inconsistent and contradictory. For example, As bioaccessibility either increased with increasing As concentrations in soils (Yang 2003), did not change with increasing As concentration (Pouschat 2006; Ruby 1996) or even decreased with increasing As concentrations (Beak 2006a; Laird 2007). Although it is not yet known why concentration only sometimes affects As bioaccessibility, operational constraints on dissolution (i.e., insufficient residence time, low Liquid: Soil ratio) are likely explanations since both are dependent upon mineralogy and soil properties. *In vitro* models often utilize residence times of 2 hours for the stomach, 2 hours for the small intestine and, if included, 18 hours for the colon. These reflect the residence times commonly observed in humans, 0.5 - 2 hours for stomach, 2 – 6 hours for small intestine and 12 – 24 hours for the colon. The residence times of *in vitro* GI models were selected to approximate human physiological residence times and also to be convenient for laboratory practice. However, if residence time is found to be a limiting factor of As bioaccessibility *in vitro*, then the upper range of human estimates should likely be adopted to increase conservatism. Similarly, the LSR (i.e., the ratio of fluid volume to soil mass) of *in vitro* GI models range between 40:1 and 100:1 (Van de Wiele 2007). However, this LSR is substantially below the range typically encountered for humans of up to 12,500:1 to 50,000:1 (Richardson 2006). It is impractical to use a LSR of 12,500 in a normal laboratory setting because analytical error increases substantially as one measures soil masses below 100 mg and

technical issues dominate when GI models are 1.25 to 5 L in size. However, if LSR controls As bioaccessibility then it may be necessary to use the 50,000:1 ratio experienced by humans.

The objective of this research is to investigate the mechanism(s) responsible for an inverse relationship between As concentration and bioaccessibility. By doing so we will be able to determine if residence time or LSR is critical in determining As bioaccessibility. In addition, we will evaluate the mechanism by which GI microbes potentially increase As bioaccessibility in the colon. We synthesized poorly crystalline scorodite to serve as a surrogate iron mineral with As covalently incorporated into the mineral structure. Subsequently, we used isotherm analysis to investigate the mechanisms controlling As dissolution from scorodite. Additionally, we repeated our work with sterilized (i.e., autoclaved) SHIME solution to investigate the mechanism by which GI microbes increase As bioaccessibility.

5.2 Materials and Methods

5.2.1 Mineral synthesis

Poorly crystalline scorodite was synthesized by mixing 0.2 M sodium arsenate (500 mL) with 0.1 M ferric sulfate (EMD). The scorodite precipitate was collected by five successive rinse (0.1 M sodium nitrate, EMD) and centrifuge (4000 g) cycles and the mineral was subsequently freeze dried. The identity of the mineral as scorodite was verified using X-Ray Diffraction. Ferrihydrite (2-line) was synthesized via the addition of equimolar concentrations of ferric nitrate (EMD) and potassium hydroxide (EMD). Potassium hydroxide was added drop-wise to ferric nitrate to bring and maintain the mineral suspension pH of 7.0 +/- 0.1 for one hour. As with the scorodite, the ferrihydrite precipitate underwent five successive rinse and centrifuge cycles prior to freeze-drying. An As concentration gradient was established by diluting freeze-dried As-bearing scorodite with freeze-dried ferrihydrite to the following concentrations (% scorodite, wt/wt): 1, 5, 10, 25, 50, 75, and 100%. The lowest As concentration was set to 1% since this amount (30

mg scorodite) was as low as could be reliably and precisely weighed into the individual SHIME extraction vessels.

5.2.2 Dynamic Simulator of the Human Intestinal Microbial Ecosystem

As described in **Section 3.2.2**, a three compartment dynamic *in vitro* GI model maintained a microbial community representative of that found in the human large intestine. The large intestine microbial community was established by incubating the supernatant of a centrifuged mixture containing feces and a phosphate/thioglycolate buffer (De Boever 2000). The fecal phosphate/thioglycolate buffer supernatant (40 mL) was added to a carbohydrate-based nutrition solution (600 mL) in the third compartment of a series of double-jacketed reactor flasks. The carbohydrate solution contained 5.01 g KHCO3 (MP Biomedicals), 2.92 g NaCl (EMD), 0.5 g arabinogalactan (Fluka), 1.0 g pectin (ICN Biomedicals), 0.5 g xylan (Sigma), 2.1 g potato starch (EMD), 0.2 g glucose (EMD), 1.5 g yeast extract (BD Bacto™), 0.5 g peptone (BD Bacto™), 2.0 g mucin (Sigma), 0.25 g L-cysteine (EM Science) per liter of Milli-Q™ water. Nutrition solution (100 mL), acidified to pH 2 via the addition 12 M HCl (EMD), was added to the stomach compartment. After 2 hours in the stomach, the acidified nutrition solution was pumped into the small intestine compartment where it was mixed with a pancreatic enzyme solution (50 mL) containing 12.5 g NaHCO3 (EMD), 6.0 g Oxgall (BD Difco™), and 0.9 g pancreatin (Sigma) per liter. After two hours, the nutrition-enzyme mixture was pumped into the colon containing the intestinal microbial community. This feeding cycle was repeated every 8 hours. The SHIME was maintained at 37°C by circulating water from a heated water bath through the walls of the double-jacketed reactor flasks. Additionally, the anaerobic status of the dynamic SHIME was maintained by flushing the headspace of the reactor flasks twice daily with nitrogen. The microbial community contained 10^8 anaerobes and 10^8 aerobes, and included Fecal Coliforms, Staphylococci, Enterococci, Clostridia, and Bifidobacteria. The microbial community

maintained in the colon of the dynamic SHIME was utilized in batch digests of scorodite/ferrihydrite mixtures.

5.2.3 Static Simulator of the Human Intestinal Microbial Ecosystem

As described in **Section 3.2.3**, synthesized mineral mixture (scorodite + ferrihydrite, total mass= 0.3 g) was added to 50 mL polypropylene conical bottom centrifuge tubes. Stomach solution (12 mL, pH 1.5) was added to each experimental unit and the pH was corrected to 1.5 +/- 0.05 via the dropwise addition of 0.5 M HCl (EMD OmniTrace^{TM})/NaOH (EMD). Subsequently, the samples were incubated with horizontal shaking for 2 hours at 37°C to mimic the physiological conditions found in the stomach. After the stomach incubation, 6 mL of small intestinal solution (see Dynamic SHIME) was added to the scorodite stomach digests and the pH was corrected to 6.5 via dropwise addition of 0.5 M NaOH (EMD). Subsequently, the centrifuge tube headspace was replaced with N_2 (Praxair) in an anaerobic chamber and the samples were returned to the incubator shaking for an addition 2 hours in the small intestine. After the small intestine residence time, 12 mL of active (i.e., non-autoclaved) or sterile (i.e., previously autoclaved and cooled) colon suspension from the dynamic SHIME was added to the small intestinal SHIME digest and the pH was adjusted to 6.3. Subsequently, the headspace of the centrifuge tube was replaced with N_2 and the colon SHIME digests were returned to the incubator shaker for a final 18 hours. Following each GI stage, samples were syringe filtered (GMF, 0.45 um) and then a 3.5 mL aliquot of the filtered solution was passed through a 10 kDa membrane of an ultrafiltration centrifuge cartridge (Microsep, Pall).

5.2.4 Chemical analysis and Quality Assurance – Quality Control

As described in **Section 4.2.5**, total arsenic (As^{V} + As^{III}) was measured using Hydride Generation Atomic Fluorescence Spectroscopy (HGAFS) (Millenium Excalibur, PSA) (Rahman 2000). The 10 kDa fraction (0.5 mL) was diluted to 9 mL gravimetrically with MilliQ Water in

50 mL polypropylene centrifuge tubes. Each sample then received 6 mL of OmniTrace HCl (12 M), 4.5 mL of Milli-Q water, and 0.4 mL of 3.01 M potassium iodide (EMD) containing 0.57 M ascorbic acid (JT Baker). The potassium iodide As^{V} present to As^{III} for measurement via HGAFS. Additionally, the speciation of inorganic As (i.e., percent As in the form of As^{III}) was measured by diluting and acidifying a second aliquot (0.5 mL) of the 10 kDa bioaccessible As via the addition of 6 mL of OmniTrace HCl (12 M) and 4.9 mL of Milli-Q ultrapure water. The fluorescence measured from this second aliquot reflects the concentration of bioaccessible As^{III} and a portion (~20%) of bioaccessible As^{V}. Every 10 samples, the As concentration in a blank (MQ water), standard, and duplicate was measured.

5.2.5 Arsenic K-Edge X-Ray Absorption Near Edge Spectroscopy

Arsenic K-Edge XANES spectroscopy was carried out at the HXMA beamline of the Canadian Light Source in Saskatoon, Canada. The ring was operating at 2.9 GeV and 250 mA, and data was collected in transmission mode due to the relatively high As concentrations in both samples and standards. SHIME samples, sorption samples, and mineral standards were analyzed as freeze-dried solids, whereas As^{V} and As^{III} aqueous standards were prepared in a N_2 atmosphere using a glovebox and then analyzed in sealed 15 mL Falcon tubes at the beamline. Multiple scans were averaged to obtain suitable signal to noise ratio, and an Au foil standard was placed downstream from the sample to verify that no shifts in energy were occurring during the run. Data analysis was conducted with WINXAS 3.1: a linear baseline was subtracted from the data and the edge jump was normalized to 1.0.

5.2.6 Statistical analysis

Nonlinear regression analysis (NLS) was performed using R (v 2.6.0, The R Foundation for Statistical Computing) to evaluate the presence of an inverse relationship between As bioaccessibility and concentration. For the regression analysis a null intercept only model

(Bioaccessibility~a) was compared to a 2-parameter reciprocal model (Bioaccessibility~a*exp(-b*Concentration)). An F-test (α=0.05) was used to test the hypothesis that the alternative reciprocal model better explained the variance than the null intercept model via residual sum of squares. Similarly, nonlinear regression analysis (NLS) was performed using (version 2.6.0, The R Foundation for Statistical Computing) to determine if the As dissolution isotherms deviated from the equation of a straight line. For the regression analysis a null linear model was compared to a 2-parameter exponential rise to a maxima model using (version 2.6.0, The R Foundation for Statistical Computing) for the stomach, small intestine, and sterile colon isotherm. In contrast for the active colon, a 3-parameter logistic sigmoidal alternative model was selected since it best explained the variance. An F-test (α =0.05) tested the hypothesis that the alternative exponential and sigmoidal models better explained the variance via residual sum of squares. Finally, a Fisher's Protected LSD (α = 0.05) was performed to compare As bioaccessibility and percent of bioaccessible As^{III} with or without GI microbial activity.

5.3 Results

An inverse relationship between As concentration and bioaccessibility was observed in the stomach ($F_{1,12}$=126; P=1x10^{-7}), small intestine ($F_{1,12}$=1061; P=4x10^{-13}), and colon ($F_{1,12}$=362; P=2x10^{-10}) following the digestion of scorodite using the SHIME (**Figure 5.1**). Scorodite As bioaccessibility ranged between 0.1 to 6.6% in the stomach, 1.4 to 83.5% in the small intestine, and 2.4 to 94.8% in the colon. Similarly, an inverse relationship was observed between As bioaccessibility and concentration in the stomach, small intestine, and colon following the SHIME digestion of As adsorbed on ferrihydrite, an iron hydroxide (**Figure 5.2**). As bioaccessibility from synthesized scorodite was as much as 14-fold higher than previously reported from natural samples (Jamieson 2006; Laird 2007), likely due to the relatively low crystallinity of the freshly-synthesized scorodite used for this study.

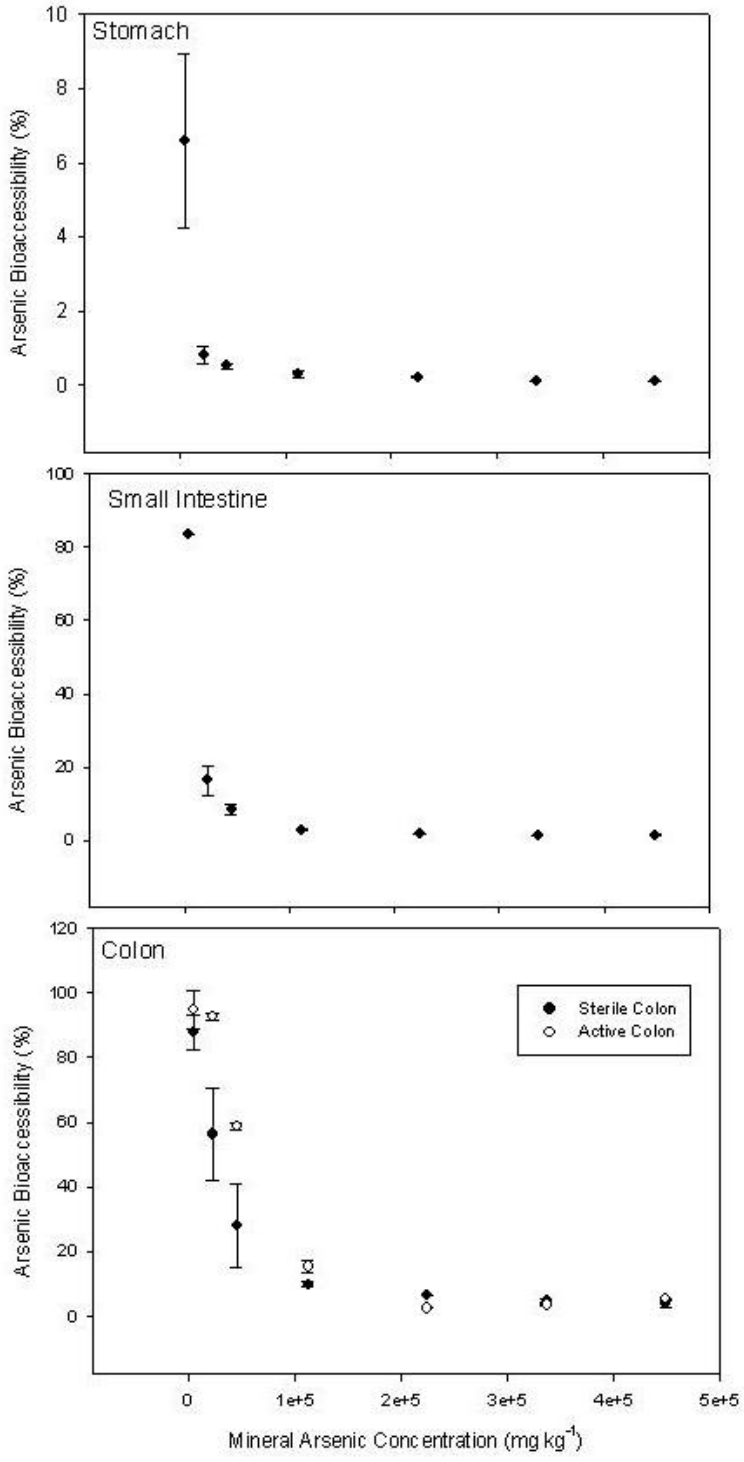

Figure 5.1 Inverse relationships were observed between As percent bioaccessibility (± standard deviation) and total As concentration (X-axis) of synthesized poorly crystalline scorodite in stomach (F=126; $P=1x10^{-1}$), small intestine (F=1061; $P=4x10^{-3}$) and colon (F=362; $P=2x10^{-10}$) stages of the Simulator of the Human Intestinal Microbial Ecosystem.

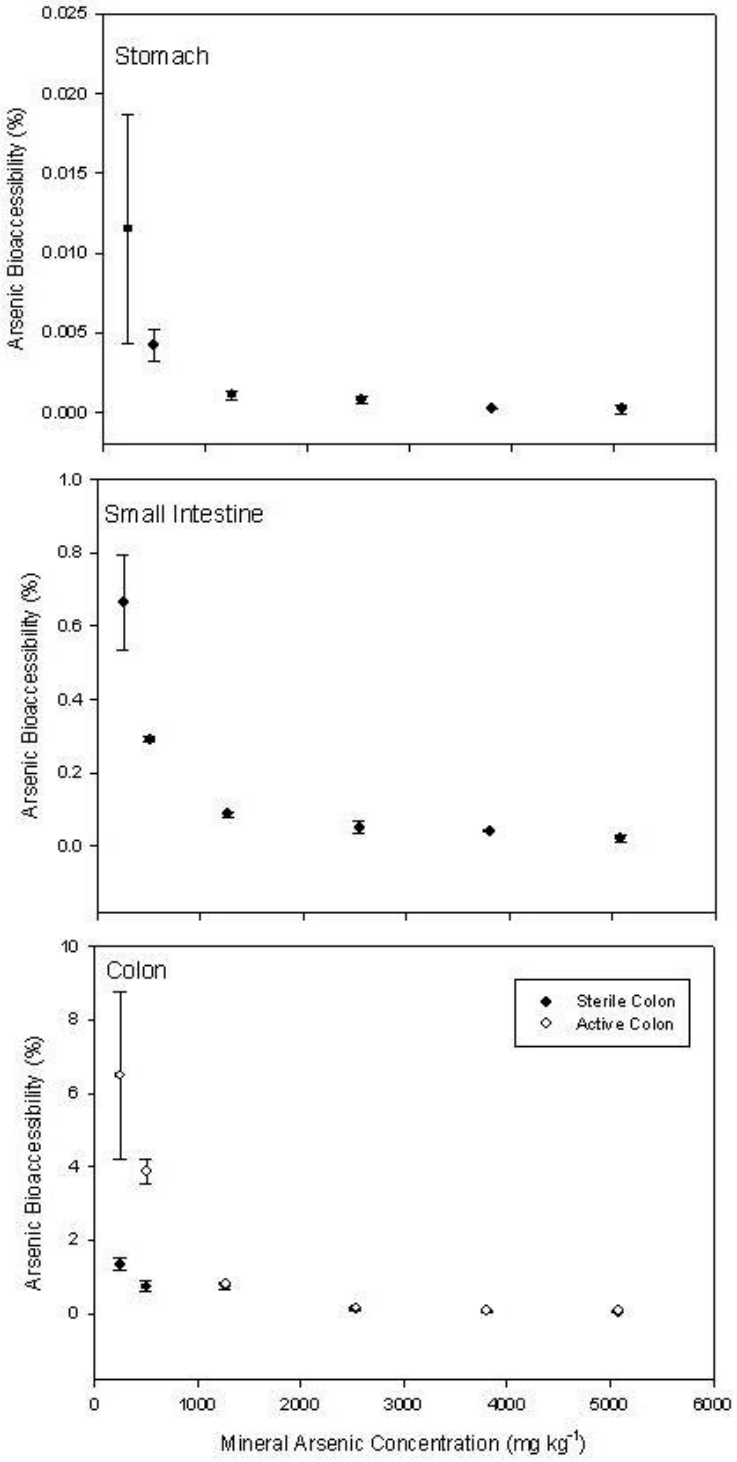

Figure 5.2. Mean Arsenic bioaccessibility (± standard deviation) of Arsenic-bearing 2-line ferrihydrite is inversely related to total As concentration in the Simulator of the Human Intestinal Microbial Ecosystem.

Naturally occurring scorodite is typically more crystalline and less soluble (Harvey 2006), thereby causing lower As bioaccessibility. Despite these differences in solubility between natural occurring scorodite and the poorly crystalline scorodite, the inverse relationship between As concentration and bioaccessibility is consistent with that of natural samples (Laird 2007). As shown in **Figure 5.3**, the dissolution isotherm of scorodite describing the release of As into the stomach fluids is best explained by a linear relationship ($F_{1,12}$=2.52; P=0.14). Therefore, all of the reactions controlling the dissolution of As from scorodite may have reached equilibrium under simulated stomach conditions without reaching a asymptotic solubility limit. Consequentially, stomach As bioaccessibility from scorodite was not likely limited by kinetic constraints under simulated stomach conditions. However, it is important to note that non-linear isotherms are also possible at equilibrium. Additionally, these results suggest that increasing the LSR (i.e., adding more gastric fluid) may increase As solubilization and bioaccessibility. Therefore, researchers should measure As bioaccessibility at higher LSR when an inverse relationship is observed between concentration and bioaccessibility, in agreement with previous work (Richardson 2006).

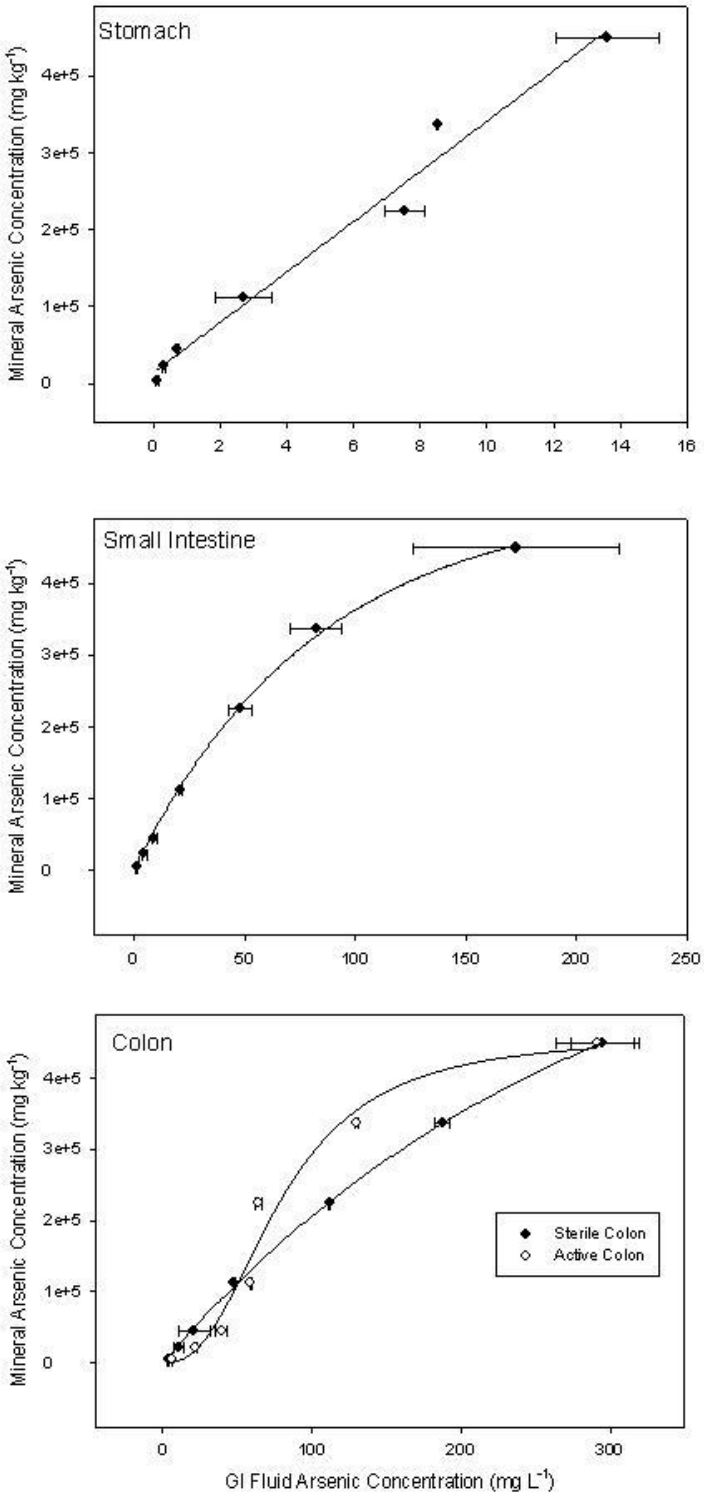

Figure 5.3 Isotherms depicting the relationships between scorodite As concentration (Y-axis) and the bioaccessible As concentration (± standard deviation) using the Simulator of the Human Intestinal Microbial Ecosystem. Isotherms were best represented by a straight line in the stomach stage (P=0.14), exponential rise to a maxima in the small intestine ($P=2x10^{-9}$) and sterile colon ($P=2x10^{-7}$) stages, and a logistic sigmoidal model for the active colon stage ($P=2x10^{-7}$).

In contrast to the stomach, a 2-parameter exponential rise to a maxima model better explained the release of As from scorodite in the small intestine isotherm ($F_{1,12}$=246; P=2x10^{-9}). The isotherm's deviation from linearity suggests that equilibrium was not reached following small intestinal digestion of scorodite through the SHIME. Therefore, the As dissolution in the small intestine is potentially limited by residence time instead of LSR. The isotherm of As dissolution in the active colon of the SHIME (i.e., with microbial activity) was better explained by a 3 parameter logistic sigmoidal model ($F_{2,11}$=25.8; P=7x10^{-5}) than a linear model. In contrast, the isotherm of As dissolution in the sterile colon of the SHIME (i.e., without microbial activity) was better explained by a 2-parameter exponential rise to a maxima than with a linear model ($F_{1,12}$ =108; P=2x10^{-7}). This suggests that microbial activity alters the dissolution kinetics of As from scorodite; however, the long colon residence time does not facilitate all dissolution processes to reach equilibrium. Based upon the isotherm of scorodite in the stomach, small intestine, and colon, only evaluating the role of LSR without considering the effect of residence time upon As bioaccessibility may cause the systematic underestimation of As bioaccessibility when *in vitro* As dissolution is limited by reaction kinetics (i.e., when the isotherm is nonlinear).

Depending upon As concentration in the sample, As bioaccessibility was greater in the colon than in the small intestine which was greater than observed in the stomach, reflecting the chemical and microbiological conditions of each stage. Solid phase As K-Edge XANES spectroscopy that the As spectra of scorodite digested through the SHIME was most similar (e.g position of edge, shape of post-edge spectral fingerprint) to As^{V} adsorbed on ferrihydrite (**Figure 5.4**). This result is consistent with scorodite dissolution and subsequent re-precipitation reactions as follows. The low pH of the stomach SHIME facilitated the dissolution of scorodite (Harvey

2006), thereby releasing As into gastric fluids. Subsequently, the adsorption of aqueous As onto existing or newly precipitated ferrihydrite

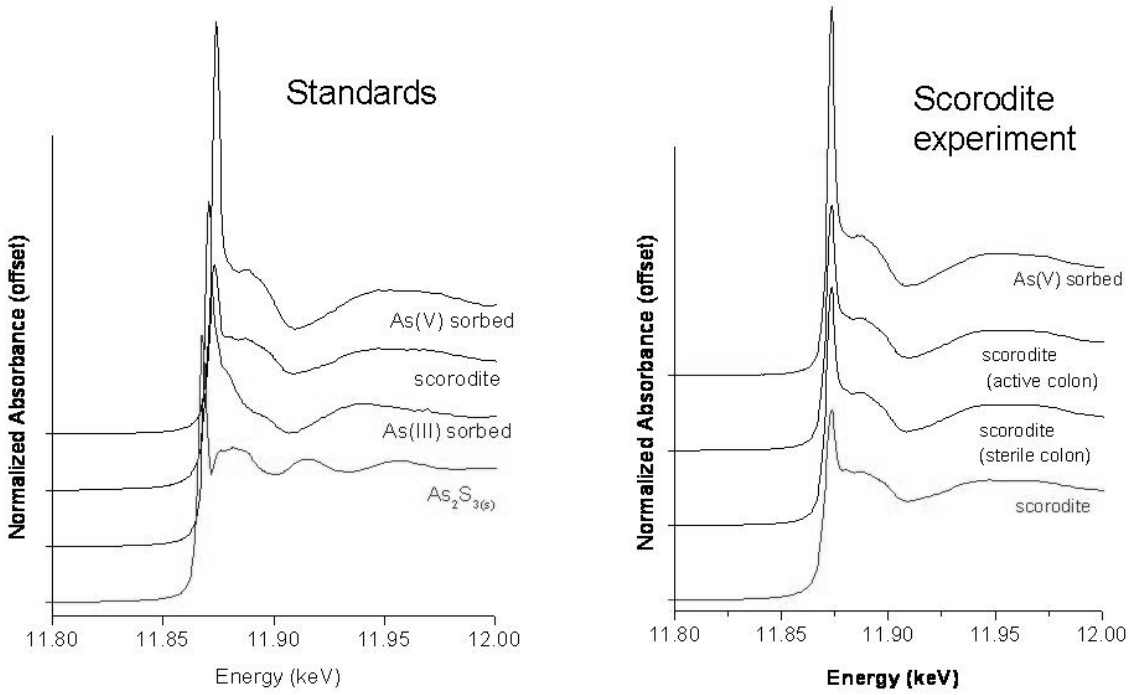

Figure 5.4 X-Ray Absorption Near-Edge Spectroscopy (XANES) of As bearing minerals digested through the Simulator of the Human Intestinal Microbial Ecosystem (SHIME). The left panel depicts several arsenic-bearing standards whereas the right panel shows scorodite digested through the SHIME.

potentially caused the relatively low As bioaccessibility observed in the stomach. The addition of HCO_3^- from the small intestine raises pH and also competes with As^V for sorption sites, thereby explaining the increased As bioaccessibility in the SHIME small intestine. The displacement of As^V with bicarbonate (Stachowicz 2007) on ferrihydrite may have caused, in part, the inverse relationship between As concentration and small intestinal As bioaccessibility since

displacement would be higher (via simple mass action) at low As surface loadings relative to high As surface loadings.

5.4 Discussion

Microbial activity increased scorodite As bioaccessibility up to two-fold relative to sterile controls; however, the impact of microbial activity depended upon the As concentration of the sample (**Figure 5.1**). At the lowest concentration ($5x10^3$ mg kg^{-1}), As bioaccessibility from scorodite in the active and sterile colon of the SHIME were equivalent (Fisher Protected LSD, $P>0.05$). Similarly, at very high As concentrations (> $1x10^5$ mg kg^{-1}) there was no difference (Fisher Protected LSD, $P>0.05$) in As bioaccessibility from scorodite with or without the activity of GI microorganisms. In contrast, when scorodite As concentrations were between $5x10^3$ mg kg^{-1} and $1x10^5$ mg kg^{-1} the bioaccessibility of As increased (Fisher Protected LSD, $P<0.05$) between 56 and 110% relative to sterile controls. These results are in contrast to those observed from natural scorodite from gold mine tailings, containing between 380 and $1x10^5$ mg kg^{-1} As, where GI microbes increased As bioaccessibility between 140 and 300% (Laird 2007). The diminished effect of GI microbes on synthesized scorodite As bioaccessibility at sample As concentrations less than $5x10^3$ mg kg^{-1} was likely due to the high small intestinal bioaccessibility observed from the freshly synthesized scorodite in comparison to more crystalline mine tailings. In contrast, GI microorganisms negligible affect on As bioaccessibility from freshly synthesized scorodite with high As concentrations (> $1x10^5$ mg kg^{-1}) may be due to As-induced toxicity to the GI microorganisms.

Bioaccessible arsenite was observed in both the active and sterile colon following the digestion of scorodite containing between $2x10^4$ and $3x10^5$ mg kg^{-1} As through the SHIME despite all As from scorodite originating as As^V (**Figure 5.5**). Therefore, the reducing conditions

of the GI tract, even without the activity of GI microorganisms, may reduce As^{V} to As^{III}. As^{V} reduction to As^{III} tended to be higher in the presence of GI microorganisms at As concentrations less than $3x10^{5}$ mg kg^{-1} in the sample; however, the difference was not significant ($F_{1,6}$=0.46; P=0.52). Furthermore, the main peak energy of the unaltered scorodite, scorodite digested through the active colon, and scorodite digested through the sterile colon corresponds to As^{V} being dominant in all samples (**Figure 5.4**). Consequentially, As^{III} was not measurably precipitated in Fe(II) or Fe(III) minerals in the colon SHIME. Notably, the transmission XANES spectra of scorodite digested through the active and sterile SHIME colon are similar to that of As^{V} adsorbed on ferrihydrite. This reflects the dissolution of scorodite and subsequent adsorption of As^{V} on the sparingly soluble ferrihydrite. Therefore, although As^{V} may be reduced to As^{III} in the SHIME, the reduction is neither associated with microbial activity nor related to the increased As bioaccessibility observed in the SHIME colon.

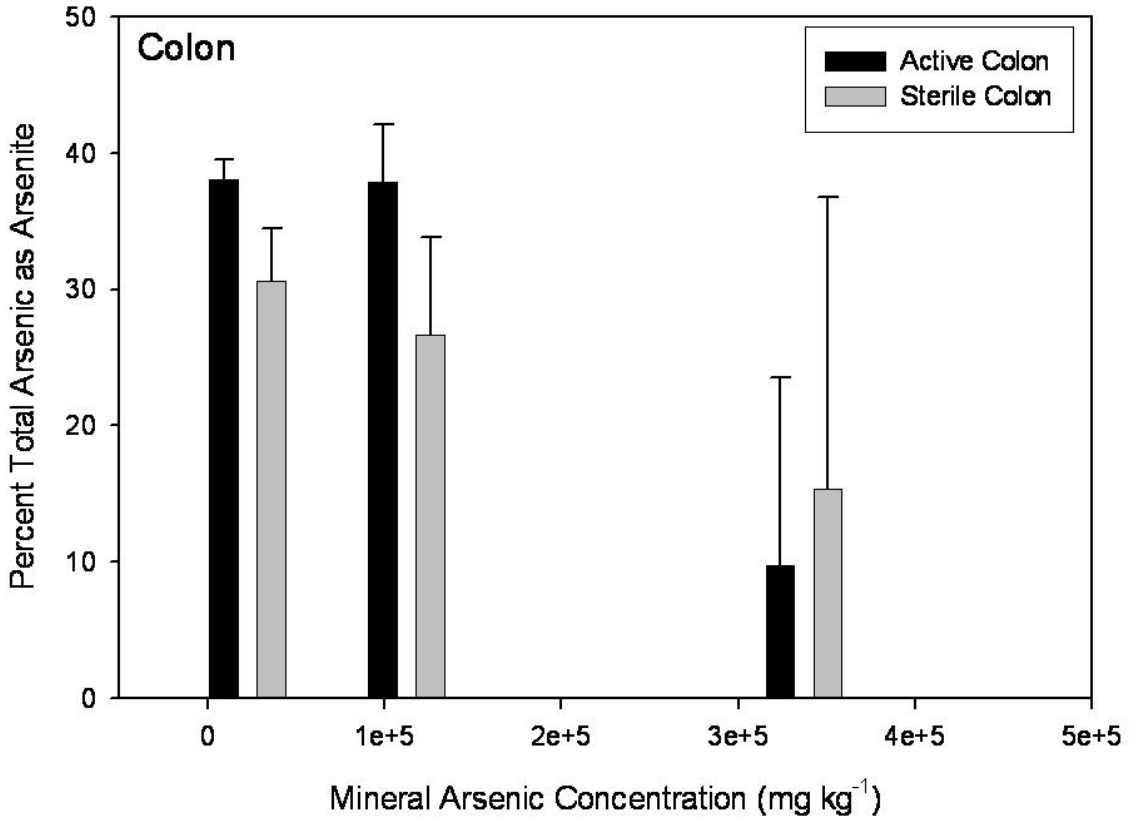

Figure 5.5 Gastrointestinal microbes did not increase the percent of bioaccessible As as As^{III} (± standard deviation) for scorodite digested in the Simulator of the Human Intestinal Microbial Ecosystem (F=0.46; P=0.52).

This research demonstrated that inverse relationships between concentration and bioaccessibility are possible both for particles with surficially adsorbed As (ferrihydrite) and particles with As incorporated into its structure (scorodite). The isotherms for As dissolution from scorodite indicated that only evaluating the role of LSR without considering residence time may cause the systematic underestimation of As bioaccessibility when *in vitro* As dissolution is limited by reaction kinetics. Therefore, we recommend researchers evaluate the effect of residence time and LSR on As bioaccessibility as a regular part of *in vitro* bioaccessibility QA-QC. Since As reducing bacteria were not responsible for the increases in As bioaccessibility, dissimilatory iron reduction is the most likely alternative mechanism explaining increased As

bioaccessibility in the colon. Future research will be conducted to confirm that iron reducing bacteria are responsible for increased As bioaccessibility observed in the SHIME.

6 INCREASED ARSENIC BIOACCESSIBILITY DUE TO MICROBIAL ACTIVITY DOES NOT AFFECT ARSENIC BIOAVAILABILITY

6.1 Introduction

Elevated soil arsenic (As) concentrations due to industrial and mining operations can present human health risk to affected communities via the accidental ingestion of contaminated soil of individuals, especially children. Oral As exposure due to the accidental ingestion of contaminated soils is calculated according to the soil As concentration (C_s), the soil ingestion rate (IR_s), and As relative bioavailability (RBA) (Casteel 2001; Health Canada 2004a; Ruby 1999). As RBA is defined as the fraction of ingested As from contaminated soil that reaches systemic circulation divided by the fraction of ingested As from sodium arsenate that reaches systemic circulation. Preliminary Quantitative Risk Assessments typically require the conservative and protective assumption that the RBA of ingested As is 100% (Health Canada 2004a). However, in more detailed risk assessments (e.g., Site Specific Risk Assessments or SSRA), risk assessors may account for limitations on GI absorption when calculating oral As exposure (US EPA Region 8 2005). As RBA is measured according to *in vivo* dosing trials to derive the As Urinary Excretion Fraction (UEF), which represents the fraction of ingested As excreted into the urine of exposed animals (Casteel 2001; Rodriguez 1999). The As RBA is then calculated by dividing the UEF of animals exposed to As from soil by the UEF of animals exposed to sodium arsenate. The ratio of the UEF_{soil}:$UEF_{arsenate}$ is equivalent to the As RBA since the extent of tissue binding, biliary excretion, and excretion in hair is assumed equivalent between the soil and sodium arsenate treatment groups.

The bioaccessibility of As, defined as the fraction of ingested As solubilized into gastrointestinal (GI) fluids, is a conservative surrogate and *in vitro* alternative to bioavailability trials that rely upon animal testing. *In vitro* GI models (e.g., Physiologically Based Extraction

Test or PBET; Simple Bioaccessibility Extraction Test or SBET; Ohio State University *In Vitro* Gastrointestinal or OSU-IVG method; and Simulator of the Human Intestinal Microbial Ecosystem or SHIME, etc) are often used for measuring As bioaccessibility by simulating the physical, chemical, and enzymatic conditions of the human GI tract (Basta 2007; Juhasz 2007; Ruby 1996; Van de Wiele 2007). Of the *in vitro* methods listed above, only the SHIME incorporates the function of aerobic and anaerobic microorganisms present in the distal GI tract (De Boever 2000). This could be of particularly importance for estimating As bioaccessibility since GI microorganisms may affect ingested As speciation (Kuroda 2001), thereby altering As toxicokinetics (Cohen 2006). In addition, *in vitro* GI microbial activity under simulated colon conditions often increases As bioaccessibility from soils, as demonstrated for: mine tailings, a contaminated agricultural soil, an ironstone soil containing elevated arsenic concentrations, synthesized amorphous scorodite (FeAsS), and synthesized 2-line ferrihydrite with surficially adsorbed arsenate (Laird 2010; Laird 2007; Laird 2009). Consequentially, *in vitro* GI models that neglect to incorporate microbial activity may underestimate As bioaccessibility for human health risk assessment.

Although *in vitro* microbes commonly increase As bioaccessibility under simulated colon conditions; incorporating microbial activity into *in vitro* GI models has not been widely adopted since the relevance of colon As bioaccessibility is unknown (Laird 2007). Specifically, there is little evidence as to whether As that is mobilized by microbial activity is, thereafter, absorbed into systemic circulation. Recent publications describing the role of GI microbial activity on As bioaccessibility from soils have utilized microbial communities and physiological conditions representative of those found in the human large intestine (Laird 2010; Laird 2007; Laird 2009). However, it is the small intestine that is thought to be the dominant site of GI As absorption due

to its large surface area and high efficiency of phosphate absorption (Kayne 1993), which is a surrogate for As absorption (Rosen 2002; Wu 2007). However, even without colonic As uptake, GI microbial activity may contribute to the bioavailability of ingested soil-borne As since the distal small intestine (e.g., ileum), like the large intestine, possesses a large and diverse community of aerobic and anaerobic bacteria under reducing conditions (Booijink 2007; Quintanilha 2007). Furthermore, the sizeable surface area and expression of the phosphate transporter NaP_iIIb in the ileum (Sabbagh 2009) would likely make microbially-mediated increases in As bioaccessibility under simulated ileal conditions to be of utmost toxicological relevance.

The primary objective of the research presented in this manuscript was to evaluate whether or not it is necessary for measures of As bioaccessibility to account for GI microbial activity. To do this, we conducted *in vitro* and *in vivo* experiments to evaluate the toxicological relevance of GI microbes to As bioaccessibility and bioavailability. For the *in vitro* experiment, we compared As bioaccessibility with and without an ileal microbial community to assess the role of microbes in a GI stage known to contribute to bioavailable As. For the *in vivo* experiment, we compared the urinary As excretion of juvenile swine (with and without oral antibiotic treatment) orally exposed to soil-borne As. We hypothesized that both *in vitro* As bioaccessibility and *in vivo* As bioavailability are affected by GI microbial activity, indicating that *in vitro* models should include GI microbes to ensure SSRAs are sufficiently protective of human health.

6.2 Materials and Methods

6.2.1 Soils

For the *in vitro* Ileum SHIME experiment, twenty soils were collected from urban residential neighborhoods in Hoboken, Belgium that were historically contaminated from industrial operations. Three of the Hoboken soils (H5, H18, and H50) containing elevated As

concentrations (i.e., greater than the As CCME Soil Quality Guideline (SQG) of 12 mg kg^{-1}) (CCME 2007) were selected for the research described in this manuscript. The soils were dry-sieved (<100 µm) to better reflect the size fraction most likely to adhere and then be accidentally ingested through hand-to-mouth behaviour (Bright 2006; Siciliano 2009). Additionally, a standard reference material (BGS 102) was obtained from the British Geological Survey. For the *in vivo* juvenile swine dosing trial, overburden from an abandoned gold and silver mine in Mount Nansen, YK was collected and sieved to <250 µm. The overburden (TM1) was collected as part of a comprehensive human and environmental risk assessment conducted for the site. Arsenic concentrations in each of the soils are listed in **Table 6.1**.

Table 6.1 Arsenic concentration in the bulk and <100 µm fractions of three Hoboken Soils (H5, H18, and H50), one standard reference soil (BGS 102), and one mine overburden (TM1). *YK1, used for the in vivo dosing trial, was sieved to <250 µm.

	H5	H18	H50	BGS 102	TM1
Bulk Soil	33	27	48	101	3940*
100 µm Sieved	35	35	115		

6.2.2 Dynamic Simulator of the Human Intestinal Microbial Ecosystem

As described in **Section 3.2.2**, the microbial community from the human GI tract was cultured using the SHIME according to previously published procedures (De Boever 2000). In brief, the SHIME microbial community was established by mixing human fecal matter, which was freshly voided by an adult male volunteer, in a phosphate/thioglycolate buffer. The fecal mixture was subsequently centrifuged and the supernatant (40 mL) was added to a carbohydrate-based nutrition solution (600 mL) in the third compartment of a series of double-jacketed reactor flasks. The carbohydrate nutrition contained per liter of distilled water: 5.01 g $KHCO_3$ (MP Biomedicals), 2.92 g NaCl (EMD), 0.5 g arabinogalacatan (Fluka), 1.0 g pectin (ICN Biomedicals), 0.5 g xylan (Sigma), 2.1 g potato starch (EMD), 0.2 g glucose (EMD), 1.5 g yeast

extract (BD Bacto™), 0.5 g peptone (BD Bacto™), 2.0 g mucin (Sigma), and 0.25 g L-cysteine hydrochloride (EMD). The SHIME microbial community was fed by pumping 100 mL of pH 2 nutrition into the first compartment (i.e., stomach stage) of the double-jacketed reactor flask. After two hours in the simulated stomach stage, the solution was pumped into the second compartment (i.e., duodenum stage). Therein, the carbohydrate solution was mixed with a pancreatic enzyme solution (50 mL) containing per liter: 12.5 g $NaHCO_3$ (EMD), 6.0 g Oxgall (BD Difco™) (dehydrated bile salts), and 0.9 g pancreatin (Sigma). Following an additional 2 hours in the simulated duodenum stage, the mixture was pumped into the third compartment (i.e., colon stage) containing the human fecal microbial community. This feeding schedule was repeated every 8 hours.

The pH of the simulated colon compartment was maintained between 5.9 and 6.3 using a pH controller (Consort R305). The temperature of the SHIME was set to 37°C using a circulating heated water bath and the anaerobic status (-196 ± 9 mV) of the system was maintained via daily headspace purges with N_2 gas. The colon microbial community contained 8.7 log CFU mL^{-1} total aerobes and 8.8 log CFU mL^{-1} total anaerobes and included Lactobacilli, Fecal Coliforms, Clostridia, Staphylococci, Enterococci, Bifidobacteria, Klebsiella, and Veillonella.

6.2.3 Static Simulator of the Human Intestinal Microbial Ecosystem

The bioaccessibility of As from the three Hoboken soils and BGS 102 was measured in the Stomach SHIME, Duodenum SHIME, Ileum SHIME, and Colon SHIME using a batch design as described in **Section 3.2.3**. Previously published SHIME procedures (Laird 2010; Van de Wiele 2007) were modified to include a novel microbially-active stage representative of conditions present in the distal human small intestine (i.e. ileum). In brief, 0.3 g of each soil was added to acid-washed 150-mL glass bottles for the 4 stages of the SHIME. For the Stomach SHIME, 30 mL of pH 1.4 HCl was added to each bottle and the pH of the soil slurry was then adjusted to 1.5

± 0.1 via the dropwise addition of 0.5 M HCl (EMD)/NaOH (EMD). The bottles were then capped, flushed with N_2 (30 min, 1 bar), sealed, and then incubated at 37°C with horizontal shaking (130 rpm). After two hours of incubation in the Stomach SHIME, 15 mL of pancreatic enzyme solution was added to each bottle and the pH was adjusted to 6.5 ± 0.2. The bottles were subsequently flushed with N_2 and incubated with shaking for an additional 1, 2, or 3 hours for the Duodenum SHIME. Thereafter, 15 mL of ileum suspension (8.6 g L^{-1} K_2HPO_4 (EMD), 6.8 g L^{-1} KH_2PO_4 (EMD), 3 g L^{-1} Oxgall (BD Difco™), 1 g L^{-1} mucin (Sigma), 0.5 g L^{-1} L-cysteine HCl (EMD), and 0.15 mL dynamic SHIME colon suspension) was added to each bottle. The pH of the solution was adjusted to 6.8 ± 0.2 and the bottles were flushed with N2 and incubated for an additional 5, 4, or 3 hours for the Ileum SHIME. Three small intestinal residence time treatments were performed (e.g., 3 hr Duodenum SHIME + 3 hr Ileum SHIME, 2 hr Duodenum SHIME + 4 hr Ileum SHIME, or 1 hr Duodenum SHIME + 5 hr Ileum SHIME) to determine whether increasing microbial activity duration increases As bioaccessibility. After the completion of the Ileum SHIME, 30 mL of colon suspension from the dynamic SHIME was added to each bottle and the pH of the solution was adjusted to 6.3±0.2. The bottles were once again, capped, flushed with N_2, and incubated for a final 18 hours.

After each of the 4 stages of the SHIME (Stomach, Duodenum, Ileum, and Colon), the bioaccessible arsenic fraction was separated with syringe microfiltration (0.45 μm, Whatman, GMF) and a 10 mL aliquot of the bioaccessible fraction was acidified via 0.5 mL of 12 M HCl (EMD, OmniTrace™). The acidified samples were frozen until measured for aqueous total As concentrations. The role of microbial activity on As bioaccessibility was evaluated by repeating Ileum SHIME treatments using aliquots of SHIME suspension that were previously autoclaved.

Each treatment was conducted in triplicate for the three Hoboken soils whereas the treatments were unreplicated for BGS 102.

6.2.4 Animals, housing, and feed

The *in vivo* As dosing procedures were based upon methods developed by Dr Stan W Casteel at the University of Missouri (Basta 2007; Casteel 2001; US EPA Region 8 2005). Eleven healthy male swine (8 week-old; initial body weight ~24 kg) were obtained from the Prairie Swine Center, Inc (PSCI). The animals were housed in individual metabolic cages (1.5 x 1.5 x 0.9 m) at the PSCI equipped with screened collection pans to facilitate the separation and quantitative collection of urine. The pens were maintained in the thermo-neutral zone of the pigs with the lights on a 14:10 hr (light:dark) cycle. The swine were previously weaned onto an antibiotic-free stage 3 starter ration pig chow provided by the PSCI and municipal drinking water was provided to each animal *ad libitum*. During the three-week experiment, each animal was fed two equal portions of feed daily in the morning and evening. Their daily feed intake was restricted to 4% body weight to encourage them to completely consume their molasses-ball dosing vehicle.

6.2.5 In vivo arsenic exposures

The health of the animals was monitored for six days prior to the onset of arsenic exposure to ensure each animal successfully adjusted to their enclosures. After the six day monitoring period, the animals were randomly assigned to one of three treatment groups: arsenic (via TM1) plus antibiotics (AB2-TM1; n=4), arsenic (via TM1) without antibiotics (AB0-TM1 n=4), and a negative control group (AB0-CON; n=3). The negative control group animals were administered neither antibiotic nor arsenic. The molasses treats used to administer the antibiotics/As were made by thoroughly mixing equal portions of pig chow, rolled oats, and molasses into a thick malleable dough. These molasses treats were used as a dosing vehicle rather than the more

commonly used dough-ball (Basta 2007) since molasses was better able to mask the bitter flavor of metronidazole.

The antibiotic treatment procedure used to modify the GI microbial communities of the juvenile swine was designed in consultation with Dr. Chris Clark (DVM, University of Saskatchewan). Neomycin (Soluble Neomix Powder) was selected because it is commonly given orally to pigs to treat enteric infections and is broadly effective against gram-negative bacteria and some gram-positive bacteria. Furthermore, neomycin was previously used to successfully show the importance of GI microorganisms for Hg toxicokinetics (Rowland 1980). Metronidazole (Apotex Inc, DIN 00545066) was also included in the antibiotic treatment regimen due to its high efficacy against anaerobic intestinal microflora (Lofmark 2010). Since this same group of microbes (i.e., faculative anaerobes, namely DIRPs) was thought to be responsible for the mobilization of metals from contaminated sediments (Oremland 2005), metronidazole was deemed likely to cause microbial community shifts relevant to As toxicokinetics. It must be remembered, however, that metronidazole is not approved for treatment of swine since it is not food-safe. Therefore, special approval was obtained to treat the swine with metronidazole on the condition that all animals would be destroyed at the end of the study and not enter the food supply. The dosages of the two antibiotics were initially set at 30% above the therapeutic dose for neomycin (30 mg kg^{-1} d^{-1}) (Drugs.com 2010) and metronidazole (53 mg kg^{-1} d^{-1}) (Clark 2010). After 7 days of treatment, the antibiotic dosages were increased to twice the therapeutic doses for neomycin (45 mg kg^{-1} d^{-1}) and metronidazole (80 mg kg^{-1} d^{-1}) to maximize the effects upon the GI microbial communities of the juvenile swine.

For animals in the AB2-TM1 treatment group, neomycin and metronidazole was hand-mixed into the molasses treat mixture described above. Similarly, TM1 was added to the molasses treat

mixture for animals in the AB2-TM1 and AB0-TM1 treatment groups. AB2-TM1 and AB0-TM1 animals were orally exposed to 160 μg As kg BW^{-1} day^{-1} from the Yukon overburden mixed into the molasses-balls. The molasses treat mixture containing antibiotic and/or As doses were divided into two equal portions, the first of which was administered (by hand) prior to morning feeding whereas the second was administered prior to evening feeding. This dosing procedure was continued for 14 days to facilitate urinary As excretion to reach steady state.

During the 14-day exposure period, swine urine was collected over three 48-hr intervals (D7-8, D10-11, and D13-14) into large polypropylene containers. The volume of excreted urine for each animal was measured for each of the three urine collection periods. Subsequently, two 50-mL aliquots of urine were transferred into disposable polypropylene centrifuge tubes and were acidified (2% HCl) and frozen until microwave digestion and As quantification. After the 14-day exposure period, the animals were euthanized via captive-bolt and exsanguination. Subsequently, the animals were dissected and a 10-cm segment of the proximal colon was isolated and excised. Three 40-mL subsamples of intestinal contents were collected and transferred into disposable polypropylene centrifuge tubes. The tubes were immediately flash-frozen in liquid nitrogen and stored at -80°C until subsequent denaturing gradient gel electrophoresis (DGGE) analysis.

6.2.6 Arsenic quantification

The acidified SHIME fluids for BGS 102 and the three Hoboken soils were microwave digested prior to As quantification via Hydride Generation Atomic Fluorescence Spectroscopy (HGAFS). The microwave digestion procedure required 2 mL of the acidified SHIME samples to be added to 10 mL of 12 M OmniTrace HCl (EMD) in Teflon-coated digestion tubes (Xpress, CEM). H2O2 (EMD, 30%, 1 mL) was added to each of the digestion tubes to initiate the oxidation of precipitated organic matter. After allowing 15 min for the reaction to subside, another 1 mL aliquot of H_2O_2 was added to each tube. The tubes were capped, sealed, and then

digested at 100% power (1600 Watts, 100°C, $Time_{ramp}$ = 10 min, $Time_{hold}$ = 40 min) in a MARS-5 microwave (CEM). The microwave digested SHIME solutions were diluted and transferred to disposable polypropylene centrifuge tubes for analysis via HGAFS. Bioaccessible As from the three Hoboken soils and BGS 102 were measured via HGAFS using a previously published procedure (Rahman 2000). In brief, inorganic As in the microwave digested SHIME samples was reduced to arsenite (AsIII) via the addition of a KI solution (50% w/v, EMD) containing 10% ascorbic acid (JT Baker). Thereafter, the aqueous As concentration in each of the microwave digested SHIME samples was measured using a Millenium Excalibur (PS Analytical). Percent As bioaccessibility was calculated according to the equation:

$$Bioaccessibility\ (\%) = \frac{Arsenic\ Released\ in\ Solution\ \ (\mu g)}{Arsenic\ Present\ in\ Soil\ Sample\ (\mu g)} \times 100\% \qquad \text{(Eqn 6.1)}$$

The acidified urine of the juvenile swine collected during the *in vivo* As dosing trial was microwave digested prior to quantification via Inductively Coupled Plasma Mass Spectrometry (ICPMS). Pig urine (2 mL) was added to 3 mL of HNO_3 (16 M, EMD OmniTrace) and 0.5 mL of H_2O_2 (EMD) in Teflon-coated digestion tubes (Xpress, CEM). The tubes were capped, sealed, and transferred into MARS-5 microwave (CEM) and digested using a 2-stage procedure at 100% power (1600 Watts, $Time_{Ramp\text{-}1\&2}$=15 min, $Time_{Hold\text{-}1\&2}$=30 min, $Temp_1$=180°C, $Temp_2$=160°C). The digested samples were transferred to 15-mL polypropylene centrifuge tubes and diluted to 2% HNO_3 (EMD) for ICPMS analysis. Swine, like humans and other mammals, convert ingested inorganic arsenic into methylated arsenic metabolites (Cohen 2006). Since such methylarsenicals do not form arsenic hydrides when mixed with sodium borohydride, the use of HGAFS (as described in Chapter 4 and 5) does not provide adequate recovery of arsenic in pig urine (ie. a large fraction of the excreted As is not detected). Therefore, urinary As concentrations were

measured using ICPMS to ensure complete recovery of methylated As metabolites present in the swine urine. Urinary As excretion was calculated according to the equation:

$$Excretion\,(\mu g/48\ hr) = Urine_{volume}\left(mL/48\ hr\right) \times Arsenic\ Concentration_{urine}\left(\mu g/mL\right) \quad \text{(Eqn 6.2)}$$

6.2.7 Statistical analysis

Normality (Kolmogorov-Smirnov, $p>0.05$) and homogeneity of variance (Levene Median, $p>0.05$) assumptions for As bioaccessibility and urinary As excretion were confirmed using SigmaStat v3.1. Two-way Analysis of Variance (ANOVA) was used to evaluate the effects of microbial activity and ileum residence time on the bioaccessibility of As from each of the three Hoboken soils (SigmaStat v3.1). Pairwise multiple comparisons were performed using the Holm-Sidak method (overall significance level = 0.05). Similarly, a two-way ANOVA was used to evaluate the role of antibiotic co-administration and urine collection period on the urinary As excretion of swine orally exposed to TM1.

6.2.8 Quality Assurance – Quality Control

The standard reference material BGS 102 was digested in the SHIME as a positive control for each treatment batch of Hoboken soils to check the consistency of As bioaccessibility results with those of previous studies. Each SHIME treatment batch also included a blank (i.e., no soil added) and sodium arsenate spike. The methodological detection limit (MDL) of As in the SHIME fluid was 181 ng L^{-1}. Average spike recovery of sodium arsenate was 81%. HGAFS instrumental precision was monitored by re-measuring one for every 15 samples. Mean deviation of duplicates was 3.0%. The bioaccessibility of As from BGS 102 and the three Hoboken soils was corrected according to the As concentrations measured in the procedural blanks. The spike recovery of MMA and DMA in swine urine measured via ICPMS was 118 and 121%, respectively. The MDL of arsenic in swine urine collected at the PSCI was 292 ng L^{-1}.

6.3 Results

Percent As bioaccessibility of the three Hoboken soils ranged between 17 - 66% in the Stomach SHIME, 14 - 51% in the Duodenum SHIME, 21 - 68% in the Ileum SHIME, and 26 – 77% in the Colon SHIME (**Table 6.2**). In contrast, the As bioaccessibility of BGS 102 was considerably lower in the Stomach SHIME (2.0 %), Duodenum SHIME (1.0 %) and Ileum SHIME (3.1 %). However, the As bioaccessibility of BGS 102 in the Colon SHIME increased to values similar as those observed for the Hoboken soils. Notably, the Hoboken soil with the greatest As bioaccessibility (H50) also had the greatest As enrichment (i.e. ($sieved_{As}$-$bulk_{As}$)/$bulk_{As}$) and As concentration.

Table 6.2 Mean (± *standard error*) percent bioaccessibility of three Hoboken soils (H5, H18, H50; n=3) and one standard reference material (BGS 102; n=1* or 3) digested through successive stages of the Simulator of the Human Intestinal Microbial Ecosystem.

GI Stage	H5	H18	H50	BGS 102
Stomach SHIME	24.6 (*0.6*)	16.5 (*0.8*)	66.4 (*4.8*)	2.0*
Duodenum SHIME	15.7 (*1.6*)	13.8 (*3.2*)	36.8 (*5.5*)	1.0*
Ileum SHIME	23.5 (*0.5*)	21.0 (*0.4*)	67.6 (*1.5*)	3.1*
Colon SHIME	26.4 (*1.0*)	28.5 (*1.1*)	77.3 (*3.8*)	29.6 (*3.4*)

When the Ileum SHIME residence time was 4 hours, microbial activity increased the release of As from H5 (Holm-Sidak, $p<0.001$), H18 (Holm-Sidak, $p=0.001$) and H50 (Holm-Sidak, $p=0.004$), thereby increasing percent As bioaccessibility (**Figure 6.1**). This microbial activity was associated with the formation of black precipitate, as previously observed in the Colon SHIME (Laird 2007), despite the Ileum SHIME's considerably shorter residence time and less negative redox status (**Table 6.3**). The size of the microbial effect upon As bioaccessibility after the 4 hour incubation was smaller than typically observed in the Colon SHIME, ranging between 5 and 11%. These microbially-mediated increases in Ileum SHIME As bioaccessibility were not

consistently observed when residence times were reduced to three hours or extended to five hours (**Figure 6.1)**. This suggests that any microbial effects upon arsenic bioaccessibility under small intestinal conditions are transient. These results also indicate that BGS 102 is a better test material with which to evaluate microbial effects on As bioavailability. However, this soil has a relatively low As concentration thus impeding its use in an *in vivo* swine dosing trial. Therefore, the *in vivo* work described herein used the overburden test material from Mount Nansen, YK since this test material contains nearly 20-fold greater As concentrations and also shows a microbial effect (2.9-fold increase) on As bioaccessibility under simulated colon conditions.

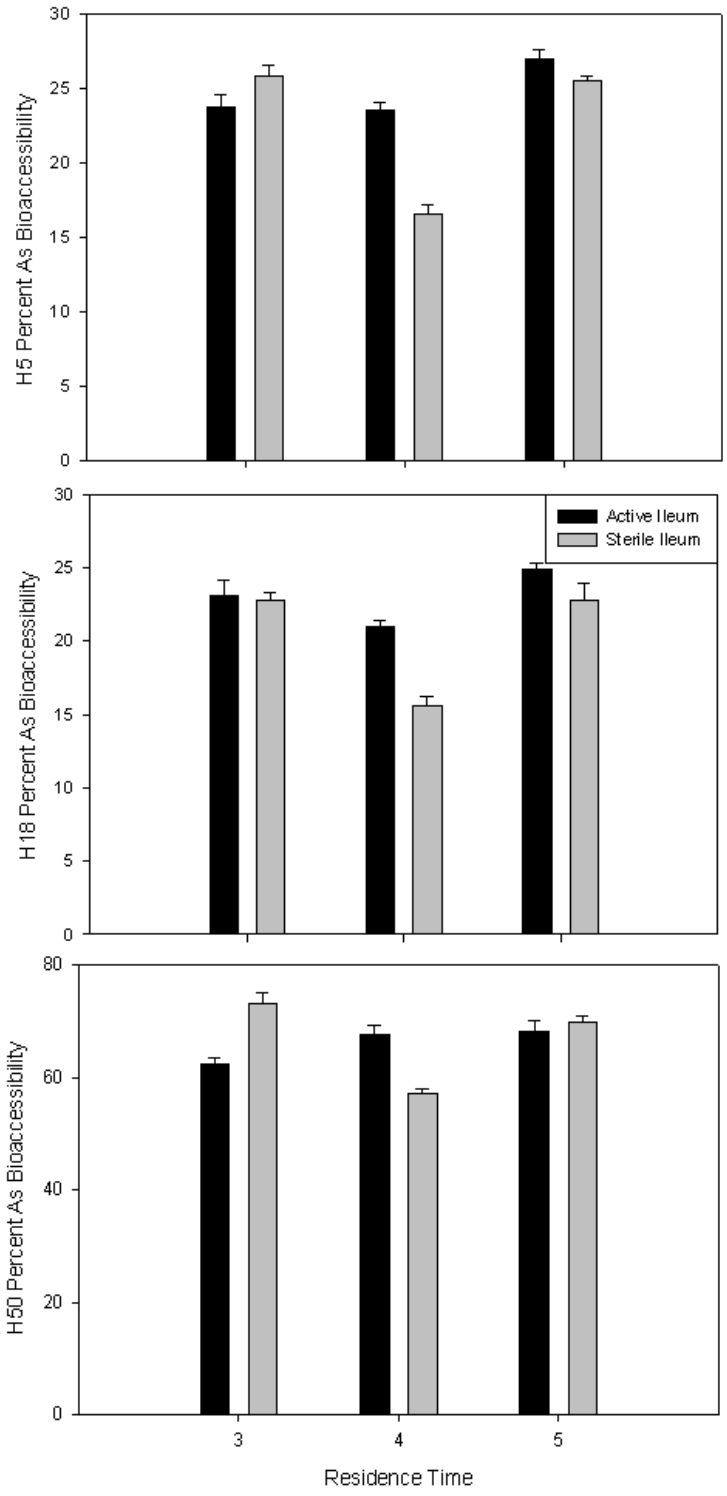

Figure 6.1 Mean percent As bioaccessibility (± standard error) from three Hoboken soils (H5, H18, H50; n=3) following sample extraction in the Ileum stage of the Simulator of the Human Intestinal Microbial Ecosystem with (i.e., Active) and without (i.e., Sterile) microbial activity. When incubation time was 4 hours, ileal microbial activity increased (Holm-Sidak Test) As bioaccessibility for Hoboken soils H5 ($P<0.001$), H18 ($P=0.001$), and H50 ($P=0.004$).

Table 6.3 The residence time and redox status of the Ileum and Colon stages of the Simulator of the Human Intestinal Microbial Ecosystem digests of the standard reference soil BGS 102. Values in parentheses represent the standard error of the mean.

GI Stage	Redox (mV)	Residence Time (hr)
Ileum SHIME	-30.7 (3.2)	4
Colon SHIME	-305.0 (6.2)	18

The GI microbial community of 3 of the 4 juvenile swine (p15, p18, and p13) treated with oral antibiotics (AB2-TM1) clustered separately from the microbial communities of swine not treated with antibiotics (AB0-TM1) (**Figure 6.2)**. There was, however, considerable inter-individual variation in the DGGE banding-patterns of the GI microbial communities in the swine proximal colon. For example, there was no more than 50% overlap in the banding-patterns of the oral-antibiotic treated swine. Furthermore, the banding-patterns of the microbial communities of untreated swine diverged as much as 40%.

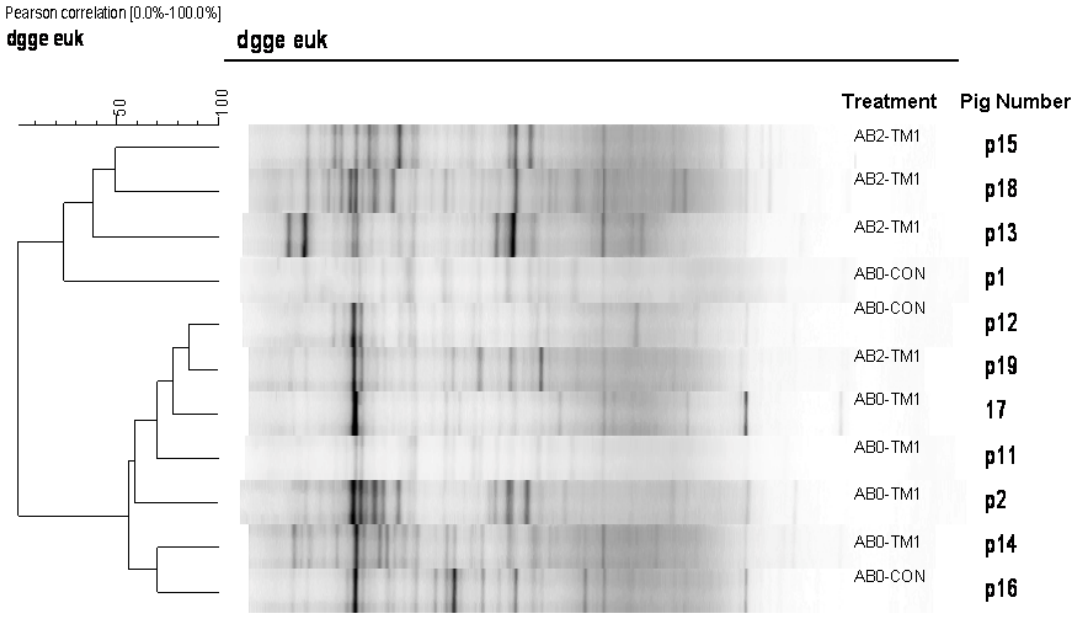

Figure 6.2 Pearson correlation cluster analysis of Denaturing Gradient Gel Electrophoresis results demonstrates that oral antibiotic treatment (neomycin and metronidazole) alters the normal microbial community of the juvenile swine proximal colon. The arsenic/antibiotic treatments of each lane is also noted: AB0-CON (neither antibiotic nor arsenic), AB2-TM1 (both arsenic and antibiotic), and AB0-TM1 (arsenic but not antibiotic).

The urinary As excretion for the first collection period was significantly lower than the second (p=0.00433) and third (p=0.0149) urine collection periods. This indicates that urinary As excretion had not reached steady state until the 10th day of As exposure (i.e., D10). The urinary As excretion of swine and the absolute bioavailability of arsenic in the test material were equivalent (Holm-Sidak, P=0.930) in AB2-TM1 (i.e., arsenic + antibiotics) and AB0-TM1 (i.e., arsenic - antibiotics) (**Table 6.4 & Table 6.5**). Furthermore, the urinary As excretion of the p19 (AB2-TM1), whose GI microbial community clustered with AB0-TM1 animals (Figure 6.2), was equivalent to the other animals in AB2-TM1. These observations support the conclusion that antibiotic-treatment induced changes in the GI microbial community did not affect the arsenic bioavailability for the Yukon mine overburden TM1. According to ethical guidelines calling for the reduction of animal use in toxicological research, the positive control (e.g., sodium arsenate) dosing group was dropped from the experimental design once it became apparent that antibiotic treatment did not alter urinary arsenic excretion. Including the positive control treatment groups, required for the calculation of RBA_{As}, would have doubled the number of animals euthanized while not augmenting our ability to test the primary hypothesis of the study (i.e., colon bioaccessibility is toxicologically relevant). Consequentially, the authors decided that proceeding with the positive control treatment groups was contrary to the three R's of animal research and, thus, inappropriate for this study. Therefore, the RBA_{As} of TM1 cannot be calculated at this time.

Table 6.4 The effect of antibiotic (neomycin and metronidazole) co-administration on the urinary As excretion (± standard error) of swine orally exposed to As from the overburden of an abandoned gold and silver mine.

Treatment Group	Description	Urinary Arsenic Excretion (µg/48 hr) Coll Period #1 D7-D8	Coll Period #2 D10-D11	Coll Period #3 D13-14
AB0-CON	-Antibiotics -Arsenic	325 (*16*)	368 (*18*)	417 (*11*)
AB0-TM1	-Antibiotics +Arsenic	1457 (*98*)	1732 (*28*)	1673 (*95*)
AB2-TM1	+Antibiotics +Arsenic	1282 (*225*)	1865 (*161*)	1743 (*71*)

Table 6.5 The effect of antibiotic (neomycin and metronidazole) co-administration on the arsenic absolute bioavailability (± standard error) of the overburden of an abandoned gold and silver mine following the oral exposure of swine.

Treatment Group	Description	Arsenic Absolute Bioavailability (%) Coll Period #1 D7-D8	Coll Period #2 D10-D11	Coll Period #3 D13-14
AB0-TM1	-Antibiotics +Arsenic	12.1 (0.9)	14.2 (0.3)	13.2 (1.0)
AB2-TM1	+Antibiotics +Arsenic	10.2 (2.1)	15.6 (1.7)	13.9 (0.8)

6.4 Discussion

The *in vitro* SHIME results described herein demonstrate that As bioaccessibility is typically substantially less than the 100% assumed during PQRA. This research finding is in agreement with numerous articles describing the calculation of soil As bioaccessibility for human health risk assessment (Carrizales 2006; Hamel 1998; Ljung 2007; Navarro 2006; Oomen 2002; Pouschat 2006; Rodriguez 1999). Arsenic enrichment in the <100 µm fraction of H5 (5%), H18 (29%) and H50 (140%) were considerably lower than those reported for the adhered fraction of sandy brownfield soils (Siciliano 2009) and the <90-µm fraction of a sandy soil collected near a Copper Chromium Arsenate (CCA)-treated utility pole (Girouard 2009). The effect of particle

size fraction on soil As concentration was inconsistent between soils, perhaps reflecting differences in inorganic mineral (e.g., carbonate) surfaces between soil samples. Percent As bioaccessibility is often negatively correlated with soil As concentration due to thermodynamic or kinetic constraints on dissolution (Laird 2010). In contrast, the percent As bioaccessibility of the <100 μm fraction of H50 was substantially greater than that of the other Hoboken soils even though it possessed the greatest As concentration. These observations emphasize the importance of measuring the total As concentration and percent bioaccessibility from several size fractions on a site-specific basis when characterizing human exposure to soil contaminants (Girouard 2009).

Microbially-mediated increases in As bioaccessibility are likely of greatest toxicological relevance in the human small intestine since this organ is thought to be responsible for the majority of As uptake. However, the magnitude of the microbial effect upon soil As bioaccessibility in the Ileum SHIME (5-11%) was substantially lower than previously reported in the Colon SHIME (2 to 3-fold). Furthermore, the transient nature of the Ileum SHIME microbial effect on As bioaccessibility suggests that small intestinal GI microbial activity plays a negligible role in As toxicokinetics.

In lieu of performing costly gnotobiotic research (Stuyven 2010), which requires experimental subjects to be born via cesarean section and subsequently housed in sterile environments, *in vivo* antibiotic and prebiotic co-administration studies have successfully been used to illustrate the role of GI microbial activity on the toxicokinetics of ingested contaminants (Rowland 1980; Rowland 1978). In one of these studies, the GI microbial community was eliminated within three days of oral antibiotic treatment (Rowland 1980). In contrast, the oral antibiotic treatment (at twice the therapeutic dosage of two antibiotics) described in this manuscript altered the

composition of the GI microbial community but certainly did not eliminate it. Despite this, it is reasonable to assume that the structural changes observed in the GI microbial community (**Figure 6.1**) were relevant to As toxicokinetics since metronidazole targets anaerobic bacteria likely responsible for alterations in As bioaccessibility (Lofmark 2010).

Since the majority of bioavailable As is rapidly metabolized and excreted in the urine, urinary As excretion is directly proportional to RBA_{As} (Casteel 2001). Consequentially, assuming that antibiotic treatment did not affect the metabolism, distribution, or excretion of As, differences between the urinary As excretion in treatment groups AB2-TM1 and AB0-TM1 would indicate that GI microbes affect RBA_{As}. However, the urinary As excretion of swine orally exposed to TM1 was identical with and without antibiotic co-administration (**Table 6.4**). Since *in vitro* results showed GI microbial activity to cause a 2.9-fold increase in the As bioaccessibility of TM1, the *in vivo* results described herein suggest that microbes do not affect As RBA even when they alter bioaccessibility. Of course, these results may prove to be specific to TM1 since only one soil was included in the experimental design. It should be noted that urinary As excretion underestimates As absorption since it does not account for As that is excreted in feces via the bile or deposited in other tissues (e.g., skin, hair, liver, kidney). Therefore, the interpretation of the results are somewhat impeded by the study design which prevented the calculation of the RBA_{As} of TM1. In addition, the experimental design of the *in vivo* swine study did not directly evaluate whether GI microorganisms altered the speciation of ingested As (e.g., via the formation of the highly toxic AsH_3 gas in the colon).

This research provides the strongest evidence to date indicating that GI microbial activity is of limited relevance to the calculation of soil As bioaccessibility in human health risk assessment. This conclusion, is in agreement with inter-laboratory comparisons that demonstrated that the As

bioaccessibility of standard reference soils and the Yukon overburden as measured using PBET or SBET (without microbes) is equivalent to that of the SHIME (with microbes) (Laird 2009). This indicates that *in vitro* GI models that neglect to include a microbially active intestinal stage do not necessarily systemically underestimate the health risks posed by soil arsenic contamination. Therefore *in vitro* GI models that do not include microbial activity (e.g., PBET) may prove more efficient than the SHIME for the calculation of As bioaccessibility since such models are typically simpler and less expensive. These results, however, may prove specific to the small number of soils used in the *in vitro* and *in vivo* experiments described in this manuscript. In addition, it is not immediately apparent whether bioaccessibility estimates garnered from *in vitro* models that do not include microbial activity adequately reflect the health risks realized by the biotransformation of inorganic arsenic to more toxic forms (trivalent methylated species or AsH_3 gas). Therefore, further work will be necessary before it is possible to discount the importance of GI microorganisms for the calculation of As bioaccessibility and bioavailability.

7 PREDICTING METAL BIOACCESSIBILITY ACCORDING TO LABILITY VIA WATER EXCHANGE RATE CONSTANTS

7.1 Introduction

Risk assessors use gastrointestinal (GI) models for the calculation of *in vitro* bioaccessibility to estimate the percentage of an ingested contaminant likely to be solubilized into GI fluids (Ruby 1999). In contrast, the oral bioavailability of a contaminant, estimated via an animal dosing trial, refers to the percentage of an ingested contaminant that is absorbed across the GI epithelium and reaches systemic circulation (Basta 2007). Since dissolution into GI fluids is a requisite precursor for the intestinal absorption of metal contaminants, bioaccessibility is often a conservative predictor of oral bioavailability (Oomen 2003). Thus, incorporating bioaccessibility and bioavailability into exposure assessments enables risk assessors to account for limitations on the release and subsequent absorption of contaminants due to the accidental ingestion of contaminated particles (Roussel 2010).

The development of *in vitro* GI models for the calculation of metal bioaccessibility has focused upon single elements most likely to pose health risks when at elevated soil concentrations. Thus, *in vitro* bioaccessibility of contaminated soils was first investigated for lead (Ruby 1993)(Ruby 1993), then for arsenic (Hamel 1999; Ruby 1996), and more recently for several other metals (Lamb 2009; Ljung 2007; Rasmussen 2008; Roussel 2010). For each of lead and arsenic, a common strategy requiring years of research was employed before bioaccessibility measurements were widely adopted into risk assessment. First, investigators examined which GI model parameters (e.g., pH, incubation time, LS ratio, microbial activity) affected metal release into simulated gastric and/or intestinal fluids (Drexler 2007; Hamel 1999; Laird 2007). Subsequently, investigators selected physiologically relevant GI model parameters that

reproducibly maximized bioaccessibility of the metal and attempted to validate their model according to its correlation with *in vivo* bioavailability (Casteel 2001; Rodriguez 1999).

Multi-contaminant approaches to the evaluation of bioaccessibility may streamline the development of *in vitro* GI models by reducing the need for the repetition of each of these procedures for all metals regulated via Canadian Council of Ministers of the Environment Soil Quality Guidelines (CCME SQG) (CCME 2007). It may prove advantageous to group metals and metalloids according to their fundamental chemical properties (e.g., ionic charge, ionic or hydrated radius, electronegativity, absolute hardness). Thereafter, *in vitro* GI model parameters may be extended and applied to soils contaminated with metals and metalloids with similar chemical properties. Additionally, establishing the role of fundamental chemical properties in determining the release of metals from soils into GI fluids may assist in the development of a predictive model for *in vitro* bioaccessibility.

The primary objective of the research described in this manuscript was to evaluate the bioaccessibility of numerous metals from bulk soils collected in an urban Arctic brownfield in Iqaluit, NV. The Iqaluit soil samples were selected for this research since they not only possess elevated soil concentrations of several metal contaminants, but also their soil properties were previously established (Siciliano 2009). We examined differences in the bioaccessibility between metals and assessed the correlation between bioaccessibility and chemical properties to determine which chemical characteristics may prove useful in the prediction of metal bioaccessibility.

7.2 Materials and Methods

7.2.1 Soil collection

In July 2007 approximately 200 soil samples were collected into 250-mL glass amber jars from the surface (10 cm) and sub-surface (50-100 cm) of an urban brownfield located in a

residential neighborhood of Iqaluit, NV. Soil samples from the brownfield, in addition to soil blanks and soil spikes, were shipped via air cargo from Iqaluit to Saskatoon. Upon arrival in Saskatoon, the sample jars were transferred to a freezer where they were stored at -20°C until subsequent analysis. Metal, total petroleum hydrocarbons, and polycyclic aromatic hydrocarbon concentrations and bioaccessibility were measured from the bulk soil (<250 μm) to construct a contaminant map of the area. Nineteen of the 200 collected soil samples were selected for the research described in this manuscript. Metal enrichment in addition to soil properties of the bulk and adhered (i.e., particles that adhered to human hands) fractions of Iqaluit soils (**Table 7.1**) were previously described (Siciliano 2009).

Table 7.1 Select properties of soils collected from an urban Arctic brownfield in Iqaluit, Nunavut.

	>2mm Gravel (%)	<2 mm Total Organic Carbon (%)	Bulk Total Organic Carbon (%)	Adhered Total Organic Carbon (%)	Bulk Average Particle Size (μm)	Adhered Average Particle Size (μm)	Mass Per Hand-Washing (g)	Soil pH
Mean (*CV%*)	16.8 (*27.7*)	0.5 (*70.5*)	0.4 (*71.2*)	0.7 (*38.1*)	486 (*52.0*)	164 (*75.7*)	0.4 (*42.0*)	8.0 (*7.4*)
Min	3.7	0.2	0.2	0.4	33.0	26.1	0.2	6.6
Max	26.7	1.6	1.3	1.2	924	480	0.9	8.7

7.2.2 Measurement of soil metal concentration

Each of the 19 bulk soil samples were microwave digested before being syringe filtered (0.45 μm) and diluted to 2% HNO_3 to facilitate a multi-element scan of 24 elements using Inductively Coupled Plasma-Mass Spectrometry (ICP-MS). The total soil concentrations for beryllium (Be), aluminum (Al), titanium (Ti), vanadium (V), chromium (Cr), manganese (Mn), iron (Fe), cobalt (Co), nickel (Ni), copper (Cu), zinc (Zn), arsenic (As), selenium (Se), strontium (Sr), molybdenum (Mo), silver (Ag), cadmium (Cd), tin (Sn), antimony (Sb), barium (Ba), mercury

(Hg), thallium (Tl), lead (Pb), and uranium (U) from each of the 19 bulk soil samples were measured using ICP-MS (**Table 7.2**). The authors note that both As and Sb are more accurately described as metalloids; however, they have been labeled as metals for the remainder of the manuscript for the sake of brevity.

Table 7.2 Limit of Detection (LOD_{soil}) and total metal concentrations of soils collected from Iqaluit, NV.

Metal	Soil Concentration Range (mg kg^{-1})	LOD_{soil} (mg kg^{-1})
Be	0.9 – 1.4	0.3
Al	32000 – 63000	18
Ti	2900 – 6700	17
V	43 – 95	0.06
Cr	30 – 62	3.5
Mn	520 – 940	0.6
Fe	29000 – 58000	27
Co	12 – 19	0.05
Ni	9.1 – 16.0	0.6
Cu	6.4 – 27	0.6
Zn	64- 1000	3.1
As	1.0 – 2.2	0.8
Se	< LOD	0.03
Sr	160 – 280	0.23
Mo	0.8 – 5.6	0.05
Ag	< LOD – 1.2	0.10
Cd	1.3 – 8.5	0.01
Sn	0.7 – 11	0.03
Sb	0.02 – 0.37	0.01
Ba	890 – 1200	0.26
Hg	0.12 – 0.51	0.06
Tl	0.29 – 0.49	0.002
Pb	17 – 45	0.08
U	0.44 – 1.4	0.02

The microwave digestion required the addition of 100 mg of soil into Teflon-coated tubes (Xpress, CEM). Subsequently, 3 mL of HNO_3 (16 M, EMD, Omnitrace) and 0.5 mL of H_20_2 (EMD) were added to each tube. After the H_2O_2-mediated reaction subsided, 1.5 mL of 40% HF (EMD) was added to each tube. The tubes were then capped, sealed and placed in a microwave

(MARS-5, CEM) in which the samples were digested at 1600 W (100%) power at 200°C for 3 hours. This heating cycle was repeated until all of the soil particles in each of the tubes were completely digested. Boric acid (EMD, 5%, 12 mL) was then added to each of the tubes, which were then returned to the microwave for 30 minutes to deactivate the HF. The acid digests were subsequently syringe filtered and diluted to 2% HNO_3 for metal quantification.

7.2.3 Static Simulator of the Human Intestinal Microbial Ecosystem

As described in **Section 3.2.3**, metal bioaccessibility was measured for each of the 24 elements listed above using the stomach and duodenum stages of the Simulator of the Human Intestinal Microbial Ecosystem or SHIME (Laird 2010). In brief, 900 mg of each of the 19 Iqaluit soils was added to labeled glass serum bottles for both the stomach (1 bottle per soil sample) and duodenum (1 bottle per soil sample) stages of the SHIME. Dilute HCl (EMD, Omnitrace) in milli-Q H_2O (36 mL, pH 1.4) was added to the serum bottles and the pH of the soil suspension was then adjusted to pH 1.5±0.1 via dropwise addition of 0.5 M NaOH/HCl. The samples were then incubated at 37°C for 2 hours with horizontal shaking (70 rpm). Next, the bioaccessible fraction was separated from each Stomach SHIME sample whereas 18 mL of pancreatic solution (12.5 g L^{-1} sodium bicarbonate (EMD), 6 g L^{-1} Oxgall (BD Difco^{TM}), and 0.9 g L^{-1} pancreatin (Sigma)) was added to each Duodenum SHIME sample. Subsequently, the pH of the Duodenum SHIME samples was adjusted to 6.5±0.1. The Duodenum SHIME samples were then returned to the incubator/shaker for an additional 2 hours of shaking. After the Duodenum SHIME incubation, the bioaccessible fraction was separated for each of the samples. The separation of the bioaccessible fractions for the Stomach SHIME and Duodenum SHIME utilized syringe filtration (0.45 µm Whatman GMF). The bioaccessible (i.e., syringe filtered) SHIME fluid was then acidified (2% HNO_3) and the aqueous concentration of each of the 24 metals listed above was measured using ICP-MS. Percent bioaccessibility (Equation 7.1) of each metal was

calculated by expressing the micrograms of blank corrected bioaccessible metal as a percentage of the total amount of the metal contained with the 900 mg soil sample:

$$Bioaccessibility\,(\%) = \frac{Metal\ Released\ \ in\ Solution\ \ (\mu g)}{Metal\ Present\ \ in\ Soil\ Sample\ \ (\mu g)} \times 100\% \qquad \text{(Eqn 7.1)}$$

7.2.4 Quality Assurance - Quality Control

Percent bioaccessibility for each metal in each soil was only included for samples containing detectable concentrations of that metal in both the soil sample and the bioaccessible SHIME liquid fraction. Notably, soil and simulated GI fluid concentrations of selenium were below detection limits in each sample; therefore, selenium was discarded from further analysis. Two of the 19 Iqaluit soil samples were extracted in duplicate in the Stomach SHIME to evaluate the repeatability of the results. The median percent deviation of duplicates for metal bioaccessibility was 10.5%.

7.2.5 Statistical analysis

Median absolute deviation (MAD), which is defined as the median of the absolute deviations about the median of a dataset (Teng 2010), for metal bioaccessibility was calculated using the equation $MAD = median_i\ (|X_i - median_j(X_j)|)$. The 90th percentile of metal bioaccessibility was determined using the cumulative frequency distribution calculated according to the Parkhurst Equation ($P = (100 \times i/(n+1))$ where P is the probability of the i^{th} the rank in a distribution of n values (Parkhurst 1996). Correlations between metal bioaccessibility and fundamental chemical properties were evaluated using Spearman Rank Order (SigmaStat v3.1) and were defined as significant when $p<0.05$. Stomach SHIME and Duodenum SHIME isotherms (Laird 2010) were created for each metal, excluding non-detects, by plotting the relationship between the metal concentration in the soil (Y-axis) and the metal concentration in the bioaccessible fraction of the

SHIME liquid (X-axis). Isotherm linearity was assessed using linear regression (ANOVA, $p<0.05$) after checking normality and homogeneity of variance assumptions (SigmaStat v3.1).

7.3 Results

Median *in vitro* bioaccessibility of was highly variable between metals, spanning three orders of magnitude (Stomach SHIME: < 1% and 41%; Duodenum SHIME: <1% - 63%). The 90th centile for metal percent bioaccessibility was 74% in the Stomach SHIME and 82% in the Duodenum SHIME, thereby providing further evidence that assuming 100% GI absorption is overprotective (**Figure 7.1**). Al, Ti, and Cr, each thought to be poorly absorbed across the GI epithelium (ATSDR 2008; Calabrese 1996), demonstrated negligible release into simulated GI fluids. However, it is notable that the bioaccessibility of these poorly absorbed metals is similar to the bioaccessibility of Tl, which is readily absorbed following oral exposure (ATSDR 1992).

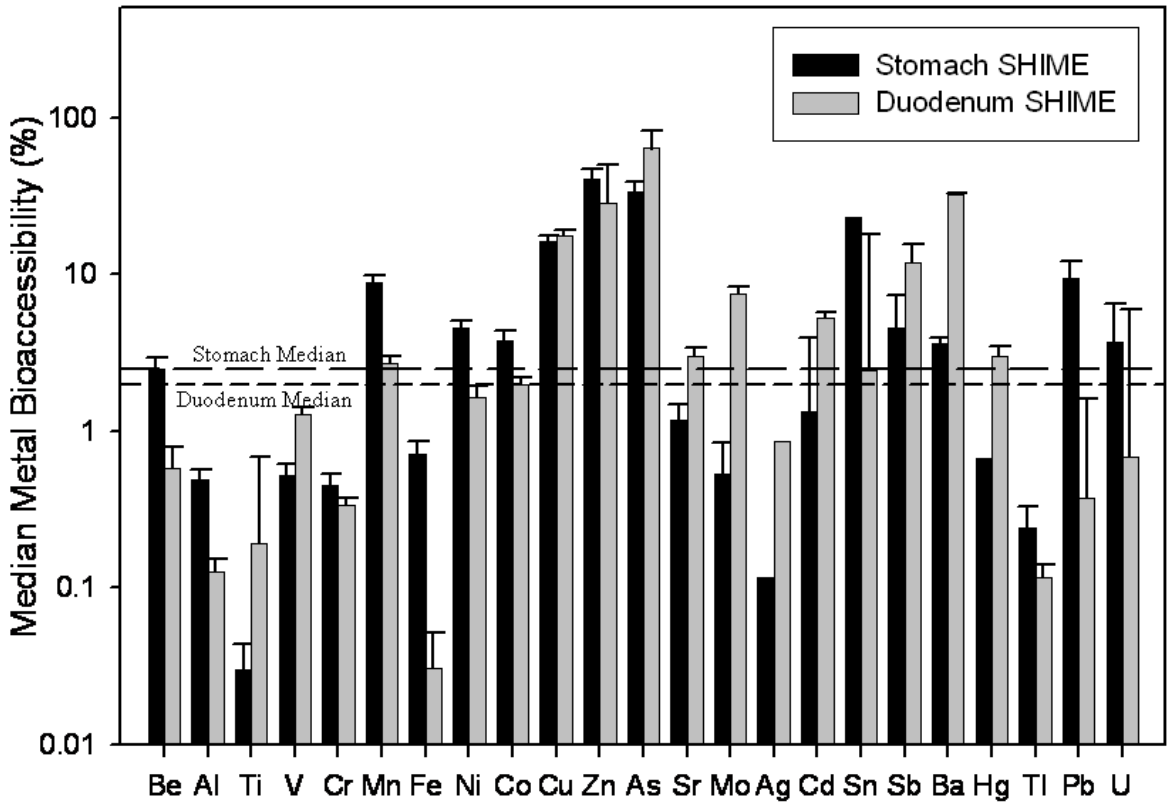

Figure 7.1 Median percent bioaccessibility (±standard error) of 23 metals extracted from 19 soils collected from a brownfield in Iqlauit, NV using the gastric and duodenum stages of the Simulator of the Human Intestinal Microbial Ecosystem.

In vitro metal bioaccessibility was occasionally correlated with the limited set of soil properties included for the purposes of this study (e.g., soil pH, particle size, total organic carbon (TOC), and > 2mm percent gravel) (**Table 7.3**). Bioaccessibility of Cr and Sr increased with increasing soil pH in the Stomach SHIME and/or Duodenum SHIME. In addition, Al, V, and Fe bioaccessibility increased with increasing Mass Per Handwashing (MPHW; i.e., the amount of soil that adhered to the skin of human adult hands) in the Stomach SHIME whereas Cd bioaccessibility decreased with increasing MPHW in the Duodenum SHIME. Correlations

between particle size and bioaccessibility were more commonly observed in the Duodenum SHIME than in the Stomach SHIME. For example, smaller particle sizes (bulk soils) were associated with higher metal bioaccessibility for Ni and Tl whereas smaller particles demonstrated lower metal bioaccessibility for Cr and Cd. In contrast, no correlations ($p>0.05$) were observed between metal bioaccessibility and bulk soil particle size in the Stomach SHIME. Few inter-correlations were observed between soil properties and these correlations were most apparent between the various measures of TOC and pH. For example, TOC for the < 2 mm fraction was correlated with the TOC adhered ($R=0.779$; $p<0.001$) and bulk ($R=0.993$; $p<0.001$) fraction of the Iqaluit soils. Concordantly, the TOC for the adhered fraction correlated with the TOC of the bulk soils ($R=0.779$; $p<0.001$). Additionally, the TOC of the adhered soil was negatively correlated with soil pH ($R=-0.825$; $p<0.001$). This relationship is supported by the fact that soil organic matter is typically composed of weak acids (carboxyl & quinone functional groups (Sparks 2003).

Table 7.3 Correlation (Spearman Rank Order) between metal bioaccessibility in the Stomach and Duodenum stages of the Simulator of the Human Intestinal Microbial Ecosystem (SHIME) and soil properties. P-values < 0.05, <0.01, and 0.001 are denoted by *, **, and ***, respectively.

Soil Properties	Stomach SHIME	Duodenum SHIME
pH	Cr (R=0.686)** Sr (R=0.800)***	Sr (R=0.721)**
Mass Per Hand-washing	Al (R=0.529)* V (R=0.582)* Fe (R=0.614)*	Cd (R=-0.536)*
Average Adhered Particle Size	Al (R=-0.604)*	V (R=0.532)*
Average Bulk Particle Size	No Correlations Observed	Cr (R=0.529)* Ni (R=-0.786)* Cd (R=0.664)** Tl (R=-0.783)**
Adhered Total Organic Carbon (%)	Sr (R=-0.661)**	Ni (R=-0.690)* Sr (R=-0.650)** Mo (R=-0.611)* Tl (R=0.833)**
Bulk Total Organic Carbon (%)	As (R=0.648)*	Ni (R=-0.810)** Tl (R=0.683)*
< 2 mm Total Organic Carbon (%)	As (R=0.661)*	Ni (R=-0.833)**
>2 mm Gravel (%)	Be (R=0.706)** Cd (R=0.857)***	No Correlations Observed

No significant correlations (Spearman Rank Order) were observed between metal bioaccessibility and (a) electronegativity (R=0.299; p=0.162), (b) covalent radius (R=-0.177; p=0.600), or (c) enrichment (R=-0.0791; p=0.716) (**Figure 7.2**). Similarly, when solubility and adsorption stability constants were compiled from Mineql 4.5 and several texts (Irving 1953; McBride 2000; Stumm 1996), no significant correlations were observed. For example, metal bioaccessibility under simulated stomach conditions was independent of stability constants for metal adsorption on oxides (R=-0.190; p=0.619) and carbonates (R=-0.236; p=0.490) (**Table**

7.4). Similarly, metal bioaccessibility was independent of the solubilities of metal carbonates, sulfides, and hydroxides (**Table 7.4**). It must be noted that each set of metal stability and solubility constants was obtained from a single source.

Table 7.4 Metal bioaccessibility under simulated stomach and small intestinal conditions was independent (Spearman Rank Correlation; $p>0.05$) of metal stability and solubility constant obtained from Mineql 4.5 and several texts (Irving 1953; McBride 2000; Stumm 1996). Each set of metal stability and solubility constants was obtained from a single source.

	Bioaccessibility		**Solubility**			**Metal**		
Me	**Stom (%)**	**Duo (%)**	**Stom (mM)**	**Duo (mM)**	**$MeCO_3$ (log k)**	**Ligand (log k)**	**MeS (log k)**	**$Me(OH)_3$ (log k)**
Mn	8.8	2.7	2.1e-2	3.4e-1	-10.7	3.2	-14.8	
Fe	0.7	0.03	1.3e-1	1.8e-1	-10.5		-26.9	
Co	3.8	2.0	2.5e-4	5.6e-3	-12.9	4.4	-22.2	-0.46
Ni	4.5	1.6	2.0e-4	4.0e-3	-8.2	4.8	-20.5	0.37
Cu	16.4	17.7	7.2e-4	3.4e-2	-9.9	6.1	-36.1	2.89
Zn	40.6	28.3	1.2e-2	3.4	-10.9	4.5	-24.7	0.99
Cd	1.3	5.3	4.9e-6	1.7e-3	-11.3		-27.8	0.47
Sr	1.2	3.0	7.6e-4	1.1e-1	-9.0	2.8		5.01
Pb	9.3	0.4	2.4e-4	1.3e-3	-13.1		-27.5	4.65
Ba	3.6	32.0	7.7e-3	6.3	-8.3	2.3		5.46

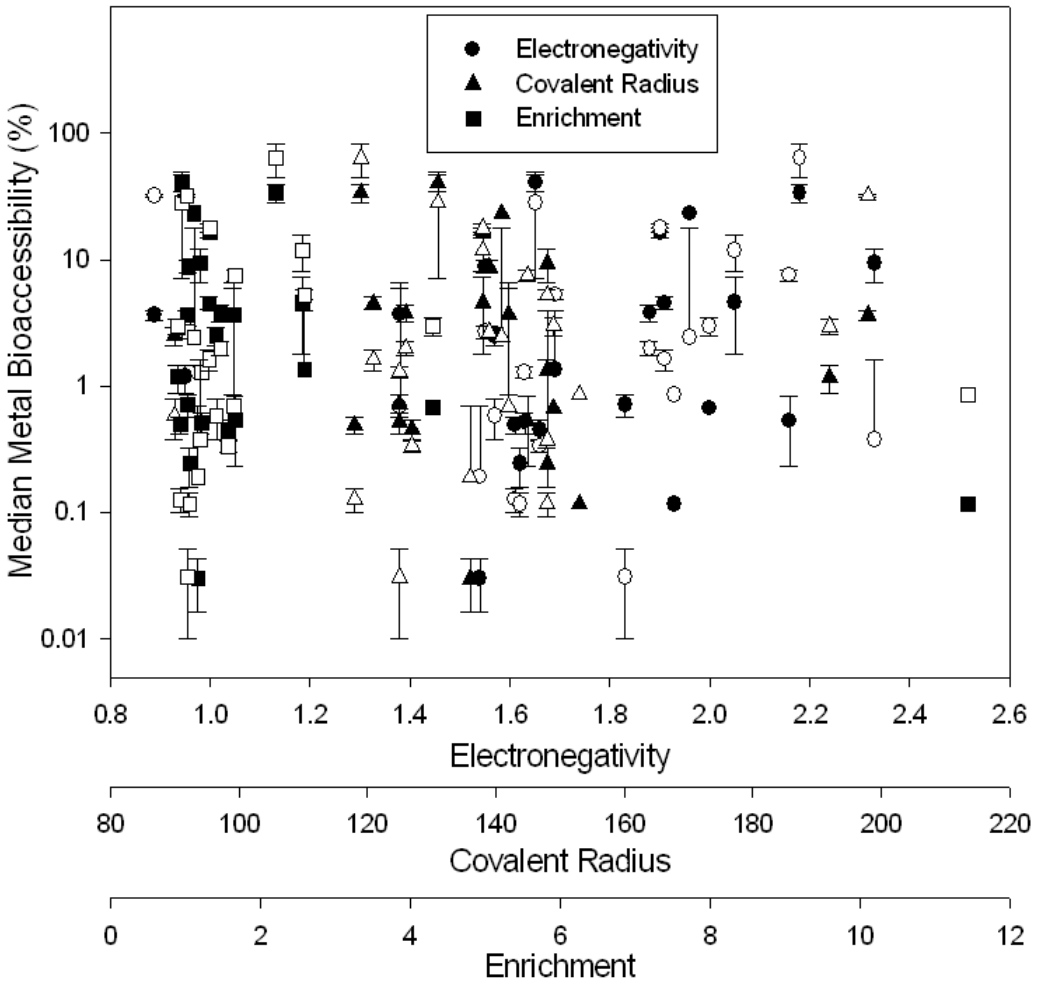

Figure 7.2 No relationships were observed between bioaccessibility (Stomach Stage: Open Symbols; Duodenum Stage: Closed Symbols) and electronegativity, covalent radius, or enrichment. Each point represents the bioaccessibility of a single metal.

Instead, differences between the median bioaccessibility of metals were explained by the linearity of the soil-solution isotherms. Metals with isotherms that demonstrated a positive ($p<0.05$) linear slope were more bioaccessible than metals a non-significant slope (**Figure 7.3**). Although it is possible for non-linear isotherms to describe systems that reach steady state (Sparks 2003), the absence of straight-line isotherms may indicate that the dissolution processes (eg. cation exchange) failed to reach steady state for those metals without straight-line isotherms. In other words, poorly bioaccessible metals may not have reached steady state and hence may

have been impeded by kinetic constraints on dissolution. Indeed, metal lability, as predicted from water exchange rates (k_{H20ex}) (Richens 1997; Taube 1952), was positively correlated with the bioaccessibility of V, Fe, Ni, Co, Mn, Zn, Cu, Al, Be, & U in the Stomach SHIME and the bioaccessibility of Fe, Ni, Co, Mn, Zn, Cu, Be, U, Cd, & Ba in the Duodenum SHIME (**Figure 7.4**). Including soil properties with k_{H20ex} did not improve the prediction of metal bioaccessibility according to multiple linear regression (stepwise forward). Similarly, previously published bioaccessibility values increased with increasing k_{H20ex} for Al, Ni, Zn, and Cu under simulated stomach conditions (**Figure 7.5**). However, in contrast to the results for the Iqaluit soils, metal bioaccessibility was independent of k_{H20ex} under simulated duodenal conditions (**Figure 7.6**).

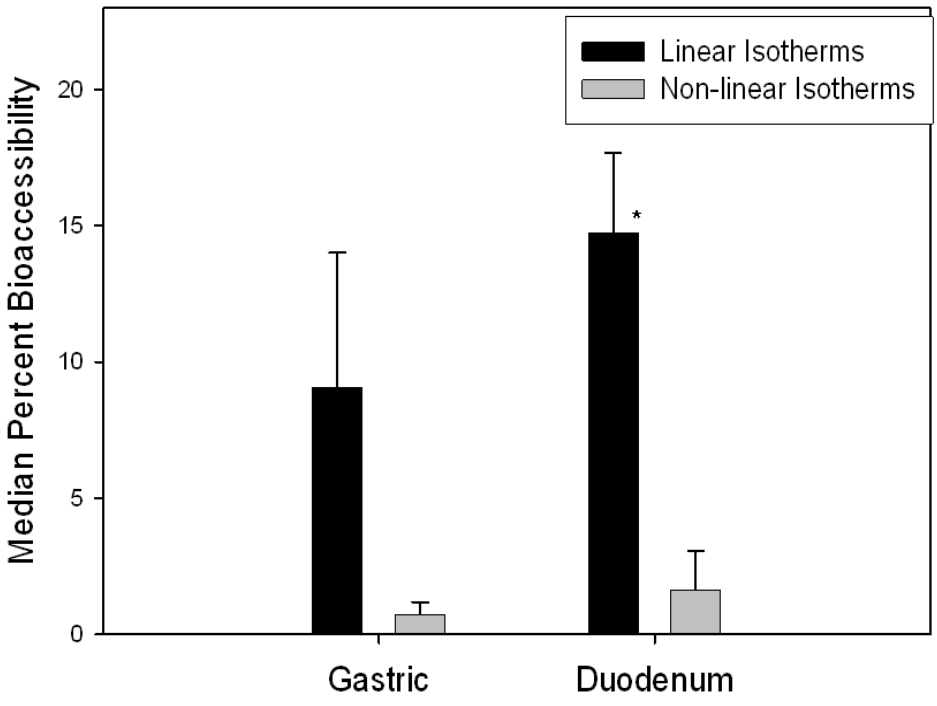

Figure 7.3 Median percent bioaccessibility (± Median Absolute Deviation) of metals with linear isotherms was higher than for those metals without a linear isotherm in the Stomach and Duodenum stages of the Simulator of the Human Intestinal Microbial Ecosystem. Significant differences in bioaccessibility (denoted with an asterisk, $p<0.05$) were defined using the Kruskal-Wallis One-Way ANOVA test.

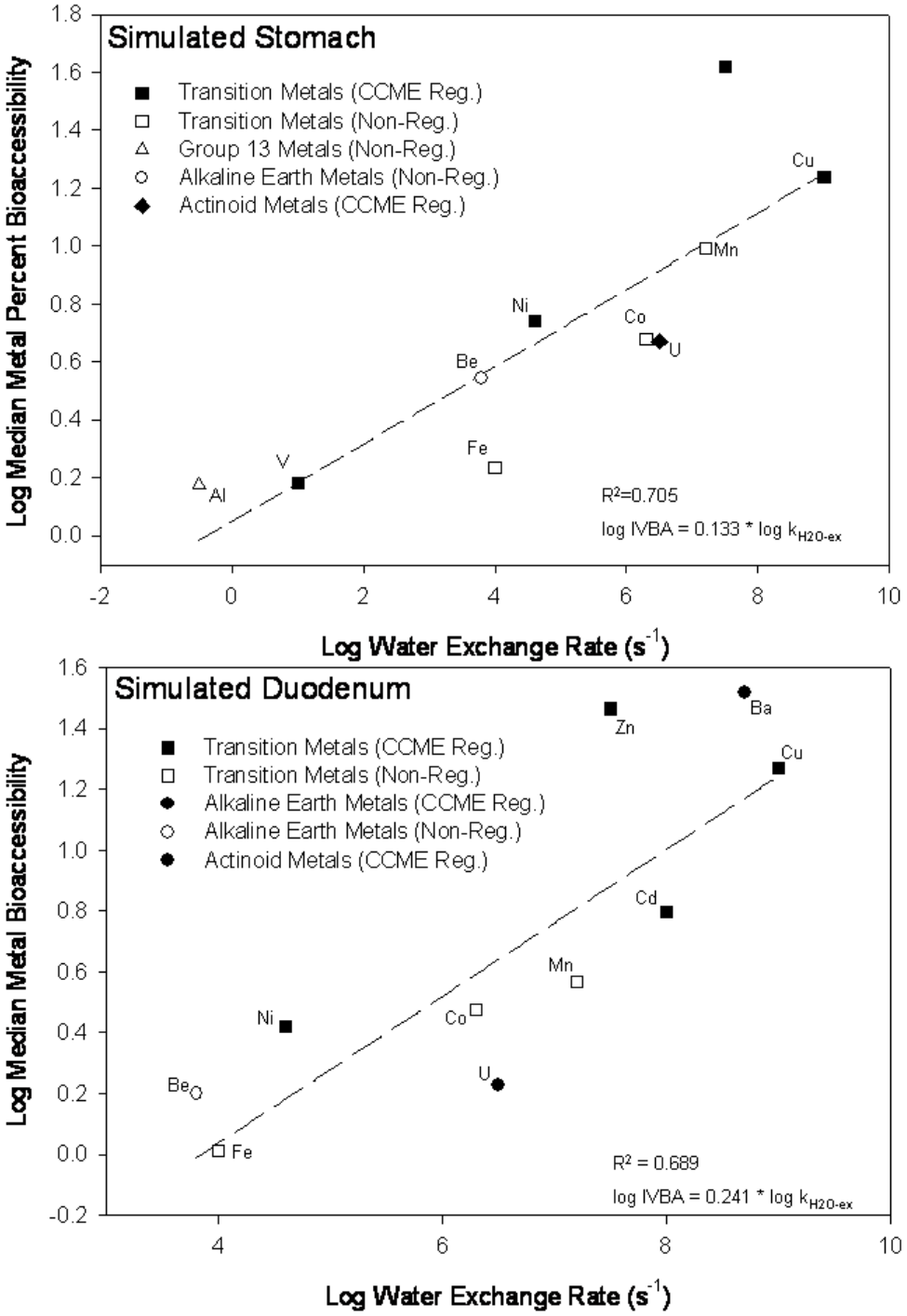

Figure 7.4 Effect of water exchange rate (k_{H20ex}) on median metal bioaccessibility in the Stomach and Duodenum stages of the Simulator of the Human Intestinal Microbial Ecosystem. Each data point represents the bioaccessibility of a metal cation either regulated (CCME Reg) or not-regulated (Non-Reg) by the Canadian Council of Ministers of Environment.

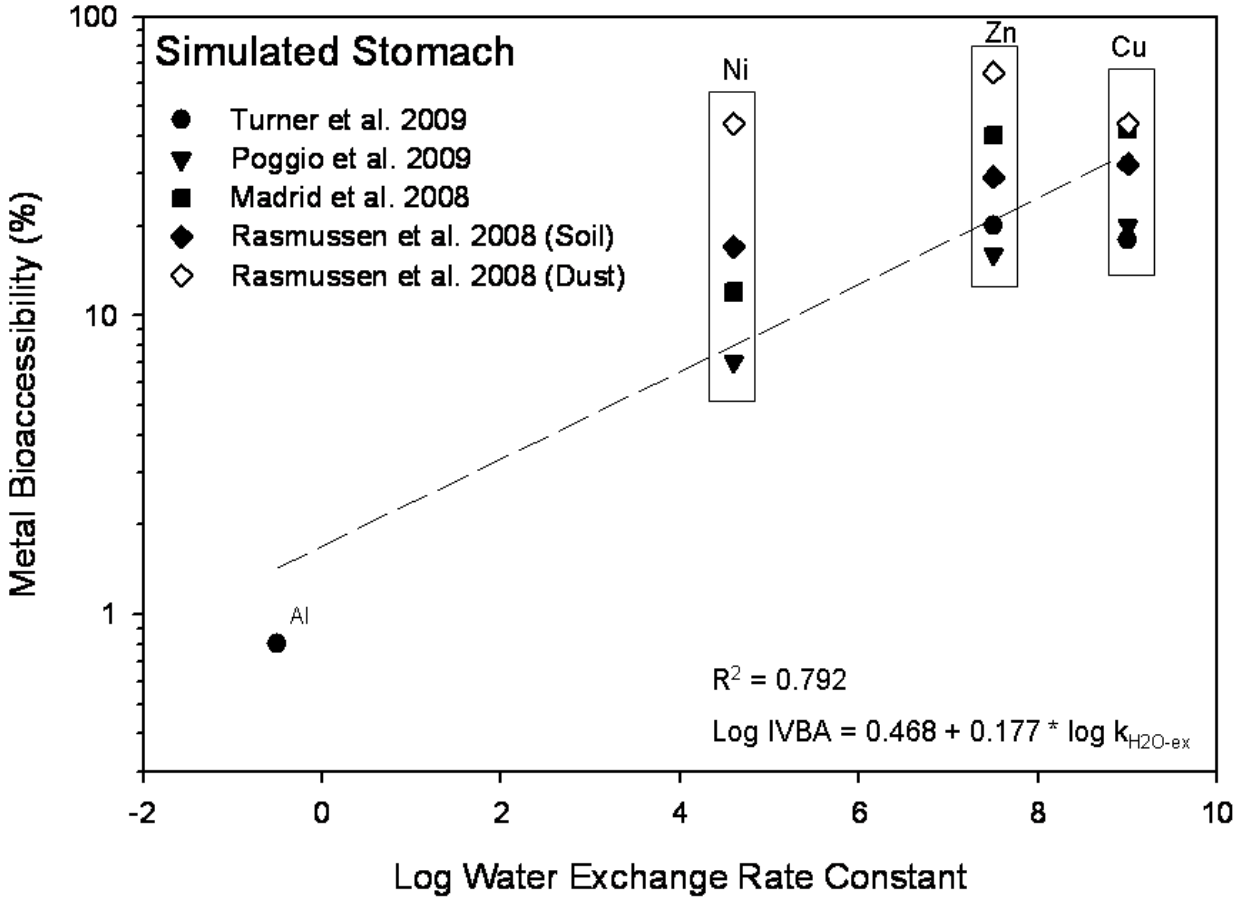

Figure 7.5 A positive relationship was observed between water exchange rate (k_{H2Oex}) and the bioaccessibility of metals from a diverse range of contaminated soils described in previous peer-reviewed manuscripts. Bioaccessibility results represent those calculated under simulated stomach conditions.

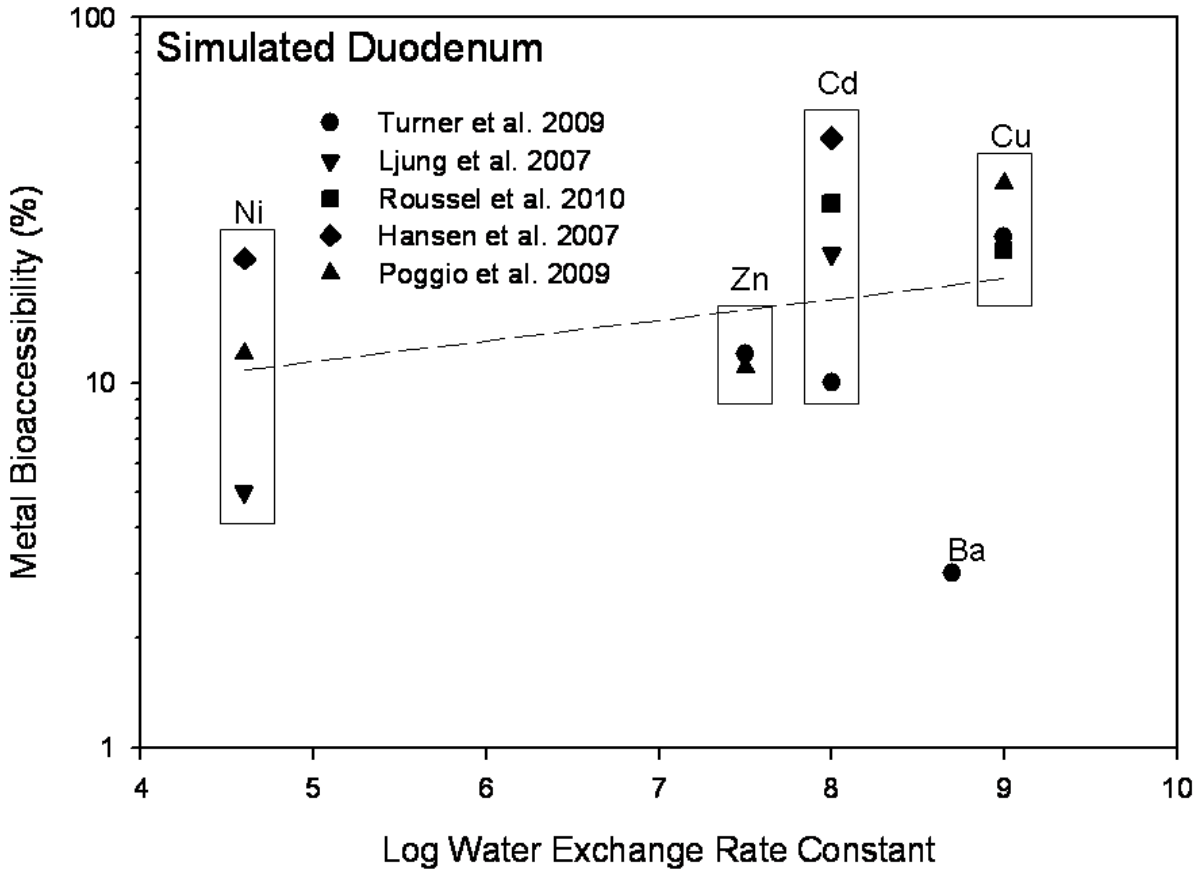

Figure 7.6 No relationship was observed between water exchange rate (k_{H2Oex}) and the previously published metal bioaccessibility values under simulated duodenum conditions. The bioaccessibility data was obtained from previously published peer-reviewed manuscripts describing a diverse range of contaminated sites distributed across the globe.

7.4 Discussion

In the dried soils, metals are present either as discrete insoluble phases (carbonates, oxides, and hydroxides) or bound in either ion exchange on the negative surfaces of soil organic matter and inorganic minerals (CEC or cation exchange capacity) or potentially as inner-sphere complexes (**Figure 7.7**). When the soil is placed in the Stomach SHIME, it is forced into equilibrium with 0.1M HCl. This results in dissolution of some metal precipitates, proton saturation of soil surfaces, and reactive surface functional groups becoming positively charged

(as Al-OH^{2+}) or neutral (R-OH or Si-OH). At this pH, CEC decreases markedly and soils develop anion exchange capacity (AEC) instead and Cl^- will displace cations at the surface. Some metals are known to form ternary complexes with mineral surfaces and Cl^-, and these metals will preferentially be retained under these conditions (**Figure 7.7**). Metals may display lower-than-expected solubilities if they are present in phases (e.g. oxides or as substituents in clay minerals) that don't fully dissolve over the reaction time in the stomach reactor.

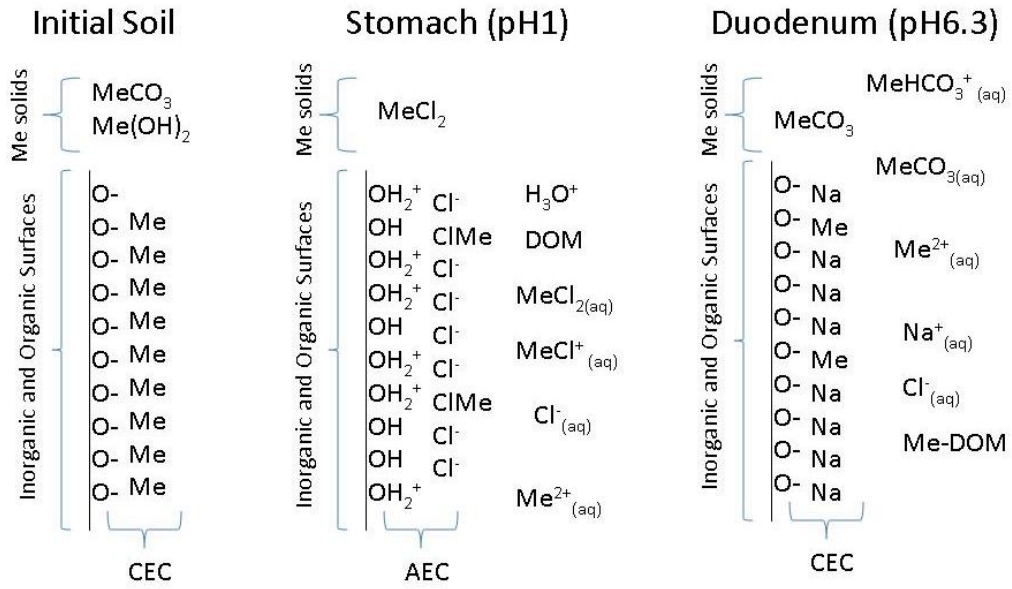

Figure 7.7 Schematic of surface site and solution changes as soils move through the Simulator of the Human Intestinal Microbial Ecosystem. Note that cations in general are shown as Me for sake of brevity. DOM represents dissolved organic matter, CEC represents cation exchange capacity, and AEC represents anion exchange capacity. Ternary metal complexes are denoted by R-OH2+-Cl-Me.

In the small intestine, the chemistry drastically changes again as the system is buffered to pH 6.5 using 0.5 M $NaHCO_3$. Under these solution conditions, the surface may again becomes negatively charged and CEC increases according to the point of zero charge of the soil components (**Table 7.5**). Cations (and specifically Na^+ due to its concentration) will displace Cl^- at the soil surface, and soluble Me-carbonate complexes and metal carbonate precipitates may form. One side effect of Na saturation of soils is dispersion of soil particles. This will increase

the bioaccessibility of metals with small hydrated radii, as they previously may have been found in internal micropores of soil aggregates which become exposed to the soil solution in Na solutions.

Table 7.5 Point of zero charge of soil components (reproduced with modifications from Sparks 2003).

Soil Component	Point of Zero Charge
$Fe(OH)_3$	8.5
$Al(OH)_3$	5.0
Fe_2O_3	6.7
SiO_2	2.0
Soil Organic Matter	~ 3

In the Stomach SHIME, due in part to the considerable variation of metal bioaccessibility between different test materials, the percent bioaccessibilities of Al, Cr, Mn, Zn, As, Sb, Ba, & Hg in the Iqaluit soils were similar (eg. within 10%) of the bioaccessibility of metals reported in (a) soils contaminated with marine paints (Turner 2009), (b) tundra soils (Shock 2007), (c) Basic Oxygen Furnace Slag (Proctor 2002), (d) urban soils collected in Torino (Madrid 2008) and Ottawa (Rasmussen 2008), (e) chat from Tar Creek Superfund Site (Schaider 2007), smelter impacted soils (Carrizales 2006), (f) mining waste effected soils (Denys 2008), and (g) a soil collected near a chlor-alkali plant in Eastern Canada (Welfringer 2009). However, the gastric bioaccessibilities of several other metals (e.g., Be, V, Ni, Cu, Cd, & Pb) in the Iqaluit soils were substantially lower than values reported in the literature (Bannon 2009; Hamel 1998; Oomen 2002; Proctor 2002; Roussel 2010). This is also consistent with previous work that showed metal bioaccessibility measured using the SHIME is often lower than values generated using different *in vitro* models (Oomen 2002; Van de Wiele 2007). In the Duodenum SHIME, Iqaluit soil metal bioaccessibilities of Cu (Roussel 2010), Sb (Denys 2008), Hg (Barnett 2001), U (Frelon 2007), Al, Cr, and Sn (Turner 2009) were equivalent (eg. within 10%) to previously reported values.

Previous research examining these same Iqaluit soils showed there to be a strong correlation between metal enrichment in the fine (i.e., <45 μm) fraction and adsorption stability constants onto mineral surfaces (Siciliano 2009). Consequentially, adsorption is likely an important mechanism by which metals are retained in these soils. Despite this, metal bioaccessibility was independent ($R^2 < 0.2$) of stability constants for metal adsorption on oxides and carbonates. Similarly, metal bioaccessibility was independent ($R^2 < 0.2$) of the (a) solubilities of metal carbonates, sulfides, and hydroxides and (b) stability constants of metal-ligands. However, the applicability of thermodynamic solubility and stability constants for metal bioaccessibility in the SHIME depends upon adsorptive and desorptive processes reaching steady state (Stumm 1970). Therefore, the failure of thermodynamic measures to reflect differences in metal bioaccessibility in the SHIME could be due to the failure of metal sorption to achieve steady state.

One approach to estimating the effect of kinetics on metal bioaccessibility is to correlate them with metal lability via water exchange rates (k_{H2Oex}). The process of water exchange is a simple substitution reaction in which a solvent molecule (e.g. H_2O) in the ion solvation shell is replaced with another H_2O molecule from the bulk solvent (Burgess 1999). More labile metal centers have higher rates of water exchange in solution, which in our system should be correlated with increasing release into solution and greater bioaccessibility. The strong log-linear relationship observed between k_{H20ex} and metal bioaccessibility in the SHIME supports the hypothesis that dissolution kinetics are useful for the prediction of soil metal bioaccessibility in human health risk assessment. This result is consistent with previous work that demonstrated that As release from synthesized amorphous scorodite fails to reach steady state in the intestinal stages of the SHIME (Laird 2009).

Water exchange rate was useful for the prediction of the metal (e.g., V, Ni, Zn, Cu, U, Cd, & Ba) bioaccessibility in the Stomach SHIME and/or Duodenum SHIME for 7 of the 13 metals regulated according to CCME SQG in Canada (CCME 2007). Additionally, k_{H2Oex} was associated with the bioaccessibility of several metals which do not have a CCME SQG (e.g., Fe, Co, Mn, & Be). This indicates that k_{H20ex} may serve as the foundation of a predictive model that facilitates risk assessors to estimate the bioaccessibility of select metals in human health risk assessment. However, it is crucial to remember that the results described in this manuscript are based upon the rather limited set of soils collected from the Iqaluit brownfield. Furthermore, a high *in vitro* bioaccessibility does not necessarily denote a high *in vivo* bioavailability. Therefore, these results must first be confirmed (*in vitro* and *in vivo*) for a wider set of soils with larger range of metal concentrations before it would be appropriate for this model to be broadly applied to ingestion exposure estimates for human health risk assessment.

Water exchange rates are, however, not universally able to predict the bioaccessibility of all metals. For example, k_{H20ex} over-predicts Pb bioaccessibility of the Iqaluit soils, likely due to the formation of ternary chloride complexes in the Stomach SHIME (Mineql+) and the precipitation of Pb carbonates in the Duodenum SHIME (Pokrovsky 2002). Similarly, CdCl complexes are also likely to form in the Stomach SHIME but not Duodenum SHIME which is consistent with Cd bioaccessibility being associated with its k_{H20ex} in the Duodenum SHIME but not Stomach SHIME (**Table 7.6**).

Table 7.6 Chloride complex speciation of metal cations in the Stomach stage of the Simulator of the Human Intestinal Microbial Ecosystem according to Mineql+ to evaluate whether or not the metal is likely to form ternary chloride complexes resulting in reduced bioaccessibility. Metal cations are denoted Me for the sake of brevity whereas metal preciptates are denoted Ppt.

Metal	Soil Concentration ($mg\ kg^{-1}$)	Solution Metal (mM)	Percent of Metal in Form of:				Fe-Me-Cl Ternary Complexes
			Me^{2+}	$MeCl^{+}$	$MeCl_2$	Ppt	
Ba^{2+}	1200	0.22	100	*	*	*	*
Cd^{2+}	8.5	1.9e-3	65	17	18	0	Yes
Zn^{2+}	1000	0.38	93	7	0	0	No
Pb^{2+}	45	5.4e-3	35	55	10	0	Yes
Tl^{+}	0.49	6.0e-5	80	20	0	0	No

The bioaccessibility of Tl is also over-predicted by its k_{H20ex}; however, Tl-Cl ternary complexes are unlikely since the majority of bioaccessible Tl is the free metal ion (Mineql+). Instead, the bioaccessibility of Tl in the SHIME may be limited by its physical inaccessibility to solvation since the likely source of Tl in our samples are the interlayers of feldspars and micas (Siciliano 2009). It should also be noted that k_{H20ex} cannot be used to predict the bioaccessibility of metals that predominately form oxyanions under the physiological conditions of the GI tract (e.g., As, Cr, Se) since little is known of k_{H20ex} of anions (Burgess 1999). Despite this, uranium fit the relationship between bioaccessibility and k_{H2Oex} in both the simulated stomach and small intestine whereas vanadium fit the relationship between bioaccessibility and k_{H2Oex} in only the simulated stomach. This is surprising since both these elements could be, like As, Cr, and Se, present as oxyanions. However, vanadium could also be present as V^{2+} or V^{3+} since such vanadium cations substitute into aluminum oxides. Similarly, uranium, if present as U(VI), can also be present as an oxocation. Until further work is performed evaluating the speciation of

vanadium and uranium in both the Iqaluit soils and simulated gastrointestinal fluids, the precise reason why uranium and vanadium appear to fit the relationship with k_{H2Oex} will, unfortunately, remain unknown and the realm of speculation.

Since metal bioaccessibility often varies with soil properties and mineralogy, it would be inappropriate to use a single bioaccessibility predicted by the relationship between k_{H20ex} and bioaccessibility for all soils. Instead, as outlined in Equation 7.2, previously unknown bioaccessibilities (i.e. Stomach ABA and Duodenum ABA) of metal cations can be approximated using the bioaccessibility of another metal (i.e., ABA_{known}) in that same soil sample, the slope of the relationship between log k_{H20ex} and log bioaccessibility, and the difference between the k_{H20ex} of the known and unknown metals:

$$Stomach\ ABA(\%) = 10^{(\log ABA_{known} + 0.133 \times (k_{H20ex\text{-}unknown} - k_{H2Oex\text{-}known}))}$$
$$Duodenum\ ABA(\%) = 10^{(\log ABA_{known} + 0.241 \times (k_{H20ex\text{-}unknown} - k_{H2Oex\text{-}known}))} \quad \text{(Eqn 7.2)}$$

We tested the accuracy of the model using previously published (Hansen 2007; Ljung 2007; Madrid 2008; Poggio 2009; Rasmussen 2008; Roussel 2010; Turner 2009) bioaccessibilities of Ni, Cu, Zn, and Al (Simulated Stomach) and Cd, Ni, Cu, Zn, and Ba (Simulated Duodenum). The model was effective in predicting metal bioaccessibility under simulated stomach conditions with an overall median residual of absolute metal bioaccessibility of 0.5% (**Table 7.7**). In contrast, the model was less effective under simulated duodenal conditions, often over-predicting metal bioaccessibility (overall median residual of absolute metal bioaccessibility = 8.0%). In conclusion, the concepts of metal lability and water exchange rates may provide risk assessors with a useful tool to predict the bioaccessibility of metal contaminants in soil.

Table 7.7 Residual (%) for predicted *in vitro* bioaccessibility (IVBA), calculated as $IVBA_{predicted} - IVBA_{actual}$, of metals under simulated stomach and duodenal conditions according to k_{H20ex} model (Equation 7.2).

Simulated Stomach Conditions					
Known Metal	Al		Cu	Zn	
Unknown Metal	Zn	Cu	Ni	Cu	Ni
Median Residual	-10.7	-3.3	-1.8	+13.7	-0.5
Min Residual	NA	NA	-8.7	+5.3	-0.5
Max Residual	NA	NA	-1.1	+21.3	+4.5
Reference	1	1	2, 3, 4	1, 2, 3, 4	2, 3, 4

Simulated Duodenal Conditions									
Known Metal	Cd				Cu		Zn	Ni	
Unknown Metal	Cu	Zn	Ba	Ni	Zn	Ba	Ba	Cu	Zn
Median Residual	+11.7	-4.4	+11.7	-8.1	+1.6	+18.2	+20.4	+102.9	+49.0
Min Residual	-7.6	NA	NA	-14.7	-1.1	NA	NA	NA	NA
Max Residual	+31.0	NA	NA	-1.6	+4.2	NA	NA	NA	NA
Reference	1, 5	1	1	6, 7	1, 4	1	1	4	4

(1) (Turner 2009)
(2) (Rasmussen 2008)
(3) (Madrid 2008)
(4) (Poggio 2009)
(5) (Roussel 2010)
(6) (Ljung 2007)
(7) (Hansen 2007)

8 SUMMARY DISCUSSION, CONCLUSIONS, AND FUTURE WORK

Risk assessment guidelines currently employed for contaminated sites use a default relative bioavailability (RBA) of 100% when estimating oral exposure (Health Canada 2004a). If RBA, as measured using *in vivo* animal experiments, differs from the RBA of the toxicological reference value (TRV) material, then the default assumption of 100% bioavailability is likely to overestimate health risk (Richardson 2006; Shock 2007). Such inaccuracies in exposure and risk assessments can thereby lead to the implementation of very costly yet unnecessary cleanup strategies. These decisions can divert resources from contaminated sites that pose legitimate threats to human health. The high cost (both time and financial) of *in vivo* animal experiments has provided incentive for the development of *in vitro* techniques that measure bioaccessibility (IVBA) (Ruby 1993). The three principal goals of this research project are (1) to evaluate the IVBA of arsenic (As) from soils and the IVBA of mercury (Hg) from traditional northern country foods, (2) to determine whether it is necessary to include a microbially active stage when measuring contaminant bioaccessibility of As and Hg, and (3) to ascertain what factors make some metal more bioaccessible than others. Fundamental research questions being addressed through these studies include: is the IVBA of ingested contaminants typically less than 100%? Is percent IVBA independent of the concentration of the contaminant in the exposure matrix? Does microbial activity affect metal IVBA and/or speciation? And if so, are these microbial effects relevant to estimates of ingestion exposure? Lastly, and perhaps most importantly, what chemical properties make some metals more bioaccessible than others?

8.1 Principal Findings

Arsenic IVBA from contaminated soils and Hg IVBA from traditional northern country foods were often substantially less than 100%, thus confirming the global hypothesis of this research project. For example, mean HgT IVBA from 16 country foods was 35% (**Table 3.1**). Similarly,

the IVBA (under simulated small intestinal conditions) of As from mine tailings ranged between 2 and 20% (**Figure 4.1**) whereas the IVBA of As from poorly crystalline scorodite ranged between 1 and 84% (**Figure 5.1**). Furthermore, the median bioaccessibility of metals collected from an urban Arctic brownfield was 2% (**Figure 7.1**). Each of these studies demonstrated that the IVBA of metals rarely approaches the default value of 100%. Therefore, higher-tier risk assessments (e.g., SSRAs) should include estimates of IVBA or RBA to better balance realism with precaution. This recommendation is in agreement with recommendations presented in D.J. Paustenbach's (1989) seminal text: The Risk Assessment of Environmental and Human Health Hazards. In this text, Paustenbach (1989) cautioned risk assessors from over-zealously utilizing worst-case assumptions. For example, systematically selecting the 95th percentile of the distributions for each variable used in the calculation of ingestion exposure (e.g., C_S, IR_S, BW, AF_{GIT}) compounds the level of confidence to 99.9994% (e.g., [1-(0.05^4)]=0.999994), far above the desired level of 95% (Paustenbach 1989).

Arsenic IVBA is inversely related to test material As concentration for mine tailings (**Figure 4.1**), scorodite (**Figure 5.1**), and arsenate (As^V) adsorbed on ferrihydrite (**Figure 5.2**). In contrast, no such relationship was observed for either Hg in traditional country foods (**Table 3.1**) or As in Hoboken soils (**Table 6.2**). Such inconsistency between and within contaminants of potential concern is in agreement with previous research. For example, contaminant IVBA occasionally decreased with increasing concentration (Beak 2006b; OME 2002; Richardson 2006) whereas IVBA was independent of contaminant concentration for other studies (Barnett 2001; Pouschat 2006; Ruby 1996). The inconsistency of these results is further complicated by the fact that such inverse relationships are potentially artifacts of the *in vitro* GI model procedures. Specifically, inverse relationships between IVBA and concentration may strictly be a

function of inadequacies of the *in vitro* GI model methodologies. For example, the ratio of gastrointestinal (GI) fluid volume to soil mass (i.e., LS ratio) is commonly two orders-of-magnitude lower *in vitro* than observed *in vivo* (Richardson 2006). This practice can result in soil contaminants (e.g. As, Ni, Pb) reaching their solubility limits, thus saturating simulated GI fluids (Hamel 1998) and underestimating the health risk posed by the contaminant (Richardson 2006). Additionally, contaminant IVBA can be limited by kinetic constraints on dissolution *in vitro* that are not present *in vivo* (Laird 2010). Alternatively, inverse relationships between IVBA and concentration may occasionally reflect *in vivo* toxicokinetic data (i.e., the inverse relationship is a real phenomena). In these cases, the dose for ingestion exposure (Eqn 2.3) does not linearly increase with increasing soil concentration. Consequentially, decreases in IVBA may negate the effect of increased soil concentration on ingestion exposure. Discerning whether an inverse relationship between IVBA and concentration is a true phenomenon or if it is an artifact of the *in vitro* model requires expert judgment and careful peer-review. The accuracy of human health risk assessments and the consequent appropriateness of remedial actions are contingent upon correctly identifying and explaining site specific relationships between IVBA and concentration.

Gastrointestinal microbial activity decreased HgT IVBA from most of the studied northern country foods, but increased HgT IVBA from seal intestine and Arctic char flesh and did not alter HgT IVBA from narwhal skin (**Figure 3.1**). In contrast, GI microbial activity under simulated colon conditions increased As IVBA for the bulk and <38 μm fraction of mine tailings (**Figure 4.2**), synthesized scorodite (**Figure 5.1**), some loadings of As^{V} adsorbed ferrihydrite (**Figure 5.2**), an As contaminated ironstone soil (Laird 2009), an agricultural soil containing elevated As concentrations (Laird 2009), and an uncontaminated agricultural soil (Laird 2009). Since Dissimilatory Iron Reducing Prokaryotes (DIRPs) (Oremland 2005) are likely responsible

for the mobilization of As from contaminated soils and soil-like particles (**Figure 5.4** & **Figure 5.5**), the IVBA of other metals known to adsorb on iron oxides and oxyhydroxides may also be similarly affected by microbial activity. These results indicate the use of IVBA results that don't account for colon microbial activity may systematically underestimate health risks from the ingestion of metal contaminated soil. In contrast, the ability of GI microbes to decrease Hg IVBA from foods indicates that neglecting to include microbial activity into estimates of Hg IVBA activity may overestimate health risks from dietary Hg.

In vitro GI microbial activity altered the speciation of Hg from traditional country foods (**Figure 3.1**) but did not affect the speciation of As from contaminated soils. For example, GI microbes decreased the proportion of bioaccessible Hg as MeHg up to 100%, perhaps due to microbial demethylation of MeHg in the country foods. This observation is in agreement with previous *in vitro* (Ludwicki 1990) and *in vivo* (Rowland 1980) research. In contrast, GI microbial activity was not responsible for the transformation of As^{V} to the more acutely toxic As^{III} (**Figures 5.4** & **5.5**). Additionally, the incubation of As contaminated Hoboken soils with GI microbes did not lead to the formation of methylated arsenicals. This observation is in stark contrast to a recent publication that demonstrated substantial *in vitro* biotransformation (including biomethylation) of As from sodium arsenate and contaminated soils (Van de Wiele 2010). The discrepancy between these studies may be due to differences between the GI microbial communities obtained for each study. Alternatively, the discrepancy could be due to differences in soil properties or could be a function of the lower As concentrations present in the Hoboken soils.

The toxicological relevance, however, of the microbially-mediated changes on As IVBA and speciation may be dependent upon such effects being reflected in contaminant RBA. Or, in other

words, metal contaminants mobilized by GI microbial activity must be subsequently absorbed across the intestinal epithelium (i.e., bioavailable) for the microbial effect to be important to the calculation of ingestion exposure. Consistent and significant microbially-mediated effects on As IVBA were only observed under simulated colon conditions (**Figure 4.2**, **Figure 5.1**, **Figure 5.2**); however, the colon is not the primary site of As absorption (Otani 1957). In contrast, microbial activity under simulated small intestinal conditions, the primary site of As absorption (Kayne 1993; Otani 1957), caused only slight and transient effects on As IVBA (**Table 6.2**) and no effects upon As speciation. Furthermore, a juvenile swine study in which animals were orally exposed to soil-borne As with and without co-administration of oral antibiotics indicated that GI microbial activity does not effect As RBA (**Table 6.5**). These *in vitro* and *in vivo* results provide the strongest evidence to date that, contrary to the hypothesis of this thesis, GI microbial activity may be irrelevant to the actual bioavailability of ingested arsenic and hence irrelevant to the calculation of As IVBA. However, the ultimate importance of GI microbes to the estimation of ingestion exposure and consequent health risk cannot yet be dismissed since pre-systemic biotransformation of ingested As is not observed for all soils (Van de Wiele 2010). Thus, the results from the juvenile swine study reported herein may have been specific to the contaminated soil used in the trial and should not be extrapolated to other test materials or contaminated site risk assessment in general.

Previous research developing *in vitro* GI models for the measurement of contaminant IVBA has largely proceeded on an *ad hoc* basis one contaminant at a time. Therefore, although there exists extensive peer-reviewed literature describing the IVBA of ingested As and Pb, this information cannot be applied to other contaminants of concern. One recent publication demonstrated that the IVBA of PAHs from contaminated soils was correlated with the Z_{water} (i.e.,

fugacity capacity of water) for each individual PAH (Siciliano 2010). Similarly, metal IVBA was strongly correlated with the water exchange rate constant ($k_{H20ex)}$ for each individual metal (**Figure 7.4**). These studies represent the first attempts to correlate IVBA with fundamental chemical properties of the contaminant of concern and offer tremendous potential for the development of predictive models (e.g., **Table 7.7**) of contaminant bioaccessibility. It is notable that the determining factor of PAH bioaccessibility (i..e., Z_{water}) is a thermodynamic-based variable (Reichenberg 2006) whereas the determining factor for metal cation bioaccessibility is a kinetic-based variable (Burgess 1999). None of the thermodynamic-based stability constants seen to be determinants of metal enrichment (Siciliano 2009) were important factors for metal IVBA from the Iqaluit soils. This suggests, for the soils included in these studies, that steady-state and equilibrium may be more readily achievable for the dissolution processes of lipophilic organic contaminants such as PAHs than for metal cation contaminants. It remains to be seen whether this principle will hold true for other contaminants in soils from additional sites.

8.2 Future Directions

Future studies of metal IVBA from contaminated soils and foodstuffs should better characterize the speciation of the bioaccessible metals since the toxicokinetics and toxicodynamics of some metals are dependent upon speciation (Clarkson 2006; Cohen 2006; Denys 2008). For example, only a small fraction of Hg^{II}, between 7 and 15% (ATSDR 1999), is absorbed across the GI epithelium into systemic circulation via a dose-dependent and saturable mechanism (Hoyle 2005). In contrast, both human and animal studies indicate near complete (~95%) absorption of ingested MeHg (ATSDR 1999). The higher bioavailability of ingested MeHg is due to its absorption via an energy dependent active transport mechanism based upon its structural homology, when complexed to cysteine, with methionine (Clarkson et al., 2007). Similarly, the absorption efficiency, metabolism, and genotoxicity of ingested As is dependent

upon the speciation of the bioavailable As (ATSDR 2007; Cohen 2006; Vahter 2002). For example, inorganic arsenate in drinking water is nearly completely absorbed into systemic circulation(ATSDR 2007) whereas at most 85% of an ingested dose of methylated arsenicals (MMA or DMA) is absorbed (ATSDR 2007). Not only are these methylated arsenical differently absorbed than inorganic arsenicals, but also they are differently metabolized (Cohen 2006). Furthermore, the toxicity and reactivity of the trivalent methylated arsenicals formed by colon microorganisms (Van de Wiele 2010) in the intestinal lumen may pose health risks (eg. colon cancer) even without absorption into systemic circulation. Consequentially, the IVBA of ingested HgT and As, as presented in the research herein, is of limited value to risk assessors when the bioaccessible metal is present as a mixture of species with varying toxicological profiles. Therefore, future calculations of Hg IVBA should calculate both Hg^{II} and MeHg IVBA separately while As IVBA should differentiate between inorganic As and trivalent methylated As metabolites. Similarly, IVBA estimates for several other metals (eg., chromium and nickel) should also characterize the percent contribution of each metal species present in the bioaccessible fraction since metal speciation will determine the TRV used in the human health risk assessment of contaminated sites (Health Canada 2004b). It must be noted though, that the utility of speciation data for the bioaccessible fractions of some metals and metalloids (e.g., arsenic and antimony) is contingent upon further toxicological research enabling the definition of TRVs that account for variations of the toxicity of differing metal species.

Evaluations of contaminant bioaccessibility in the colon stage of the Simulator of the Human Intestinal Microbial Ecosystem (SHIME) do not as of yet account for inter-individual variation in the composition of GI microbiota. For example, current SHIME techniques for measuring contaminant bioaccessibility are conducted in triplicate by removing three aliquots of microbial

suspension obtained from the dynamic SHIME (see **Section 3.2.2**). However, the microbial community cultured in the colon stage of the SHIME was derived from a single human fecal sample that is not necessarily representative of the enteric microflora of other individuals or populations. Since the GI microbial community used in the SHIME was obtained from a fecal specimen provided by an individual with a typical western urban diet (dominated by cereals, legumes, dairy products, fruits, vegetables, and meat), it is unknown whether the results obtained are relevant to the receptors of the risk assessment (i.e. Inuit adults and children). Thus, future studies investigating the role of GI microbial activity on Hg IVBA should utilize fecal samples collected from an Inuit volunteer with a traditional diet rich in country foods. Additionally, bioaccessibility studies using the colon stage of the SHIME should be repeated using fecal specimens obtained from several individuals. This strategy will provide researchers with information regarding the consistency of microbial effects upon metal bioaccessibility between individuals with varying diets, ages, and genders.

Before estimates of contaminant bioaccessibility can be used in human health risk assessment they must be validated against accepted *in vivo* protocols (Casteel 2001; US EPA Region 8 2005). The necessity of these validation studies is no less imperative for *in silico* predictive models based upon fugacity and/or metal lability than they are for *in vitro* models like the SHIME. In addition, future work must evaluate the mechanisms by which soil properties and mineralogy interacts with k_{H20ex} before it will be possible to conclude whether metal IVBA may be predicted according to its lability without the prior knowledge of the bioaccessibility of another metal from the test material. Thereafter, the completion of *in vivo* validation studies will facilitate the use of concepts such as fugacity and metal lability as predictors of AF_{GIT} for human health risk assessment. Additionally, the importance of enteric microbes to the calculation of

metal bioaccessibility should be further investigated for a larger variety of test materials using *in vivo* validation studies of IVBA with and without GI microbial activity.

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