A TOOL TO REDUCE UNCERTAINTY IN RISK CHARACTERIZATION: COMBINING *IN VITRO* EXTRACTION METHODS AND A CELLULAR BIOASSAY

by

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A dissertation submitted to the

Graduate School-New Brunswick

and

The Graduate School of Biomedical Sciences

Rutgers, The State University of New Jersey

In partial fulfillment of the requirements

For the degree of

Doctor of Philosophy

Graduate Program in Environmental Sciences

Written under the direction of

Paul J. Lioy, Ph.D.

And approved by

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New Brunswick, New Jersey

MAY, 2016

ABSTRACT OF THE DISSERTATION

A tool to reduce uncertainty in risk characterization: combining *in vitro* extraction methods and a cellular bioassay

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Risk assessors have utilized worst-case scenarios and the default assumption of 100% bioavailability to make ingestion exposure estimates. This leads to overestimation of risk by miscalculating the likelihood of a contaminant becoming bioavailable after exposure. Furthermore, bioavailability studies often involve animal models, which are time consuming and expensive. As a result, *in vitro* gastrointestinal models have been incorporated into risk characterization for the calculation of bioaccessibility. However, these models do not provide information on the effects of chemicals on the human body in the presence of low-level, chronic exposures, which are typical of environmental contaminants. The primary objectives of this research are two-fold: first, to examine how a cellular system is affected by heavy metal-contaminated soil after extraction by *in vitro* bioaccessibility techniques, and second to identify the utility of a hepatocellular model as a complementary tool for *in vitro* bioaccessibility models in risk assessment.

The percent bioaccessibility measured for five metals, Pb, As, Cd, Ni and Cr, across nine soils, using an *in vitro* system that incorporated synthetic saliva, gastric and intestinal fluids, varied from <10% to nearly 100% with the bioaccessibility of most metals declining from the saliva/gastric fluid to the intestinal fluid due to the higher pH of the latter. However, no single generalization predicted the association across all metals in the various soils, indicative of the need to evaluate multiple metals' bioaccessibility when estimating risk from ingestion of soil. Subsequent to *in vitro* extraction, the toxicity of the bioaccessible fraction of nine soils was assessed using an *in vitro* hepatocellular model. A multiple regression linear model that predicts hepatotoxicity from bioaccessible metal concentration accounted for more than 80% of the variability in our predictive model, highlighting the potential of exposing an *in vitro* hepatocellar (or other cell type) model to the bioaccessible fluid fraction derived from soil as a complimentary and precursor screening tool for more expensive *in vivo* examinations. Conversely, Ni only accounted for 26%, Cr for 28%, Pb for 0.4%, Cd for 2% and As for 11% of the model variability on an individual basis if toxicity of all metals are independent of each other.

The use a human cellular system as a complimentary tool in risk assessment allows for the application of a mixed metal contaminant system as a more biologically relevant model than total metal content or metal bioaccessibility alone. Results from this study provided evidence of the utility of cellular model responses to bioaccessible fluids as a tool to evaluate contaminants since it examines mixture effects rather than single elements. Risk

studies that evaluate exposure to mixtures of metals rather than individual metals better reflect real-world exposures to soils, which is of particular importance when assessing risk.

ACKNOWLEDGEMENTS

This dissertation would not have been able to reach completion without the guidance and help of my advisor, committee members, and the encouragement from family and friends.

I am indebted to Dr. Paul J. Lioy for his constant support, guidance, patience and “tough love” in the completion of this dissertation work. I am also grateful to my thesis committee members who were helpful throughout my entire graduate career: Dr. Brian Buckley who developed my background in experimental chemistry; Dr. Clifford Weisel for always having an open door and an open ear; Dr. Jeffery Laskin for finding me a “home” in his toxicology lab; and Dr. Timothy Buckley for allowing me to travel down to North Carolina and offering me an outside perspective to my dissertation research.

This work could not be completed without the help of analytical staff member Elizabeth McCandlish who was always willing to help with any ICP-MS need or question. Mrs. Teresa Boutillette, thank you for constant support, encouragement, and “gentle” reminders.

To “Grumpy”, you helped me through good times and bad and for that I appreciate you.

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This research was supported in part by the NIEHS sponsored Rutgers Center for Environmental Exposures and Disease, Grant #: NIEHS P30ES005022, and Fellowship support from the NIEHS training grant 5T32ES019854

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LIST OF ABBREVIATIONS

ADME	Absorption, Distribution, Metabolism, and Excretion
As	Arsenic
As V	Inorganic arsenate
As III	Inorganic arsenite
ATSDR	Agency for Toxic Substances and Disease Registry
Cd	Cadmium
CERCLA	Comprehensive Environmental Response, Compensation and Liability Act
Cr	Chromium
Cr III	Chromium with oxidation +3
Cr VI	Chromium with oxidation +6
CPSC	Consumer Product Safety Commission
DRA	Dose-Response Assessment
EA	Exposure Assessment
EOHSI	Environmental and Occupational Health Sciences Institute
EPA	United States Environmental Protection Agency
FDA	United States Food and Drug Administration
GI	Gastrointestinal
HAP	Hazardous Air Pollution
HBSS	Hanks Buffered Saline Solution
HI	Hazard Identification
HHRA	Human Health Risk Assessment
HMBSR	Hamel Mass Balance and Soil Recapture
HUD	U.S. Department of Housing and Urban Development

IARC	International Agency For Research on Cancer
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
IVBA	*In vitro* bioaccessibility
Ni	Nickel
NIOSH	National Institute for Occupational Safety and Health
NIST	National Institute of Sciences and Technology
NJDEP	New Jersey Department of Environmental Protection
NPL	National Priorities List
NRC	National Research Council
OSHA	Occupational Safety and Health Administration
RI/FS	Remedial Investigation and Feasibility Study
Pb	Lead
PBET	Physiologically Based Extraction Test
PBS	Phosphate Buffered Saline
RBA	Relative Bioavailability
SBA	Surrogate Bioavailability
SPBET	Simplified Physiologically Based Extraction Test
SBRC	Solubility/Bioavailability Research Consortium
SRM	Standard Reference Material
U.S.	United States
WHO	World Health Organization

Chapter 1: Introduction

Risk assessors use *in vitro* bioaccessibility (IVBA) measurements to improve site-specific accuracy of oral exposure estimates of ingested organic and inorganic material likely to dissolve in the gastrointestinal system and be transferred to other organ systems. Oral IVBA systems use gastrointestinal (GI) models that mimic stomach and intestinal fluid dynamics in order to calculate the percentage of contaminants likely to solubilize in the GI system. In contrast, oral *in vivo* bioavailability measurements use animal models to calculate the percentage of ingested contaminant that is able to cross the gastrointestinal epithelium and enter into systemic circulation and be transported to other organs and cellular systems in the body. IVBA measurements provide an improvement over worst-case scenario approaches (total acid digestion and x-ray techniques) for collecting data needed by risk assessors because dissolution in the gastric and intestinal fluids are a precursor to passage across the intestinal lumen (Riegelman & Rowland, 1973).

By incorporating bioaccessibility and/or bioavailability into oral exposure assessments, risk assessors can begin to address questions surrounding the release and absorption of metals present in contaminated soils within the digestive system. While important research has validated IVBA methods (Ellickson et al., 2001; Oomen et al., 2003; Ruby et al., 1999), a number of data gaps limit risk assessors from incorporating IVBA measurements on a wider scale. One data gap includes determining the consequences of the inclusion or exclusion of the intestinal compartment and whether its presence influences

estimates of metal bioavailability. In addition, most IVBA measurements and validation efforts have focused on lead (Pb) and arsenic (As), limiting application of the results of the studies to a case-by-case basis and to aged or specific soil/waste types.

The PhD research presented herein addresses the following hypothesis.

1.1 Hypothesis

Human exposure and risk associated with environmental contamination of metals is overestimated when it is assumed that 100% of the bioaccessible fraction of various metals becomes bioavailable. The liver plays a role in the regulation of cellular absorption *in vivo* and the inclusion of an *in vitro* liver model may help relate bioaccessibility results to subsequent potential toxic effects. Two aims will support this hypothesis.

1. To investigate the risk that contaminated soils may pose to human health using a sequential extraction method to estimate oral bioaccessibility.
2. To develop an *in vitro* cellular viability assay to further investigate human health risk and to use it as a comparison point between two techniques for estimating risk (IVBA and cellular viability).

1.2 Definitions and Calculations

1.2.1 Oral Bioaccessibility for this thesis

is defined as the amount of metal that dissolves in saliva, gastric and intestinal fluid.

1.2.2 Bioavailability

The bioavailability is defined as the amount of metal that is able to cross the gastrointestinal epithelium and reach systemic circulation after ingestion.

1.2.3 Relative Bioavailability (RBA)

RBA is defined as the absorption ratio of the dose able to reach systemic circulation.

1.2.4 Extractable/Leachable Metal

The leachable metal is determined by the amount of metal determined using EPA method 3051A. This method provides acid-extractable concentrations that may not reflect total, bulk, concentration.

Chapter 2: Literature Review/Background

2.1 Overview: Soil and the Assessment of Risk

Soil is concomitant with everything around us and has many vital functions necessary for sustaining human existence on Earth. It performs many important roles such as filtering water, providing a medium for growth, and housing antibiotic producing microorganisms. Thus, the soil ecosystem functions as a critical foundation for life. As a result, maintaining healthy soil is crucial for its use as a fundamental resource.

When soil quality is compromised by the introduction of physical, chemical and biological impurities, limiting human contact and exposure as well as facilitating contaminated site cleanup becomes an important concern. Pollutants deposited at high enough levels in or on soil may require governmental intervention to meet health or safety regulations. The legacy of industrial waste releases into the air and deposits in water and on soils throughout the United States resulted in major environmental legislation including the Clean Air Act, the Clean Water Act of 1970, and the National Environmental Policy Act (NEPA) of 1970, which established the Environmental Protection Agency (EPA) (EPA, 2013, 2014, 2015). It also led to the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA) (also known as the Superfund Act) of 1980 that shifted focus toward human risk reduction by developing tools to help prioritize cleanup and subsequently remediating contaminated sites (NRC, 2003).

The EPA and other organizations, e.g. contractors, conduct remedial investigations and feasibility studies (RI/FS), once a site is listed on the National

Priorities List (NPL). The remedial investigation allows the EPA and other organization to collect data on the following: (1) site conditions; (2) nature of waste; (3) environmental and human health assessment of risk; and (4) treatability testing to discern costs and treatment options (EPA, 2011). Whereas the feasibility study is the means by which alternative remedial actions are evaluated and developed. The sequential nature of the multi-phase RI/FS approach allows the data collected to influence each phase in the process. It is at various points during the RI/FS process, especially during environmental and human health risk assessment, that there lies potential for bioaccessibility measurements to help better define risk.

There are inherent complications with remedying hazardous waste sites. Beyond the many legal issues, which tend to drive the process (Greenberg et al., 1998), the first science and engineering consideration is that considerable time and money are needed to remediate contaminated sites. Furthermore, it's difficult to know how bulk concentrations in soil correlate with actual risk. Factors such as the soil matrix and contaminant type (e.g., organic vs. inorganic) can have widely different impacts on toxicant concentrations, from bulk to internal, thereby placing limitations on systemic absorption (A. G. Oomen et al., 2002; Zia et al., 2011). The correlation between contamination and risk cannot be done without first completing a site investigation that establishes the horizontal and vertical extent of contamination, which is accomplished during a remedial investigation and then completing a preliminary risk assessment.

The above provides a crude indication of the potential for exposure and risk. However, over the past 20+ years investigators (Bradham et al., 2011; Casteel et

al., 1997; Ellickson et al., 2001; Hamel et al., 1998; A. G. Oomen et al., 2002; Oomen et al., 2003; Ruby et al., 1999; Zia et al., 2011) have focused on the contaminant fraction of a soil or other medium that reach systemic circulation otherwise known as the bioavailable fraction. Thus, instead of just relying only on the bulk concentration data obtained during a remedial investigation, a more realistic characterization of exposure and risk can be completed to expand remediation options.

The concept of bioavailability/accessibility is an important addition to waste management because it has significant repercussions on the type of cleanup needed for contaminated soils. Further, risk assessors rely on bioavailability estimates to understand exposure and toxicity issues (Lioy & Burke, 2010; NRC, 2012). In practice, it is difficult to ascertain human bioavailability because it has normally required animal studies which can be expensive and time consuming (Ruby et al., 1996). Further, given the complicated path a potential toxicant can face as it travels through various systemic physiological systems (e.g. respiratory and digestive), it is difficult to account for all the factors that will impact the fate of a contaminant. To account for the intrinsic human properties and characteristics that determine the fate of a pollutant in a soil matrix, *in vivo* bioavailability studies with laboratory animals have been developed and used to predict human exposures (Casteel et al., 1997; G. B. Freeman et al., 1995). However, as mentioned above, *in vivo* animal studies prove inefficient due to cost, time constraints, and due to the challenges faced when scaling between animal surrogates and the human system. For example, Freireich et al. (1966)

quantitatively compared the toxicity of anticancer agents in mouse, rat, hamster, dog, monkey and human and found that surface area adjustments helped with interspecies scaling. Yet uncertainties are magnified when the toxicity of a substance is not dependent on the surface area. Issues like these make it difficult for interspecies scaling to account for all the different chemical, biological and physical properties that may impact toxicity. In the case of a soil matrix, there are a myriad of contaminants that can induce toxicity endpoints.

The use of an *in vitro* fluid analogue has been developed to bridge the gap between *in vivo* animal models and oral exposure by presenting a clearer picture as to what may happen post ingestion because this approach can establish the bioaccessibility of a toxicant. Given the latter concept, *in vitro* alternates that are rapid and cost-effective, such as gastrointestinal and respiratory fluid analogues that mimic the human system, have been developed as an alternative to their *in vivo* animal assay counterparts (Ruby et al., 1999).

The bioaccessible fraction is the fraction of contaminant that is able to, for example, dissolve in simulations of gastrointestinal fluids and subsequently become available to pass the intestinal lumen to become bioavailable. This is also important to risk management due to the relationship of bioaccessibility with bioavailability (NRC, 2003). The importance of bioaccessibility to bioavailability is driven by the need for cheaper, higher throughput and more rapid tests. Surrogates for bioavailability such as *in vitro* extraction techniques are often easier to put into practice and are often cheaper assessments of risk (Deshommes et al., 2012). This is because i*n vivo* bioavailability studies often require the purchase of animals

such as mice, monkey, and juvenile swine as well as the materials to support such experiments. On the other hand, bioaccessibility studies often only require basic laboratory equipment and materials to assess risk.

In theory, measurements of bioaccessibility will almost always over-estimate bioavailability but be much less than estimates derived from leachable extraction techniques (Zia et al., 2011). This is associated with the fact that the acidic pH of the stomach allows for higher dissolution of the metals into the gastric fluid, but as the pH increases when it moves from the stomach to the intestines, the solubility decreases creating insoluble metal that is excreted after passing through the large intestine along with other insoluble waste. However, consideration must also still be given to the matrix material (e.g., soil), contaminant type and/or species, solubility factors, etc. Due to the fact that, in the case of most metals, bioaccessibility is defined as the dissolved contaminant fraction in human gastric and intestinal fluid, factors that influence solubility and the ability of a contaminant to dissolve in each fluid compartment will most affect bioaccessibility.

2.2. Heavy Metals in Soil

At least eight of the top twenty-five (25) most frequently detected hazardous contaminants in groundwater and soil are metals. These metals are lead (Pb), chromium (Cr), zinc (Zn), arsenic (As), cadmium (Cd), copper (Cu), nickel (Ni) and mercury (Hg). Together with antimony (Sb), beryllium (Be), selenium (Se), silver (Ag), and thallium (Tl), these elements constitute the United States Environmental Protection Agency's (U.S. EPA) "priority pollutant metals" that have potential threat

to human health (NRC, 2003). Most priority pollutant metals rarely occur in a matrix in single form, but rather as a complex mixture in various complexes and oxidation states of various chemicals with varying matrix properties (e.g. particle size and morphology). The complexity of pollutant metals in various matrices can vary widely and underscores the uncertainty and variability that often arises in metal measurement studies.

The continued environmental concern and awareness of heavy metals in soils, especially in industrial and waste disposal sites, is evidenced by recent legislation whose primary aim is management and remediation. For example, in 1980 the United States passed the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA) or better known as the Superfund Act to ensure proper management of waste disposal sites. Under the umbrella of the Superfund Act, the EPA uses the National Priorities List (NPL) to identify the most serious hazardous waste sites in the nation. Once placed on the list, sites are targeted for long-term federal cleanup and thoroughly evaluated to assess the extent of human and/or environmental hazard caused by toxic agents.

2.3 Sources of metals in soils: natural and anthropogenic

Heavy metals generally exist naturally in soils well below levels that would raise alarm for human wellbeing with certain exceptions including metals such as arsenic. Any major increases in concentrations above background are usually due to anthropogenic sources while natural sources such as volcanoes are also a concern. Urbanization, militarization, and industrialization are among the reasons

that pollution caused by heavy metals has become a serious environmental problem. From smelters to landfills to industrial waste sites and pesticides in agricultural settings, varied opportunities exist for metals to make their way into the soil ecosystem.

2.4 Exposure Characterization

Human health risk assessments (HHRA) for ingestion exposure to heavy metals in soils seek to estimate the likelihood of adverse health effects in humans post ingestion. Standardized protocols govern the implementation of HHRA in order to systematize remediation efforts and the data that govern public policy. To begin HHRA, risk assessors make preliminary judgments regarding the scope and approach to be used. Subsequently, the four (4) basic steps govern HHRA (EPA 2012).

- **Hazard Identification (HI):** Hazard is defined as any biological, chemical or physical agent that has the potential to cause harm in humans and/or ecological systems. The process of determining hazard examines available scientific data to weigh evidence that characterizes the link between negative health outcomes and the chemical. Scientific data can come in the form of epidemiological studies involving the statistical evaluation of a specific population to examine associations between exposure and human health effect. When human studies are not available for a chemical stressor, risk assessors rely on animal studies such as mice, rabbits and monkeys to gather information about a potential hazard. From these studies, qualitative

thresholds such as “carcinogenic to humans” are used to describe adverse human health outcomes. HI happens in the risk management phase.

- **Dose-Response Assessment (DRA):** Dose-response assessment examines the relationship between effect and exposure. This relationship describes the potential for adverse health effects, or the response, based on exposure to a contaminant (dose). The shape of the dose-response curve is dictated by several factors such as the contaminant type, response endpoint (i.e. cancer, death, etc.), and the type of experiment (i.e. epidemiological, animal). Similar to HI, human subject data are limited and dose-response relationships are anchored in animal studies. However, such studies are limited in their scope and several extrapolations must occur. First, animal studies often involve doses that are higher than seen in humans, so extrapolations to lower doses must be performed. Next, extrapolation must be made from a specific animal species to humans in order to accurately predict a human DRA relationship. These extrapolations compound to introduce a number of uncertainties in the DRA analyses.
- **Exposure Assessment (EA):** Exposure is defined as contact with a chemical, physical or biological agent. Exposure investigations, measure the frequency, time, and amount of human exposure to an environmental agent with considerations to the nature and susceptible populations that could be exposed to the contaminant. The exposure pathway as well as the exposure route is both considered in EA. The exposure route is described by the method of intake or uptake (i.e. oral, dermal, inhalation) whereas the

exposure pathway is described by the path a contaminant takes from the source to subsequent human contact. Mathematically, exposure is defined by:

$$\boldsymbol{E = \int_{t1}^{t2} C(t)\, dt}$$

where E is exposure and C(t) is the concentration that varies with time. For all routes over specific exposure durations (e.g. hourly, daily, or annually), the mathematical exposure equation is:

$$E = \sum (\int_{t1}^{t2} C(t)dt \ldots \int_{ty}^{tz} C(t)dt)$$

- **Risk Characterization:** Subsequent to hazard identification, dose-response assessment, and exposure assessment, risk assessors convey the nature and absence/presence of risk.

The assessment of exposure considers the path an agent takes from its source to human contact, i.e. exposure pathway, and the means of entry into the body, i.e., exposure route.

Oral exposure is the primary route for soil, especially for young children. Once the contaminant crosses a barrier, in this case the mouth, the amount ingested travels to the gastrointestinal (G.I.) tract. The external exposure, or previously called potential dose, is the amount of agent that is inhaled, ingested or dermally absorbed. Once a barrier is crossed, the external exposure becomes the internal exposure. When considering the oral route, internal exposure is defined by the dissolvent of an agent in the G.I. tract allowing it to subsequently become available for absorption across the intestinal lumen into systemic circulation. The amount of contaminant that is available for interaction with a

specific organ or cell is the biologically effective dose. This is the dose that could potentially impart health effects. The internal exposures and biologically effective doses are assessed via specific exposure parameters and scenarios that determine bioaccessibility and bioavailability in humans in order to obtain more accurate risk estimates. For external exposures, the units are usually, mg of toxicant/kg of body weight per day.

2.5 Hazard Identification of Selected Metals

Regardless of the source, heavy metal accumulation can diminish soil quality, reduce agricultural yield and negatively impact the health of humans, animals and the ecosystem. While all these effects of heavy metal buildup are cause for concern, particular focus has traditionally been paid to the human health impact of metals in soils by organizations such as the United States Environmental Protection Agency (U.S. EPA), National Research Council (NRC) and the World Health Organization (WHO). Once an exposure occurs, heavy metals that have accumulated in soils at toxic levels can lead to chronic health effects. For example, arsenic and chromium can negatively impact the kidney and liver while lead is more likely to affect the heart, kidneys, bones, intestines, reproductive and nervous system.

There are several exposure pathways through which humans can be exposed to an environmental contaminant. However, for contaminated soil, incidental ingestion is often the primary route of exposure. For example a HHRA of dioxin in residential soils found that soil ingestion had the largest effect on the

soil criteria and that 75% of one's lifetime exposure occurred before the age of nine (9) (Paustenbach et al., 2005). This fact drives risk assessment of NPL sites in the United States. Incidental ingestion of soil is thought to be greatest in children due to their tendency for hand-to-mouth behavior leading to increased ingestion rates compared to adults (Moya & Phillips, 2014).

2.5.1 Effects on Children

Children are particularly susceptible to the toxic effects of different metals due to their still developing nervous system and brain, their proportionally higher intake of contaminants than adults, and their socio-behavioral activities (Au, 2002). In the case of soil-derived exposure to heavy metals, socio-behavioral activities such as hand-to-mouth tendencies and proclivity for playing on the ground are the main activities leading to exposure.

Our understanding of toxicity of environmental contaminants is based mainly on studies using adults (work environments) and animals (Au, 2002; Casteel et al., 1997; Donaldson et al., 2006). Thus, our knowledge regarding how children are impacted by ingestion of contaminants is impacted by methods for toxicity tests leading to amendments of universal policies that used to apply both to children and adults. Evidence indicated that there are profound differences between children and adults. This has led to enacting legislation, such as the signing of an executive order in 1997 called the Protection of Children from Environmental Health Risk and Safety Risk and the Food Quality Protection Act of 1996, aimed at protecting children's health.

2.6 Selected Heavy Metals

The metals most frequently detected in groundwater and soil are lead (Pb), chromium (Cr), zinc (Zn), arsenic (As), cadmium (Cd), copper (Cu), nickel (Ni) and mercury (Hg). These metals are among the elements that constitute the United States Environmental Protection Agency's (U.S. EPA) "priority pollutant metals" that have potential threat to human health (NRC, 2003). Pb, Cr, As, Cd and Ni were selected for evaluation in this thesis due to their being in higher concentrations in our soil samples, their potential impact on human health, and the wealth of literature that exist regarding oral exposure.

2.6.1 Lead

In the United States, the average arithmetic mean concentration of lead in uncontaminated soil is 19 mg/kg (n=1319) (Ruby et al., 1999). Native lead can occur in varying forms such as lead sulfide (PbS), lead sulfate ($PbSO_4$) and lead carbonate ($PbCO_3$). Lead sulfide can be found primarily at mining, milling, and smelting and ore-handling sites. Lead sulfate and lead carbonate are more commonly a result of precipitation reactions in soil with the formation of $PbSO_4$ being favored in acidic soils and $PbCO_3$ being favored in alkaline soils. Lead can also be sorbed to various mineral phases such as iron and manganese oxides, iron sulfates, and phosphate minerals as a result of weathering.

Soil laden with lead comes from historical anthropogenic use and sources include gasoline additives, paint, batteries, and ammunition. The most common forms released by these sources are lead oxide and carbonate species, which are

highly soluble forms of lead. In general, the solubility of a metal is important as the toxicant must dissolve in the specified physiological fluid to become bioaccessible and thereby bioavailable (Hillwalker & Anderson, 2014; Wragg et al., 2003).

2.6.1.1 Legislation

Lead is a naturally occurring element that has been distributed into the environment by its historic widespread use in products such as gasoline, household paint and pipes. Significant production and redistribution of lead use has been documented as early as 3000 BC. In fact, the Roman Empire used lead widely and lead's slightly sweet taste made it an attractive additive for Roman wine. Lead was also added to paint to increase durability where the sweet taste was also appealing to young children. Although the use of lead in many capacities has been phased out, the residues of lead products still remain in our soil ecosystem. Currently, several measures and federal laws are in place to limit lead exposure, especially in young children. The Center for Disease Control (CDC) currently sets a reference level of 5 µg/dL to identify children with dangerous blood lead levels (BLLs). This reference level is based on protecting children one (1) to five (5) years of age (CDC, 2013).

Lead has been actively monitored as one of the six criteria pollutants since the passing of the Clean Air Act of 1970, and subsequent EPA establishment of a national ambient air quality standard (NAAQS) of 1.5 µg/m^3 (maximum averaged quarterly) as part of the Clean Air Act Amendment of 1977. The reduction in usage of lead in gasoline was primarily related to increased awareness of the negative

health effects of lead, particularly in children, and the invention of the catalytic converter present in all car models built after 1975. Lead poisoned catalysts used to reduce ozone precursors and was phased out because of that, and serendipity led to Pb reductions in the air (Lioy & Georgopoulos, 2011). In 1978, lead in household paint was eventually banned and phased out by the Consumer Product Safety Commission (CSPC), while the U.S. EPA requires that homes built before then be tested for lead and remediated if lead paint is present. Whereas the U.S. Housing Department of Urban Development (HUD) requires federally assisted housing to notify, evaluate and reduce lead-based paint hazards. Additionally, in 1988 the Lead Contamination Control Act required the CPSC, U.S. EPA and states monitor lead in schools and fix levels that are deemed excessive.

Presently, the U.S. EPA and U.S. Department of Housing and Urban Development (HUD) has established a 400 ppm standard for children's play areas and an average of 1200 ppm standard in the remainder of the yard (EPA, 2001). The U.S. EPA also believes that more than 12 million homes exceed a 400ppm yard-wide standard while 4.7 million homes exceed the standard 1200 ppm Pb threshold (Zia et al., 2011). Implications for high yard Pb level in US homes are significant. For example, concentrations of Pb in house dusts is significantly correlated to Pb in garden soil (Thornton et al., 1985) and a meta-analysis of contributions from soil versus house dusts to BLLs has shown that house dusts have a considerably higher contribution than soil (Lanphear et al., 1998). However, it is not always practical or possible to collect house dusts. When the correlation between garden soil and house dusts is taken into consideration,

assessment of soil provides useful basis and possible surrogate for area/regional risk assessment of Pb.

2.6.1.2 Impact on children

Children under the age of six are particularly susceptible to the consequences of lead exposures as it is extremely damaging to their still developing neurological system and incomplete development of the blood-brain barrier (fetuses and children under three (3) years of age). Almost all children are exposed to lead; six (6) % of children in the U.S. under the age of two (2) have blood lead levels (BLLs) above 10 µg/dL and the percentage of children in the toxic BLL range is 11% for black, non-Hispanic children under the age of five (5) (CDC, 2013). Even at a level below 5 µg/dL, children my exhibit hyperactivity, delayed growth and hearing loss. However, as the exposure is more prolonged, lead can cause permanent brain damage and even death.

2.6.2 Arsenic

Attention has been given to understanding the mechanisms and factors that control and affect arsenic bioavailability. Arsenic is the second most abundant contaminant at U.S. EPA Superfund sites (EPA, 2001). In the U.S., arsenic's arithmetic mean concentration is 7.2 mg/kg (n=1257) (Ruby et al., 1999). Arsenic occurs naturally in two valence states: III and V. Anthropogenic sources of arsenic are mining and smelting, agricultural use of insecticides and pesticides, feed additives, tanning, coal burning, etc. Arsenic is also a naturally occurring

contaminant in water, especially in NJ. Arsenic can occur as sulfide minerals or more commonly co-precipitate with iron. Arsenic commonly co-occurs with lead and like lead, it's solubility, especially in the anionic form, makes it quite mobile and soluble and thereby bioavailable in certain types of soil matrices.

As with lead, ingestion is the predominant route of exposure to As with diet and drinking water being the largest source for adults. Hand-to-mouth activity also contributes to oral ingestion in children. Once ingested, arsenate (As V) partially reduces to arsenite (As III), which yields a mixture of both valence states in the blood. In the liver, As III undergoes methylation to form MMA and DMA, monomethylarsonic and dimethylarsonic, respectively. Most inorganic arsenic is excreted in the urine and a smaller amount in the feces. In humans, MMA and DMA are not readily metabolized and do not readily enter the cell, leaving them unchanged as they are excreted in urine. Due to the latter, measurement of urinary arsenic is commonly used as a biomarker of recent arsenic exposure.

Arsenic is most extensively absorbed in the gastrointestinal tract from drinking water. Relative to its presence in water in a water-soluble form, arsenic contaminants in soil may be present in water-insoluble forms or not absorbable due to their reaction with other constituents.

2.6.2.1 Legislation

Under the Clean Air Act of 1970, arsenic is defined as a hazardous air pollutant (HAP), i.e. a substance that may cause serious harm or death in humans following substantial exposure (EPA, 2007). Additionally, under CERCLA, the EPA and Agency for Toxic Substances and Disease Registry (ATSDR) is required to

prepare a substance priority list of substances commonly found at sites on the NPL. Arsenic is ranked number one (1) on the ATSDR substance priorities list and in 1986, National Emission Standards were created for arsenic plants, glass manufacturing plants and primary copper smelters that are known to emit inorganic arsenic. There is no ambient air standard for arsenic. However, in drinking water, the allowable level of As is 10 ppb. In food, the United States Food and Drug Administration (U.S. FDA) established allowable levels of arsenic that range from 0.5 to 2ppm depending on the source. Additionally, the International Agency for Research on Cancer (IARC) determined that inorganic arsenic is a group 1 known human carcinogen.

2.6.2.3 Impacts on Children

Children may be exposed to arsenic in soil much in same way that they are exposed to lead: via hand-to-mouth contact. However, the biggest source of arsenic exposure is through drinking water. The effects of inorganic arsenic have been recognized and documented since ancient times with large oral doses resulting in death. Smaller doses of arsenic may result in vomiting, diarrhea and nausea. Additionally, long-term exposure to arsenic may result in lower IQ. Exposure to high levels of arsenic exhibits symptoms similar to those in adults such as cardiovascular, neurological and dermal effects. However, unlike lead, it is unclear if there is a difference between adult and children inorganic arsenic adsorption in the gut. Some evidence suggests that children metabolize inorganic arsenic less efficiently than adults.

2.6.3 Chromium

Chromium is released into the environment from industries such as electroplating, leather tanning and textile production. Releases from industries that use chromium, with the addition of chromium released from the burning of natural gas, oil or coal, can be found in air, soil, and water. Of the 1,699 current or former NPL sites, chromium has been found in at least 1,127 sites targeted for long-term federal cleanup (ATSDR, 2012). Chromium occurs naturally in rocks, animals, plants and soil, existing in a combination with other elements in two main forms: chromium III (Cr III) and chromium VI (Cr VI).

Exposure to trace levels of chromium in air can occur from industrial releases and cigarette smoke. Cigarette smoke can contaminate indoor air at levels 10-400 times greater than outdoor air concentrations. Increased potential exposure to chromium in air can occur in the workplace, specifically to workers in metallurgy and tanning industries. Chromium in the atmosphere is usually deposited into soil and water. The general population may be exposes via drinking water or food. Low levels of Cr III occur naturally in various food items and Cr III is considered essential to human health in small amounts.

Once chromium enters the body, Cr VI is changed to Cr III. Most of the chromium will leave the body in the urine and some will remain in cells for at least several years (ATSDR, 2012). In workers, where work environment air concentrations are much higher than levels found normally in ambient air, the most common health effects involves the respiratory tract such as irritation of the nose lining, asthma, cough and wheezing. Inhalation of Cr VI has been shown to

cause lung cancer in workers. These respiratory tract issues in humans were similar to issues seen in animals exposed to chromium in air. In contrast, studies involving populations living in areas with high levels of Cr VI has shown mixed results with cancer as an endpoint (ATSDR, 2012). Oral ingestion animal studies have shown Cr VI causes tumors in the stomach and intestinal tract. The health effects of chromium are mainly based on animal studies where Cr III has minimal health effects and Cr VI causes irritation to the stomach and small intestine and blood. The IARC has determined that Cr VI is carcinogenic to humans.

Maximum levels for total chromium in drinking water, set by the EPA, and bottled water, set by the FDA, have been established at levels of 0.1 mg/L. In workplace air, the Occupational Safety and Health Administration (OSHA), has set a legal limit for Cr VI of 0.005 mg/m^3 and 0.5 mg/m^3 for Cr III averaged over an eight (8) -hour workday. While the National Institute for Occupational Safety and Health (NIOSH) recommends an exposure limit of 0.5 mg/m^3 for Cr III and Cr VI compounds in air averaged over an 8-hour workday.

Northern New Jersey was the chromite/chromate capital in the world during the first half of the 1900s where 2 to 3 million tons of chromite ore processing residue were produced in Hudson County (Lioy et al., 2008). In Hudson County, 200 chromium waste sites have been identified and documented by the New Jersey Department of Environmental Protection where most sites have been remediated and continue to be monitored. One such site included Liberty State Park/NIST SRM 2701 (NIST, 2013) soil that is used extensively in this dissertation. By the mid 1970's, NJDEP started buying the land and

eventually opened Liberty State Park. Since then, its historic use as a landfill has made it the target of remedial investigations (RIs). Remedial action as an outcome of the RIs resulted in contaminated soil sites being delineated, capped or transitioned to a suitable use that does not pose a risk to human health. Furthermore, various groups (Fagliano et al., 1997; N. C. G. Freeman et al., 1997; P. Lioy et al., 1992; Stern et al., 1998) have demonstrated the connection between house dusts and Jersey City and Bayonne waste sites prior to their clean up.

2.6.4 Nickel

Nickel occurs naturally and is found in all soil. It is emitted from volcanoes, discharged from industries into wastewater, and released into the air by coal-burning power plants. Nickel is found in over half of the 1,662 current and/or former NPL sites. Nickel has many properties that make it desirable to be combined with other metals to form alloys with iron, copper, chromium and zinc (ATSDR, 2005). Nickel alloys are used to make items such as metal coins, jewelry, valves and heat exchangers and Ni is used to make stainless steel, plating, color ceramics and some batteries. In addition, nickel is present in high concentrations in residual oil combustion emissions from oil-fired power plants (Lippmann et al., 2006).

Most of the Ni released into the environment ends up in soil or sediment where it strongly attaches to iron or manganese (ATSDR, 2007). In the U.S., the concentration of nickel in soil is between 4 to 80 ppm. The highest concentrations

of nickel (on order of 9,000 ppm) are found near nickel ore processing sites where nickel is extracted from its ore and used to make various consumer and industrial products such as stainless steel, batteries and guitar strings. The general population is exposed to nickel in their food on a daily basis, which is the primary route of exposure. Approximately one hundred and seventy (170) micrograms of nickel is consumed every day and Ni is naturally high in chocolate, soybeans, nuts and oatmeal. Daily intake of water accounts for two (2) micrograms of the daily consumption rate. After nickel enters the body, it distributes to each organ system, but it mainly ends up in the kidney and any nickel that enters into the bloodstream is quickly excreted in the urine and feces.

Allergy is the most common adverse health outcomes in humans with about ten (10) to twenty (20) % of the population having a nickel allergy. It takes a sufficiently large amount of nickel contact to induce harmful health effects. Higher dosages of nickel can cause adverse effects in the blood and kidneys via increased red blood cells and increased urine proteins, respectively. Oral ingestion of high levels of nickel is rare; therefore negative oral health outcomes are often based on animal studies. Nickel produces lung disease in dogs and rats and affects the stomach, blood, liver, kidneys and immune system in rats and mice. Despite the lack of human toxicity data, the IARC has classified metallic nickel as a group 2B (possible human carcinogen) compound and nickel compounds as group 1 (carcinogenic to humans) materials.

The health effects of acute oral toxicity are derived from reports of accidental exposures. When accidental ingestion of water containing 7.1 to 35.7

mg Ni/kg nickel sulfate and nickel chloride occurred, workers reported gastrointestinal upset and neurological symptoms such as vomiting and weariness, respectively. Available data regarding nickel toxicity is limited and insufficient to establish toxicity thresholds. Chronic exposure derived MRLs (minimal risk levels in humans), LOAELs (lowest observed adverse effects level), and NOAELs (no observed adverse effect levels) vary widely, ≥1.3 mg Ni/kg/day, 1.3 to 90 mg Ni/kg/day, and 2.2 to 45 mg Ni/kg/day, respectively, due to the limited number of animal studies (ATSDR, 2005).

Nickel is an essential trace element in animals; however, whether it is essential in humans has not been established. Nickel deficiency in various animal species such as rats, chicks, cows and goats is manifested primarily in the liver with effects including abnormal cellular morphology and oxidative metabolism. It is unknown whether children and adults differ in their susceptibility to nickel. However animal studies have found increased newborn deaths and decreased newborn weight after nickel ingestion at doses 1000 times higher than found in drinking water. Children can also be exposed to nickel through household dust, which poses increased risk in younger children who have greater contact with the floor.

2.6.5 Cadmium

Cadmium is emitted into soil, water and air by metal mining and refining (non-ferrous), fossil fuel combustion, waste disposal and incineration, and phosphate fertilizer (manufacture and application). Cd is found in the earth's

crust and is also used for batteries, pigments, as a plastic stabilizer, and for coatings and plating. Cadmium is commonly associated with zinc, lead, and copper ores and is also a natural constituent in ocean water. Cd can be transported long distances in the air before being deposited onto soil and into water. In soil, cadmium is mostly immobile in soil due to its tendency to strongly bind to organic matter. Cd is taken up by plant life and eventually enters the food supply.

In the U.S., the primary source of Cd in non-smokers is from food with levels as high as 0.05-0.12 mg/day. Since tobacco leaves accumulate high levels of cadmium the levels absorbed from smoking one pack a day can reach 1-5 µg/day. In uncontaminated soil, concentrations of cadmium varies between 0.6 and 1.1 mg/kg (Faroon et al., 2012). Inhalation and oral ingestion of Cd-contaminated water is not expected to be a major concern except for people living near cadmium-emitting industries.

Approximately 1-10% of the cadmium in food and water will be absorbed by the body and once inside, most of the cadmium that enters goes to the kidney and liver where it remains for years at a time. A small portion of Cd will slowly be excreted in urine and feces. In humans, oral ingestion of high levels of cadmium can lead to stomach irritation, vomiting and diarrhea, and possibly death. In laboratory animals, kidney and bone effects have been seen including but not limited to anemia, liver disease, and nerve or brain damage. The IARC considers cadmium to be a human carcinogen while the EPA considers cadmium a probable carcinogen.

A few animal studies show that younger animals absorb more cadmium than adults. Animal studies also indicate that the young are more susceptible to loss of bone and loss of bone strength. Studies in animals exposed to high levels of cadmium during pregnancy resulted in reduced body weights and skeletons in the young as well as targeting the central nervous system (Faroon et al., 2012). Cadmium can be found in breast milk and may expose an infant through breastfeeding.

The EPA has determined that exposure to cadmium in water should not cause adverse health effects in children as long as 40 µg/L in 10 days is not exceeded. In bottled water, the FDA has set cadmium levels at 5 µg/L and OSHA has set a legal limit of 5 $\mu g/m^3$ averaged over an 8-hr workday.

2.7 The Gastrointestinal System

Understanding the gastrointestinal system and how pollutants, particularly metals, act once ingested is a necessary component to assessing contaminated soil risk and, from the stand point of this thesis, to appreciate the basis of *in vitro* gastrointestinal surrogates.

As food and/or nonfood items (NFIs) enter the digestive system via the mouth, they are masticated or chewed to break down the material and increase the surface area. The material is then swallowed and transported through the esophagus by peristalsis (wavelike muscular contractions) to the stomach where it is digested and broken down even further mechanically and chemically. Materials are then transferred via absorption through the intestinal lumen and into the blood

stream. Indigestible waste products are then discharged from the body through defecation. Organs such as the liver and pancreas aid in the digestive process by producing and secreting resources that aid in digestion while the colon absorbs water and electrolytes from the material. From this description we can see that as ingested soil moves through the gastrointestinal system, it interacts with a series of fluid compositions with fluctuating pH and residence times. Table 2.1 summarizes the order and conditions a soil material would possibly face in each gastrointestinal compartment.

Table 2. 1 Modified from (Oomen et al., 2002). Residence time and pH of four (4) digestive compartments.

Order of interaction	Compartment	Residence Time	pH
1	Oral	Seconds to minutes	6.5
2	Stomach	Fasting ½ life: 8-15 min Fed ½ life: 0.5-3 hr	1-2 2-5
3	Small Intestine Duodenum Jejunum Ileum	 0.5-0.75 hr 1.5-2 hr 5-7 hr	 4-5.5 5.5-7 7-7.5
4	Colon	15-60 hr	6-7.5

Many transformations can occur between an exposure to a contaminant and the manifestation of adverse health effects. Further, once ingested, contaminants may face a complicated path before entering systemic circulation or before being eliminated in feces and/or urine. Understanding this path and the conditions that will impact internal dose is anchored in understating the human physiological systems they will encounter.

Among the routes available for exposure for heavy metals, inadvertent and advertent ingestion is considered a major concern compared to dermal and inhalation (Ruby et al., 1999; Zia et al., 2011). Exposure can occur via inhalation of heavy metal-containing particles in the air where a small amount is actually swallowed instead of inhaled. Contact frequently happens after ingestion of food that contains heavy metals from contaminated soil. However, the most direct route of exposure to heavy metals is direct ingestion of contaminated soil and/dust themselves.

2.7.1 Gastrointestinal factors that influence *in vitro* bioaccessibility methods.

In vitro methods for oral bioaccessibility seek to mimic the major processes in the gastrointestinal tract. The mouth, the stomach and the (small) intestines have all been identified as major components in the digestive process. However, while the mouth is an essential component in the digestion process, the fact that the time materials remain in this compartment is transient leads this compartment to being consistently excluded from many bioaccessibility methods development and applications.

The stomach compartment is more routinely mimicked in *in vitro* extraction methods than any other compartment in the gastrointestinal system. This compartment is largely regulated by pH, with most *in vitro* methods seeking to mimic fasting conditions between pH 1 and 2. The use of the gastric compartment only is not truly appropriate for the *in vitro* extraction of metals since the pH

increases significantly once it moves from the gastric compartment to the intestine. This change in pH significantly reduces (impacts) metal solubility. Clearly, the gastric compartment selection for bioaccessibility experiments is a matter of convenience in methods application, not for achieving the best representation of bioaccessibility.

Most bioaccessibility studies have been validated for lead and a few experiments included the intestinal compartment because the solubility of Pb significantly decreases with increasing pH. As the pH becomes near neutral (pH 7), this change renders most of the Pb insoluble and thereby excreted in waste, and not contributing to risk. Accounting for just the dissolution of a toxicant in gastric fluid provides only the “worst-case” scenario, and is no better than leachable extractions where acids are used to extract metals into a fluid for analysis in representing the bioaccessible mass. Furthermore, by using just the gastric fluid, a closer simulation of human physiology is traded for simplicity, which creates models that are not representative of intestinal absorption and eventual transport into systemic circulation. Conversely, methods that include the intestinal component often mimic the three compartments of the small intestine (duodenum, jejunum and ileum) by adding fluids composed of enzymes, bile salts and bicarbonate.

The decision to include or omit one or more of these compartments shown in Table 2.1 will affect the measured bioaccessibility and subsequent absorption of each metal differently. Inclusion or omission (fed or fasting conditions) of food also affects bioaccessibility by way of impacting the pH of the fluid in each

compartment. Presently very few gastrointestinal models consider the small intestine and even fewer include the colon in their analogues. These omissions, especially the intestine, can have significant impacts due to the significant differences in physiochemical conditions within each gastrointestinal compartment and the high bacteria biome of the colon and intestine.

While there are many indirect methods available to measure lead bioaccessibility in soils, regardless of the chosen procedure it is largely controlled by pH, phosphates, organic matter, and iron content. In the human digestive tract, the pH can vary widely from a pH of 1.0 to 8.5. This variation in pH has proven difficult to standardize for oral bioavailability because it not only varies from person to person, but also varies with fast/fed states, diet, and age making a gastric only system a poor methodology. For example, as the pH of a gastrointestinal fluid increases, the bioavailability Pb decreases. Phosphates precipitate Pb whereas organic matter and iron oxides provide chelation sites for Pb binding. Most indirect methods for bioaccessibility/bioavailability use pH as a control for heavy metal dissolution in gastrointestinal fluids as it is the easiest to control and monitor and is the best compromise between ease of use and biological application.

2.8 Oral Bioavailability and Bioaccessibility

2.8.1 *In vitro/in vivo* correlations

Given that oral bioavailability is determined by the amount of contaminant that is able to cross the intestinal mucosa and enter into systemic circulation, *in vivo* animal trials are carried out in order to assess the amount of metals that end

up in the blood and/or organs. Animal surrogates such as immature swine are often used due to the presence of similar gastrointestinal traits to humans. Other species tested include rats (Ellickson et al., 2001), rabbits (Davis et al. 1992; Freeman et al. 1993) and monkeys (Freeman et al 1995). Physiological differences exist between humans and animal surrogates thereby making it difficult to interpret data and their relationship with human health. Despite extrapolation uncertainties, *in vivo* systems can still provide useful information regarding bioavailability and toxicity endpoints; something that cannot be assessed using *in vitro* systems alone.

2.8.1.1 Human Studies of Oral Bioavailability

To date, there is one human trial for oral lead bioavailability involving soil. This study was completed by Maddaloni et al. (1998) where isotopic measurements were taken to study the blood lead uptake from adults dosed with soil contaminated by mining activity in the area. On average, 26.2% of the total lead was taken into systemic circulation. This experiment has become the basis for subsequent measures of bioavailability and is often used as reference for ingestion soil lead bioavailability in adults.

There have been several studies on the prevalence of ingestion of soil and dust by children. Early studies were based on survey responses that were used to estimate the prevalence of dust and soil ingestion. Tracer element methodology is a biomarker method that has also been used to quantify soil and dust ingestion where it is assumed that tracers are assumed to not be metabolized or absorbed

in large quantities in the human body (Moya & Phillips, 2014). The last method that is commonly used is the biokinetic model that predicts soil ingestion based on biomarker measurements in the blood and urine for age groups. In particular, Gavrelis et al. (2011) did a U.S. nationwide study which evaluated ~21,000 individuals between the ages of 1 to 74 years of age and ~25,000 individuals between 0.5 and 74 years of age. The prevalence of non-food item consumption was 21% for the 1 to 3 year of age.

2.8.1.1.2 Arsenic

MEALEY et al. (1959) used radiolabeled arsenic (As^{74}) to follow plasma arsenic concentrations in four (4) human subjects for ten (10) days. After an initial rapid clearance in the first few hours, arsenic clearance occurred at a much slower rate with a terminal elimination rate that appeared after 7 days (half-life of 86 hours). Urinary arsenic recovery over 9 days was 57 to 90% of the intravenous dose. Ducoff et al. (1948) administered sodium arsenite to two (2) human subjects intravenously and collected urine and feces over a period of seven (7) days. For the two subjects, urine recovery was 65.7 and 59.1% and feces recovery was 0.9 and 0.5% (G. B. Freeman et al., 1995). The average urinary arsenic excretory dose in the Bettley and O'Shea (1975) study was 52% and ≤3.5% for fecal excretion. Dose recovery from six subjects yielded 62.3 ± 4.0% arsenic dose excreted in urine and 6.1 ± 2.8% of arsenic dose eliminated in feces. For risk assessment purposes, bioavailability is often evaluated in terms of total arsenic rather than its form in systemic circulation. While the decision to use total arsenic rather than its specific

form may lead to assessing all possible sources of As risk, it may also lead to unreasonable estimates of bioavailability.

2.8.1.2 Bioavailability Studies: Mice

Mice are often chosen as a test species due to their low cost of purchase and low cost of husbandry, handling ease, increased assay sample size, and widespread usage (Bradham et al., 2011). Physiologically, mice are well characterized and their gastrointestinal system can be manipulated biologically to determine absorption of metals and metalloids. While humans and mice have different metabolism, several studies have determined that the similarities between them are sufficient enough in physiologically based pharmacokinetic models to allow for scaling between human and mouse assays (Evans et al., 2008; Hughes et al., 2008)

2.8.1.3 Arsenic bioavailability

2.8.1.3.1 in Mice

Urine is the dominant route for arsenic clearance post oral ingestion of inorganic As, which makes it the ideal candidate for estimating the extent of dietary absorption. In mouse assays, the amount of As excreted in urine and feces during the experimental period is used to approximate recovery of As. However, total As recovery will not be achieved since these estimates do not include As retained in the tissues of the mice. On the other hand, it could be part of the biologically effective dose.

When we compare the dietary component of the mouse assay with the average human diet in more developed countries, human diets get more calories from fat, less from fiber, and less than optimal vitamin and mineral compositions. Dietary variations can affect gastrointestinal absorption in many ways, most importantly by altering the microbiota of the gastrointestinal tract. However, since most *in vitro* assays extraction assays do not take the microbiota into account, the dietary component (i.e. changes produced by the dietary composition) is mainly examined in *in vitro* animal models.

2.8.1.3.2 Juvenile Swine and Monkeys As Bioavailability

Soil RBA for As in juvenile swine and monkeys has a range between 0% and 52% (Casteel et al., 1997; G. B. Freeman et al., 1995; Rees et al., 2009; S. M. Roberts et al., 2007; Rodriguez et al., 1999). When compared to the mice assay, differences in experimental design and dosing levels highlight the physiological variances between species. However, there are very few soil experiments involving both species warranting further studies to evaluate possible sources of variability (Stephen M. Roberts et al., 2002).

In vivo assays are necessary surrogates because of severe ethical issues with controlled human toxicity studies involving toxic chemicals. While useful, animal assays are relatively expensive, lengthy and cannot be easily adapted into a high throughput assay. These factors have given rise to the development of *in vitro* bioaccessibility methods that mimic human physiological characteristics. Given the lack of human bioavailability data, *in vitro* assays are often validated by

in vivo animal models, which can create a closer link between *in vitro* bioaccessibility and *in vivo* bioavailability. Furthermore, in 2007 the National Research Council (NRC) reported and recommended the development and validation of *in vitro* assays that can replace *in vivo* assays. The focus of this recommendation lies in reliable and accurate data that can reduce uncertainty in risk assessment as well as reduce dependence on animal studies.

Table 2.2 outlines previous studies involving As bioavailability using animal models. Relative bioavailability (RBA) values ranged from averaged 48% in a rabbit model, ranged from 0-78% in a swine model and 10-28% in a monkey system. The primate system shows a lower RBA for contaminated soil compared to a swine and rabbit model.

Table 2. 2 Literature Reports of Arsenic Relative Bioavailability. Modified from (Stephen M. Roberts et al., 2002)

Study	Animal	Soil Type	Relative bioavailability
G. Freeman et al. (1993)	Rabbit	Smelter area soils	48%
Lorenzana et al. (1996)	Swine	Mining area soils Mining area slag	78% 42%
Casteel et al. (1997)	Swine	Soil and mining area wastes	0-50%
G. B. Freeman et al. (1995)	Monkey	Mining area soils Mining area dusts	20% 28%
Stephen M. Roberts et al. (2002)	Monkey	Contaminated Soil	10-25%

2.8.2 Current *In Vitro* Methods for Bioaccessibility.

The utility of *in vitro* protocols for gastrointestinal bioaccessibility of harmful contaminants present in soil has been consistently evaluated as a rapid and inexpensive alternative to costlier *in vivo* experiments. Current methodologies vary widely in approach. However, two strategies seem to underlie each tactic:

- Chemical extractions at low pH
- Mimicking human gastrointestinal, biological and chemical conditions

Each line of experimentation for assessing bioaccessibility has its own advantages and disadvantages. Furthermore, research needs to be performed in order to validate and standardize *in vitro* results and methodologies for use in risk assessment (Wragg et al., 2003).

Worst-case scenarios and assumptions are easy to use with little risk of being too incautious, especially with soil sites that can be quite complex due to soil quality of different matrices, contaminant and contaminant species, and site conditions. It is often assumed that all of the metals present in soil become bioavailable, representing the conservative approach. *In vivo* tests on soil, however, indicate that only a fraction of the contaminants in the soil matrix becomes bioaccessible. By using *in vitro* methodologies, it is possible to quantify the bioaccessible fraction post ingestion of contaminated soils. However, the conservative approach sacrifices biological relevance for ease of use and protective capabilities. The addition of the intestinal compartment and subsequently the colon reduces simplicity of the *in vitro* system while increases

biological relevance. Furthermore, *in vitro* studies may adequately predict bioavailability; however, they do not provide absolute bioavailability data, which, at present, can only be achieved via *in vivo* testing. Yet, exclusive use of *in vivo* testing is not feasible on a global scale; thus, creating a need for widely applicable *in vitro* studies.

Young children are considered particularly susceptible to ingestion exposure given their hand-to-mouth activity and increased susceptibility to accidental ingestion. Additionally, children absorb an increased percentage of toxicants through their digestive system as compared to adults. In order to be useful to risk assessors, bioaccessibility tests must be simple to use and generally applicable to multiple soil matrices and metals/metalloids (NRC, 2003). The latter represents a complex caveat given the wide variety of soil types, conditions, as well as the range of contaminants, contaminant species and mixtures.

In vitro bioaccessibility is calculated as the follows:

$$\%IVBA = \frac{in\ vitro\ extractable\ mg_{metal}/kg_{soil}}{total\ contaminant\ mg_{metal}/kg_{soil}} \text{ x } 100$$

where the denominator is calculated using an acid-extractable digestion method for total concentration (EPA method 3051A) or in the case of a standard reference material (SRM), the denominator is the certified concentration.

There are a wide variety of *in vitro* bioaccessibility methods that exist in the literature. However, in searching for a bioaccessibility assay, several factors were taken into account:

- Validation method
- Calibrated for a fasted state instead of fed
- Ease of use
- Biological relevance

In consideration of these factors, four (4) *in vitro* bioaccessibility assays described below were reviewed for use in this dissertation.

2.8.2.1 The Physiologically Based Extraction Test (PBET)

Developed by Ruby et al. 1993, the PBET test simulates the leaching of a solid in the gastrointestinal tract and subsequently the fraction available for transport across the intestinal membrane. This two-stage sequential extraction (gastric and intestinal) was designed for a toddler due to their tendency to ingest soil at a higher rate than other age groups. The extraction is carried out at 37ºC and argon purged to keep the system under anoxic conditions and extract metal concentrations are subsequently measured by Inductively Coupled Plasma Spectroscopy.

Data obtained from this model has been linearly correlated with results from Sprague-Dawley rats, for an r^2=0.93 between the *in vivo* and *in vitro* model. When compared to rabbit and primate models (Ruby et al 1996), arsenic was found to be over-predictive of bioavailability using the PBET test. In 1999, Ruby et al. compared blood levels of children in homes with leaded house dust and found that the PBET model was useful in predicting ingestion bioaccessibility for lead. However, the utility of the PBET model is hampered by the fact that the

procedure is cumbrous, making it difficult to execute with large sample batches that would be associated with a RI.

2.8.2.2 Simplified Bioaccessibility Extraction Test (SBET)

Medlin 1997 simplified the PBET extraction procedure specifically for lead. Using only the stomach phase of the PBET technique, Medlin obtained a correlation coefficient of 0.85, which was later shown to correlate well with lead in young swine and weanling rats. Drexler 1999 further refined Medlin's findings to develop what is now called the SBET test. At the request of U.S. EPA Region VIII and the need for simple bioaccessibility testing regimes, the SBET test has undergone extensive validation for lead and was developed by testing soils that had previously been studied in swine and other animal models. Given that this method simplified the reagents and uses a single extraction fluid, this extraction is ideal for large batch samples. However, the SBET method *only* utilizes the stomach compartment of the gastrointestinal system and is considered an accurate model system for lead since at pH values above 5.5 lead is insoluble and would therefore be excreted as a non-soluble product. This method is undergoing further validation for arsenic, but unquantifiable losses of arsenic in the system leads to questions in the validity of the swine model. Additionally, the *in vivo* database for arsenic pales in comparison to the database for lead. This limits the test in its applicability outside of lead and will overestimate lead bioaccessibility. This method is the basis of the standard operating procedure for an *in vitro* bioaccessibility assay for lead in soil that is currently adopted by the

EPA where *in vivo* RBA values are calculated using test animals such as juvenile swine and mice.

2.8.2.3 Hamel Mass Balance and Soil Recapture (HMBSR)

This method uses a three-compartment model to simulate the leaching of a solid matrix as it moves through the gastrointestinal tract. The artificial saliva used was described by Fusyama et al. 1963 and the US Pharmacopoeia formula for the gastric fluid was used. The intestinal fluid was simplified to a 0.2 M sodium carbonate solution. The combination of these three fluids in a sequential extraction at 37º C intends to mimic the *in vivo* processes that occur before a given contaminant crosses the intestinal barrier. Hamel compared her results from the 1999 study to the Maddaloni et al. 1998 that characterized soil bioavailability of soil in human subjects. The HMBSR method overestimated the bioavailable fraction found in Maddaloni et al.'s study indicating more aggressive leaching from the HMBSR method.

Ellickson et al. 2001 validated the HMBSR method using a NIST SRM soil in Sprague-Dawley rats to compare bioaccessibility with bioavailability. In this study bioaccessibility did not correlate on a one-to-one basis with bioavailability compared to previous studies (Ruby et al. 1996). Rather, due to interspecies variation and the limitation of the HMBSR in mimicking intestinal absorption, bioaccessibility produces larger results when compared to bioavailability. There are advantages and disadvantages associated with all models for bio accessibility. However, consideration must be given to validation methods and

the differences between human and animal physiology that drive GI absorption and subsequent system-wide circulation. These considerations should drive decision-making with regard to method of choice.

2.8.2.4 Solubility/Bioavailability Research Consortium (SBRC) assay

This method uses the gastric compartment for a single-phase extraction. One (1) gram of test substrate is added to 100 ml of gastric fluid consisting of 0.4 M glycine at pH 1.5 (adjusted with nitric acid, HNO_3). Each sample is then placed into an end over end rotator in a 37°C water bath for 1 hr.

The SBRC assay is validated against a mouse and juvenile swine. For arsenic, there exists a high correlation (R^2=0.92; Pearson Correlation=0.96) between SBRC bioaccessible As and the RBA estimate from juvenile swine. A high correlation also exists between the SBRC assay and the juvenile swine assay for As (R^2=0.75; Pearson Correlation=0.87). This high correlation between *in vitro* and *in vivo* assays suggests the utility of the SBRC assay to predict bioaccessibility for Pb and As. However, this method lacks applicability on a widespread basis because this method trades biological relevance for simplicity with the exclusion of the intestinal compartment. Further, only testing for two metals precludes the use of this method on new metals without knowing the rate of dissolution in the intestinal fluid, at a minimum.

Another factor that influenced the selection criteria of an *in vitro* extraction method was the ability to use the raw extracts in a cellular-based assay. The use of a gastric only system was quickly eliminated as the acidic nature of simulated

gastric fluids that would quickly kill cells in our assay. A more attractive GI fluid alternative was the use of the raw intestinal fluid extracts (often a combination of saliva, gastric and intestinal fluids) whose pH was near neutral. An examination of viability of our cells in our assay with selected buffers (PBS, HBSS), cellular media, simulated gastric fluid, and simulated intestinal fluid showed that simulated gastric fluid had the most significant decrease in viability compared to other media.

Considering all of these factors, biological relevance, applicability on a wide scale, and usability in a cellular-based assay, the HMBSR method was chosen as the IVBA method for this dissertation.

2.9 Biologically Effective Dose

An important part of a risk assessment is the effect that the biological dose has on the target organ. In the case of metals, the liver is considered to be among the organs targeted as the liver plays an important role in the detoxification of contaminants. The first-pass effect metabolizes many agents, thereby greatly reducing the bioavailability as toxicants are carried through the portal vein into the liver before it reaches systemic circulation. The liver maintains homeostasis thereby protecting the human body against ingested toxins. Due to the liver's vital role in digestion and detoxification, this organ was chosen for further research herein in this dissertation to analyze the link between bioaccessibility and liver cytotoxicity. While this choice limits the test's applicability, it was reasoned as the best organ model choice for this system.

The liver is the largest gland and organ in the human body. It is connected to the hepatic artery and the portal vein, which carries oxygen rich blood from the aorta and blood containing digested nutrients from the GI tract, spleen and pancreas, respectively. The blood vessels are subdivided into sinusoids, or small capillaries, and lobules made of hepatic cells.

Hepatocytes, liver epithelial cells, make up 70 to 85 % of the liver's mass and are involved in important roles such as the detoxification and excretion of contaminants and the initiation of the formation and secretion of bile that aids in the digestion of lipids in the small intestine. The epithelial barrier that is between the capillary sinusoids that connect the portal vein to the central vein is often only one cell thick. Sinusoids carry blood to the central vein and are lined by phagocytic cells or macrophages called Kupffer cells and sinusoid cells that line the blood vessels.

Hepatocyte cultures are commonly used in the cell biology and in the pharmaceutical industry as *in vitro* model system for the study of liver metabolism and contaminant toxicity (Guillouzo et al., 2007). This makes hepatocytes an attractive *in vitro* model to measure the effects of a potential biologically effective dose in the liver. Absorption, distribution, metabolism, followed by a contaminant's subsequent excretion (ADME) underlies the processes that govern oral bioavailability. Solubility measures the ability of a compound to dissolve in the gastric compartment while the permeability measures the ability of a compound to cross the intestinal membrane and subsequently become bioavailable. First-pass removal of a contaminant can result in poor oral

bioavailability. Conversely, if sufficient contaminant concentration is reached, liver injury may occur.

2.10 HepG2 cells

The main function of the liver within the digestive system is to aid in the processing of nutrients absorbed in the small intestine. Additionally, the liver takes the absorbed materials from the small intestine to make the chemicals needed for the body to function and detoxifies harmful chemicals. Given this role, hepatocyte cultures like the hepatocellular cell line HepG2 are frequently used in *in vitro* models for human biotransformation.

HepG2 cells are frequently used in drug metabolism studies in which drugs are converted into water-soluble metabolites by cells to allow elimination in urine or bile. Xenobiotics that enter the body through the gastrointestinal tract are absorbed through the intestinal lumen and are transferred to the liver via the portal vein to prevent a potential toxicant from entering into systemic circulation. Heavy metals are in part treated similarly and are detoxified in the liver. Due to this fact, HepG2 cells represent an attractive *in vitro* surrogate to determine the effects heavy metal bioavailability on the human body.

Chapter 3: Materials and Methods

3.1 Introduction

The goal of this dissertation is to determine the soluble fraction of metals in human gastrointestinal fluids, i.e. the bioaccessible fraction and coupling this extractable fraction to an *in vitro* liver assay in order to narrow data gaps such as risk uncertainties that currently exist in the data that drive risk assessment. The bioaccessible fraction will represent the maximal concentration that is available for transport across the intestinal mucosa. The second goal of this dissertation is to analyze the relationship between the maximal soluble fraction and the bioavailable fraction that is able to reach its target organ, herein the liver.

To meet this objective, the HMBSR three-compartment sequential extraction *in vitro* method for bioaccessibility was selected from the four *in vitro* extraction methods described above and was slightly modified to estimate human exposure to metals in soils. Then, an *in vitro* hepatic cellular system was subsequently employed to represent target organ metal toxicity in the liver. The selected cellular based assay was used to quantitatively link bioaccessibility results for the selected metals to an estimate of a biologically effective dose of the extracted metals that could induce adverse health effects. Since it was hypothesized that incorporation of this endpoint into an already existing model for bioaccessibility will help to further elucidate the fate of bioaccessible metals and their impact on human health, the methods described below lead to that end.

Standard Reference Materials (SRM) was chosen to potentially allow for inter-laboratory comparison of NIST SRM 2710 and 2709. Two local New Jersey (NJ) soils were chosen for analysis as site-specific soils with high levels of Cr. These Cr-rich soils are also representative of a remediated Superfund site. In addition, six (6) unidentified soils were provided by the EPA and used to examine the applicability of our bioaccessibility/cell assay when no information is known about the soil in question. Thus, examining its possibility for use as a cursory test prior to completing a more involved risk assessment.

3.2 Soil Materials

National Institute of Standards and Technology (NIST) is a source of standardized reference materials (SRM) of known contaminant concentrations. Standard reference materials have become a particularly useful source of materials that are easily accessible and ideal for use in inter-laboratory comparison studies. NIST SRM 2709, and 2710 were utilized in this dissertation to represent a suite of soils from varying locations, contaminant concentrations and field conditions. Additionally, one local soil, herein referred to as Liberty State Park Soil, and five (5) unknown soils (herein referred to as unkwn1, unkwn2, etc.) received from the Environmental Protection Agency in Research Triangle Park, NC (EPA-NC) were utilized in *in vitro* extraction procedures and cell exposure assays. Table 3.1 shows the soil parameters of each NIST SRM soil.

Table 3. 1 Summary of Selected Soil Parameters

Material	Particle Size (μm)	Source	Drying
SRM 2710	<74	USGS: top 4 inches of pasture land along Silver Bow Creek (Butte, Montana)	Oven-dried for 3 days at room temperature
SRM 2709	<74	USGS: top 7.5 to 13 inches of plowed field in Central San Joaquin Valley	Oven-dried for 3 days at room temperature
SRM 2701	<74	USGS: Chromite ore-contaminated soil from Hudson County, New Jersey	Oven-dried for 3 days at room temperature
Liberty State Park	<45 45-125 125-250 250-500	V. Kista: top 2 cm of chromite ore contaminated soil in Hudson County, New Jersey	Air-dried for 64 hours at room temperature

3.2.1 NIST SRM 2710

NIST SRM 2710 is a highly contaminated soil collected in Montana that was oven-dried, sieved, sterilized and homogenized at 50 grams per unit. The U.S. Geological Survey (USGS) collected NIST SRM 2710 from the top four (4) inches of pasture land in Butte, Montana along Silver Bow Creek, approximately nine miles east of the local Anaconda smelting plant and 6.5 miles south of settling pond that feed the creek. Copper, manganese and zinc were high in concentration due to periodic flooding of the creek. Samples were collected and shipped back to the USGS. There, they were dried in an oven at room temperature for three days. A vibrating 2 mm screen was used to remove large debris and subsequently used to disaggregate large chunks of soil. The leftover material was ground in a ball mill, passed through a 74-μm screen and blended for twenty-four (24) hours. Homogeneity was ensured using twenty grab samples

that were measured using X-ray fluorescence spectrometry (major oxides) and inductively coupled plasma atomic emission (ICP-AE) analysis (trace elements). Final homogeneity testing was conducted randomly on 50 g bottled units.

Certified values are weighted means from two or more analytical methods or the mean of one definitive method. NIST assigned nine (9) Polish laboratories the task of corroborating certified values. Table 3.2 shows the certified and/or noncertified values and certification methods of selected metals.

Table 3. 2 NIST 2710 Mass Fraction and Certification Method for Selected Metals

Certified Status	Element	Mass Fraction (mg/kg)	Certification Method*
Certified	Lead (Pb)	5532 ± 80	POLAR, ICP
Certified	Arsenic (As)	626 ± 38	ICP, INAA
Certified	Nickel (Ni)	14.3 ± 1.0	INAA
Certified	Cadmium (Cd)	21.8 ± 0.2	ID ICPMS, RNAA
Noncertified	Chromium (Cr)	39	INAA, DCP, ICP

*DCP: Direct current plasma atomic emission spectrometry; lithium metaborate fusion
ICP: Inductively coupled plasma atomic emission spectrometry; acid digestion
ID ICPMS: Isotope dilution inductively coupled plasma mass spectrometry; mixed acid digestion
INAA: Instrumental neutron activation analysis
POLAR: Polarography
RNAA: Radiochemical neutron activation analysis; mixed acid digestion

3.2.2 NIST SRM 2709

NIST SRM 2709 is an agricultural soil that has been oven-dried, sieved, sterilized and blended to achieve homogeneity. One unit of NIST 2709 consists of 50 grams of dried material. The soil was collected in Central California San Joaquin Valley from a plowed field in the Panoche fan between the Panoche and

Cantu creek beds. The top 3 to 5 inches of soil were removed (containing sticks and plant debris) and the sample was collected from the remaining 5 inches down to 18 inches. After shipment to the USGS laboratory for processing, the sample was air-dried in an oven for three days at room temperature. Subsequently, the soil was processed in a NIST standardized manner (sieved twice through a 2mm screen, ground in ball mill, sieved to <74 microns, blended, and homogeneity ensured with 20 grab samples).

Certified values are a result of two or more independent analytical methods, or a single definitive method. Table 3.3 shows the certified and/or noncertified mass fraction values and certification methods for selected metals.

Table 3. 3 NIST 2709 Mass Fraction and Certification Method for Selected Metals

Certified Status	Element	Mass Fraction (µg/g)	Certification Method*
Certified	Lead (Pb)	18.9 ± 0.5	ID-TIMS
Certified	Arsenic (As)	17.7 ± 0.8	INAA
Certified	Nickel (Ni)	88 ± 5	INAA
Certified	Cadmium (Cd)	0.38 ± 0.01	ID-ICPMS, RNAA
Certified	Chromium (Cr)	130 ± 4	ICP

*ICP: Inductively coupled plasma atomic emission spectrometry; acid digestion
ID-ICPMS: Isotope dilution inductively coupled plasma mass spectrometry; mixed acid digestion
ID-TIMS: Isotope dilution thermal ionization mass spectrometry; mixed acid digestion
INAA: Instrumental neutron activation analysis
POLAR: Polarography
RNAA: Radiochemical neutron activation analysis; mixed acid digestion

3.2.3 NIST SRM 2701/Liberty State Park

Between 1905 and 1976, two million tons of chromium ore residues from a chromite ore-processing site in Jersey City was generated and disposed of in soil

and meadowlands in the surrounding area (NJDEP, 2010). Concern for public health was generated due to the fact that the processing residue contained trivalent (Cr III) and hexavalent (Cr VI) chromium. This concern has increased over the years as many of these sites have been eventually converted to residential, public (i.e. state parks) and industrial sites.

In May 1991, V. Kista collected slag material from Liberty State Park, Hudson County, New Jersey. An 8x8 m^2 sampling grid yielded twenty-one (21) samples. The top two (2) cm of soil was gathered and air-dried at room temperature for 64 hours and then stored in plastic containers. The material (Liberty State Park Soil) was retrieved from the cold room and air dried for 3 days (72 hours) then sieved by S. Hylton in 2012 into three (3) size fractions: <45 μm, 45-125 μm , and 125-500 μm. In 2014, S. Hylton further fractionated the 125-500 μm size fraction into two: 125-250 μm and 250 to 500 μm.

NIST SRM 2701 was sourced from a location in Hudson County, New Jersey (chromite ore-contaminated soil) from a three-acre site behind the Interpretative Center at the Liberty Science Center in Liberty State Park, Jersey City, NJ. Samples were collected, sealed and shipped under the direction of Stuart Nagourney of NJDEP to the USGS (Lakewood, CO) as part of a multi-agency agreement for processing. The soil was air-dried, sieved, radiation-sterilized and homogenized to create SRM 2701 (Hexavalent Chromium in Contaminated Soil). Given that this soil collection was essentially in the same area as the Liberty State Park Soil, NIST 2701 is considered closely linked to Liberty State Park soil described earlier and serves as a basis for comparison of

raw soil versus processed homogenized SRM materials. NIST 2701 is significant because it was the first soil to be speciated for chromium

A unit consists of 75 grams of dried, sterilized soil in an amber screw-capped glass bottle (herein known as NIST 2701). The certified concentrations for hexavalent chromium, total chromium, iron, and manganese are based on the agreement of results from two or more independent analytical methods. The certified value for hexavalent chromium is the un-weighted mean of analytical measurements by NIST and collaborating laboratories using the US EPA SW-846 analytical method (Method 3060A). Reference concentrations are noncertified values that are a best estimate and do not meet the NIST criteria for certification. Twenty (20) bottles were selected randomly to assess homogeneity. For chromium, the relative standard deviation (RSD) was less than 1% (170 mg sample size). EPA Method 3050 was used to determine the leachable % mass fraction recovery of total Cr for a value of 9.6%. Table 3.4 shows the mass fraction and method certification values of selected metals in NIST 2701 and Table 3.5 show the information values for selected species and properties.

Table 3. 4 NIST SRM 2701 Mass Fraction Values and Method Certification

Certified Status	Element	Mass Fraction (mg/kg)	Mass Fraction (%)	Method*
Certified	Hexavalent Cr	551.2 ± 34.5		3060A, 6800, SID-ICP-MS
Certified	Total Cr		4.26 ± 0.12	ID-ICP-MS, XRF, INAA
Certified	Fe		23.73 ± 0.19	XRF, INAA, PGAA
Certified	Mn		0.2137 ± 0.0014	XRF, INAA

*3060A: EPA Method 3060A
6800: EPA Method 6800
ID-ICP-MS: Isotope dilution inductively couple plasma mass spectrometry
INAA: Instrumental neutron activation analysis
PGAA: Prompt gamma activation analysis
SID-ICP-MS: Speciated isotope dilution inductively coupled plasma mass spectrometry
XRF: X-ray fluorescence spectrometry (XRF) following borate fusion preparation

Table 3. 5 Information Values for Selected Species and Properties

Species/Property	Value	Unit
Chemical Oxygen Demand (COD)	<10	mg/kg
Redox Potential	526	mV
Sulfide	<10	mg/kg
Total Organic Carbon	36900	Mg/kg
pH	9.6	

3.2.4 Liberty State Park Size Comparison

Three size fractions LSP 125-500, 45-125, and >45 microns were tested for differences in extractable metal concentrations for Pb, As, Ni, Cr, and Cd. The results of the differences are presented in Figures 3.1 to 3.5. There were no differences between means for each metal except for As which was significantly different from LSP 125-500 microns on a P 0.05 level. Smith et al. (2009) found mean arsenic concentrations to be generally uniform across larger particle size

fractions, but found a marked increase in arsenic concentration in the < 2.5-micron soil fraction. This increase in concentration in the lower size range could account for the differences in As means.

Figure 3. 1 Differences between size distributions for Pb (Liberty State Park Soil)

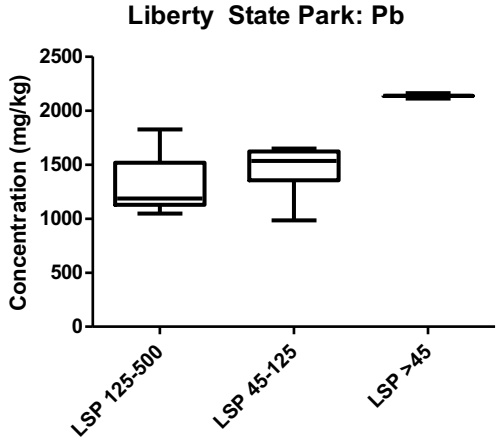


Figure 3. 2 Differences between size distributions for As (Liberty State Park Soil)

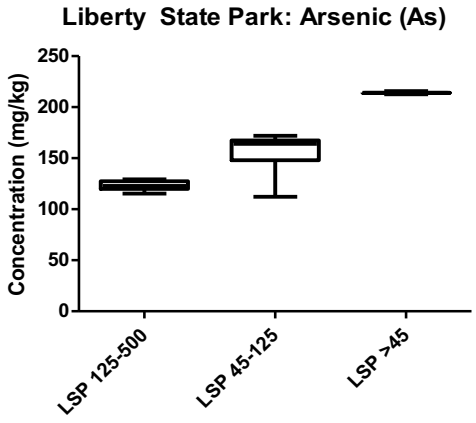


Figure 3. 3 Differences between size distributions for Cd (Liberty State Park Soil)

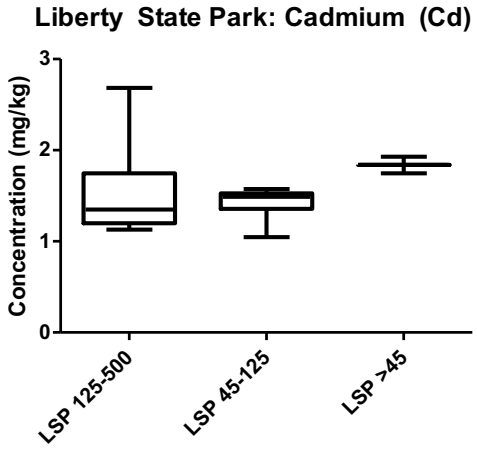


Figure 3. 4 Differences between size distributions for Ni (Liberty State Park Soil)

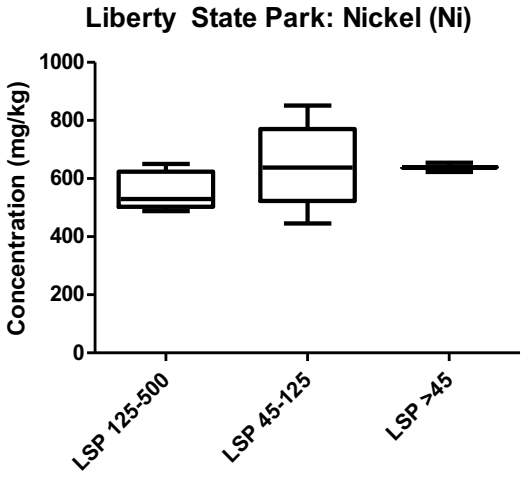
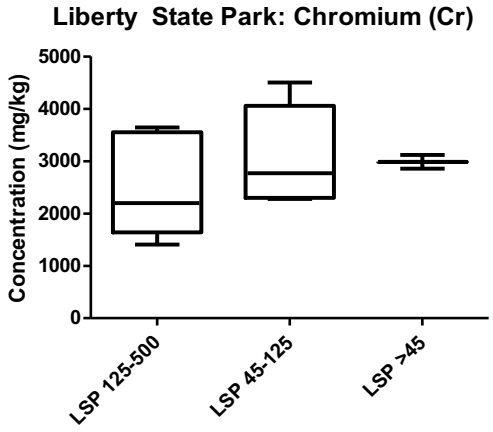


Figure 3. 5 Differences between size distributions for Cr (Liberty State Park Soil)



3.2.5 Labile/Leachable Concentrations

The certified concentrations in essentially all NIST SRMs are given as total concentrations. For environmental purposes, and this dissertation, the extractable or labile concentrations are more useful. The U.S. EPA has established a number of leach protocols for determining extractable elements. Acid digestion Method 3051 was performed on all soils prior to *in vitro* extraction (results shown in Chapter 4). Percent leach recovery is calculated using the following equation:

$$100\ x\ \frac{Median\ Value}{Certified\ or\ Information\ Value}$$

In addition, NIST performed leach recovery for NIST 2710 and NIST 2709 using the above equation. Certified values are weighted means of one or more methods whereas informational values cannot be certified by NIST due to bias. For soils without NIST certified or information values, results from acid digestion (method 3051) were used. Tables 3.6 and 3.7 show the percent leach recovery

for selected metals found by NIST. Leach recovery values were not certified for NIST 2701 outside of total chromium (9.6%).

Table 3. 6 NIST 2710 Leach Recovery for Selected Metals

Element	Range Mg/kg		Median	N	% Leach Recovery
Arsenic	490	600	590	3	94
Cadmium	13	26	20	8	92
Chromium	15	23	19	6	(49)
Lead	4300	7000	5100	8	92
Nickel	8.8	15	10.1	8	71

() indicates that information value was used
-- at or below detection limit
** no % Leach Recovery calculated

Table 3. 7 NIST 2709 Leach Recovery Values for Selected Metals

Element	Range Mg/kg		Median	N	% Leach Recovery
Arsenic	--	--	<20	2	**
Cadmium	--	--	<1	5	**
Chromium	60	115	79	5	61
Lead	12	18	13	5	69
Nickel	65	90	78	7	89

-- at or below detection limit
** no % Leach Recovery calculated

3.2.6 Unknown Soils

Five (5) unknown soils samples and one NIST SRM soil material (NIST 2710a) were received from the EPA's National Exposure Research Lab in Research Triangle Park, NC. The only information received with the soil samples was a sheet that gave a coded number or name for each soil sample and the size fraction. NIST SRM 2710a is similar to its predecessor NIST 2710, described above; however, it was collected along a different part of the Panoche

fan creek in San Joaquin Valley. Selected metals mass fraction concentrations for NIST 2710a are within ten (10) percent of NIST 2710 certified mass fraction values. Table 3.8 shows the subsequent recoding of the samples. From herein, all samples will be referred to by their sample number (including NIST 2710a).

Table 3. 8 Parameters for a Suite of Soils Received from the EPA

Sample #	Size (μm)
Unknown 1 (unkwn1)	<250
Unknown 2 (unkwn2)	<250
Unknown 3 (unkwn3)	<250
Unknown 4 (unkwn4)	<74
Unknown 5 (unkwn 5)	<74
Unknown 6 (unkwn 6)	<250

In order to calculate the *in vitro* bioaccessibility, the following equation is used:

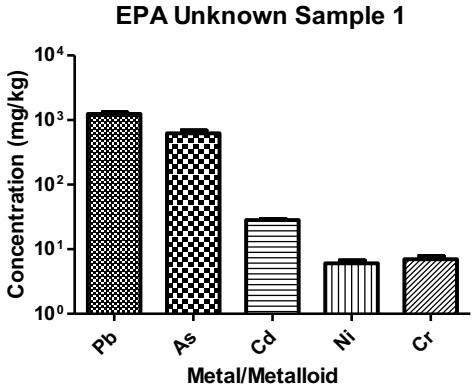
$$\%IVBA = \frac{in\ vitro\ extractable\ mg_{metal/kg_{soil}}}{total\ contaminant\ mg_{metal/kg_{soil}}} \text{ x } 100$$

Inductively Coupled Plasma Mass Spectrometry (ICP-MS) following sequential extraction in gastrointestinal fluids determines the in vitro extractable metal. For SRM materials, the denominator is the certified or reference value of the contaminant. For unknown soils or field-collected soils, the denominator in the %IVBA equation is determined using EPA method 3051. This method may underestimate total concentrations as it is intended by the EPA as a rapid multi-element dissolution method that aids in the analysis and subsequent decision making about materials and site cleanup levels. While the extractable concentrations of this method may not reflect bulk concentrations, this method is relatively inexpensive, fast, easy to use, and widely accepted making it attractive

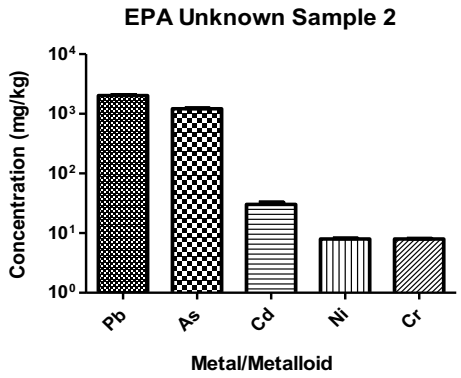
alternative to bulk concentration analysis methods. In addition, EPA 3051 extractable concentrations for our selected metals in our SRM soils generally fall within 20% of total certified or reference values (Figure 3.6). Furthermore, comparison of SRM 2701 (Liberty State Park Soil) and non-standardized Liberty State Park Soil (LSP) shows that leachable concentrations and total concentrations fall to within 20% of each other (except for Cr). From this we postulate that the 3051 method will not grossly underestimate %IVBA for As, Cd, Ni and Pb in our unknown soils.

Figure 3. 6 (a-f) EPA 3051A results for EPA unknown soil samples

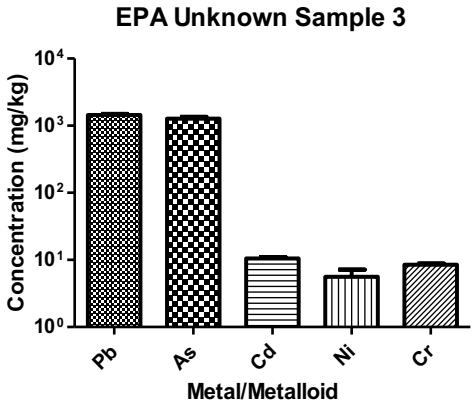
a.



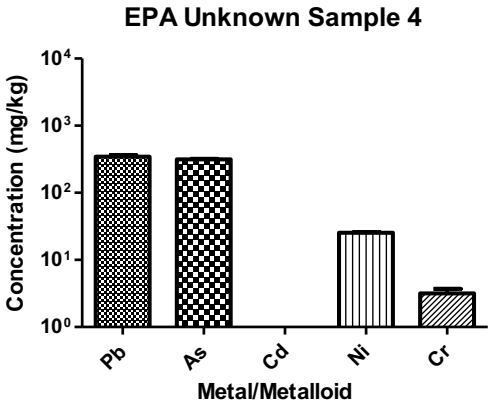
b.



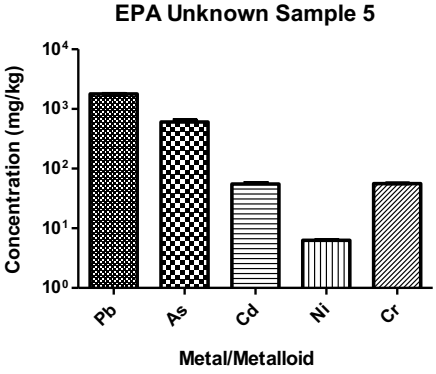
c.



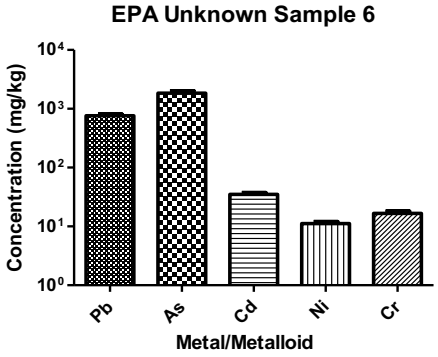
d.



e.



f.



3.3 Acid Digestion Procedure: EPA Method 3051A

Leachable or labile elemental concentrations were determined using the EPA 3051A method. A minimum of 250 mg representative sample was individually digested in 10 ml of concentrated ultrapure nitric acid, which is the NIST recommended minimum sample mass (200 mg for NIST 2701) that should be used for analytical determinations related to certified values. Minimum sample requirements for EPA 3051 were 500 mg of soil. This recommendation was used for EPA 3051 analysis unless otherwise stated.

Samples are placed in acid-rinsed Teflon vessel with 10 ml ultrapure nitric acid. Samples are allowed to sit for a period of time up to overnight to allow the formation of gases from the decomposition of organic matter to dissipate. The samples were then capped, and run according to EPA 3051 method guidelines. After extraction, the samples were allowed to cool and transferred to clean 50 ml polypropylene tubes and filled to the 50 ml meniscus with deionized water for a dilution factor (Df) of 5. The latter samples were then diluted 4-fold using 5 % high purity nitric acid and transferred to 15 ml tubes. Further dilutions depended on several factors such as the presence of undissolved particles in the sample and high concentration of chosen metals. The samples were analyzed via Inductively Coupled Plasma-Mass Spectroscopy, ICP-MS, to obtain concentrations in ppb (µg/L or ng/ml).

3.4 Instrumentation

3.4.1 Inductively Coupled Plasma-Spectroscopy

All soil samples were analyzed using a Thermo-elemental X5 model Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) system (Thermo Elemental Inc.). Metal standards for elements of interest were prepared using solutions purchased from High Purity Standards (Charleston, SC) at 10 µg/mL unless otherwise stated. Standard curve regression lines with an r^2 of 0.99 or greater were deemed acceptable for the measured isotopes of each element.

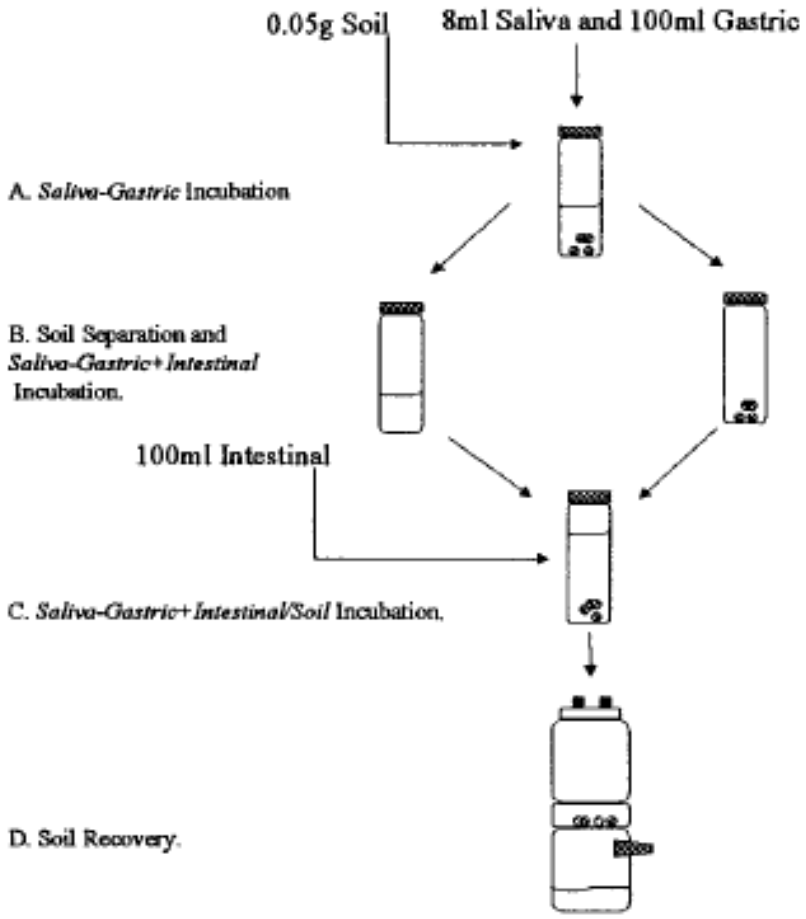
Quality Assurance (QC) checks were run approximately every 10 samples on the ICP-MS using a High Purity Standard solution that differed from the solution used for the calibration curve. QC measurements were deemed acceptable if they were within 20% of the NIST acceptable values. QC included blank standards, sample repeats and SRM standards.

3.5 *In vitro* Extraction

Figure 3.7 shows the steps of the HMBSR *in vitro* extraction method. First, 50 mg of soil sample was added to 8 ml of simulated saliva and 100 ml of simulated gastric fluid and shaken for two (2) hours at 37°C. After the end of the first incubation period, the soil sample was separated from the saliva/gastric fluid and 100 ml of simulated intestinal fluid was added and shaken for two (2) hours at 37°C. Next, the soil sample was reintroduced to the saliva/gastric-intestinal fluids for a final incubation shaken for two (2) hours at 37°C. Soil recovery was performed at the end of the extraction period and aliquots were taken after the

first and third incubation period with the second aliquot also used for HepG2 cell exposure (Figure 3.7).

Figure 3. 7 Taken from (Ellickson et al., 2001)



Artificial Saliva Solution:

- 0.004 M Calcium Chloride
- 0.4% (w/v) Mucin
- 0.005 M Potassium Chloride

- 0.007 M Sodium Chloride
- 0.004 M Sodium Phosphate
- 0.017 M Urea

Artificial Gastric Fluid:

- 0.03 M Sodium Chloride
- 0.084 M Hydrochloric Acid
- 0.32% (w/v) Pepsin

Artificial Intestinal Fluid:

- 0.2 M Sodium Bicarbonate

3.6 HepG2 Cells

Human hepatocellular carcinomas, HepG2 cells purchased from American Type Culture Collection (ATCC), were used for incorporation into an existing model of oral bioaccessibility. The cells were maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin at 37°C in an atmosphere of 5% CO_2 in air. All media were purchased from Gibco or Life Technologies.

The HepG2 cells were exposed to the soil extracts in 1, 0.1, 0.01, .001 and .0001 times the extract concentration in order to represent a range of metal-extract values that could reach the liver. Results are presented as percent viability of the control. The cells were plated at a density of 2×10^4 cells per well in a 96 well plate and allowed to grow for 24 hours before exposure. After the 24-hour attachment period, the cells were washed twice with phosphate buffered

(PBS) and replaced with serum-free media containing varying concentrations of the soil extracts. The extracts and cells were incubated for 24 hours and then treated with AlamarBlue used to determine the viability.

AlamarBlue (AB) is a measure of the number of cells based on a fluorometric/colometric measure of metabolic activity. In 96-well plates, 20,000 cells per well were plated and allowed to grow for 24 hours. After exposure to soil extracts, the cells are incubated with AB for 4 hours and the wells analyzed with a spectrophotometer at 590 nm (Vmax Kinetic Microplate Reader, Molecular Devices). It is expected that there will be a dose-response relationship between the *in vitro* extract concentration and the viability of the hepatocytes. Shea et al. (2008) utilized HepG2 cells as a bioassay contamination in sediments. Metallothionein was used as a biomarker for exposure and Shea et al. (2008) found a dose response relationship between cadmium concentration and metallothionein response.

Chapter 4: Bioaccessibility of Heavy Metals in Soils Using a Sequential Extraction Protocol.

4.1 Introduction

The presence of heavy metal contamination in soil may present health risk to the human population. *In vitro* models are useful in the measurement of heavy metal bioaccessibility, i.e. the soluble fraction in GI fluids, in that they may more accurately assess risk by utilizing a more biologically relevant system. The Hamel Mass Balance and Soil Recapture (HMBSR) method is an *in vitro* simulated gastrointestinal model that mimics human physiological conditions (e.g. pH and residence times) of the mouth, stomach and small intestine and was utilized to recover the bioaccessible concentration of lead, cadmium, chromium, nickel and arsenic in contaminated soil mediums.

Lead (Pb) has been extensively researched and validated in several *in vitro* gastrointestinal models that estimate human health risk (Ellickson et al. 2001; Rodriguez et al. 2003; Ruby et al. 1996). However, investigation of the bioaccessibility of other metals such as As, Cd, Ni and Cr are not as complete. Inherent differences (i.e. pH, residence time, separation procedures and inclusion/exclusion of one or more GI compartments) in each *in vitro* model underlie the conflicting results that GI studies often produce. The use of NIST SRM materials helped elucidate these differences as SRM materials are highly homogenized and easily purchased by any laboratory.

The HMBSR method is unique from other *in vitro* models in that this method aims to recover the insoluble fraction at the end of the extraction period.

The HMBSR method also allows the user to assay the bioaccessible concentration in both the stomach and intestinal compartments. In addition, the HMBSR method incorporates the most significant compartments of the GI tract (described in Chapter 2). The insoluble fraction was not of interest in this study as (1) our endpoint is hepatotoxicity and (2) it is assumed that portion is cleared out of the body via waste. For this study, we tested the hypothesis that the percent of bioaccessible metals from each soil sample in the gastric and intestinal fluids depend on the total concentration of heavy metals in soil.

4.2 NIST SRM 2710

Percent bioaccessibility for Pb, Ni, Cd, As and Cr for NIST SRM 2710 is presented in Table 4.3**.** Percent bioaccessibility was determined by dividing the µg/g mass concentration of extractable metal by the NIST certified (noncertified in the case of chromium) concentrations (Figure 4.2). Gastric IVBA of As, Pb, Cd, Ni, and Cr were 50.9, 16.8, 58.4, 118, and 17.8%, respectively. Intestinal IVBA of As, Pb, Cd, Ni, and Cr were 24.2, 5.2, 15.4, 52.1, and 8%, respectively. The addition of the intestinal compartment in our simulated gastrointestinal model reduced the gastric IVBA value by approximately 50% for each of the metals extracted. These results indicate that the use of IVBA results that do not include the intestinal compartment overestimates health risk from the ingestion of contaminated soils as seen in previous studies using the HMBSR method (Hamel et al. 1999; Ellickson et al. 2001).

Often, certified values of heavy metals are not available for field soil samples. EPA Method 3051A is routinely used to estimate metal extractable concentrations in sediments and soils. The use of the extractable metal concentrations of soil obtained using EPA Method 3051A, can significantly impact the %IVBA given that this method doesn't always assess total concentration. Lower values in the denominator may lead to overestimation of risk, so care must be taken when choosing a denominator for a specific contaminant type. Table 4.1 shows the certified and noncertified concentrations for NIST 2710 selected metals (Pb, Ni, Cr, As, and Cd). Non-certified concentrations are concentrations that have been analyzed, but do not fit the acceptance criteria for a certified value (*Cr). Percent recoveries were performed by NIST using a modified EPA Method 3050, which uses hydrochloric acid in addition to nitric acid to perform acid digestion as opposed to method 3051A, which only uses nitric acid. Recoveries were determined upon receipt of a soil sample by performing EPA Method 3051A in triplicate in the Buckley Laboratory. Lab recoveries were lower than the NIST SRM leach recovery due to the use of hydrofluoric acid in the NIST acid digestion mixture thati may have led to a more complete digestion (Table 4.1). This variation, caused by the choice of digestion method, may underestimate bioaccessibility due to an complete digestion, which may underestimate bioaccessibility and thereby risk. (Ruby et al. 1999),

Reporting total metal mass in simulated gastrointestinal fluids in addition to % IVBA measurements can help inter-laboratory comparisons and inter-laboratory method validations. Various factors can significantly impact IVBA

values such as methodology and digestion choice (i.e denominator in %IVBA calculation) and a comparison of total metal mass may help minimize these variances and allow for a truer comparison of risk.

Table 4. 1 Certified Mass Fraction, Leachable Concentration (EPA Method 3051A) and percent recoveries of NIST 2710.

Element	Mass Fraction (mg/kg)	EPA 3051-SNH ppm	SNH % Recoverable**	% Leach Recovery-NIST ppm*
Lead (Pb)	5532 ± 80	5240.5 ± 92.7	94.7	92
Arsenic (As)	626 ± 38	494.1 ± 12.5	78.9	94
Nickel (Ni)	14.3 ± 1.0	14.8 ± 0.8	103	71
Cadmium (Cd)	21.8 ± 0.2	16.2 ± 0.2	74.3	92
Chromium (Cr)	39	14.3 ± 1.0	36.7	49

***Median value used to calculate percentage**

****Mean value used to calculate percentage**

Figure 4. 1 Extractable Metal Concentrations Using EPA Method 3051A for NIST SRM 2710 for selected metals

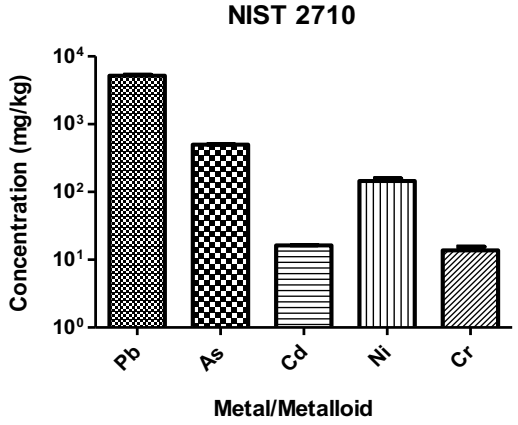


Figure 4. 2 a-b In vitro bioaccessibility results using the HMBSR method for NIST 2710

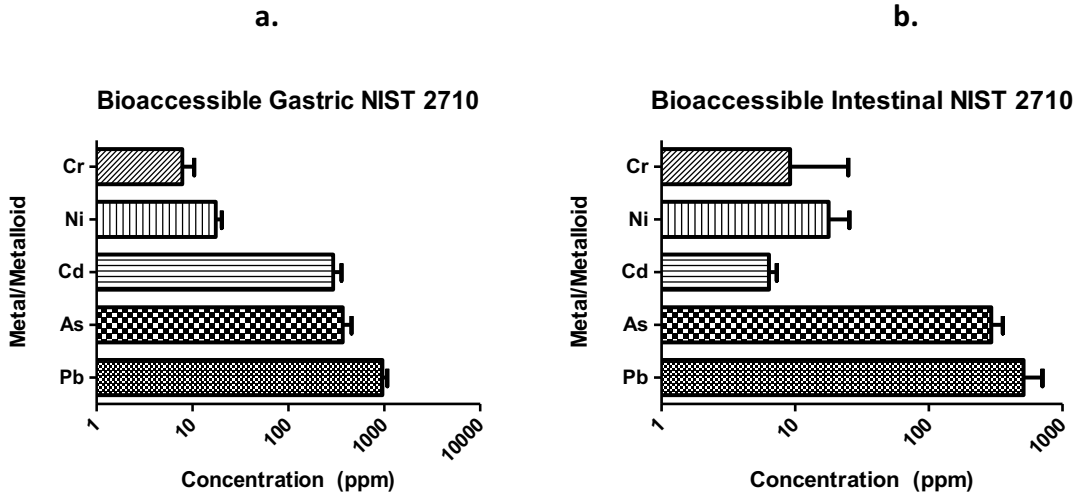


Figure 4. 3 Comparison of gastric and intestinal in vitro bioaccessibility results using the HMBSR method for NIST 2710

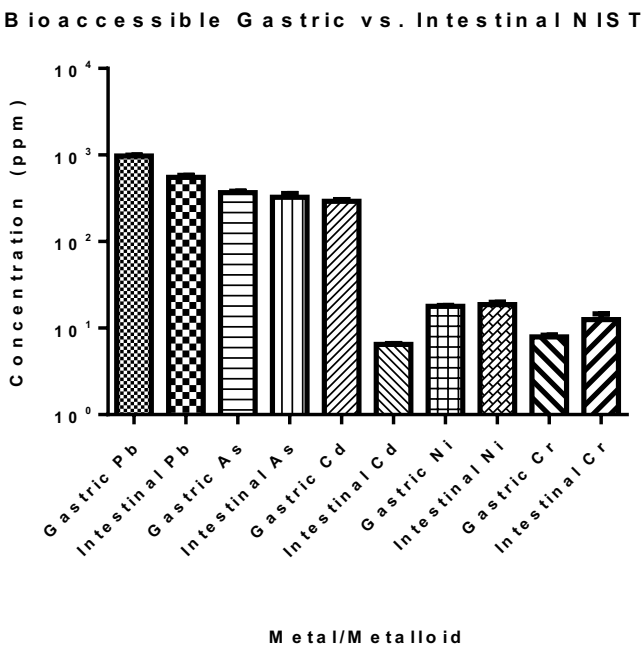


Figure 4.1 shows the extractable concentrations of Pb, As, Ni, Cd, and Cr (from highest to lowest concentration present in the NIST soil). Figures 4.2 (a-b) and 4.3 shows the in vitro bioaccessibility results for NIST 2710, given as the concentration of soluble metal, in both the intestinal and gastric compartments. While Pb had the highest total metal mass in the GI extraction system, cadmium was the most soluble in the saliva-gastric fluid and Ni was the most soluble in the saliva-gastric/intestinal fluid. As the contaminant moves from the stomach to the intestinal compartment, the bioaccessible concentrations of As, Cd, Cr and Pb were 24.2 ± 3.8, 15.4 ± 1.2, 8.0 ±0.6 and 5.2 ± 0.7 %, respectively. This suggests that the bulk concentration in soil does not necessarily correlate to % IVBA measurements in the intestinal compartment. In fact, there appears to be an inverse relationship between the bulk concentrations in the NIST 2710 SRM material where Ni has the lowest mass fraction in the bulk yet yields the highest bioaccessibility value in the intestinal compartment. Conversely, bulk Pb in the soil material was significantly higher than the other metals assayed yet Pb yielded the lowest % IVBA in the intestinal compartments. The inverse relationship between bulk soil concentration and % IVBA measurements is driven by soil properties and metal species form, representing a limitation of bioavailability estimates.

4.3 NIST SRM 2709

Percent bioaccessibility for lead, cadmium, nickel, chromium and arsenic was measured in the NIST SRM 2709 soil material and is presented in Table 4.4.

As in the preceding section, the percent bioaccessibility was determined by dividing the µg/g mass concentration of extractable metal by the NIST certified value times (x) 100 (Table 4.4). Gastric and intestinal %IVBA of As was below the detection limit (BDL) and Pb, Cd, and Cr were 5.2, 0.18, and 63%, respectively. Intestinal % IVBA Pb, Cd, and Cr were 0.7, BDL, and 31.9%, respectively. The addition of the intestinal compartment in our simulated gastrointestinal model again reduced gastric IVBA value, this time by approximately 50% for Cr and by 86% for Pb. These results again indicate that reporting of IVBA results that do not include the intestinal compartment may grossly overestimate health risk from the ingestion of soils that are not highly contaminated by as much as 80-90% for lead and by more than half for chromium.

The use of a minimally contaminated soil in the HMBSR *in vitro* extraction method resulted in As and Cd %IVBA being below the detection limit and with Pb hovering around the detection limit. This is a limitation of in vitro extraction methods where the contaminant concentration in the soil needs to be sufficiently high enough in order to be not only extracted in both the stomach and intestinal compartment, but in addition detected by our instrumentation. And as mentioned previously, elevated soil bulk concentrations do not necessarily imply elevated bioaccessibility percentages. This dependence on sufficient bulk concentration represents a data gap in our current *in vitro* extraction techniques.

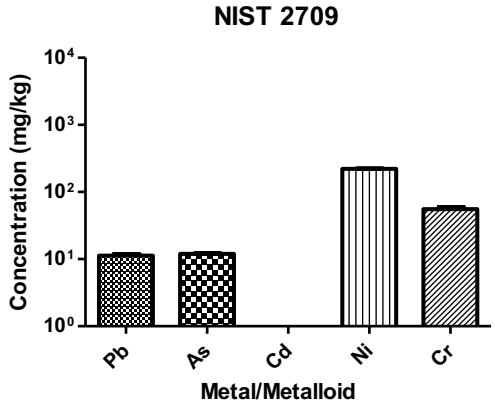
Table 4. 2 In vitro bioaccessibility results using the HMBSR method for NIST 2709

Total Metal Mass in artificial gastrointestinal fluids and soil postextraction (µg)			
	Soluble Metal in *Saliva-Gastric*	**Soluble Metal in *Saliva-Gastric+ Intestinal/Soil***	**Mass Recovered from Residual Soil***
As	<0.44	<0.81	0.5 ± 0.1
Pb	0.23 ± 0.03	0.03 ± 0.01	0.5 ± 0.05
Cd	0.01 ± 0.001	<0.04	0.02 ± 0.003
Ni	1.2 ± 0.2	0.5	9.6 ± 2.5
Cr	0.6 ± 0.08	0.3 ± 0.1	4.0 ± 1.0
***In vitro* bioaccessibility of selected metals**			
	%Soluble Metal in *Saliva-Gastric*	**% Soluble Metal in *Saliva-Gastric+ Intestinal/Soil***	**Recovery %**
As	<3.6	<3.6	50.6 ± 13.9
Pb	24.1 ± 3.5	3.3 ± 1.3	55.7 ± 5.3
Cd	55.6 ± 8.1	<0.2	80.2 ± 15
Ni	28.6 ± 4.6	11 ± 0.7	217.9 ± 57.1
Cr	9.2 ± 1.2	4.6 ± 1.8	61 ± 15.7

*n=3 for Recovery

Table 4.4 shows that chromium was the most soluble in the gastric and intestinal compartment. Arsenic and lead were already at an extremely low value in the bulk NIST SRM (at relatively equal concentrations) and As was below the extractable detection limit. While As was not extractable above the detection limit in our fluids, insoluble portions in the system were recovered. Mass recovery from the total system after the final incubation period was approximately 50.6% for As and 80.2% for Cd. Pb was recoverable in the total system at 55.7%, and IVBA for the gastric and intestinal compartment was 24.1 and 3.3%, respectively. Low recovery values could mean that some of the lead was insoluble in the system and thereby eliminated as waste via the feces.

Figure 4. 4 Extractable Metal Concentrations Using EPA Method 3051A for NIST SRM 2709 for selected metals



NIST 2709 was chosen as a background soil sample with heavy metal concentrations on average at or minimally above background. Arsenic and lead bioaccessible fractions were less than 6% in the intestinal compartments, which represent decreased risk in our system. Percent bioaccessible fraction for nickel was 28.6% in the gastric fluids and 11% in the intestinal fluids.

4.4 Liberty State Park Soils

Pre-remediation, Liberty State Park Soils were chosen due to the presence of high levels chromium. This test soil was also useful due to its relationship with NIST SRM material 2701. As mentioned previously, NIST 2701 and the Liberty State Park Soils were sampled from the same area. NIST 2701 was processed by NIST and sterilized, size fractionated (<74 microns) and standardized for chromium concentration along with Cr speciation concentrations (Cr3 and Cr6). Figures 4.5 to 4.7 show the results of EPA Method 3051A on three LSP size fractions (125-500 μm, 45-125μm, and <45 μm). These results show elevated

concentrations of lead and chromium in these soils. These results also show that there does not seem to be substantial differences in concentrations between the chosen size fractionations for lead, arsenic, chromium, nickel and cadmium.

Percent bioaccessibility for Pb, Ni, As, Cd and Ni were measured in Liberty State Park Soils and are presented in Tables 4.9 and 4.10. These soil samples were separated into three size fractions: 125-500 microns, 45-125 microns, and <45 microns. Soil particles that are less than 250 microns are considered to be the fraction that will adhere to children's hands and can subsequently be inadvertently ingested (Ruby et al. 1999). Larger size fractions (> 250 μm) can also be ingested; however not inadvertently, and most likely by children who exhibit pica. Percent bioaccessibility was determined by dividing the μg/g mass concentration of extractable metal by the value obtained using EPA Method 3051a (Figures 4.5 to 4.7). The hypothesis of this section was that soil size fraction would have an effect on the bioaccessible fraction in our *in vitro* system.

Gastric IVBA for LSP for the 125-500 micron fraction was 23% for As, 63% for Pb, 30.4% for Cd, 23.8% for Ni and 13.4% for Cr. Intestinal IVBA of As, Pb, Cd, Ni and Cr were 5.1%, 1.2%, BDL, 4.2% and 1.2%, respectively. For LSP (45-125 micron), the % gastric IVBA fraction was 24.1 for As, 75.7 for Pb, 47.1% for Cd, ~100% for Ni and 1.5% for Cr. Intestinal IVBA (LSP 45-125 microns) of As, Pb, Cd, Ni and Cr were 10.3%, 2.9%, 23.8%, 7.4% and 13.7%, respectively, with nickel having the highest bioaccessibility.

The addition of the intestinal compartment in our simulated gastrointestinal model significantly reduces the gastric % IVBA value for all the metals analyzed.

The gastric IVBA for both size fractions were comparable, however one notable difference is the increased % soluble metal in the intestinal compartment for LSP 45-125. The results indicate that the size fraction used to calculate IVBA does not have significant impacts in the gastric compartment, but may slightly misjudge health risk from the ingestion of metals in soils in the intestinal compartment. The lack of dependence on size in the gastric compartment may be due to the low pH environment that may indiscriminately extract contaminants. In the intestinal compartment, size may dominate transport and metabolism mechanisms, favoring smaller contaminants.

Figure 4. 5 EPA Method 3051A Results of Liberty State Park (125-500 microns) n=6

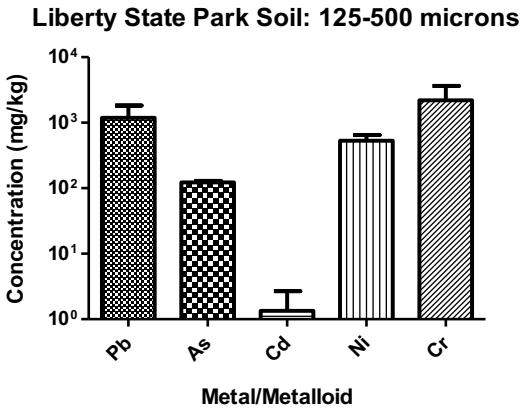


Figure 4. 6 EPA Method 3051A Results of Liberty State Park (45-125 microns) n=6

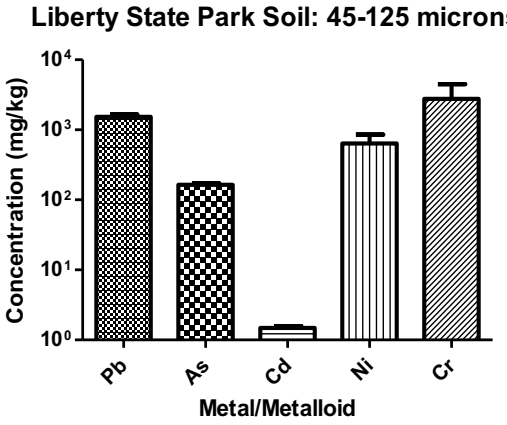


Figure 4. 7 EPA 3051 Results for Liberty State Park (<45 microns) n=2

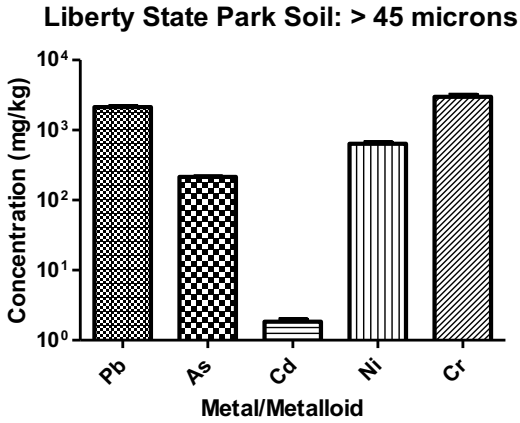


Table 4. 3 In vitro bioaccessibility results using the HMBSR method for Liberty State Park (125-500 microns) n=6

Total Metal Mass in artificial gastrointestinal fluids and soil postextraction (µg)			
	Soluble Metal in *Saliva-Gastric*	**Soluble Metal in *Saliva-Gastric+ Intestinal/Soil***	**Mass Recovered from Residual Soil***
As	1.4 ± 0.4	0.3 ± 0.2	5.6 ± 1.7
Pb	41.0 ± 11.0	0.8 ± 0.4	68.9 ± 25.1
Cd	0.02 ± 0.004	<0.4	0.1 ± 0.03
Ni	6.6 ± 1.1	1.2 ± 0.8	30.4 ± 9.2
Cr	16.4 ± 2.9	1.4 ± 0.8	174.6 ± 81.2
***In vitro* bioaccessibility of selected metals**			
	%Soluble Metal in *Saliva-Gastric*	**% Soluble Metal in *Saliva-Gastric+ Intestinal/Soil***	**Recovery %**
As	23.0 ± 6.2	5.1 ± 2.8	90.1 ± 27.4
Pb	63.0 ± 21.7	1.2 ± 0.6	105.8 ± 44.9
Cd	30.4 ± 13.3	<1.8	179 ± 89.0
Ni	23.8 ± 4.9	4.2 ± 3.0	110 ± 35.6
Cr	13.4 ± 5.7	1.2 ± 0.8	142.7 ± 86

*n=3 for Recovery

Table 4. 4 In vitro bioaccessibility results using the HMBSR method for Liberty State Park (45-125 microns) n=3

Total Metal Mass in artificial gastrointestinal fluids and soil postextraction (µg)			
	Soluble Metal in *Saliva-Gastric*	**Soluble Metal in *Saliva-Gastric+ Intestinal/Soil***	**Mass Recovered from Residual Soil**
As	1.9 ± 0.2	0.8 ± 0.2	6.15 ± 0.5
Pb	55.5 ± 1.8	2.1 ± 0.6	85.6 ± 8.0
Cd	0.03 ± 0.002	0.02	0.1 ± 0.02
Ni	45.1 ± 1.4	2.4 ± 0.2	30.7 ± 7.3
Cr	21.2 ± 1.4	2.4 ± 0.24	232.2 ± 65.1
***In vitro* bioaccessibility of selected metals**			
	%Soluble Metal in *Saliva-Gastric*	**% Soluble Metal in *Saliva-Gastric+ Intestinal/Soil***	**Recovery %**
As	24.1 ± 4.3	10.3 ± 3.0	78.7 ± 13.1
Pb	75.7 ± 12.8	2.9 ± 1.0	116.7 ± 22.3
Cd	47.1 ± 7.4	23.8	163.9 ± 32.6
Ni	139.9 ± 31.6	7.4 ± 1.8	95.5 ± 31.2
Cr	13.7 ± 4.3	1.5 ± 0.5	150.1 ± 62.5

4.5 Spike and Blank

Spike and nitric acid blanks were included in each EPA 3051A method. Analysis was carried through the complete process as quality controls. Nitric acid was spiked with a 10ppm As, Pb, Cd, Ni and Cr standard. Recovery values for the assayed metals were between 6.74 and 7.75 ppm (Table 4.11). Nitric Acid Blanks were also run through the EPA method 3051A process and the results were below the detection limit, as expected.

Table 4. 5 EPA 3051 Results Acid Spike Recovery (spiked at 10ppm) n=3

	Pb	As	Cd	Ni	Cr
	ppm	Ppm	ppm	ppm	ppm
	7.56	6.67	6.54	7.16	6.87
	8.01	7.07	6.86	7.53	7.20
	7.67	BDL	6.80	7.91	7.45
Average	**7.75**	**6.87**	**6.74**	**7.53**	**7.17**
Stdev	**0.23**	**0.28**	**0.17**	**0.37**	**0.29**

Table 4. 6 EPA 3051 Results for Nitric Acid Blank (n=2)

Pb	As	Cd	Ni	Cr
ppm	ppm	ppm	ppm	ppm
<0.005	<0.08	<0.008	<0.07	<0.02
<0.005	<0.08	<0.008	<0.07	<0.02

Spike and nitric acid blanks were included biofluid extraction batch analysis and were carried through the complete biofluid extraction process as quality controls to ensure method quality and control. Simulated gastrointestinal biofluids were spiked with a 20ppm As, Pb, Cd, Ni and Cr standard. Extract recovery values for the assayed metals were between 10 and 12 ppm for the spiked sample in the gastric compartment and 5-6ppm in the intestinal compartment. Biofluid blanks were also run through the complete extraction to ensure that background levels of metals were not significantly high. Biofluid blanks were consistently at or below the detection limit for each batch experiment extraction (see Chapter 5). These results show that background metal concentrations were not significantly impacting our EPA 3051 results and that the five metals chosen were sufficiently recoverable using this method.

4.5 EPA Soils

Six soil samples were received from the EPA. The soils received were comprised of five unknown soil samples and one NIST soil sample. Table 4.13 shows the only information received with the soil samples. These samples were used to see if the use of SRM materials that are homogenized and sterilized generated different results from unprocessed soil samples (other than sieving).

NIST SRM 2710a serves as a replacement to NIST SRM 2710. The original collection site used for NIST 2710 was unavailable due to remediation efforts by the Montana Department of Environmental Quality. From herein, NIST 2710a is described as unknown 3.

Table 4. 7 Parameters for unknown soil samples 1-6

Sample	Size (µm)	Notes
Unknown 1	< 250	
Unknown 2	< 250	
Unknown 3	<74	NIST 2710a
Unknown 4	< 250	
Unknown 5	< 250	
Unknown 6	< 250	

Table 4. 8 Comparison of Certified values of NIST SRM 2710 and NIST 2710a

Certified Status	Element	Mass Fraction In NIST 2710 (mg/kg)	*Mass Fraction in NIST 2710a (mg/kg)
Certified	Lead (Pb)	18.9 ± 0.5	0.552 ± 0.003 %
Certified	Arsenic (As)	17.7 ± 0.8	0.154 ± 0.01 %
Certified	Nickel (Ni)	88 ± 5	8 ± 1
Certified	Cadmium (Cd)	0.38 ± 0.01	12.3 ± 0.3
Certified	Chromium (Cr)	130 ± 4	23 ± 6

*unless otherwise noted as %

EPA Method 3051A was performed on unknown sample 1. Results from this method yielded 126 ppm for lead, 625 ppm for arsenic, 28.4 ppm for cadmium and 6.1 ppm and 6.9 ppm for Ni and Cr, respectively (Table 4.15). Similar bulk concentrations were seen in unknown sample 2 where Pb and As were significantly higher than Cd, Ni, and Cr at 2030 and 1220 ppm, and 28.4, 6.1 and 6.9 ppm, respectively. The major difference between unknown 1 and 2 is that unknown 2 has twice as much Pb and As, whereas the concentrations of Cd, Ni and Arsenic are close in concentration.

Bioaccessibility was measured using the HMBSR method for each of the six EPA soil samples. Unknown sample 1 demonstrated a % soluble metal concentration range from 56 to 99.1% in the gastric compartment and a range of 36.7 and 99.4% in the intestinal compartment. Unlike the previous soil samples, the bioaccessibility was not as significantly reduced in the intestinal fluid. While lead, arsenic, nickel and cadmium showed reduction in the bioaccessible fraction from the stomach to the small intestine. One metal that is of particular interest is chromium, which was not reduced in the intestine. In fact, the entire bulk concentration of chromium was completely bioaccessible in both parts of the GI tract.

In unknown sample 2, the concentration of lead and arsenic is almost twice the concentration in unknown sample 1. Similar IVBA results can be seen from unknown sample 2 where bioaccessibility is better estimated using the intestinal tract data. Similarly, chromium was completely bioaccessible (100% IVBA) in the gastric system and reduced in the intestine to 71.1%.

Table 4. 9 EPA Method 3051A Results on EPA Sample UNKNOWN 1

	Pb	As	Cd	Ni	Cr
	ppm	ppm	ppm	ppm	ppm
	1310	664	29.0	6.3	7.3
	1260	658	29.0	6.5	7.6
	1170	554	27.1	5.4	6.1
Average	**1250**	**625**	**28.4**	**6.1**	**6.9**
Stdev	**70**	**62**	**1.1**	**0.6**	**0.8**

Table 4. 10 In vitro bioaccessibility results using the HMBSR method for EPA Unknown Sample 1 (n≥6)

***In vitro* bioaccessibility of EPA Unknown Sample 1**		
	%Soluble Metal in *Saliva-Gastric*	**% Soluble Metal in *Saliva-Gastric*+ *Intestinal/Soil***
As	56 ± 10	44.9 ± 5.2
Pb	80.0 ± 6.8	66.0 ± 11.7
Cd	77.6 ± 7.4	36.7 ± 5.2
Ni	79.2 ± 39.3	57.6 ± 6.8
Cr	99.1 ± 12.7	99.4 ± 50.1

Table 4. 11 EPA Method 3051A Results on EPA Sample UNKNOWN 2

	Pb	As	Cd	Ni	Cr
	ppm	ppm	ppm	ppm	ppm
	2010	1220	30.3	7.7	7.8
	2080	1230	32.6	7.9	7.9
	2010	1220	30.3	8.1	8.1
Average	**2040**	**1220**	**31.1**	**7.9**	**7.9**
Stdev	**42**	**0.9**	**1.2**	**0.2**	**0.2**

Table 4. 12 In vitro bioaccessibility results using the HMBSR method for EPA Unknown Sample 2 (n≥6)

***In vitro* bioaccessibility of selected metals of EPA Unknown Sample 2**		
	%Soluble Metal in *Saliva-Gastric*	**% Soluble Metal in *Saliva-Gastric*+ *Intestinal/Soil***
As	54 ± 3.2	41.9 ± 6.5
Pb	57.3 ± 21.6	34.6 ± 8.9
Cd	64.4 ± 26.2	27.1 ± 4.9
Ni	61.2 ± 30.0	44.4 ± 3.1
Cr	101.6 ± 6.9	71.1 ± 3.5

Lead and arsenic are present at relatively equal concentrations in unknown samples 3 and 4 with Ni being the highest of all six samples with a concentration of at 25.3 ppm (Tables 4.19 and 4.20). Percent IVBA results for unknown sample 3 were similar in each compartment for As, Ni, and Cr, whereas the soluble Pb concentration decreased from 53.3 to 31.7% and soluble Cd concentration decreased from 41.7 to 16.2 % in the intestinal tract for unknown sample 3. For unknown sample 4, significant reductions of concentration in the intestinal compartment can be seen for Cd and Ni. Lead, chromium, and arsenic IVBA concentrations were similar in both compartments. For both unknown samples 3 and 4, the chromium concentration was not attenuated as it moved through the GI tract. For unknown sample 4, the chromium percent recovery was between 212 and 247 %. The significant increase in *in vitro* bioaccessibility beyond the maximum percentage of 100% may be due the usage of acid digestion (EPA Method 3051A) for assessment of total chromium, which may underestimate total chromium. The latter would cause a reduced denominator in our equation for bioaccessibility, but would not impact bioaccessible chromium in our system. Additionally, the concentration of Cd was

extremely low in the bulk sample, so the % IVBA concentration while detectable in the gastric fluid was not detectable in the intestinal compartment as it decreased in concentration.

Table 4. 13 EPA Method 3051A Results on EPA Sample UNKWN 3

	Pb	As	Cd	Ni	Cr
	ppm	ppm	ppm	ppm	ppm
	1440	1290	10.6	4.8	8.0
	1370	1170	10	4.6	8.6
	1490	1340	11	7.4	8.8
Average	**1440**	**1270**	**10.5**	**5.6**	**8.5**
Stdev	**62**	**86**	**0.50**	**1.6**	**0.4**

Table 4. 14 In vitro bioaccessibility results using the HMBSR method for EPA Unkwn Sample 3 (n≥6)

***In vitro* bioaccessibility of selected metals of EPA Unkwn Sample 3**		
	%Soluble Metal in *Saliva-Gastric*	**% Soluble Metal in *Saliva-Gastric+ Intestinal/Soil***
As	52 ± 14.2	40.9 ± 3.7
Pb	53.3 ± 2.5	31.7 ± 3.4
Cd	41.7 ± 2.3	16.2 ± 2.1
Ni	57.5 ± 16.9	45.6 ± 13.3
Cr	85.6 ± 4.1	91.7 ± 22.8

Table 4. 15 EPA Method 3051A Results on EPA Sample UNKWN 4

	Pb	As	Cd	Ni	Cr
	ppm	ppm	ppm	ppm	ppm
	364	321	0.25	24.7	3.1
	346	316	0.23	25.9	3.1
	334	308	0.22	25.3	3.6
Average	**348**	**315**	**0.23**	**25.3**	**3.3**
Stdev	**15**	**6**	**0.01**	**0.6**	**0.3**

Table 4. 16 In vitro bioaccessibility results using the HMBSR method for EPA Unknown Sample 4 (n≥6)

In vitro bioaccessibility of selected metals of EPA Unkwn Sample 4		
ug	**%Soluble Metal in *Saliva-Gastric***	**% Soluble Metal in *Saliva-Gastric*+ *Intestinal/Soil***
As	38 ± 2.8	35.6 ± 7.5
Pb	88.7 ± 15.5	80.9 ± 19.0
Cd	89.6 ± 24.7	<0.04
Ni	12.6 ± 0.8	9.7 ± 0.9
Cr	247 ± 26	213. ± 49

Tables 4.21 and 4.22 show the ppm concentrations of lead, arsenic, cadmium, nickel and chromium in the bulk soil sample. Cadmium and chromium are present at relatively equal concentrations in unknown sample 5, while As is present at 1881ppm (the highest arsenic concentration of all six soil samples). Again, the chromium % IVBA concentrations were the same in both fluid compartments as well as the arsenic soluble metal concentrations. This could be due to the fact that neither the gastric nor intestinal fluid is entirely reducing Cr (VI) to Cr (III), which is more readily absorbed whereas Cr (III) is virtually insoluble and eliminated rather quickly from the body.

Table 4. 17 EPA Method 3051A Results on EPA Sample UNKWN 5

	Lead	As	Cd	Ni	Cr
	ppm	**ppm**	**ppm**	**ppm**	**ppm**
	1810	607	55.1	6.32	55.8
	1700	544	54.0	6.30	56.5
	1790	646	57.8	5.14	54.9
Average	**1760**	**599**	**56**	**5.9**	**55.7**
Stdev	**56.6**	**52**	**1.9**	**0.67**	**0.8**

Table 4. 18 In vitro bioaccessibility results using the HMBSR method for EPA Unknown Sample 5 (n≥6)

In vitro bioaccessibility of selected metals of EPA Unkwn Sample 5		
	%Soluble Metal in *Saliva-Gastric*	**% Soluble Metal in *Saliva-Gastric*+ *Intestinal/Soil***
As	25 ± 2.7	21.7 ± 5.1
Pb	64.6 ± 7.2	52.2 ± 8.1
Cd	33.5 ± 1.4	15.3 ± 1.2
Ni	68.8 ± 9.5	54.4 ± 9.2
Cr	12.5 ± 1.1	12.7 ± 2.2

Table 4. 19 EPA Method 3051A Results on EPA Sample UNKWN 6

	Lead	As	Cd	Ni	Cr
	ppm	ppm	ppm	ppm	ppm
	810	2020	37.7	12.1	18.1
	756	1840	35.0	11.1	16.6
	759	1780	34.5	9.88	14.3
Average	**775**	**1880**	**35.7**	**11.0**	**16.3**
Stdev	**30**	**125**	**1.7**	**1.1**	**1.9**

Table 4. 20 In vitro bioaccessibility results using the HMBSR method for EPA Unknown Sample 6 (n≥6)

In vitro bioaccessibility of selected metals of EPA Unkwn Sample 6		
	%Soluble Metal in *Saliva-Gastric*	**% Soluble Metal in *Saliva-Gastric*+ *Intestinal/Soil***
As	64 ± 5.8	41.6 ± 16.3
Pb	73.9 ± 5.7	17.8 ± 2.1
Cd	27.0 ± 1.6	8.2 ± 0.4
Ni	48.5 ± 5.7	37.2 ± 4.2
Cr	52.8 ± 6.6	44.7 ± 12.2

The varying relationships between bulk metal concentrations and percent bioaccessible fractions underscore several complicated relationships. First, only five metals were evaluated for this dissertation and soil samples often contain many heavier metal contaminants, making it is possible that another metal may affect the inherent properties of the observed metals. Second, the varying relationships between the metals may alter the bioavailability of individual metals present in the system. The soil ecosystem is a complicated matrix that is comprised on many constituents such as organic compounds, inorganic species and soil microbiota, any of which that could impact bioavailability and consequently harmful effects on a system. Last, the differences in soil metal species, etc. mandates the need for a reliable system to measure these attributes in each sample.

Chapter 5: *In Vitro* Cell Bioassay- HepG2 Viability

5.1 Introduction

Currently, risk assessment guidelines and bioaccessibility/bioavailability models are primarily based on animal studies, which are limited in their applicability to a broad range of metals and/or contaminants. Not only are animal studies costly, but animal models are limited in the inherent differences between animal/human species and limited by the difficulty in obtaining quantifiable values in target organs or fluids. Subsequently, intra-species extrapolations can lead to uncertainties and errors in interpretation.

Data gaps in current *in vitro* bioaccessibility studies are associated with the need to link the results to *in vitro* human model systems that can give a better understanding of the potential human toxicity at various concentrations in the final metals concentrations extracted from the soil samples. Several factors have also been taken into consideration when designing a tool to assess risk such as the route of exposure to toxicity endpoint and final bioavailability. The former has a better chance of reducing uncertainties in an *in vitro* system. In addition, any test that may assess risk should be easily reproducible and relatively inexpensive to utilize in typical contaminated soil applications.

Cytotoxicity testing is most commonly used in the pharmaceutical industry and there are many cell parameters that can be used to assess cell viability. For example, the Trypan blue dye exclusion assay tests cell membrane integrity to indicate live versus dead cells. Tetrazolium salts such as MTT are converted to a formazan product by metabolically active to give a colorimetric readout. Within

the scope of this dissertation, it is important to develop an inexpensive, non-toxic to the user, reproducible and accurate assay that can be automated to a high-throughput system. In many instances, human cell lines can act as a reasonable *in vitro* surrogate for humans. Considering the latter, it is important to select a relevant cell line or tissue type and endpoint in order to improve the results from the risk assessment process. HepG2 cells are often used in toxicity studies due to their ability to secrete and synthesize several normal plasma proteins, thus retaining many of the characteristics of normal hepatocytes.

As described in Chapter 2, ingestion of contaminated soil will comprise the greatest exposure route for contaminated soil. As ingested soil moves through the GI tract, the liver plays a vital part in metabolizing, storing, distributing and detoxifying metal and organic contaminants. Exposure to heavy metals could also cause nephrotoxicity and neurotoxicity. However, given the role of the liver in the detoxification of contaminants, the liver would therefore be subjected to potentially high concentration and prolonged contact with heavy metals derived from ingestion. As a result, liver cells were chosen as an *in vitro* surrogate for potential toxicity to the bioaccessible material from soil to the human liver. A study by Dehn et al. (2004) utilized HepG2 cells in order to assess the utility of these cells to reasonably mimic *in vitro* and *in vivo* responses to cadmium. The cells showed a dose-response to increasing concentrations of cadmium and the authors determined that the cell line was a useful *in vitro* model.

5.2 Exposure of HepG2 Cells to soil extracts

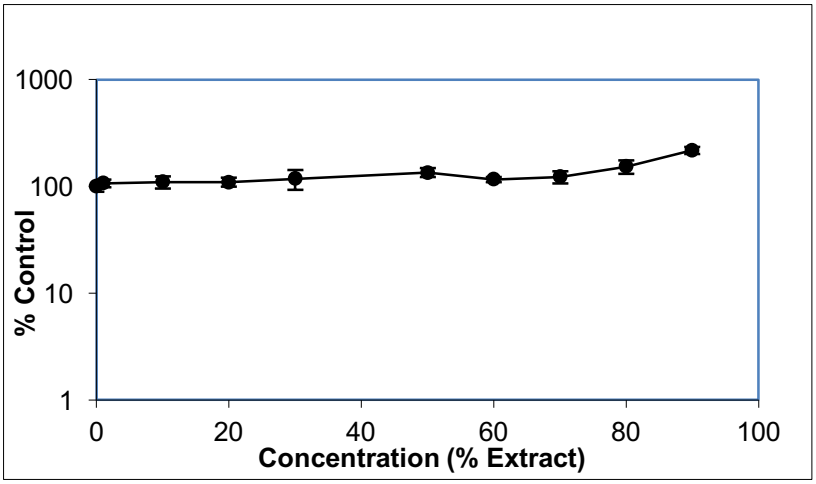
In order to establish HepG2 cells as a suitable toxicity bioassay, it was necessary to verify that the cells were sufficiently sensitive at environmentally and biologically relevant concentrations. Viability was chosen as an endpoint as it is easy to perform, reproduce and standardize. AlamarBlue was chosen as a simple fluorometric/colorimetric method of viability assessment for the same reasons. In addition, AlamarBlue is non-toxic to cells and the user, making it an attractive option versus more harmful chemicals.

Plating density and incubation time are the two variables that most affect the cell lines response to AlamarBlue and prior to use, it is recommended that for each new cell line a concentration-time curve is generated (see Appendix). HepG2 cells are seeded into 96-well plates at a density of $2x10^4$ cells per well. This density was chosen to allow the plate to reach 80-90% confluence during the overnight incubation period. At this concentration, between 1 and 6 hours are within the linear range of the fluorescence curve.

Soil extracts from the *in vitro* intestinal (versus gastric) extraction of soil samples were used in the HepG2 bioassay given the near neutral pH of the intestinal soil extract. Given the use of raw intestinal extracts, this was also a factor in the determination of a time endpoint as longer incubation periods could lead to cell contamination that would impact fluorescence intensity. Subsequent to exposure to bioaccessible extracts from each soil, cell viability was tested and measured using AlamarBlue, cell media blank corrected and plotted as a function of control.

In order to assess matrix effects, the saliva-gastric/intestinal fluid (S-G/I) was seeded at increasing concentrations and analyzed for viability after 24 hours to assess a level at which the raw intestinal extracts would not induce an adverse viability outcome. The S-G/I fluid showed little to no variation until higher concentrations were reached (e.g. 80% of S-G/I extract and 20% DMEM cell culture media) (Figure 5.1). Increases in fluorescence units (FU) as the S-G/I extract concentration reached 100% may be due to interferences with the fluorescence or color saturation of A/B.

Figure 5. 1 Raw saliva-gastric/intestinal extract w/o metals exposed to HepG2 cells



5.3 Results

Subsequent to the completed *in vitro* bioaccessibility test, raw intestinal extracts were exposed to HegG2 cells for 16 hours at concentrations of 0.0, 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, and 1 times the raw bioavailability extract aliquot concentration in order to assess the impact of a wide range of possible

bioavailable concentrations for different contaminants. The results of the Hepg2 cell exposure are presented in Figures 5.2 and 5.5. The data are presented as a viability percentage of control cells that were not exposed to extracts and maintained in serum free media. Viability significantly decreases as we move from 0.025x to 0.05x and appears to plateau at around 30 percent viability for NIST 2709 and NIST 2710 and plateaus at 30% as we move from 0.25x to 0.5x for LSP 125-500 microns and 45-125 microns.

The full extract exposure concentration yields a result of 26.7% NIST 2710, 14.7% for NIST 2709, 42.4% for LSP 125-500 and 28.2% LSP 45-125. These viability percentages are within the % bioaccessible intestinal fraction range of As and overestimates % intestinal IVBA for Pb, Cd and Cr. Additionally, the results for NIST 2710 and LSP 45-125 are close to the average, 26.2%, of total lead taken up into systemic circulation by the (Maddaloni et al., 1998) study where a human trial was conducted for the oral bioavailability involving soil. Overestimation of percent bioaccessibility in our system is preferable as it reduces the chance of underestimating risk, thereby still protective of human health.

These results show the possibility for use of an *in vitro* cell line as an alternative/complementary method to traditional measurements of metal clean-up levels given that the results of the cellular bioassay also show that with sufficient concentration dilution, there is a level below which no potential adverse reactions are expected to occur. Cell viability results also suggest the possibility of minimizing the time it takes to generate bioaccessible extract concentration

values. The results of the cell culture method could also be used as a possible screening tool for heavy metal contaminated soils in order to rank contaminated soil that could warrant further intensive review. However, the references in vitro bioaccessible concentrations are still needed in order to determine a safe level that constitutes a safe level without safety factors.

To be complete for validations, *in vivo* studies are needed in order to fully assess the ability of HepG2 cells to reliably correlate the target organ toxicity and bioaccessibility values. However, despite this need, HepG2 cells can provide an idea of what may happen to exposure to multiple metals at once in the liver post oral ingestion, and at what level there is no adverse response, without safety factors.

Figure 5. 2 Biofluid Extract Exposure NIST SRM 2710

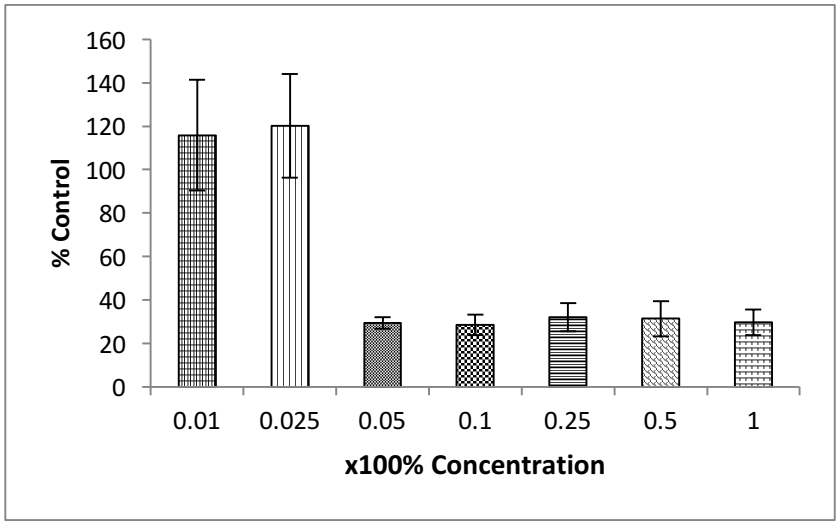


Table 5. 1 Concentration Units in ppm of soil extract exposure for NIST 2710

Metal	**0.01**	**0.025**	**0.05**	**0.1**	**0.25**	**0.5**	**1**
Pb	9.28	23.2	46.4	92.9	232	464	928
Ni	0.17	0.42	0.85	1.70	4.24	8.48	17
As	3.19	7.97	15.9	31.9	79.7	159	319
Cd	0.13	0.32	0.64	1.27	3.18	6.37	12.7
Cr	0.08	0.19	0.38	0.75	1.89	3.77	7.55

Figure 5. 3 Biofluid Extract Exposure NIST SRM 2709

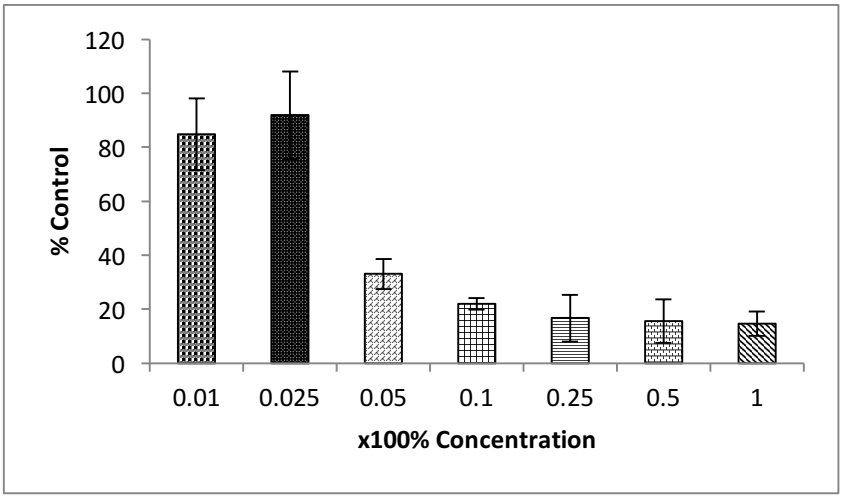


Table 5. 2 Concentration Units in ppm of soil extract exposure for NIST 2709

Metal	**0.01**	**0.025**	**0.05**	**0.1**	**0.25**	**0.5**	**1**
Pb	0.006	0.02	0.03	0.06	0.16	0.31	0.62
Ni	0.097	0.24	0.49	0.97	2.43	4.86	9.72
As	<0.006	<0.02	<0.03	<0.06	<0.14	<0.28	<0.56
Cd	0.002	0.005	0.01	0.02	0.05	0.11	0.21
Cr	0.06	0.15	0.30	0.60	1.51	3.01	6.02

Figure 5. 4 Biofluid Extract Exposure Liberty State Park 125-500 microns

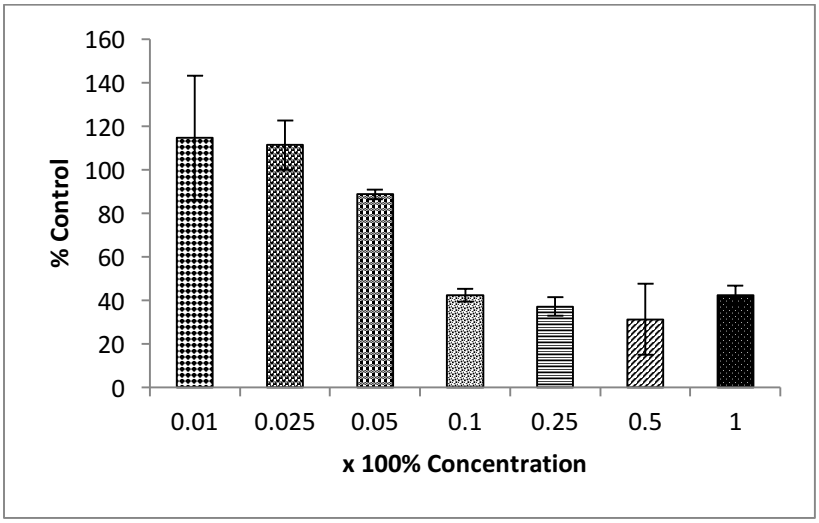


Table 5. 3 Concentration Units in ppm of soil extract exposure for LSP 125-500

Metal	**0.01**	**0.025**	**0.05**	**0.1**	**0.25**	**0.5**	**1**
Pb	0.16	0.39	0.79	1.58	3.95	7.90	15.7
Ni	0.23	0.58	1.16	2.33	5.82	11.6	23.2
As	0.06	0.16	0.32	0.63	1.58	3.17	6.3
Cd	<0.004	<0.01	<0.02	<0.04	<0.11	<0.21	<0.42
Cr	0.29	0.72	1.44	2.88	7.20	14.39	28.7

Figure 5. 5 Biofluid Extract Exposure Liberty State Park 45-125 microns

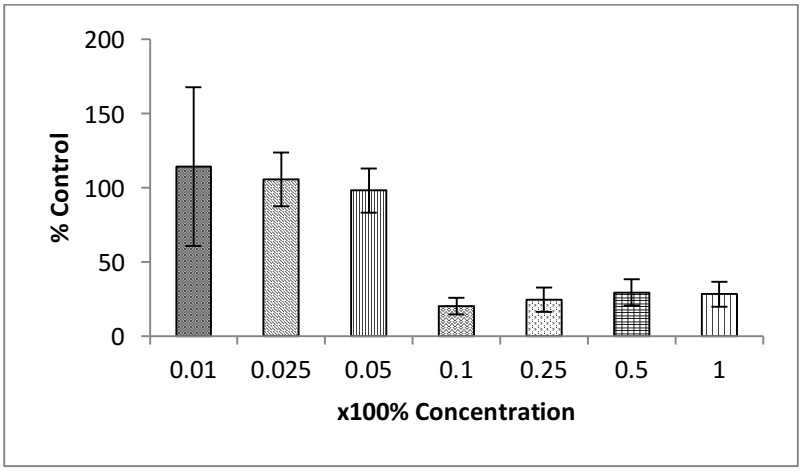


Table 5. 4 Concentration Units in ppm of soil extract exposure for LSP 45-125

Metal	**0.01**	**0.025**	**0.05**	**0.1**	**0.25**	**0.5**	**1**
Pb	0.42	1.06	2.12	4.24	10.6	21.2	42.4
Ni	0.47	1.18	2.37	4.74	11.8	23.7	47.4
As	0.16	0.40	0.80	1.61	4.01	8.03	16.1
Cd	0.003	0.01	0.02	0.03	0.08	0.17	0.33
Cr	0.47	1.18	2.37	4.74	11.8	23.7	47.4

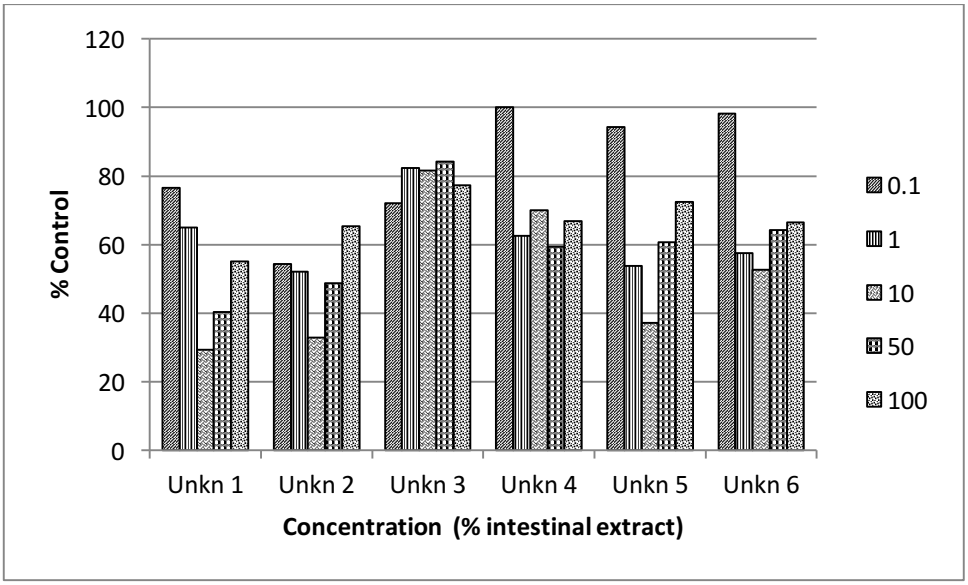
5.4 Discussion

The use of raw biofluid extracts from contaminated soil more closely represents real-world environmental conditions where contaminants, such as heavy metals, rarely occur as a single contaminant. However, it is a much better representation of the levels that can potentially induce toxicity in the liver (in our test system). It is important to note that in all four soil viability assays, Figures 5.4 to 5.7, the HepG2 maintained normal viability levels between (+/- 20% of control) at 1 and 2.5 % of the heavy metal extract concentration. The LSP soils maintained normal viability levels with exposure to concentrations up to 5% of

extract concentration. As the concentration of intestinal extract increases there is a sharp decrease in cell viability from 2.5% to 5% for NIST 2709 and NIST 2710 and from 5% to 10% for Liberty State Park Soils. Sharp decreases in soil extract viability as the concentration increases indicates a dilution concentration limit below which no adverse human effects are expected, which can be used for site-specific heavy meal cleanup. This response underscores the primary principle that underlies risk management: concentration reduction, which thereby reduces bioavailability.

Figure 5.6 shows the *in vitro* biofluid extraction exposure concentration for all six unknown soils. Unknown soil sample 1, which had similar acid-extractable concentrations compared to the five other soils, showed the greatest reduction in % IVBA extract exposure measurements to HepG2 cells. While the contaminant concentration of the bulk sample was not particularly high in unknown sample 1, the % IVBA measurements for the bioaccessible fractions was more than any measurement for each assay.

Figure 5. 6 Raw extract exposure of Intestinal % IVBA in an in vitro cellular assay.



Given the unknown nature of the samples, the viability of the the unknowns are compared to the bioaccessible fraction of each of the soil samples. In unknown sample1, the viability results of the HepG2 assay at 100% extract concentration were within the range of bioaccessibility for lead, nickel and chromium and are outside and above the range for arsenic and cadmium in the intestinal compartment. For unknown sample 2, at 100% extract concentration the cell model for percent viability does not fall within the range of % bioaccessibility for chromium for all other metals. In unknown sample 3, % intestinal bioaccessible chromium again fell within the range of the cell model viability while all other metals were outside and below the range. Unknown sample 4 showed an underestimation of chromium intestinal bioaccessibility whereas As, Cd, and Ni were outside the range and overestimated. Lead was within the % bioaccessible intestinal range for unknown sample 4. The percent

cell viability was outside and above all five metal percent bioaccessible intestinal concentrations for unknown sample 5. For sample 6, percent cell viability was within the range for arsenic and chromium, whereas lead, cadmium and nickel were overestimated and outside the % bioaccessible range.

Cell viability results from our unknown samples produce similar results to those shown by our well-characterized NIST SRM soil samples and highly studied Liberty State Park Soils when compared to intestinal percent bioaccessibility. There is a concentration dilution, below which no adverse outcomes are expected. These results allow the possibility of the usage of this method on field sample soils where the *in vitro* extraction/cell assay could be employed before further intensive bulk soil analysis is conducted. Information about risk could be more rapidly and readily available prior to more exhaustive testing. Several key factors may have contributed to the lack of significant differences in soil exposure pass the initial threshold. First, the soil extracts may prove to have undermined consequences on the bioassay environment that are independent of contaminant concentration. Only a suite of 5 metals were analyzed in the soil bulk system making is possible that another heavy metal could be responsible for changes in HepG2 viability. In addition, the bioavailable fraction of heavy metal contaminants can also decrease as concentration increases due to absorption mechanistic saturation (Ellickson et al. 2001; Ruby 1996), with the latter being more likely in our system.

Chapter 6: *In Vitro* Cell Bioassay- HepG2 Glutathione

6.1 Introduction

The aim of this chapter is to evaluate a biomarker for oxidative stress, in addition to the HepG2 viability results presented in Chapter 5 of this dissertation, as a possible supplement to risk assessment and management. Oxidative stress is thought to be an aspect of many diseases such as lung cancer, asthma, neurodegenerative disorders and diabetes (Rahman et al., 2006). Glutathione (GSH) is a tripeptide thiol antioxidant present in mammalian cells and plays a key role in the detoxification of cells by scavenging cellular hydrogen peroxide and conjugation of electrophilic metabolites of xenobiotics. Glutathione is mostly concentrated in the liver and intracellular concentrations can be an indicator of oxidative stress, which has a significant impact on the balance of cellular thiols, i.e. reactive oxygen species and xenobiotic interactions can cause a drop in GSH levels. Additionally, increases in oxidative stress can cause upregulation of cellular defense mechanisms such as the antioxidant GSH. Thus, measuring GSH levels can assess toxicological responses that promote oxidative stress leading to apoptosis and cell death (Griffith & Meister, 1985).

Almost all metals bind to thiol groups and under the right conditions reduced metals might undergo a Fenton reaction to form highly reactive hydroxyl radicals (Jozefczak et al., 2012). GSH mediates metal toxicity by binding to metals and affecting their transport, deposition and overall availability. Metals such as chromium, cadmium, arsenic and lead can bind to GSH. When GSH

binds to metals, the complex can be more readily eliminated from the human body. Conversely, metal-GSH complexes can increase toxicity by facilitating the metal ion's easier transport across biological membranes (Nordberg et al., 2014). In order to elucidate the differences between metals and its subsequent impact on GSH levels, metal salts of lead, arsenic, cadmium and chromium were used instead of pure *in vitro* intestinal extracts.

6.2 GSH Levels in HepG2 Cells exposed to heavy metals

GSH levels were analyzed following a method outlined in Rahman et al. (2006). Briefly, GSH was determined spectrophotometrically using a popular method that includes the use of 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), otherwise known as the recycling assay (Rahman et al., 2006). Cells were plated in a 48-well plate and grown to approximately 80% confluence and assayed after 16-hour exposure duration.

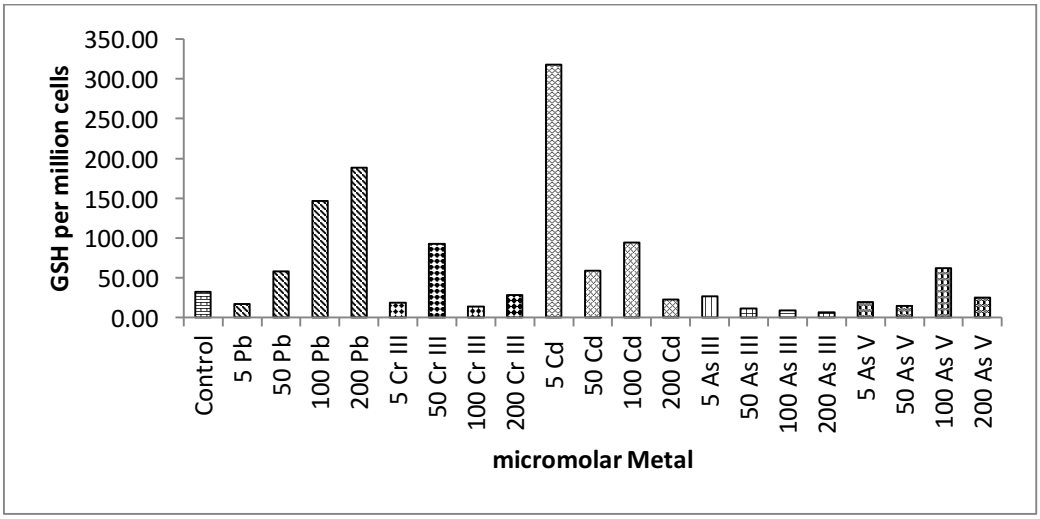
Figure 6.1 shows the GSH levels in response to lead acetate, chromium chloride, sodium arsenate and arsenite, and cadmium chloride. Nickel was not evaluated for GSH levels as previous studies have shown that the direct impact of nickel on GSH level is limited and that decreases of GSH levels are thought to be a secondary effect of nickel-generated ROS (Sigel et al., 2007). Cr III was chosen for exposure because in the human body, Cr VI is readily reduced to Cr III. Results are presented as micromolar GSH levels per 1,000,000 cells. HepG2 cells were exposed to a range of 0 to 200 micromolar metal concentration. This range was chosen in order to generate a response but was not expected to be high enough to induce cell death.

Low-level lead (5ppb) shows depletion of GSH levels versus control (HepG2 cells not exposed to any metal salts) (Figure 6.1). GSH levels increased with increasing Pb concentration suggesting the involvement of oxidative stress and free radicals in the pathogenesis of lead poisoning (Adegbesan & Adenuga, 2007). The inverse relationship is seen for cadmium where low levels initiate an increased protective response whereas an increase in cadmium concentration

depletes GSH. This cadmium relationship with GSH is consistent with previous studies such as Gaubin et al. (2000) who evaluated the glutathione mechanisms in a human lung cell-line (A549 cells) exposed to low-level cadmium. Arsenic III and V overall depletes GSH which can induce oxidative stress and is consistent with previous *in vitro* and rodent studies (Hall et al., 2013). Chromium GSH levels were depleted for every concentration tested except for an upregulation at 50 µM.

These GSH results show that hepatotoxic response is dependent on several factors including the type of heavy metal and its concentration as well as speciation. These results are in accordance with the viability assay which shows that low levels of heavy metals may induce a greater hepatotoxic effect.

Figure 6. 1 GSH response to selected metals (Pb, Cr, Cd, As) in HepG2 Cells



Chapter 7: Discussion

7.1 Summary of Results

The research presented in this dissertation examined the bioaccessibility of lead, nickel, cadmium, arsenic and chromium in a suite of NIST SRM soils and a suite of unidentified soils. This research was anchored on the following objectives:

- Analysis of bioaccessibility of heavy metal contaminated soil using a sequential simulated biofluid sequential extraction *in vitro* method.
- Development of an *in vitro* HepG2 cellular model for incorporation into an existing *in vitro* method for bioaccessibility.
- Development of a rapid cellular bioassay to further investigate human health risk and to optimize its use as a preliminary screening tool for human toxicity.

7.2 Bioaccessibility Results

Previous studies have measured absorption of soluble metal after ingestion. Gastrointestinal uptake rates derived from human studies for nickel indicate that absorption across the GI tract varies from 1 to 30% depending on the fed or fasting state. There are no human studies for nickel metal (Buchet et al., 1981; Sunderman et al., 1989). Oral uptake of cadmium is considered to be low, on order the of 5 to 10%, with most cadmium ingestion occurring after incorporation into mineral rich food (Buchet et al., 1981). Oral ingestion of lead has been investigated more extensively than other metals and its bioavailability has been

recognized as a function of multiple factors such as chemical speciation and level of exposure. Maddaloni et al. (1998), one of the only human studies involving oral ingestion of lead laden soil, found on average 26.2% ± 8.1 of the administered dose was absorbed. Arsenates and arsenites are readily absorbed across the GI tract. Bettley and O'Shea (1975) estimated absorption across the GI tract for arsenic from fecal excretion in humans and oral doses of arsenite and found at least 95% of arsenite was absorbed. Studies based on urinary excretion in humans were able to account for 55-87% of daily oral intakes of arsenate or arsenite (Buchet et al., 1981; Tam et al., 1979). Chromium compounds were estimated by its excretion in urine. Trivalent chromium compounds are poorly absorbed (0.6%) while hexavalent chromium is more readily absorbed at 6.9% (Kerger et al., 1996).

Chapter 4, Table 4.1 shows the certified mass fraction concentrations of lead, nickel, arsenic, chromium, and cadmium for NIST 2710. While lead had the highest reported concentration in the bulk sample, it had the lowest percent bioaccessibility in both the gastric and intestinal compartment. Conversely, nickel had the third highest reported concentration out of the five metals assayed in the bulk sample and was the highest percent bioaccessibility. Cadmium and chromium were significantly lower in concentration when compared to lead, however bioaccessibility in both the gastric and intestinal compartments were significantly higher than the bioaccessibility for lead. Arsenic concentration ranked second in the bulk, while the % bioaccessibility ranked third in the intestinal fluid and second in the intestinal fluid. Chapter 4, Table 4.4 shows the

certified mass fraction concentration of lead, nickel, arsenic, chromium and cadmium for NIST 2709. Unlike NIST 2710, the highest and lowest percent metal bioaccessibility values correlate with the highest and lowest bulk concentration values, chromium and cadmium, respectively.

The same relationship between percent bioaccessibility and bulk soil concentrations seen in NIST SRM 2710 can be seen in the Liberty State Park Soils where chromium has the highest concentration in the bulk soil and the lowest % bioaccessible values in both the intestinal and gastric compartment. Additionally, bioaccessibility analysis of our suite of unknown soil samples showed a lack of a relationship between bulk soil concentration and bioaccessibility results. Given the unknown nature of the samples, bulk soil concentration represents the majority of the information available. However, the relationship between bulk concentration and bioaccessibility is primarily driven by metal form and solubility.

As shown in previous studies, the incorporation of bioaccessibility values versus the use of bulk soil concentrations greatly affects risk assessment. Additionally, the use of the NIST SRM Materials can provide a baseline for method comparison, as more metals outside of arsenic and lead are analyzed and validated using *in vitro* extraction methods. A comparison of NIST SRM Materials and unidentified soil samples, show a similar relationship between the bulk and bioaccessible fraction, showing the utility of the HMBSR *in vitro* extraction protocol to reasonably predict bioaccessibility for a suite of soil matrices.

7.3 *In vitro* Cell Model: HepG2

In all but one instance in our comparison between % intestinal bioaccessibility and % viability in our cell model, the % cell viability overestimated or fell within the potential % metal bioaccessibility range. This shows the possibility of *in vitro* bioaccesibility and the dilution level necessary for contaminant cleanup. It is well known that total bulk metal content in soils is a poor indicator of risk as it leads to gross estimations. It is also well known that *in vitro* bioaccessibility results may provide risk assessors a more reliable estimate of risk when compared to the use of bulk concentration estimates. The results of the HepG2 viability assay in this dissertation more reasonably predicted risk when compared to bulk concentration estimates while overestimating risk when compared to %IVBA results. And for no toxicity, dilution of the bioaccessible fraction down to 2.5% of the IVBA results seems sufficient for minimizing risk.

It is reasonable that the HepG2 viability assay would underestimate estimates of risk using bulk concentrations and overestimate %IVBA measurements in its assessment of risk. First reason being that the cell viability assay is more relevant to what happens inside the human body post oral ingestion where bulk concentrations in soil do not reflect internal dose. Second, the viability assay and % IVBA results have different meanings. Bioaccessible concentrations represent the maximal concentrations that could possibly become bioavailable and enter systemic circulation and it is assumed that this concentration was fully exposed to the liver. It is more likely that a percentage of the bioavailable fraction would overburden the liver and cause liver damage or be exposed to metals not

measured by the assay in this dissertation; leading to overestimation of risk compared to IVBA measurements.

7.3.1 Cell-Based Bioassay for Environmental Risk Management: Viability

Concentration control of individual hazardous materials is one of the fundamental principles that underlie environmental risk management. However, this fundamental principle is faced with several problems. First, it is virtually impossible to set concentration standards for each and every possible contaminant at a reasonable rate. Second, environmental contaminants rarely exist as a single entity but rather as a mixture of multiple contaminants simultaneously. Cellular bioassays represent an attractive addition to conventional-contaminant specific risk management given that bioassays can assay toxicity across a spectrum of contaminants at once. In fact, Clemedson et al. (1996) found that short-term cell-based (mammalian) survival assays show a good correlation with acute lethal blood concentrations.

Many different soil remediation techniques exist, with *in situ* treatment of soil being preferred in some cases given the considerable cost of transporting contaminated soil. Remediation techniques include methods such as bioremediation, where microbes are used to degrade organic compounds and soil washing, where particle size separation occurs physically. While different thresholds exist for different sites and specific contaminants, remediation goals generally focus on reducing the bioaccessibility of contaminants and thereby the concentration in soils. The percent remaining post remediation must meet

regulatory guideline and the use of bioassay pre-screening tools can help prioritize sites for cleanup.

Risk is influenced by many factors, such as the contaminant type, medium and in the case of metals, speciation, which determine its fate and potential toxicity. By evaluating the use of viability as a test endpoint of HepG2 exposed to pure in vitro bioaccessibility extracts, we find that there is a level at which the of dilution causes no adverse outcomes (in the case of this dissertation, reduction in viability). Figure 5.2 in Chapter 5 shows results of a HepG2 viability assay for NIST SRM 2710. A 99 and 97.5 percent reduction in intestinal extract concentration show no difference in cell viability between the control and exposed cells. There is a marked decrease in viability as the concentration of intestinal extract increases to 5 (five) percent of the intestinal extract concentration. The same is true for NIST SRM 2709 whose concentrations are comparatively low to NIST 2710. Reduction in intestinal bioaccessible concentrations down to 99 and 97.5% show minimal reduction in viability compared to control. Therefore, effectively reducing the concentration of bioaccessible metal concentrations decreases the likelihood of an adverse toxicity endpoint, i.e. viability.

Both Liberty State Park soil fractions, 145-500 and 45-125 microns, respectively, show minimal decreases in viability when exposed to 1 (one) percent to 5 (five) percent of the intestinal *in vitro* bioaccessibility extract and a significant decrease of HepG2 cell viability when the bioaccessible intestinal concentration is reduced by 90%. NIST SRM soils have been homogenized, size

fractionated (< 74 microns) and sterilized. Compared to the Liberty State Park (LSP) soils, NIST SRM soils require a higher rate of dilution, down to 5%, versus down to 10% for LSP. This difference may be attributed to the differences in size where heavy metals are to be present in higher concentrations at lower size fractions.

For our six unknown soils received from the EPA they were pre-processed, but the specifics of this process are unknown. What is known is that the size fractions for unknown samples one to four were less than 250 microns and unknown samples five and six being under 74 microns. Compared to NIST 2710, NIST 2709 and the LSP soil, the six unknown soils showed less of a reduction in viability for increasing concentrations of intestinal bioaccessible extracts.

For unknown sample 1, significant reduction in viability begins at 1 % of the intestinal extract concentration, indicating that dilution of the soil down to 0.1% of the intestinal bioaccessible concentration could possibly minimize human toxicity as an endpoint. Unknown sample 2 had the highest concentration across the board of all the metals assayed (Pb, As, Ni, Cd, and Cr) and showed the most significant reduction in viability regardless of the dilution factor, with 99.9% reduction in concentration producing a reduction in viability on the order of 40%. In the case of unknown soil 2, remediation efforts beyond concentration reduction may be necessary to remove the threat of this soil to human health. Unknown sample three showed minimal reduction in viability for each bioaccessible concentration assayed. Unknown samples four through five showed almost 100

percent viability when exposed to 0.1 % of the extract concentration. When the intestinal extract concentration increased from 0.1 to 1%, the viability of unknown samples four, five and six decreased significantly.

The objective of the HepG2 viability bioassay was to investigate the potential of the utility of a cellular bioassay to as an effective addition to risk management. The results from this bioassay demonstrated that the containment of metals, or in other words effective concentration reduction in metal bioavailability, reduces potentiality for human toxicity. However, concentration reduction is site specific and given that a mixed media with a wide array of different metals was used, it is difficult to elucidate which metal or metal combination may be driving the toxicity endpoint.

Despite the latter, remediation efforts that effectively reduce the bioavailability of heavy metals will reduce impacts on human health. Of the metals assayed, cadmium is a hepatic toxin (cadmium also significantly impacts the kidney). Exposure to increasing levels of chromium can also cause impaired liver functions. Using the cadmium bioaccessible concentrations, the unknown soils would rank as follows (in order of increasing potential for human harm): Unknown 4< unknown 6< unknown 5< unknown 3< unknown 2< unknown 1. The latter hierarchy mostly holds true however when compared to the HepG2 results, unknown sample 2 would be possibly be flagged for further investigation given its significant decrease in HepG2 viability in spite of significant concentration dilution down to 2.5% of the bioaccessible concentration.

7.3.2 Cell-Based Bioassay for Environmental Risk Management: GSH

Equally, dose-response curves or concentration-responses are often used for assaying adverse effects of pure chemicals. In order to utilize a second line of evidence for the utility of a cellular bioassay as a useful addition to risk management, a biomarker for oxidative stress, GSH, was evaluated using pure metals. Pure metals were chosen in order to effectively compare GSH results to standards for individual metals.

For Pb II, increasing concentrations of lead shows an increased in GSH response. While there was a spike in GSH response at 50 micromoles for Cr III, there was not a significant increase GSH level response. As previously discussed, low levels of cadmium causes a significant protective GSH response while As II and As V cause GSH depletion. The concentration relationship using individual metals shown in Chapter 6 is not as strong as the concentration relationship shown with soil *in vitro* intestinal extracts. This difference may be due to the metal-specific nature of GSH response. Given the latter, GSH may need to be further explored as a secondary test for toxicity.

7.4 Exposure/Dose Estimate

An important part of public health protection is the prevention and/or reduction of exposures to environmental contaminants that may lead to adverse health outcomes. Exposure assessment in the risk assessment process requires quantitative determination of concentration estimates to which the human population may be exposed. Young children have high relative risk of exposures

from soil ingestion evidenced their higher rate of absorption and soil ingestion rate compared to adults. In addition, *in vitro* bioaccessibility assays, including the one utilized in this dissertation, were developed to represent human physiology with a child in mind.

The general equation of a selected exposure route, in this case ingestion, is:

$$Average\ Daily\ Dose\ (ADD) = \frac{Cx\ IR\ x\ EF\ x\ ED}{BWx\ AT}$$

Where C is concentration in soil (mg/kg), IR is ingestion rate (mg/day), EF is exposure frequency (days/year), ED is exposure duration (years), BW is body weight (kg) and AT is averaging time (days).

Non-carcinogenic risk is characterized using a hazard quotient (HQ). HQ is equal to the ratio of the ADD to the reference dose (RFD). These values are used to determine acceptable human health risk levels. The goal of this dissertation was to refine human health risk and exposure assessment of metal-contaminated sites using an in-vitro bioaccessibility model in combination with two cellular bioassays. Non-cancer risk was chosen as an endpoint given the choice of children under the age of 6 years of age as a population basis.

7.4.1 Average Daily Dose

The average daily ingestion dose was calculated for Pb, As, Cd, Ni and Cr for each soil assayed in this dissertation. The following values were used (taken

from EPA's Child-Specific Exposure Factors Handbook 2008) using the age group 1-<6 years of age. The median ingestion rate (IR) used for a contaminated soil was 200 mg/day, which is the average soil ingestion rate for a child. The soils assayed in this dissertation are from widely different regions of the U.S. and differ in soil environment (i.e. pastureland versus agricultural land vs park land). The child is assumed to live in the area where the soil was assayed giving an exposure frequency (EF) of 365 days/year and a max ED is exposure duration of 6 years. The median body weight (BW) used was 16 kg and the averaging time (AT) is 7 days a week. The average daily dose was calculated using 100% bioaccessibility, % IVBA, and HepG2 viability results (see appendix) and subsequently used to calculate hazard quotients.

The average daily dose (ADD) for each metal was calculated for our suite of soils (Tables 7.1 through Table 7.3). The equation for ADD is underscored by the concentration of the contaminant in the media. Table 7.1 shows ADD values using the concentration of metals present in the bulk soil. Table 7.2 show ADD values using IVBA bioaccessible concentrations of the metals in soils. Table 7.3 shows ADD levels calculated using the lowest observed level of potential adverse effect.

Table 7. 1 Average daily dose calculation using 100% Bioaccessibility (i.e. soil concentration) in mg/kg-day

	NIST 2710	NIST 2709	LSP 125-500	LSP 45-125	LSP >45	
Pb	5.8	0.02	1.4	1.5	2.2	
As	0.7	0.02	0.13	0.16	0.2	
Ni	0.01	0.09	0.6	0.7	0.7	
Cd	0.02	0.0004	0.002	0.001	0.002	
Cr	0.04	0.1	2.6	2.7	3.1	
	Unknown 1	**Unknown 2**	**Unknown 3**	**Unknown 4**	**Unknown 5**	**Unknown 6**
Pb	1.3	2.1	1.5	0.	1.8	0.8
As	0.7	1.3	1.3	0.33	0.6	2
Ni	0.01	0.01	0.01	0.003	0.01	0.01
Cd	0.03	0.03	0.01	0.03	0.06	0.03
Cr	0.01	0.01	0.01	0.003	0.06	0.02

Table 7. 2 Average daily dose calculation using % IVBA in mg/kg-day

	NIST 2710	NIST 2709	LSP 125-500	LSP 45-125
Pb	1	0.001	0.02	0.04
As	0.33	0.001	0.01	0.02
Ni	0.02	0.01	0.02	0.05
Cd	0.01	0.0002	0.0004	0.0003
Cr	0.01	0.01	0.03	0.05

	Unknown 1	Unknown 2	Unknown 3	Unknown 4	Unknown 5	Unknown 6
Pb	0.9	0.7	0.5	0.3	1	0.1
As	0.3	50	0.5	0.1	0.1	0.8
Ni	0.004	0.004	0.003	0.00002	0.003	0.004
Cd	0.01	0.01	0.002	0.0004	0.01	0.003
Cr	0.01	0.01	0.01	0.01	0.01	0.01

Table 7. 3 Average daily dose calculation using lowest viability response dose in mg/kg-day

	NIST 2710	NIST 2709	LSP 125-500	LSP 45-125	
Pb	0.05	0.001	0.01	0.004	
As	0.02	0.001	0.01	0.005	
Ni	0.001	0.01	0.001	0.001	
Cd	0.001	0.0002	0.0002	0.00003	
Cr	0.0004	0.01	0.02	0.005	
	Unknown 1	**Unknown 2**	**Unknown 3**	**Unknown 4**	**Unknown 6**
Pb	0.13	0.21	0.001	0.18	0.08
As	0.07	0.53	0.001	0.16	0.20
Ni	0.001	0.004	0.0001	0.00012	0.001
Cd	0.003	0.003	0.00001	0.0186	0.003
Cr	0.001	0.001	0.00001	0.002	0.002

7.4.2 Hazard Quotints

The HQ was calculated for each metal using the concentration in each soil (Table 7.4). The HQ was also calculated for each metal using the % IVBA results in each soil (Table 7.5) to give a site-specific risk assessment. HepG2 viability results were used to assess its possible use in site-specific risk assessment (Table 7.6). The concentration that most significantly reduced viability in our assay was chosen as possible surrogate the biologically effective concentration that has potential for adverse toxicological outcomes in our liver system. In certain cases, oral reference doses depend on the speciation of the metal, therefore HQs were calculated for Cr III and Cr VI. An oral reference dose does not exist for lead as there is no dose for which lead would not have an

adverse effect. Given the latter, blood lead concentration of 5 ug/dL was used in place of an oral reference dose.

Using the approach of 100% bioaccessibility from soil concentrations (Table 7.4), the results suggests that the soils assayed in this dissertation were at a level of unacceptable risk for As and Cr VI. Cr III was at unacceptable risk levels for all LSP soils. Ni had a potential for risk in all soils except NIST 2710 and unknown soils 1 through 3. Cd had minimal expectation for potential risk except for NIST 2710 (HQ=2.3). For lead, only NIST 2709 had a HQ less than 1.

Table 7. 4 HQ calculated using Soil Concentrations

	NIST 2710	NIST 2709	LSP 125-500	LSP 45-125	LSP >45
Pb	115	0.4	27	31	45
As	2176	61.5	428	543.4	745
Ni	0.7	4.6	29	34	33
Cd	2.3	0.04	0.2	0.1	0.2
Cr III	0.03	0.1	1.7	1.8	2.1
Cr VI	14	45	851	896	1039

	Unknown 1	Unknown 2	Unknown 3	Unknown 4	Unknown 5	Unknown 6
Pb	26	42.4	30	7.2	36.8	16.2
As	2173	4243	4413	1095	2084	6539
Ni	0.3	0.4	0.3	0.00012	0.3	0.6
Cd	3.0	3.2	1.1	2.6	5.8	3.4
Cr III	0.005	0.01	0.01	0.002	0.04	0.01
Cr VI	2.4	2.7	3.0	1.1	19	57

The use of *in vitro* bioaccessibility values to calculate risk in environmental risk assessment (ERA) provides a more realistic level of concern (Table 7.5). The potential for adverse effects decreased significantly for each soil assayed overall.

The results suggest that all soils were at a level of unacceptable risk for As and Cr VI. Cr III and nickel was at unacceptable risk levels for all LSP soils. Unknown soils 1 through 6 and NIST 2710 were at unacceptable risk levels for cadmium. Pb showed a significant reduction in HQ values with NIST 2709 and both LSP soils falling below an HQ of 1 (one).

Table 7. 5 HQ using %IVBA Bioaccessible Concentrations

	NIST 2710	NIST 2709	LSP 125-500	LSP 45-125
Pb	19.4	0.02	0.4	0.8
As	1108.1	2.0	21.9	55.8
Ni	0.9	0.5	1.2	2.5
Cd	1.3	0.02	0.04	0.03
Cr III	0.01	0.004	0.02	0.03
Cr VI	2.6	2.1	10.0	16.5

	Unknown 1	Unknown 2	Unknown 3	Unknown 4	Unknown 5	Unknown 6
Pb	17.2	14.6	9.4	5.8	19.2	2.8
As	976	1776	1805	390	452	2720
Ni	0.2	0.2	0.1	0.001	0.2	0.2
Cd	1.1	0.9	0.2	0.037	0.9	0.3
Cr III	0.005	0.004	0.01	0.005	0.005	0.01
Cr VI	2.4	2.0	2.7	2.4	2.5	25

The use of an *in vitro* cellular system as a potential surrogate for target organ toxicity (i.e. hepatocytes to simulate the liver) to calculate risk may provide a more easily obtained estimate of the potential for toxicity in the liver. The potential for adverse effects decreased significantly for each soil compared to the use of IVBA in HQ calculations. The results suggests that all soils were still at a level of unacceptable risk for As and Cr VI, whereas Cr III and nickel were at acceptable risk levels for all LSP soils. Cadmium had unacceptable risk for

Unknown Soil 1 and NIST 2710 (Table 7.6). This shows still shows that the sum of the metals produces overall risk. For lead, the HQ for NIST 2710 was equal to 1, and unknown samples 1, 3, 4, 5 and 6 all had HQs greater than 1 (one).

Table 7. 6 HQ using % HepG2 Viability

	NIST 2710	NIST 2709	LSP 125-500	LSP 45-125
Pb	1	0.02	0.2	0.08
As	55.4	2.0	40.5	16.5
Ni	0.04	0.5	0.2	0.1
Cd	0.1	0.02	0.02	0.003
Cr III	0.0003	0.004	0.01	0.003
Cr VI	0.1	2.1	5.0	1.6

	Unknown 1	Unknown 2	Unknown 3	Unknown 4	Unknown 5	Unknown 6
Pb	2.6	4.2	0.02	3.6	1.6	2.6
As	217	1778	4.4	547	208	654
Ni	0.03	0.2	0.0003	0.006	0.03	0.1
Cd	0.3	0.3	0.001	1.862	0.6	0.3
Cr III	0.0005	0.001	0.00004	0.001	0.004	0.001
Cr VI	0.2	0.3	0.003	0.6	1.9	5.7

The need to reduce uncertainties in numerical calculation of risk calculation has opened the door for the use of additional lines of evidence to support estimation of risk such as the use of site-specific biological responses. Site-specific biological responses may include biomarkers of exposure like glutathione (GSH) (Saunders et al., 2010). Given the latter, environmental risk assessments for 11 “sites” were evaluated and compared to GSH levels (Figures 7.1 to 7.3). Agreement between numeric risk and biomarker assessment

increased as a more site-specific/target organ specific calculation was used (i.e. soil concentration > % IVBA > %viability). However, the relationship between biomarker response (i.e. GSH depletion) and HQ calculations and its inclusion as an additional line of evidence in risk assessment is limited to the biological system utilized to assay GSH levels. Correlation is also possibly limited by the ability of the contaminants to elicit a hepatotoxic response.

A more biologically relevant biomarker of response system would be specific to the metal or contaminant assayed and given the simplicity of and rapid response time, it could be possible to create target organ specific cellular bioassays. Despite its specificity to the liver, the use of a cellular system to measure biological response can be used in site-specific and population-specific bioaccessibility risk assessment processes.

Figure 7. 1 Comparison of estimated daily hazard quotient using 100% bioaccessibility and oral reference dose.

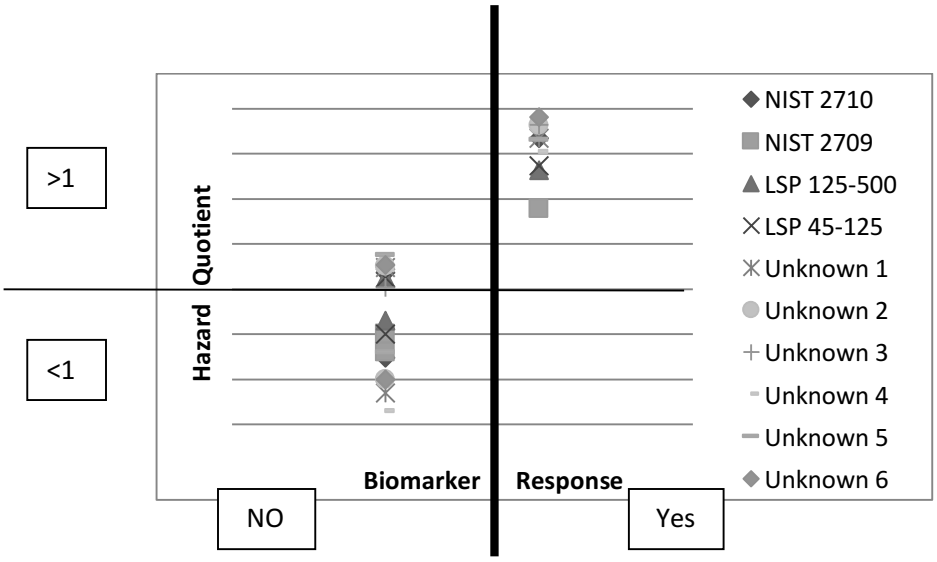


Figure 7. 2 Comparison of estimated daily hazard quotient using %IVBA and oral reference dose.

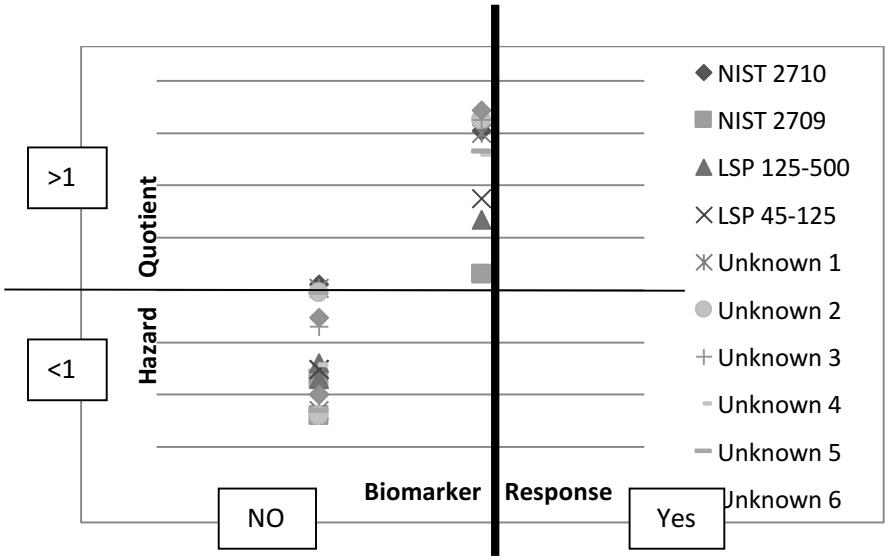
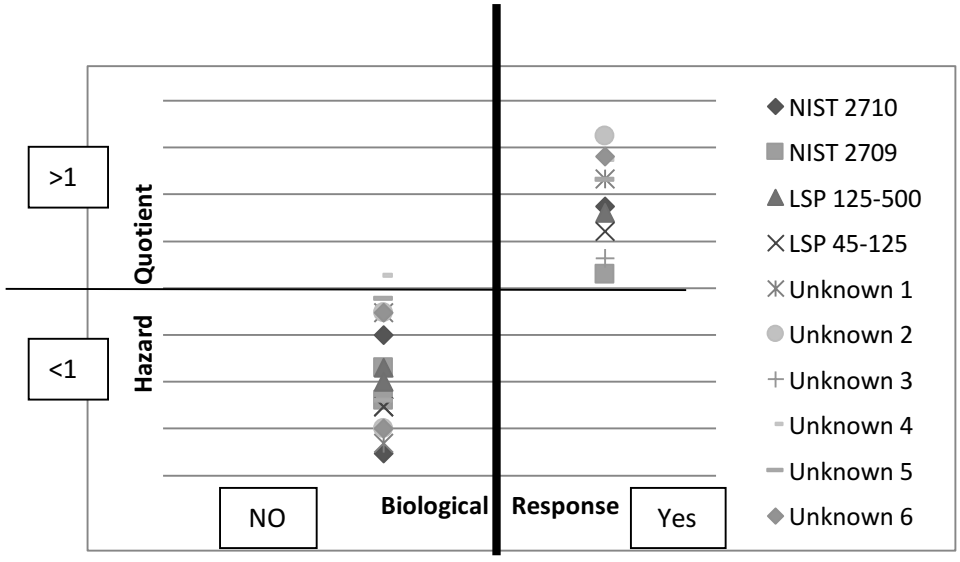


Figure 7. 3 Comparison of estimated daily hazard quotient using viability and oral reference dose.



7.5 Summary

In this study of arsenic, lead, cadmium, nickel and chromium, bioaccessibility values in the intestinal and gastric compartment of the GI tract is shown to be more predictive of risk than typical acid leaching techniques, in accordance of previous studies. The incorporation of a hepatocellular model into an existing IVBA system reasonably predicted potential risk to the liver. Cellular models may improve uncertainties and accuracy in risk, thereby improving the prioritization of contaminated sites for a suite of heavy metals. The use of a HepG2 cellular viability model showed that sufficient concentration reduction of heavy metal soil contaminants minimizes the potential for human health risk. These results also prove that the use of a gastric only model for bioaccessibility does not reasonably predict bioaccessibility as well as a model containing both the intestinal and gastric compartments.

The use of *in vitro* bioaccessibility and viability response concentrations in the calculation of risk was aligned with changes in glutathione biomarker results and representative of metal concentrations in the soil sample. This study provides evidence that using site-specific bioaccessibility and one method of possible validation using biomarkers may enhance the accuracy of predicting risk. Furthermore, both lines of evidence can be used to make informed decisions about risk and site management.

Population effects complicate environmental risk assessment and these effects cannot be completely observed in bioaccessibility measurements. In vitro cellular systems and biomarkers of response represent an attractive supplement

to risk assessment to quantitatively identify biochemical and physiological states within cells, tissues, and/or individuals. Our suite of metals has select preference for various parts of the body and may not necessarily accumulate in the liver. In fact, the liver's "first-pass" clearance may effectively remove exogenous metal concentrations depending on the effective liver dose. As a result, sufficient concentrations to cause a potential for toxic injury is hypothesized to occur when the contaminant selectively accumulates in the liver or in diseased population that diminishes liver detoxification function. In case of metals, the use of the HepG2 viability and GSH model are more appropriate to assess risk for a selective population.

For example, persons afflicted with Wilson's disease accumulate copper in the liver. Additionally, alcoholic liver disease and its subsequent progression of liver damage have been shown to selectively reduce mitochondrial hepatic glutathione content in rats. Depending on the extent of damage, hepatocytes may undergo cell death or become sensitized to cell death stimuli (Yuan & Kaplowitz, 2009). The model presented in this dissertation could represent a basis for incorporation of sensitized populations into models for risk assessment.

Chapter 8: Development of a tool for risk characterization

Evaluations of exposure from incidental and/or accidental ingestion of metal-contaminated soils depend on accurate measurements of bioavailability in order to make reliable assessments regarding human health. Animal models are routinely used to measure *in vivo* estimates of bioavailability. However, given the time-consuming and costly nature of animal assays, *in vitro* tests have been developed as a complementary tool to help determine metal bioaccessibility (a common surrogate for bioavailability) in soils. Given that assessments of heavy metal bioaccessibility and bioavailability in soils significantly impact human exposure assessments, the NRC (NRC, 2012) has advocated the development of complimentary models that would aid in the reduction of uncertainties in current risk evaluation. Furthermore, the need for the development of rapid, convenient, and inexpensive tools for risk assessment has increased as bioaccessibility and bioavailability estimates of metals in soils can have significant impacts, which can possibly change the course and cost of soil remediation

Models for bioaccessibility aim to mimic the physical and chemical environment of the gastrointestinal tract and the amount of dissolved contaminant in simulated gastrointestinal solutions is considered to be the bioaccessible fraction that is available for potential absorption into systemic circulation. Conversely, models for relative bioavailability (RBA) measure the uptake of a contaminant into the target organ from the soil matrix relative to the uptake of a contaminant in an animal model in the form of a readily soluble salt.

In order for *in vitro* bioaccessibility (IVBA) studies to transfer from a complementary tool to a surrogate tool for RBA measurements, the relationship between IVBA and RBA needs to be established and validated. Various studies have shown the potential of *in vitr*o assays to predict RBA in order to refine human health exposure assessment, particularly for arsenic (Bradham et al., 2015; Ruby et al., 1996), lead (Denys et al., 2012; Ruby et al., 1996) and cadmium (Denys et al., 2012). As, Cd and/or Pb RBA values in the aforesaid studies were measured using a swine, primate or rodent model where contaminant concentrations were measured after exposure in the blood/urine or measured by contaminant accumulation in organs/bones (Bradham et al., 2011; Casteel et al., 1997; N. C. G. Freeman et al., 1997; Lorenzana et al., 1996). However, it is unclear how these *in vitro-in vivo* correlations (IV-IVC) perform outside of the model given that these studies utilized a limited number of soils. For instance, a study conducted by (Li et al., 2015) measured As-bioaccessibility and As-RBA to determine IV-IVC for twelve (12) soils using five different (5) bioaccessibility assays. Their research showed significant differences between predicted RBA values and highlighted the inability of established IV-IVCs to predict As-RBA outside of the soils used to establish the correlations.

The ability for *in vitro* assays to accurately predict soil RBA rely on the strength of the correlation between *in vitro* and *in vivo* observations. Juhasz et al., 2013 suggested several criteria for establishing performance validation of predictive *in vitro* assays of contaminated soils. First, performance validation needs to be established by evaluating the robustness of the RBA and

bioaccessibility relationship with soils that are independent from the original soils used to establish the model, something that is rarely done when establishing IV-IVC relationships. Second, consideration needs to be taken regarding the number of data points used to validate the model, which can be limited by factors such as research outcomes, study cost and time constraints. To date, IV-IVC models have utilized between 5 and 19 soil samples, and it is still unclear from the literature how many soil samples are sufficient to permit a comparison of method performance (Juhasz et al., 2013).

Soil type and contaminant concentrations are also important when establishing *in vivo-in vitro* relationships. Soil contaminated from mining activity comprise the majority of IV-IVC studies deeming most studies relevant only in the context of refining human health exposures for populations near mining sites, furthering the need to establish models that are applicable on a wider range of soil contaminant types. In addition, the applicability of the model to "real-world" exposure scenarios are centered around the use of soils with environmentally relevant contaminant concentrations, which is paramount for the predictive nature of the IVBA model. As mentioned previously, the cost of *in vivo* animal studies associated with risk assessment greatly limit the amount and speed of RBA data being generated in peer reviewed literature. Differences in methodologies, endpoints, and animal models in the literature further complicate efforts to establish metal specific relationships between IVBA and RBA, especially when attempting to establish IV-IVC comparisons on soils from different locations and soil properties.

The development of human health-based risk estimates, while often measured using animal data, is also preferably established. When human data are unavailable or needs to be supplemented, surrogate data endpoints related to the adverse effect are often investigated. The latter are particularly useful when we refer to unintentional exposures where ethical issues are mitigated given that exposure occurs regardless of whether an endpoint is being measured. In regard to *in vitro* analyses in risk assessment processes, *in vitro* tests can be useful when a system is designed around endpoints that reflect changes post *in vivo* toxic insult (Flint, 1993). A growing number of *in vitro* tests using biological systems, such as isolated organs and cell cultures, have been developed in order to evaluate the toxicological hazard of a compound. *In vitro* systems may be useful for evaluating mechanisms of toxic action, which can be used as a basis for hazard identification especially for screening purposes (Blaauboer, 2001).

Indications of toxicity from heavy metals are mainly credited to oxidative stress, i.e. the imbalance between the production of free radicals and antioxidants, which may lead to chronic liver injury and hepatic inflammation (Garcia-Nino & Pedraza-Chaverri, 2014). Given that the liver plays an essential role in the metabolism and detoxification of xenobiotics, the liver is considered to be an important organ with regard to environmental pollutants. In addition, bioaccessible contaminants absorbed by the intestine enter the hepatic system resulting in a first pass through the liver where heavy metals may accumulate. Therefore, the evaluation of the effects of heavy metals on a human liver cell line

may help elucidated the toxic potential of bioavailable metals from contaminated soil.

In order to evaluate potential metal-induced hepatic toxicity, cytotoxicity was measured using a human liver epithelial cell line, HepG2; cytotoxicity is measured in terms of cell viability. An IV-IVC relationship was derived solely on the basis of an *in vitro* derived parameter for RBA, herein known as the surrogate bioavailability (SBA). The goal of this chapter is to establish a correlation between heavy metal bioaccessibility and an *in vitro* measure of cytotoxicity. By comparing an established *in vitro* bioaccessibility assay to an *in vitro* surrogate for target organ toxicity from heavy metal bioavailable concentrations, we may be able to determine whether an *in vitro* assay can be used to reasonably predict potential adverse effects to further improve human risk estimates and hazard calculations.

Bioaccessible concentrations of Pb, Ni, As, Cd, and Cr were evaluated using simulated gastrointestinal fluids in a sequential *in vitro* extraction method Method details are outlined in Ellickson et al., 2001. Contaminated soil was exposed to simulated saliva, gastric, and intestinal fluids in order to mimic oral ingestion and its subsequent digestion. Results for eight (8) soils and two (2) NIST SRM reference soil materials suggest that the correlation between the two *in vitro* surrogates of bioaccessibility and bioavailability may inform refinement and support the utility of *in vitro* assays in human risk estimates. It is understood that before *in vitro* assays can be used as a surrogate measurement for RBA correlation, an analysis needs to be conducted in order to establish a relationship

between bioaccessibility and *in vivo* bioavailability. By carrying out this research, a simplistic validation study was performed in order to see if cellular toxic endpoints could relate to bioaccessibility values before more expensive *in vivo* studies were conducted.

Ellickson et al., 2001 compared oral bioavailability of As and Pb measured in vitro to bioaccessibility measured in a rodent animal model. While an IV-IVC relationship was not reported, the bioaccessibility/bioavailability values for a NIST SRM soil 2710 was 65.9%/37.8% and 10.7%/0.7% for As and Pb, respectively. Furthermore, tissue retention was highest in the liver for As, with most of the As being present in the blood or excreted in the feces one, two and three days post exposure. For Pb, as expected, the greatest accumulation site was in the femurs, one, two and three days' post exposure.

In the current research, all soils tested via the *in vitro* bioaccessibility extraction assay were subsequently tested on HepG2 cells in order to measure viability response (as outlined in Chapter 5). *In vitro* bioaccessibility (IVBA) is expressed as amount of metal reaching the cell. Herein, the HepG2 viability response is referred to as the surrogate bioavailability or SBA, which is expressed on a percentage basis of the the lowest observed adverse effect on viability. As mentioned previously, *in vitro* bioaccessibility is calculated as the follows:

$$IVBA = \%IVBA\ x \frac{contaminant\ metal\ concentration\ x\ 50mg}{extraction\ liquid\ volume} \qquad \textbf{(8.1)}$$

%SBA was determined as the lowest concentration that induces a reduction to ≤80 percent in HegG2 viability. In other words, %SBA is considered to be the viable percentage of the control where we first see a significant reduction in cell viability.

In order to evaluate the relationship between %SBA and IVBA, statistical analyses were performed using R (Team, 2014). Prior to regression analysis, an initial data analysis was conducted where the data were inspected for missing values, outliers, asymmetric distributions, etc. Table 8.1 summarizes the data (IVBA for the metals measured and %SBA). Upon inspection, missing values in the data set were due to the resulting metal bioaccessibility value being less than the method detection limit of the assay. To account for missing values, the method detection limit of the corresponding extractable metal concentration was used.

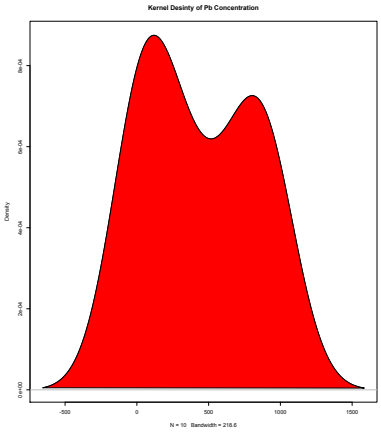
Table 8. 1 Soil ID, metals measure, %SBA and Intestinal IVBA concentration (ppm) for test soils.

	%SBA	Pb IVBA	Cd IVBA	As IVBA	Ni IVBA	Cr IVBA
NIST 2710	5	928	12.7	319	17	7.55
NIST 2709	5	0.62	0.21	0.56	9.72	6.02
LSP 125-500	10	15.7	0.42	6.3	23.2	28.7
LSP 45-125	10	42.4	0.33	16.1	47.4	47.4
Unkwn 1	10	825	10.4	281	3.51	6.86
Unkwn 2	10	706	8.43	511	3.51	5.62
Unkwn 3	0.1	456	1.70	519	2.55	7.79
Unkwn 4	1	282	0.04	112	2.45	7.03
Unkwn 5	1	919	8.51	130	3.21	7.07
Unkwn 6	1	138	2.93	782	7.29	4.09

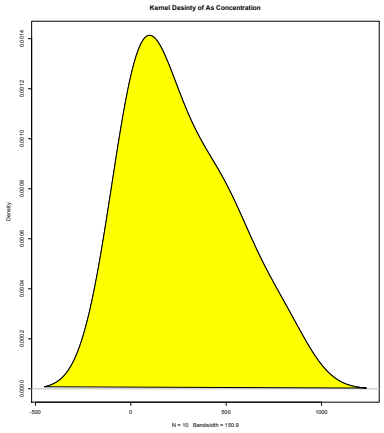
Graphical summaries of the data were created in order to evaluate qualities of the data such as the skewness, heteroskedasticity and normality. Figures 8.1a-e show the kernel density estimates of the IVBA for Pb, Ni, As, Cr and Cd. Histogram construction requires specification of the number of bins and certain bin choices may result in misleading histograms that obscures the data. For this reason, the Kernel Density Estimate plot was generated in an attempt to estimate the density directly from the data without assumptions regarding the underlying distribution. From the graphs we can see that the overall right-skewed shapes of the heavy metal concentration kernel density estimates suggest a non-normal distribution.

Figure 8. 1 Kernel Density Estimations of the metal concentrations for:

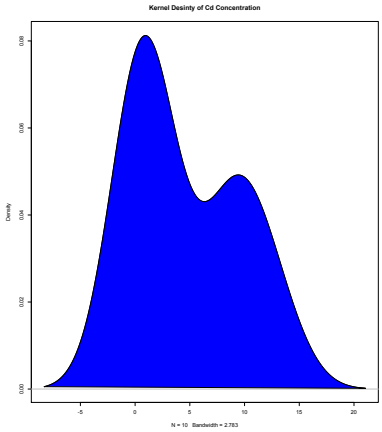
a) Lead (Pb)



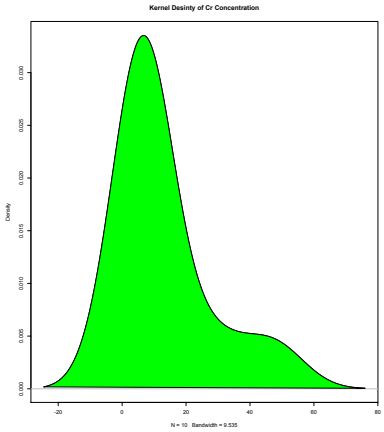
b) Arsenic (As)



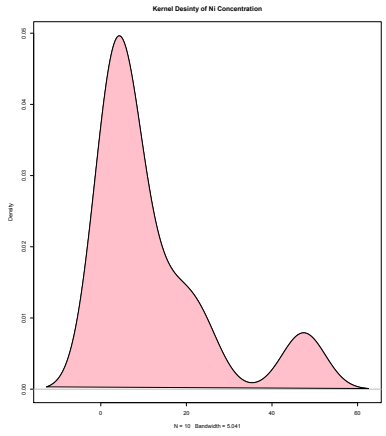
c) Cadmium (Cd)



d) Chromium (Cr)



e) Nickel (Ni)



Figures 8.2 and 8.3 show a side-by-side boxplot of the IVBA of each metal in the contaminated soil samples. Nickel and chromium have similar medians, which exceeded the cadmium's median value by a factor of two and were more than an order of magnitude less than the medians of lead and arsenic. Lead appears to be reasonably symmetric, but As, Cd and Ni is skewed to the right and chromium is skewed to the left.

Figure 8. 2 Boxplots of IVBA for Pb, Cd, Ni, Cr and As

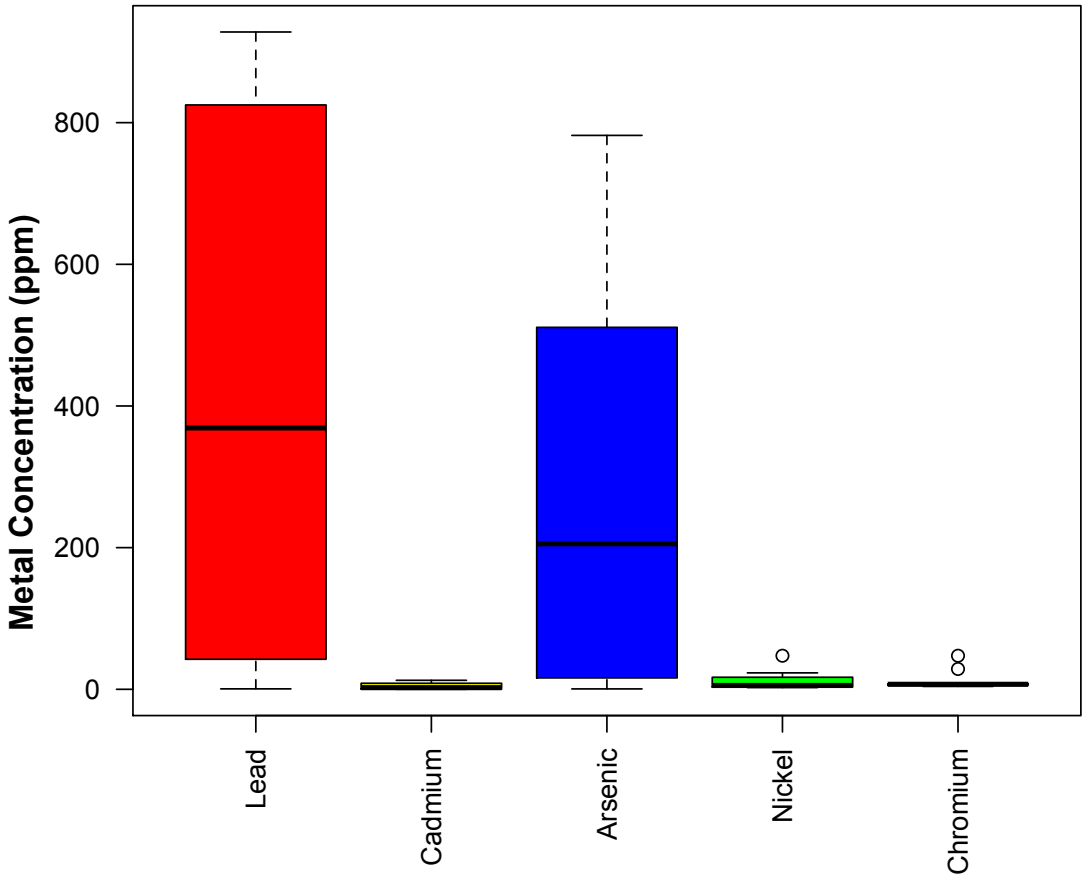
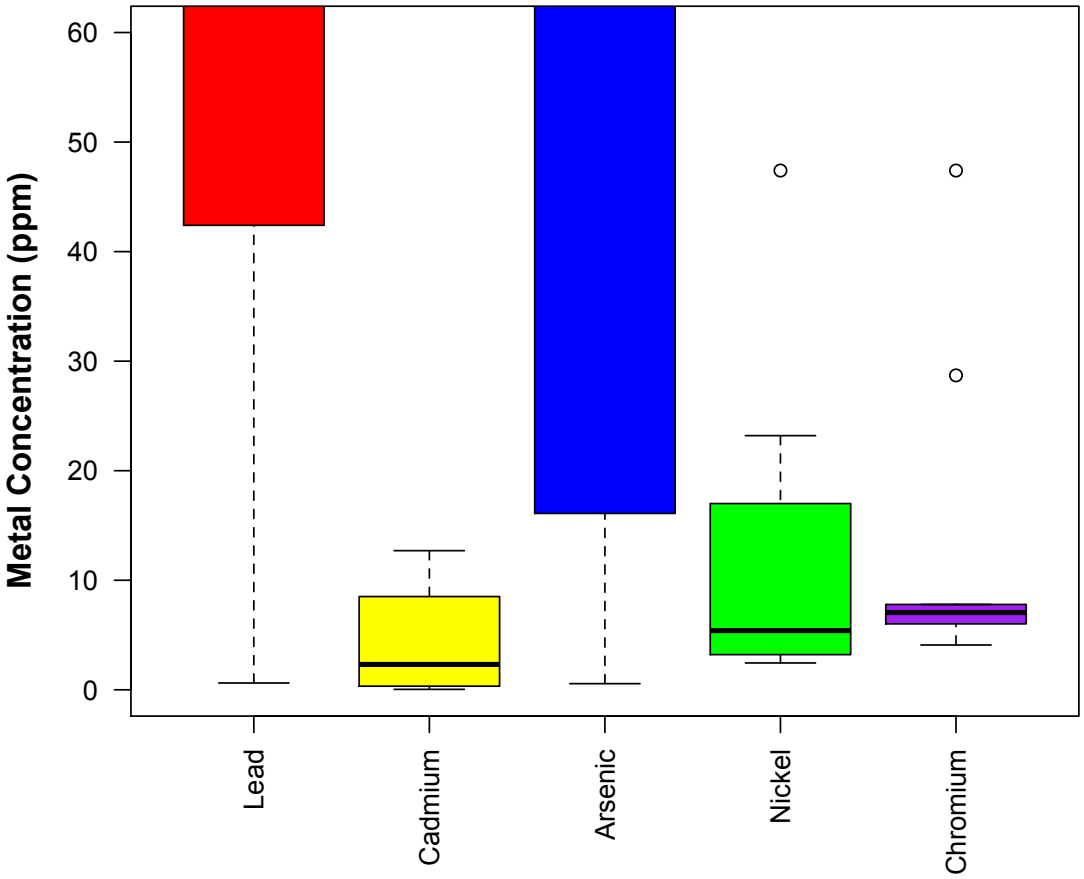


Figure 8. 3 Zoom-in on Boxplots of IVBA for Pb, Cd, Ni, Cr and As



Many statistical inferences, particularly in linear regression analysis, are based on the assumption of a normal data distribution. This assumption is often best checked using a quantile-quantile (Q-Q) plot, which checks the normality of the residuals. Normality can also be checked with tests such as the Shapiro-Wilk test, which is considered more suited for data sets less than 50. Additionally, tests for normality such as the Shapiro-Wilk test against assumptions of normality, meaning such tests can only be used to determine data sets that deviate from normality. A p-value < 0.05 would mean that you would reject the

NULL hypothesis, making it highly unlikely that the samples came from a normal distribution. Table 8.2 shows the results of the Shapiro-Wilk test on Pb, As, Cd, Ni and Cr IVBA. The test suggests that it is unlikely that cadmium, nickel and chromium IVBA are normally distributed. However, in order to confirm our suspicions regarding normality, four residual plots were evaluated for each metal assayed.

Table 8. 2 Shapiro-Wilk test for normality results ppm IVBA concentrations

	ORIGINAL	
	W	**P-value**
PB	0.86	0.09
CD	0.83	0.03
AS	0.90	0.19
NI	0.72	0.002
CR	0.61	7.12e-05

Figures 8.4 through Figure 8.8 show plots of the residuals of each of the five metal contaminants. Points 5, 6 and 7 deviate from the normality line in the Pb normal Q-Q plot. The same deviations can be seen for points 4 and 9 in the Cd normal Q-Q plot and for points 5 and 7 for the As, Ni and Cr normal Q-Q plots. Otherwise, the normal Q-Q plots for all five metals are reasonably close to normal. Also, departures from normality are not as likely to be detected when the sample size is small. Heteroskedasticity is another assumption underlying linear regression, and can be investigated by a scale location plot. The scale location plot should look seemingly random, which is difficult to ascertain given the small sample size for each of our metals. The residuals versus leverage or Cook's distance plot show which points have the greatest influence on the regression.

Generally, a cook's distance less than one is not a reasonable cause for concern. Outliers may reflect model misspecification and given that our model only accounted for five heavy metals in our soil sample, there may be other important explanatory variables (metals or otherwise) that account for the subset of possible data outliers.

Figure 8. 4 Residuals plot of Pb IVBA

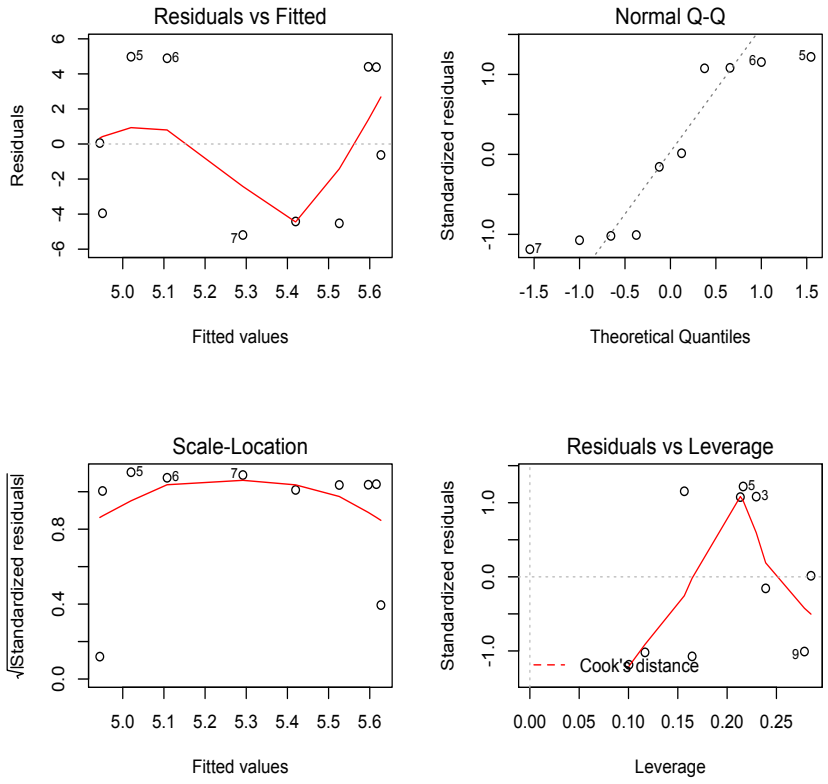


Figure 8. 5 Residuals Plot of Cd IVBA

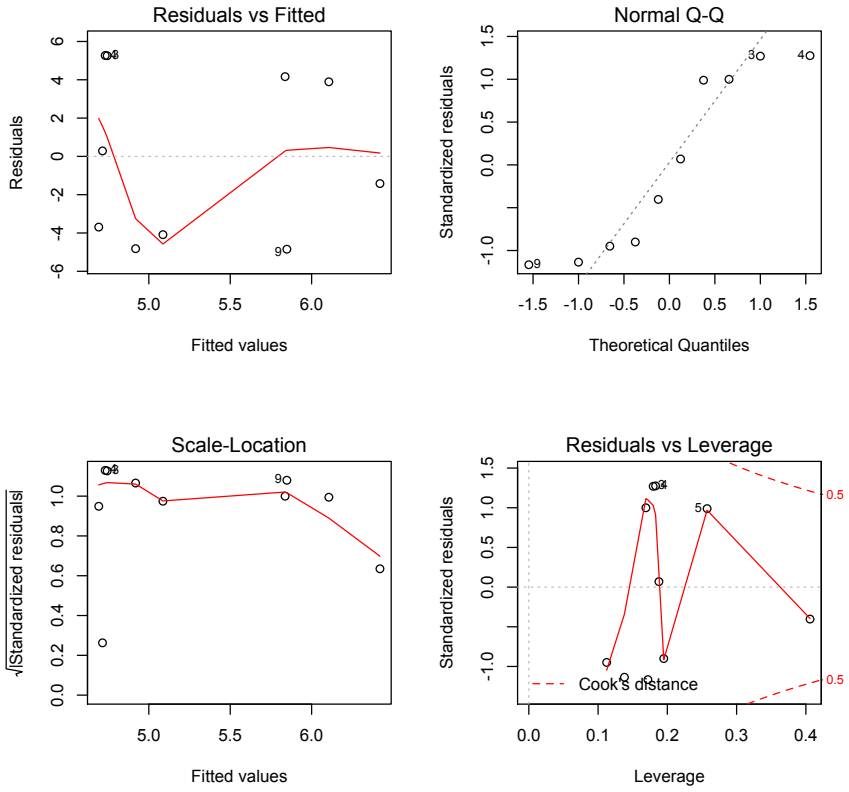


Figure 8. 6 Residuals Plot of As IVBA

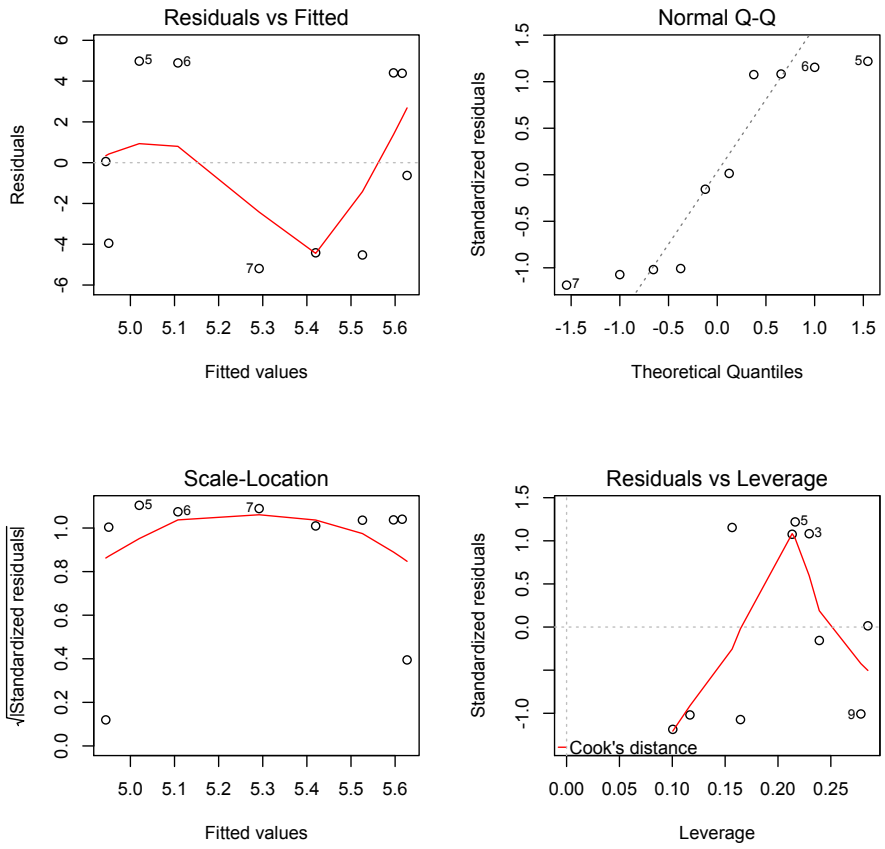


Figure 8. 7 Residuals Plot for Ni IVBA

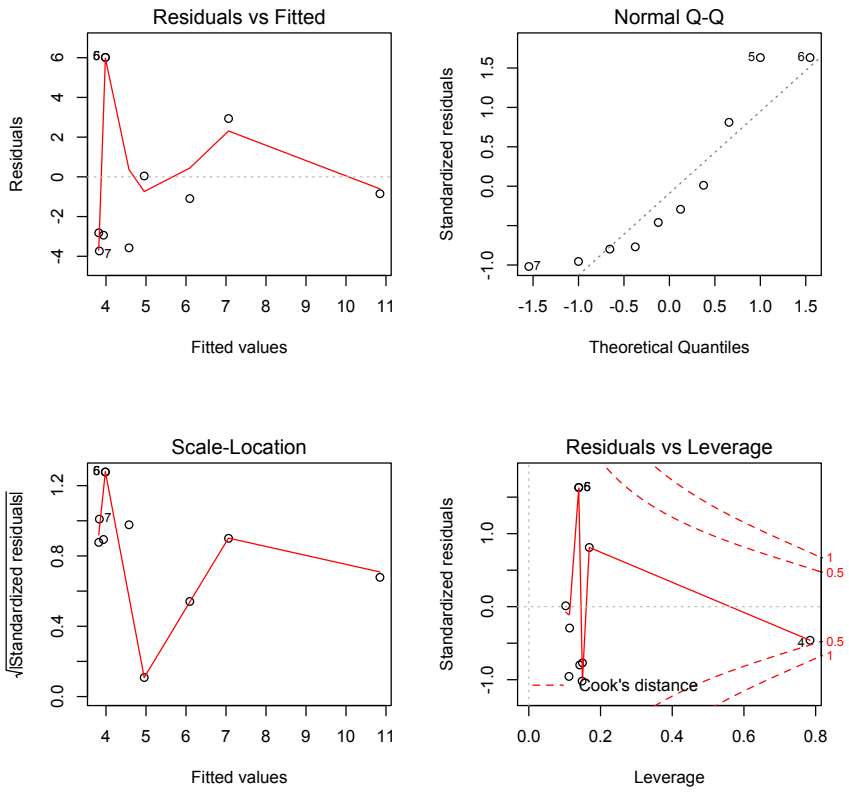
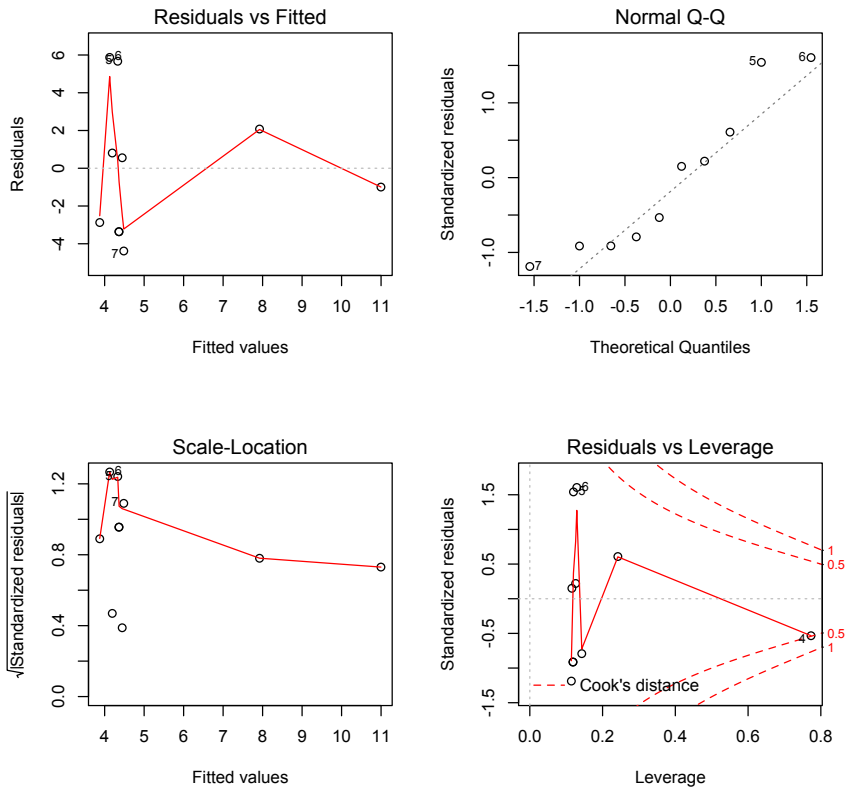


Figure 8. 8 Residuals Plot for Cr IVBA



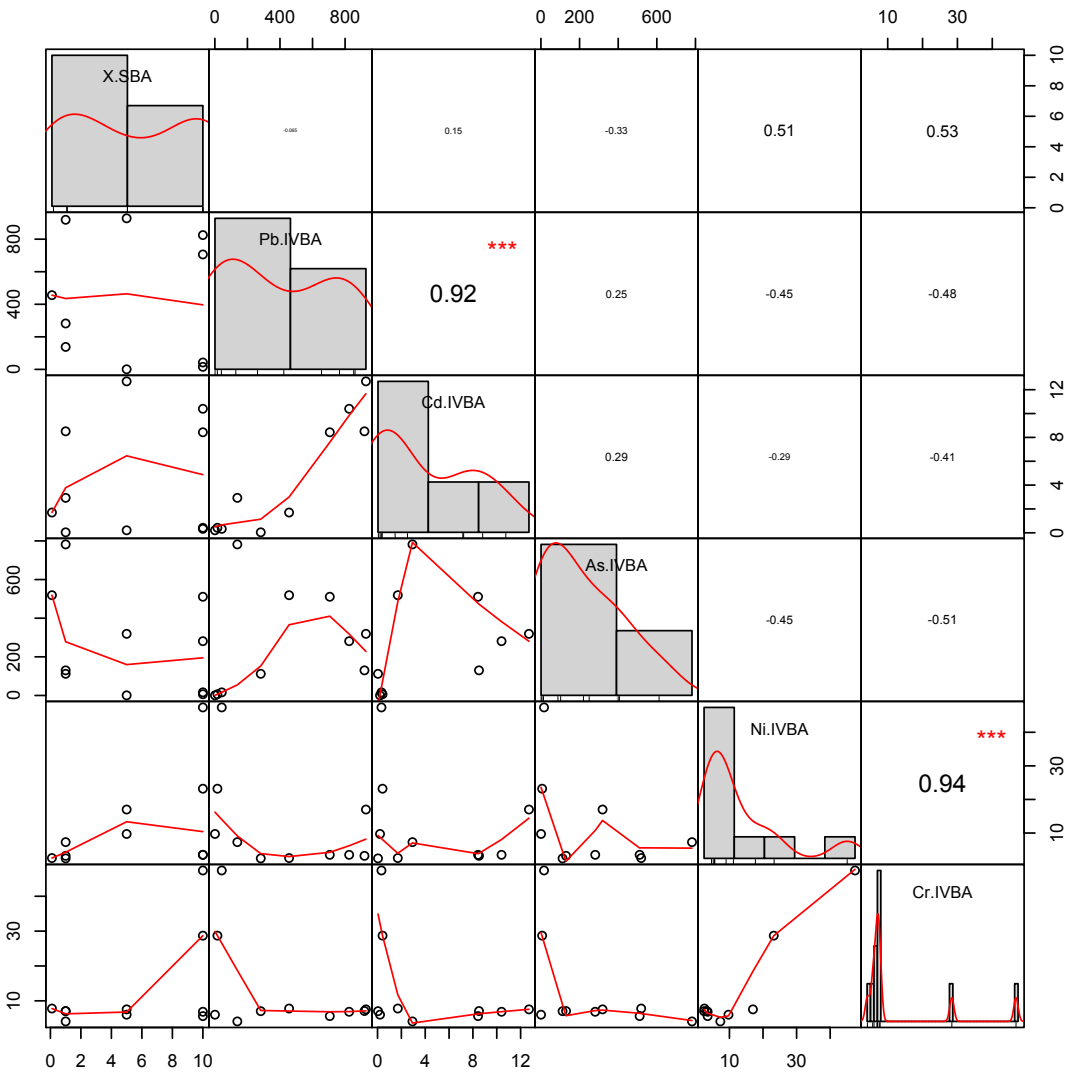
Each metal concentration was evaluated to discern their influence on %SBA. The regression analysis of fit of IVBA versus %SBA for Pb, Ni, Cr, Cd and As, respectively, show that Ni and Cr had the highest productivity of regression fit with an R^2=0.26 and R^2=0.28, respectively, accounting together for 54% of the model variance, if %SBA of all metal are independent of each other. The other metals showed significantly lower R^2, 0.004 for Pb, 0.02 for Cd and 0.11 for As, potentially capturing approximately another 11% of the model variance. Lead, cadmium, and arsenic had coefficients that were significant on a $p<0.05$ level.

The strength of linear relationships between the variables is measured by correlation coefficients, where stronger relationships correspond to higher

coefficients. For our variables, the strongest positive relationships are between lead and cadmium and between chromium and nickel with correlations coefficients of 0.92 and 0.94, respectively. Moderately strong correlations can be seen between SBA and Ni and SBA and Cr, with correlation coefficients of 0.51 and 0.53, respectively. These R^2 between metals concentration indicate that they are not independent but likely are present in common minerals across the soils. Both nickel and chromium showed the strongest correlation to the *in vitro* target organ surrogate, which was further supported by our linear regression analyses.

Figure 8.9 shows a graphical representation of the correlative relationships between data variables. Figure 8.9 also shows the histogram and the kernel density estimation of the variables and their corresponding scatterplots. Cadmium appears to be strongly positively correlated with lead and a strong positive correlation exists between nickel and chromium.

Figure 8. 9 Pair-wise scatterplot matrix.



*****significant on a p<0.001 level.**

Correlations between bioavailability and bioaccessibility have been studied most extensively for arsenic and lead. The current EPA preferred model for lead RBA is RBA=0.878*IVBA -0.028 where RBA and IVBA are expressed as fractions and not percent (EPA, 2007). This model utilizes juvenile swine and the EPA's current method for accessing bioaccessibility to make the correlation

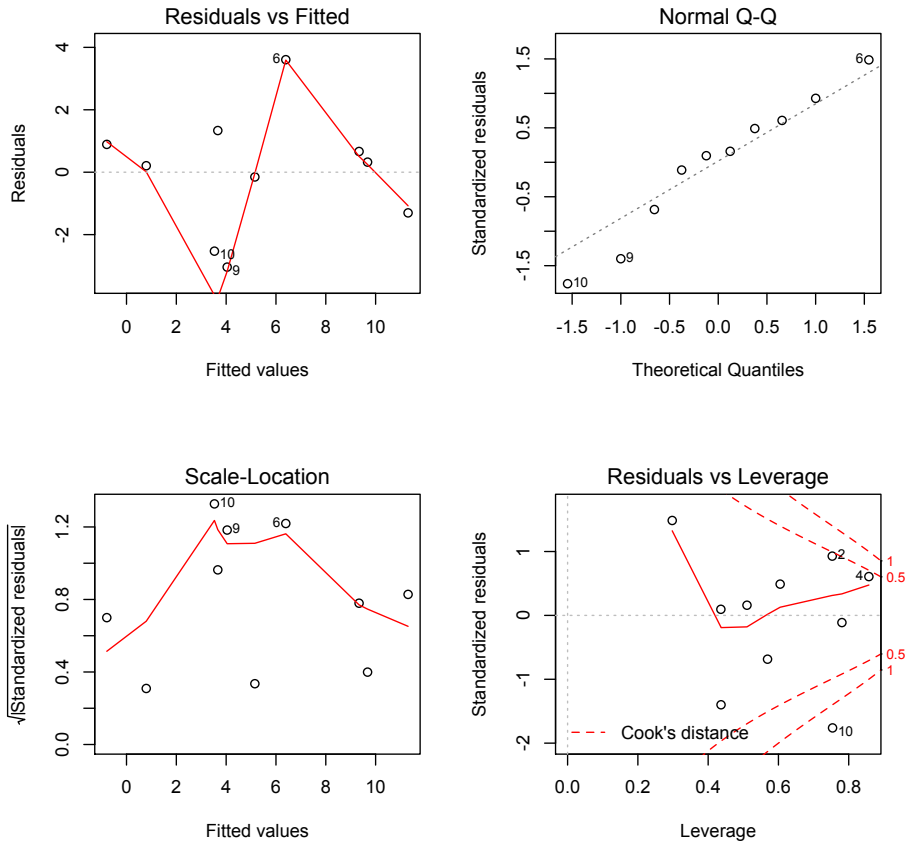
between the two methods. Bradham et al. 2011 compared the relative bioavailability of As in soils determined by a mouse assay to an *in vitro* assay for As bioaccessibility. Results suggest a relationship for As RBA of %RBA=0.72%IVBA+5.64 (R^2=0.92). However, Li et al. 2015 compared *in vivo-in vitro* relationships across different methods and different gastrointestinal compartments and found that bioaccessibility and bioavailability in contaminated soils depend on the soil type, bioaccessible method and animal model used. The relationship also depended on the phase (i.e., gastric vs. intestinal) used which leads to inherent differences in model results when comparing inter-laboratory studies.

A simple multiple regression model was evaluated using the data generated in this study. However due the low power of data set (n=10), a simple first order model without interaction terms was chosen (Equation 8.2)

$$\mathbf{Y=\beta_0 +\beta_1 A+\beta_2 B+\beta_3 C+\beta_4 D+\beta_5 E} \quad (8.2)$$

This model yields an equation of **Y=4.63 -0.02Pb + 2.25Cd -0.004As -0.56Ni + 0.66Cr** with a multiple **R^2=0.80 and an adjusted R^2=0.56**, where only the cadmium coefficient was significant on a $p<0.05$ level. Multiple linear regression models assume homoscedasticity and normality, therefore an assessment of the residuals and the q-q plot of model were again needed.

Figure 8. 10 $Y=\beta_0+\beta_1A+\beta_2B+\beta_3C+\beta_4D+\beta_5E$ Residual Plot



The concordance of IVBA and SBA estimates obtained with *in vitro* extraction methods and an *in vitro* hepatocellular assay of heavy metals in soil suggest that these methods can be used as a complement to current risk assessments. Current in vivo-in vitro correlations relate contaminants on an individual and limited basis (i.e. As and Pb). However, contaminants rarely occur alone in a media, but rather in mixtures. This proposed model accounts for mixtures.

While the correlation analysis highlighted the fact that the metals were highly correlated, given that Cd is the only coefficient that was significant and that the assay is for hepatocellular effect, the multivariate model doesn't include all

possible co-occurring metals. While Pb can have hepatotoxic effects and As can lead to carcinogenic effects in the liver, ingestion of significant amounts of Cd causes the most immediate damage to the liver in our system.

Results from this study suggest that combining *in vitro* assays yielded comparable estimates of bioavailability and provided evidence of the utility of estimates of bioaccessibility. The results also support the idea that more research needs to conducted that evaluate contaminants as mixtures effects rather than as single elements. A multiple regression linear model accounted for more than 80% (56% when adjusted) of the variability in our predictive model, highlighting the potential of *in vitro*-*in vitro* correlations as a complimentary and precursor screening tool for more expensive *in vivo* examination. As we continue to develop more tools for incidental/accidental ingestion risk estimates, future research is needed in order to compare the *in vivo* accumulation, i.e. in the liver, of our metals for a greater number of soils. In addition, this proposed model allows for metal-specific accumulation surrogate organ systems by simply changing the cell-line to the specific organ desired.

Chapter 9: Conclusions and Recommendations

In vitro bioassay models offer a potentially sensitive tool for accompaniment in toxicity assessment of contaminated soils. Standard evaluations of the effects of heavy metal contaminated soil largely focused on exposures to single metals, particularly lead and arsenic. Mixed metal exposures reflect real-world scenarios and are of particular importance when assessing risk since most soils contains a mixture of metals. The results from a cellular extract viability (CEV) assay using a mixed metal contaminant system show that there is a sufficient dilution concentration, below which no adverse health outcomes are expected in a hepatic system. Additionally, the inclusion of the intestinal compartment in *in vitro* intestinal models helps risk assessors better assess risk by getting a closer, more biologically relevant assessment of heavy metal soil exposure by estimating the dose that could potentially cause adverse effects in a target organ.

Several areas have been identified for further investigation:

- More evaluations need to be made between bioavailability measurements and estimates of bioaccessibility in order to accurately assess the relationship between heavy metal solubility and risk. In addition, the use NIST SRM materials in *in vivo* bioavailability studies can help with inter-laboratory and inter-method comparisons of bioaccessibility.
- Incorporation of other human cell lines that are more representative of each specific metal, such as human bone cell cultures for lead, may

further highlight the differences between each metal and their subsequent bioaccessibility.

- Testing the incorporation of *in vitro* cellular systems into other *in vitro* extraction methods of bioaccessibility in order to assess the ability of this model to reasonably assess liver organ burden and subsequent target organ toxicity.
- Evaluation of housedusts to more accurately assess the full potential of risk post oral ingestion given that house dust comprised an important part of oral soil intake, especially in children.

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APPENDICES

Appendix A

Inductively Coupled Plasma Mass Spectrometer (ICP-MS) Report

Chemical analyses were run to identify the heavy metal concentrations for various soils samples following three scenarios:

1. **Following EPA Method 3051 A which measures extractable/leachable metal concentrations from soils and sediments.**
2. **Following a sequential in vitro bioaccessibility extraction method: gastric and intestinal aliquots.**
3. **Following the use of filters to recover the insoluble fraction from the in vitro bioaccessibility method.**

Appropriate standards were used during each run and Quality Control (QC) standards were run approximately every 10 samples. QC recovery was deemed acceptable at a level of +/- 20% of the analyte QC concentration.

Appendix A.1 ICPMS Run 1 EPA 3051 A

Key:
SNH x-xx-xx = Analyzer initials Notebook #-page#-sample #
Acid Blank= Nitric Acid with no sample additions
Acid Spike: Nitric Acid spiked with 10 ppb of the analyte concentration

Table A.1.1 Sample Description and Weights for SNH 1-25 Liberty State Park Soils (LSP) and NIST SRM Soils

Sample	Experimental Setup	Description	Goal Weight grams	Actual Weight grams
SNH 1-25-01	**LSP (125-500 um)**	Liberty State Park Soil	0.25	0.251
SNH 1-25-02	**LSP (125-500 um)**	Liberty State Park Soil	0.25	0.2496
SNH 1-25-03	**LSP (125-500 um)**	Liberty State Park Soil	0.25	0.2513
SNH 1-25-04	**NISTSRM 2709**	NIST 2709	0.25	0.2512
SNH 1-25-05	**NISTSRM 2709**	NIST 2709	0.25	0.2507
SNH 1-25-06	**NISTSRM 2710**	NIST 2710	0.25	0.2498
SNH 1-25-07	**NISTSRM 2710**	NIST 2710	0.25	0.252
SNH 1-25-08	**Acid Blank**	HNO3		
SNH 1-25-09	**Acid Spike**	HNO3 +spike	10 ppb	
SNH 1-25-10	**Control Vessel**	Liberty State Park Soil	0.27	0.2734
SNH 1-25-11	**LSP (45-125 um)**	Liberty State Park Soil	0.25	0.254
SNH 1-25-12	**LSP (45-125 um)**	Liberty State Park Soil	0.25	0.2493
SNH 1-25-13	**LSP (45-125 um)**	Liberty State Park Soil	0.25	0.2529

Table A.1.2 Sample Description and Weights for SNH 1-26 Liberty State Park Soils (LSP)

Sample	Experimental Setup	Description	Goal Weight grams	Actual Weight grams
SNH 1-26-01	**LSP (125-500 um)**	Liberty State Park Soil	0.25	0.2494
SNH 1-26-02	**LSP (125-500 um)**	Liberty State Park Soil	0.25	0.2517
SNH 1-26-03	**NIST SRM 2709**	NIST 2709	0.25	0.2513
SNH 1-26-04	**NIST SRM 2709**	NIST 2709	0.25	0.2507
SNH 1-26-05	**NIST SRM 2710**	NIST 2710	0.25	0.2515
SNH 1-26-06	**NIST SRM 2710**	NIST 2710	0.25	0.2506
SNH 1-26-07	**Acid Blank**	HNO3		
SNH 1-26-08	**Acid Spike**	HNO3 +spike	10 ppb	
SNH 1-26-09	**Acid Spike**	HNO3 +spike	10 ppb	
SNH 1-26-10	**LSP (45-125 um)**	Liberty State Park Soil	0.25	0.2497
SNH 1-26-11	**LSP (45-125 um)**	Liberty State Park Soil	0.25	0.2508
SNH 1-26-12	**LSP (<45 um)**	Liberty State Park Soil	0.25	0.2498
SNH 1-26-13	**LSP (<45 um)**	Liberty State Park Soil	0.25	0.2509
SNH 1-26-14	**Control Vessel**	Liberty State Park Soil	0.27	0.2768

Table A.1.3 Sample Description and Weights for SNH 1-28
Liberty State Park Soils (LSP)

Sample ID	Dilution Factor	112 Cd	58 Ni	52 Cr	75 As	208 Pb
0.10	1.00	<0.00975	<0.081	<0.027	<0.1035	<0.006
0.30	1.00	0.287	0.202	0.261	0.265	0.149
0.50	1.00	0.486	0.424	0.483	0.485	0.355
1.00	1.00	0.997	0.895	0.952	0.974	0.851
5.00	1.00	5.177	5.13	5.133	5.205	4.993
10.00	1.00	9.984	9.987	9.956	10.02	9.805
30.00	1.00	29.98	29.99	30	29.96	30.08
SNH 1-26-1	800.00	1223.2	488000	1716000	126880	1180800
SNH 1-26-2	800.00	2684.8	511120	1406400	129280	1191200
SNH 1-26-3	800.00	280	223280	55408	11736	11792
SNH 1-26-4	800.00	274.4	217120	50488	11776	11512
SNH 1-26-5	800.00	16464	145040	13624	497840	5346400
SNH 1-26-6	800.00	15968	143040	13736	480160	5201600
SNH 1-26-7	800.00	<8	<65	<22	<83	<5
SNH 1-26-8	1.00	8.181	8.955	8.591	8.343	9.456
SNH 1-26-9	1.00	8.584	9.421	9.01	8.845	10.02
SNH 1-26-10	800.00	1465.6	605680	2768000	165120	1612800
SNH 1-26-11	800.00	1459.2	548720	2275200	162800	1540800
SNH 1-26-12	800.00	1928	622800	2857600	215680	2161600
SNH 1-26-13	800.00	1747.2	654240	3116800	212560	2111200
SNH 1-26-14	800.00	1129.6	507920	1920000	123360	1154400
SNH 1-25-1	800.00	1432.8	650240	3518400	121360	1826400
SNH 1-25-2	800.00	1312.8	547920	2472800	122400	1047200
SNH 1-25-3	800.00	1384	615120	3645600	115360	1416000
SNH 1-25-4	800.00	261.6	222560	59648	12152	11040
SNH 1-25-5	800.00	278.4	221600	56088	12192	11016
SNH 1-25-6	800.00	<8	<65	<22	<83	<5
SNH 1-25-7	800.00	16256	157760	15560	504240	5173600
SNH 1-25-8	800.00	<8	<65	<22	<83	14.4
SNH 1-25-9	1.00	8.51	9.888	9.314	<0.1035	9.588
SNH 1-25-10	800.00	1572.8	851200	6032800	171920	1649600
SNH 1-25-11	800.00	1509.6	670880	3609600	160080	1479200
SNH 1-25-12	800.00	1045.6	445120	2316800	112160	984800
SNH 1-25-13	800.00	1511.2	743600	4500800	166000	1528800

Appendix A.2 ICPMS Run 2

Key:
SNH x-xx-xx = Analyzer initials Notebook #-page#-sample #
Acid Blank= Nitric Acid with no sample additions
Acid Spike: Nitric Acid spiked with 18/10 ppb of the analyte concentration

Table A.2.1 Sample Description For Biofluid Extractions

Sample ID	Description	Dilution Factor	112Cd ppb	58Ni ppb	52Cr ppb	208Pb ppb	75As ppb
0.1	Standard	1.00	<0.1	<0.035	<0.1	<0.1	<0.135
0.3	Standard	1.00	0.30	0.25	0.31	0.31	0.33
0.50	Standard	1.00	0.490	0.47	0.46	0.5	0.48
1	Standard	1.00	1.02	1	0.96	0.99	0.97
5	Standard	1.00	5.10	5.17	5.14	5.06	5.06
10	Standard	1.00	9.99	10.05	10.02	9.87	10.04
30	Standard	1.00	29.99	29.96	29.97	30.03	29.98
SNH 1-35-1	LSP 125-500	2270	522.1	130752	317119	454681	30032.1
SNH 1-35-2	LSP 125-500	2270	417.68	140898.9	350488	1186075	39021.3
SNH 1-35-3	Fluid Blank	1.05	<0.105	1.575	3.6225	0.9366	<0.14175
SNH 1-35-4	Fluid Spike	540	14	15.2775	18.018	14.343	14.994
SNH 1-35-5	NIST 2709	2270	283.75	24107.4	11138.89	6163.05	431.3
SNH 1-35-6	NIST 2710	2270	12961.7	18743.39	7574.99	4528650	363427
SNH 1-35-7	LSP 125-500	4168	<417	89190.4	118227.2	133952	18374.72
SNH 1-35-8	LSP 125-500	4168	<417	81577.6	48131.2	54995.2	18037.76
SNH 1-35-9	Fluid Blank	1.05	<0.105	0.61845	1.7577	0.1323	<0.14175
SNH 1-35-10	Fluid Spike	1.05	14.112	6.43545	7.95795	5.28255	3.3705
SNH 1-35-11	NIST 2709	4168	<417	16065.92	9251.84	2342.08	<562
SNH 1-40-1	LSP 125-500	2270	628.79	182712.3	453773	954308	38839.7
SNH 1-40-2	LSP 125-500	2270	460.81	137244.2	357979	765671	26854.1
SNH 1-40--3	LSP 125-500	2270	424.49	116087.8	282161	842624	19108.86
SNH 1-40--4	LSP 125-500	2270	422.22	122239.5	303045	561825	24584.1
SNH 1-40-5	Fluid Blank	1.05	<0.105	1.5036	3.2277	0.14385	<0.14175
SNH 1-40--6	Fluid Spike	1.05	6	15.645	18.0705	15.8865	12.1065
SNH 1-40-7	NIST 2709	2270	238.35	21689.85	10530.53	5602.36	<306
SNH 1-40-8	NIST 2709	2270	227	21950.9	10607.71	4449.2	<306
SNH 1-40-9	NIST 2710	2270	11772.22	3684210	6760.06	4469630	298505
SNH 1-40-10	NIST 2710	2270	12042.35	3818140	7842.85	4621720	334371
SNH 1-40--11	LSP 125-500	4168	31635.12	26070.84	39591.83	25699.89	13258.41
SNH 1-40--12	LSP 125-500	4168	<417	26931.84	44345.6	26565.76	8444.8
SNH 1-40-13	LSP 125-500	4168	<417	26624	41554.24	26162.24	3016
SNH 1-40--14	LSP 125-500	4168	<417	19206.72	41387.84	18973.76	1252.16
SNH 1-40-15	Fluid Blank	1.05	0.1932	0.4032	1.617	0.39585	<0.14175
SNH 1-40-16	Fluid Spike	1.05	5.65	5.96505	8.1249	5.8779	<0.14175
SNH 1-40-17	NIST 2709	4168	<417	1206.4	6631.04	1272.96	<562
SNH 1-40--18	NIST 2709	4168	<417	1397.76	7375.68	1456	<562

Sample ID	Description	Dilution Factor	112Cd ppb	58Ni ppb	52Cr ppb	208Pb ppb	75As ppb
SNH 1-40-19	NIST 2710	4168	6693.808	1382784	5678.4	1663584	217817.6
SNH 1-40-20	NIST 2710	4168	7298.168	1592032	6331.52	1907360	234249.6
SNH 1-43-1	LSP 45-125	2270	683.27	933651	416999	1150890	35253.1
SNH 1-43-2	LSP 45-125	2270	653.76	926160	444693	1139313	39543.4
SNH 1-43-3	LSP 45-125	2270	740.02	981094	476246	1208094	43833.7
SNH 1-43-4	Fluid Blank	1.05	0.2961	427.56	203.28	526.05	17.2515
SNH 1-43-5	LSP 45-125	4168	<437	109574.4	98675.2	109699.2	<562
SNH 1-43-6	LSP 45-125	4168	<437	62358.4	80953.6	61318.4	25334.4
SNH 1-43-7	LSP 45-125	4168	642.096	75753.6	94515.2	74464	36616.32
SNH 1-43-8	Fluid Blank	1.05	<0.105	0.1722	2.79405	0.1617	<0.14175

Appendix A.3 ICPMS Run 3 EPA 3051A on Filter Recovery Samples

SNH x-xx-xx = Analyzer initials Notebook #-page#-sample #
Acid Blank= Nitric Acid with no sample additions

Table A.3.1 Sample Description For Filter Recovery Samples

Sample ID	Dilution Factor	Description	112Cd	58Ni	52Cr	75 As	208Pb
			ppb <0.009	ppb <0.03	ppb	ppb <0.103	ppb
0.10	1.00	Standard	75	5	<0.03	5	<0.006
0.30	1.00	Standard	0.285	0.257	0.29	0.28	0.28
0.50	1.00	Standard	0.497	0.466	0.47	0.478	0.47
1.00	1.00	Standard	1.014	0.988	0.99	0.968	0.97
5.00	1.00	Standard	5.122	5.117	5.11	5.104	5.11
10.00	1.00	Standard	10.01	10.07	10.00	10.01	10.07
30.00	1.00	Standard	29.98	29.96	29.98	29.98	29.96
SNH 1-35-14	200.00	LSP (125-500)	135.4	16058	93,740.00	2872	11,770.00
SNH 1-35-15	200.00	LSP (125-500)	86.4	17788	69,040.00	3186	32,060.00
SNH 1-35-16	200.00	NIST 2709	10.6	9060	3,744.00	427	448.40
SNH 1-35-17	200.00	NIST 2710	373	8086	2,378	7552	213,400
SNH 1-40-3/22	200.00	LSP (125-500)	100	23020	111,240	4288	64,800
SNH 1-40-5	200.00	Fluid Blank	158	503.4	920	<21	221
SNH 1-40-7	200.00	NIST 2709	14	6284	2,600	289.8	394
SNH 1-40-9	200.00	NIST 2710	369	7574	1,655	7674	218,400
SNH 1-43-9	200.00	LSP (45-125)	103	30760	240,400	5334	75,820
SNH 1-43-10	200.00	LSP (45-125)	78	24040	180,140	4954	65,340
SNH 1-43-11	200.00	LSP (45-125)	94	19056	136,680	4472	64,160
SNH 1-43-12	200.00	Fluid Blank	<2	399.2	778	<21	30
SNH 1-46-14	200.00	Filter Blank	<2	490.2	676	<21	95
SNH 1-40-21	200.00	LSP (125-500)	1,845	32260	214,000	5846	68,580

Appendix A.4 ICPMS Run 4

Key:
SNH x-xx-xx = Analyzer initials Notebook #-page#-sample #
Acid Blank= Nitric Acid with no sample additions
Acid Spike: Nitric Acid spiked with 18/10 ppb of the analyte concentration
DF is 2270 for Saliva/Gastric and 4168 for Saliva/Gastric-Intestinal

Table A.4.1 Sample Description for Biofluid Extractions

Sample ID ppb	Description	112Cd ppb	68Ni ppb	52Cr ppb	75As ppb	208Pb ppb
SNH 1-79-1	NIST 2701	270.13	412005	655803	13590.49	133748.4
SNH 1-79-2	NIST 2701	147.55	414729	668969	11676.88	2615.04
SNH 1-79-3	NIST 2710	5.89	7.3164	3.19095	160.965	1.575
SNH 1-79-4	NIST 2709	48	6993	3594.24	<105	1065.96
SNH 1-79-5	Blank	<22	3893.05	7334.37	<443	5166.52
SNH 1-79-6	Spike	18	20	22	19	3
SNH 1-79-7	NIST 2701	104	314475.6	31485.07	<813	12033.02
SNH 1-79-8	NIST 2701	<41	325937.6	33689.94	1400.448	2709.2
SNH 1-79-9	NIST 2710	7,186	15975.94	7735.808	318101.8	2379.928
SNH 1-79-10	NIST 2709	<41	17209.67	9307.144	<813	18560.1
SNH 1-79-11	Blank	3	5.27415	6.2055	2.69115	2.6187
SNH 1-79-12	Spike	3	5	6	3	1
SNH 1-80-1	NIST 2701	129	402925	647631	6710.12	110957.6
SNH 1-80-2	NIST 2701	141	446055	713007	6022.31	119038.8
SNH 1-80-3	NIST 2709	170	25060.8	11395.4	<443	4726.14
SNH 1-80-4	NIST 2710	16,262.28	20187.11	10432.92	454908	1075299
SNH 1-80-5	NIST 2701	<41	317476.6	57726.8	2663.352	3576.144
SNH 1-80-6	NIST 2701	<41	319602.2	59394	5760.176	3021.8
SNH 1-80-7	NIST 2709	41.68	17893.22	10424.17	<813	1229.56
SNH 1-80-8	NIST 2710	6,089.45	12591.53	8581.912	285924.8	561846.4
SNH 1-92-1	NIST 2710	13,082.01	17921.65	7683.95	368421	947271
SNH 1-92-2	NIST 2710	13,501.96	18477.8	8271.88	381133	938872
SNH 1-92-3	Blank	0.01	2.61975	3.4062	<0.20475	0.5628
SNH 1-92-4	Spike	17.44	19.51	21.71	16.08	15.51
SNH 1-92-5	NIST 2709	199.76	27898.3	12755.13	<443	4932.71

SNH 1-92-6	NIST 2709	238.35	30054.8	14076.27	<443	5148.36
SNH 1-92-7	NIST 2710	6,293.68	14325.42	9769.792	289175.8	582686.4
SNH 1-92-8	NIST 2710	5,739.34	12704.06	8569.408	275338.1	708143.2
SNH 1-92-9	Blank	<0.010237 5	1.27155	1.71675	<0.20475	0.21
SNH 1-92-10	Spike	4.73	7.59	8.27	6.11	3.48
SNH 1-92-11	NIST 2709	<41	15963.44	9094.576	<813	979.48
SNH 1-92-12	NIST 2709	<41	17805.7	11628.72	<813	1033.664
SNH 1-94-1	NIST 2710	13,415.70	16557.38	7125.53	372734	984726
SNH 1-94-2	NIST 2710	13,756.20	17063.59	8044.88	380452	936375
SNH 1-94-3	Blank	<0.010237 5	1.98975	3.3096	<0.20475	0.47565
SNH 1-94-4	Spike	0.02	2.11	3.38	<0.19	0.29
SNH 1-94-5	NIST 2709	190.68	24062	12487.27	<443	4242.63
SNH 1-94-6	NIST 2709	188.41	25446.7	12882.25	<443	4235.82
SNH 1-94-7	NIST 2710	6,572.94	15192.36	24974.66	358739.8	498909.6
SNH 1-94-8	NIST 2710	6,118.62	13612.69	14512.98	297970.3	510996.8
SNH 1-94-9	Blank	<0.010237 5	0.8358	3.234	<0.20475	0.0525
SNH 1-94-10	Spike	<.10	0.851	3.351	<193	0.035
SNH 1-94-11	NIST 2709	<41	15780.05	15538.3	<813	633.536
SNH 1-94-12	NIST 2709	<41	18551.77	17680.66	<813	925.296
SNH 1-95-1	NIST 2710	13,238.64	16466.58	7184.55	354574	991082
SNH 1-95-2	NIST 2710	13,806.14	17147.58	8044.88	375912	956578
SNH 1-95-3	Blank	<0	1.8585	3.52275	<0	0.1533
SNH 1-95-4	Spike	17.17	18.76	21.25	17.49	14.72
SNH 1-95-5	NIST 2709	208.84	33437.1	15122.74	<443	4244.9
SNH 1-95-6	NIST 2709	206.57	30690.4	14151.18	<443	4160.91
SNH 1-95-7	NIST 2710	6,360.37	15696.69	17284.7	319018.7	493908
SNH 1-95-8	NIST 2710	6,360.37	14804.74	21681.94	328396.7	504328
SNH 1-95-9	Blank	<0	0.9849	5.7288	<0	0.0378
SNH 1-95-10	Spike	4.84	7.46	12.00	7.47	3.58
SNH 1-95-11	NIST 2709	<41	23978.5	19322.85	<813	1008.656
SNH 1-95-12	NIST 2709	<41	25491.49	35540.54	<813	1058.672

Appendix A.5 ICPMS Run 5

Key:
SNH x-xx-xx = Analyzer initials Notebook #-page#-sample #
Acid Blank= Nitric Acid with no sample additions
Acid Spike: Nitric Acid spiked with 18/10 ppb of the analyte concentration
DF is 2270 for Saliva/Gastric and 4168 for Saliva/Gastric-Intestinal

Table A.5.1 Sample Description for Biofluid Extractions

Sample ID	Description	112Cd ppb	60 Ni ppb	52Cr ppb	75As ppb	208Pb ppb
SNH 1-79-1	EMS 113609	23017.8	8,855.27	10285.37	430,619.00	656711
SNH 1-79-2	EMS 113609	26309.3	9,860.88	9297.92	463,988.00	784512
SNH 1-79-3	Blank	<0	1.89	3.79785	<0	0.378
SNH 1-97-4	Spike	14.55	14.65	17.21	14.97	15.17
SNH 1-97-5	NIST 2710a	4,226.99	2,846.66	6,387.89	653562	1448.685
SNH 1-97-6	NIST 2710a	4,414.41	3,049.52	6,901.65	697441.5	399.105
SNH 1-97-7	EMS 113609	12,187	3,721	11,466	284480.07	11518.626
SNH 1-97-8	EMS 113609	11,729	3,437	11,008	295098.93	13597.815
SNH 1-97-9	Blank	<0.010237	1	2	<0.3339	24.465
SNH 1-97-10	Spike	5	7	9	6	9
SNH 1-97-11	NIST 2710a	1,905	2,456	8,694	529727.1	13804.518
SNH 1-97-12	NIST 2710a	1,974	2,659	9,885	565798.8	88841.76
SNH 1-99-1	EMS 113609	21,157	3,513	6,117	362722.5	3591.945
SNH 1-99-2	EMS 113609	21,680	3,914	6,666	317520	2037.42
SNH 1-99-3	Blank	0	1	3	<0.3339	21.084
SNH 1-99-4	Spike	15	16	19	15	14
SNH 1-99-5	NIST 2710a	4,423	3,374	7,812	700528.5	713317.5
SNH 1-99-6	NIST 2710a	4,584	3,616	7,916	728973	731619
SNH 1-99-7	EMS 113609	10,756.66	3,744.97	12,248.17	316377.18	540264.9
SNH 1-99-8	EMS 113609	9,463.76	3,392.36	13,123.61	258459.81	784255.5
SNH 1-99-9	Blank	<0.010237	0.52	5.04	<0.3339	0.0483
SNH 1-99-10	Spike	4.53	6.46	9.10	6.11	3.43
SNH 1-99-11	NIST 2710a	1,629.31	2,346.69	16,289.01	516757.5	619703.7
SNH 1-99-12	NIST 2710a	1,726.58	2,800.62	13,091.19	532969.5	519999.9
SNH 2-01-1	EMS 113609	20,312.46	3,552.26	6,099.03	314874	789,390.00

SNH 2-01-2	EMS 113609	21,443.63	3,814.65	6,667.92	341113.5	814,086.00
SNH 2-01-3	Blank	<0.010237	1.06	3.06	<0.3339	0.20
		ppb	ppb	ppb	ppb	ppb
SNH 2-01-4	Spike	16.45	17.58	20.33	17.24	15.46
SNH 2-01-5	EMS 11609	22,270.50	4,731.93	8,264.34	628645.5	1,146,820.50
SNH 2-01-6	EMS 11609	22,270.50	4,734.14	7,664.58	652018.5	1,274,269.50
SNH 2-01-7	NIST 2710a	4,449.69	3,223.71	7,741.76	736249.5	734,044.50
SNH 2-01-8	NIST 2710a	4,350.47	3,210.48	7,655.76	698103	711,553.50
SNH 2-01-9	B.O. MS4	191.84	3,479.49	7,668.99	121605.75	255,339.00
SNH 2-01-10	B.O. MS 4	165.38	3,159.77	7,644.74	110272.05	261,513.00
SNH 2-01-11	EPA Reference	18,010.44	4,359.29	7,082.46	150888.15	971,743.50
SNH 2-01-12	EPA Reference	18,043.52	4,103.51	7,025.13	152806.5	970,861.50
SNH 2-01-13	IKJ 525-0-2	9,395.51	4,784.85	7,574.18	1103161.5	520600.5
SNH 2-01-14	IKJ 525-0-2	9,909.27	5,360.36	8,416.49	1184305.5	533610
SNH 2-01-15	EMS 113609	9,885.27	3,603.12	15,364.92	295139.46	586063.8
SNH 2-01-16	EMS 113609	8,442.40	3,165.39	20,285.27	293639.85	815868.9
SNH 2-01-17	Blank	<0.010237	0.51	5.48	<0.3339	0.06195
SNH 2-01-18	Spike	4.26	6.27	8.49	5.72	3.29
SNH 2-01-19	EMS 11609	7,380.51	3,716.60	7,696.65	406110.6	802088.7
SNH 2-01-20	EMS 11609	8,446.45	3,566.64	8,207.33	445424.7	483117.6
SNH 2-01-21	NIST 2710a	1,564.46	2,516.91	8,539.67	492844.8	606328.8
SNH 2-01-22	NIST 2710a	1,434.76	2,541.23	8,807.17	475011.6	569851.8
SNH 2-01-23	B.O. MS4	<40	2,606.08	11,064.69	103999.98	239005.41
SNH 2-01-24	B.O. MS 4	<40	2,306.16	10,164.92	90503.49	230615.7
SNH 2-01-25	EPA Reference	7,461.57	3,384.26	8,401.87	104243.16	817895.4
SNH 2-01-26	EPA Reference	7,996.57	3,862.51	10,898.52	112673.4	726297.6
SNH 2-01-27	IKJ 525-0-2	2,983.01	4,040.84	12,069.83	865720.8	116280.57
SNH 2-01-28	IKJ 525-0-2	2,881.68	4,109.74	11,713.17	865315.5	128966.46
SNH 2-03-1	EMS 113609	20,654.24	3,442.01	6,511.37	330088.5	837238.5

SNH 2-03-2	EMS 113609	21,664.13	3,726.45	7,183.89	341113.5	806368.5
SNH 2-03-3	Blank	<0	0.89	3.04	<0	0.12285
SNH 2-03-4	Spike	16.16	17.56	20.41	17.73	14.86
SNH 2-03-5	EMS 11609	22,424.85	4,604.04	8,041.64	645844.5	1176588
SNH 2-03-6	EMS 11609	22,001.49	4,489.38	7,605.05	629307	1193566.5
SNH 2-03-7	NIST 2710a	4,295.34	3,005.42	7,600.64	684652.5	711333
SNH 2-03-8	NIST 2710a	4,244.63	3,075.98	7,770.42	686416.5	700528.5
SNH 2-03-9	B.O. MS4	178.61	3,124.49	7,697.66	119577.15	262174.5
SNH 2-03-10	B.O. MS 4	288.86	3,219.30	7,993.13	123987.15	260631

Appendix A.6 ICPMS Run 6

Key:
SNH x-xx-xx = Analyzer initials Notebook #-page#-sample #
Acid Blank= Nitric Acid with no sample additions
Acid Spike: Nitric Acid spiked with 18/10 ppb of the analyte concentration
DF is 2270 for Saliva/Gastric and 4168 for Saliva/Gastric-Intestinal

Table A.6.1 Sample Description for Biofluid Extractions

Sample ID	Description	112Cd ppb	60 Ni ppb	52Cr ppb	75As ppb	208Pb ppb
SNH 2-03-11	EPA Reference	18610.2	3,539.03	6312.915	137,040.75	1278459
SNH 2-03-12	EPA Reference	18,540	3,757	6,492	139797	1673.595
SNH 2-03-13	IKJ 525-0-2	10,379	5,078	8,227	1120581	1799.28
SNH 2-03-14	IKJ 525-0-2	10,515.65	5,364.77	8,442.95	1220467.5	3483.9
SNH 2-03-15	EMS 113609	11,753.70	2,978.96	5,884.96	268308.6	34114.1
SNH 2-03-16	EMS 113609	10,898.52	3,299.14	6,079.50	457989	14323.3
SNH 2-03-17	Blank	0.47	0.39	1.59	10.04745	9.3891
SNH 2-03-18	Spike	5.30	7.10	8.60	6.16	9.43
SNH 2-03-19	EMS 11609	7870.926	2079.189	5512.08	233371.74	39537.02
SNH 2-03-20	EMS 11609	9808.26	3060.015	5868.744	445019.4	57957.9
SNH 2-03-21	NIST 2710a	1126.734	1746.843	5402.649	480685.8	37494.3
SNH 2-03-22	NIST 2710a	1086.204	1706.313	5414.808	477443.4	36894.46
SNH 2-03-23	B.O. MS4	<40	1937.334	6047.076	94961.79	281521.4
SNH 2-03-24	B.O. MS 4	<40	2496.648	6306.468	98974.26	217038.2
SNH 2-03-25	EPA Reference	8191.113	2938.425	5929.539	103756.8	1012439
SNH 2-03-26	EPA Reference	8568.042	3680.124	6971.16	105945.42	948807.3
SNH 2-03-27	IKJ 525-0-2	3501.792	4235.385	6310.521	813842.4	153122.3
SNH 2-03-28	IKJ 525-0-2	3270.771	3911.145	6197.037	849103.5	146272.8
SNH 2-05-1	EMS 113609	22072.05	3534.615	5885.145	320607	979681.5
SNH 2-05-2	EMS 113609	22138.2	3666.915	6575.31	351918	958954.5
SNH 2-05-3	Blank	<0.0102375	1.0185	3.1416	<0.36225	<0.0063

SNH 2-05-4	Spike	17.1255	18.669	21.5775	16.9995	16.9995
SNH 2-05-5	EMS 11609	24034.5	4952.43	7818.93	734044.5	1605902
SNH 2-05-6	EMS 11609	23836.05	5060.47 5	7827.75	694354.5	1512410
SNH 2-05-7	NIST 2710a	4387.95	3329.55	7946.82	736029	865683
SNH 2-05-8	NIST 2710a	4462.92	3397.90 5	7993.12 5	680683.5	885528
SNH 2-05-9	B.O. MS4	<21	3490.51 5	7803.49 5	102179.7	350595
SNH 2-05-10	B.O. MS 4	<21	3395.7	7902.72	115387.65	351036
SNH 2-05-11	EPA Reference	19192.32	4301.95 5	7362.49 5	152586	1231272
SNH 2-05-12	EPA Reference	18552.87	4421.02 5	7545.51	153886.95	1153436
SNH 2-05-13	IKJ 525-0-2	9944.55	5115.6	8222.44 5	1234800	611667
SNH 2-05-14	IKJ 525-0-2	10213.56	5986.57 5	8934.66	1234359	582340. 5
SNH 2-05-15	EMS 113609	11401.089	3388.30 8	9135.46 2	276090.36	747373. 2
SNH 2-05-16	EMS 113609	6711.768	2448.01 2	5816.05 5	213552.57	947996. 7
SNH 2-05-17	Blank	<0.0102375	0.43785	2.97465	<0.36225	<0.0063
SNH 2-05-18	Spike	5.21848484 8	7.30997 2	13.3122 5	7.08179090 9	5.14992 8
SNH 2-05-19	EMS 11609	8867.964	3493.68 6	13451.9 1	495681.9	879501
SNH 2-05-20	EMS 11609	9771.783	3728.76	14367.8 9	551613.3	675635. 1
SNH 2-05-21	NIST 2710a	1252.377	2492.59 5	34255.9 6	663070.8	484738. 8
SNH 2-05-22	NIST 2710a	1317.225	2569.60 2	47298.5 1	747373.2	391681. 9
SNH 2-05-23	B.O. MS4	<40	2906.00 1	63956.3 4	158269.65	407326. 5
SNH 2-05-24	B.O. MS 4	<40	2022.44 7	16511.9 2	112632.87	345477. 7
SNH 2-05-25	EPA Reference	9236.787	3015.43 2	43204.9 8	158310.18	774528. 3
SNH 2-05-26	EPA Reference	9281.37	3165.39 3	30510.9 8	143840.97	907466. 7
SNH 2-05-27	IKJ 525-0-2	3631.488	4352.92 2	21371.4 7	1080124.5	150487. 9
SNH 2-05-28	IKJ 525-0-2	3355.884	4255.65	16994.2 3	1012439.4	148461. 4
SNH 2-06-1	EMS 113609	39006.072	6772.56 3	14084.1 8	673203.3	1723741
SNH 2-06-2	EMS 113609	39678.87	7352.14 2	14943.4 1	653748.9	1615931
SNH 2-06-3	Blank	<40	3611.22 3	13184.4 1	<1398	64.848
SNH 2-06-4	Spike	15.4738546 3	17.4165 9	20.8163 7	15.8138326	14.7939

SNH 2-06-5	EMS 11609	23284.8	4817.925	8731.8	660618	1459269
SNH 2-06-6	EMS 11609	11.1615	2.2911	4.2084	340.095	731.64
SNH 2-06-7	NIST 2710a	1096.578	754.11	2197.692	184671.9	215686.8
SNH 2-06-8	NIST 2710a	4121.145	2740.815	8138.655	735147	822685.5
SNH 2-06-9	B.O. MS4	<21	3067.155	8548.785	122443.65	369117
SNH 2-06-10	B.O. MS 4	<21	2978.955	8350.335	129587.85	357210
SNH 2-06-11	EPA Reference	18940.95	4118.94	7759.395	164448.9	1161153
SNH 2-06-12	EPA Reference	18881.415	4176.27	7552.125	163787.4	1208340
SNH 2-06-13	IKJ 525-0-2	10072.44	5064.885	8700.93	1280884.5	608580
SNH 2-06-14	IKJ 525-0-2	10101.105	5362.56	9155.16	1316164.5	581458.5
SNH 2-06-15	EMS 113609	10862.04	3112.704	7887.138	259310.94	1097147
SNH 2-06-16	EMS 113609	8661.261	2845.206	14440.84	266971.11	1060265
SNH 2-06-17	Blank	<0	0.31395	3.13215	<0	<0
SNH 2-06-18	Spike	4.541105051	6.621336	14.2127	7.193323232	3.304017
SNH 2-06-19	EMS 11609	7899.297	3100.545	37352.45	543507.3	1076882
SNH 2-06-20	EMS 11609	8004.675	3222.135	37056.58	626999.1	932190
SNH 2-06-21	NIST 2710a	907.872	1811.691	35832.57	625377.9	146151.2
SNH 2-06-22	NIST 2710a	988.932	1629.306	35143.56	612813.6	299111.4
SNH 2-06-23	B.O. MS4	<40	1929.228	37510.52	137315.64	283061.5
SNH 2-06-24	B.O. MS 4	<40	1710.366	8718.003	100311.75	247111.4
SNH 2-06-25	EPA Reference	8547.777	2906.001	56417.76	180439.56	1117412
SNH 2-06-26	EPA Reference	8559.936	2739.828	16686.2	130790.31	1056212
SNH 2-06-27	IKJ 525-0-2	3088.386	3728.76	9362.43	928137	121144.2

Appendix B

Summary Statistics of EPA 3051A Charts in Chapter 3

Analyzed in GraphPad Prism

B.1 EPA Method 3051 Summary Statistics

Table B.1.1 Liberty State Park Soil: 125-500 microns (mg/kg)

	Pb	As	Cd	Ni	Cr
Number of values	6	6	6	6	6
Minimum	1047	115.4	1.13	488	1406
25% Percentile	1128	119.9	1.2	502.9	1639
Median	1186	122.9	1.348	529.5	2196
75% Percentile	1519	127.5	1.746	623.9	3550
Maximum	1826	129.3	2.685	650.2	3646
Mean	1303	123.1	1.528	553.4	2447
Std. Deviation	283.4	4.816	0.5772	65.35	946.6
Std. Error	115.7	1.966	0.2356	26.68	386.4
Lower 95% CI of mean	1005	118.1	0.9221	484.8	1453
Upper 95% CI of mean	1600	128.2	2.134	622	3440
Sum	7816	738.6	9.167	3320	14679

Table B.1.2 Liberty State Park Soil: 45-125 microns (mg/kg)

	Pb	As	Cd	Ni	Cr
Number of values	6	6	6	6	5
Minimum	984.8	112.2	1.046	445.1	2275
25% Percentile	1356	148.1	1.356	522.8	2296
Median	1535	164	1.488	638.3	2768
75% Percentile	1622	167.5	1.527	770.5	4055
Maximum	1650	171.9	1.573	851.2	4501
Mean	1466	156.3	1.427	644.2	3094
Std. Deviation	243.5	22	0.1914	144	952.1
Std. Error	99.42	8.983	0.07814	58.79	425.8
Lower 95% CI of mean	1210	133.3	1.226	493.1	1912
Upper 95% CI of mean	1722	179.4	1.628	795.3	4276
Sum	8796	938.1	8.564	3865	15470

Table B.1.3 Liberty State Park >45 Microns

	Pb	As	Cd	Ni	Cr
Number of values	2	2	2	2	2
Minimum	2111	212.6	1.747	622.8	2858
25% Percentile	2111	212.6	1.747	622.8	2858
Median	2136	214.1	1.838	638.5	2987
75% Percentile	2162	215.7	1.928	654.2	3117
Maximum	2162	215.7	1.928	654.2	3117
Mean	2136	214.1	1.838	638.5	2987
Std. Deviation	35.64	2.206	0.1278	22.23	183.3
Std. Error	25.2	1.56	0.0904	15.72	129.6
Lower 95% CI of mean	1816	194.3	0.689	438.8	1340
Upper 95% CI of mean	2457	233.9	2.986	838.3	4634
Sum	4273	428.2	3.675	1277	5974

Table B.1.4 NIST 2170 EPA 3051 method

	Pb	As	Cd	Ni	Cr
Number of values	3	3	3	3	3
Minimum	5174	480.2	15.97	143	13.62
25% Percentile	5174	480.2	15.97	143	13.62
Median	5202	497.8	16.26	145	13.74
75% Percentile	5346	504.2	16.46	157.8	15.56
Maximum	5346	504.2	16.46	157.8	15.56
Mean	5241	494.1	16.23	148.6	14.31
Std. Deviation	92.75	12.47	0.2491	7.984	1.087
Std. Error	53.55	7.201	0.1438	4.61	0.6275
Lower 95% CI of mean	5010	463.1	15.61	128.8	11.61
Upper 95% CI of mean	5471	525.1	16.85	168.4	17.01
Sum	15722	1482	48.69	445.8	42.92

Appendix B.2: Liberty State Park Soils EPA 3051A extraction Summary Statistics by Metal

Table B.2.1 Lead (Pb)

	LSP 125-500	LSP 45-125	LSP >45
Number of values	6	6	2
Minimum	1047	984.8	2111
25% Percentile	1128	1356	2111
Median	1186	1535	2136
75% Percentile	1519	1622	2162
Maximum	1826	1650	2162
Mean	1303	1466	2136
Std. Deviation	283.4	243.5	35.64
Std. Error	115.7	99.42	25.2
Lower 95% CI	1005	1210	1816
Upper 95% CI	1600	1722	2457

Table B.2.2 Arsenic (As)

	LSP 125-500	LSP 45-125	LSP >45
Number of values	6	6	2
Minimum	115.4	112.2	212.6
25% Percentile	119.9	148.1	212.6
Median	122.9	164	214.1
75% Percentile	127.5	167.5	215.7
Maximum	129.3	171.9	215.7
Mean	123.1	156.3	214.1
Std. Deviation	4.816	22	2.206
Std. Error	1.966	8.983	1.56
Lower 95% CI	118.1	133.3	194.3
Upper 95% CI	128.2	179.4	233.9

Table B.2.3 Chromium (Cr)

	LSP 125-500	LSP 45-125	LSP >45
Number of values	6	6	2
Minimum	1.13	1.046	1.747
25% Percentile	1.2	1.356	1.747
Median	1.348	1.488	1.838
75% Percentile	1.746	1.527	1.928
Maximum	2.685	1.573	1.928
Mean	1.528	1.427	1.838
Std. Deviation	0.5772	0.1914	0.1278
Std. Error	0.2356	0.07814	0.0904
Lower 95% CI	0.9221	1.226	0.689
Upper 95% CI	2.134	1.628	2.986

Table B.2.4 Nickel

	LSP 125-500	LSP 45-125	LSP >45
Number of values	6	6	2
Minimum	488	445.1	622.8
25% Percentile	502.9	522.8	622.8
Median	529.5	638.3	638.5
75% Percentile	623.9	770.5	654.2
Maximum	650.2	851.2	654.2
Mean	553.4	644.2	638.5
Std. Deviation	65.35	144	22.23
Std. Error	26.68	58.79	15.72
Lower 95% CI	484.8	493.1	438.8
Upper 95% CI	622	795.3	838.3

Table B.2.5 Chromium

	LSP 125-500	LSP 45-125	LSP >45
Number of values	6	5	2
Minimum	1406	2275	2858
25% Percentile	1639	2296	2858
Median	2196	2768	2987
75% Percentile	3550	4055	3117
Maximum	3646	4501	3117
Mean	2447	3094	2987
Std. Deviation	946.6	952.1	183.3
Std. Error	386.4	425.8	129.6
Lower 95% CI	1453	1912	1340
Upper 95% CI	3440	4276	4634

Appendix C

Summary Statistics of Liberty State Park Soils ANOVA Summarized Graphically in Chapter 3 Analyzed in GraphPad Prism

Appendix C. Liberty State Park Soils ANOVA Results for EPA 3051A

Table C.1 Comparison of Means Liberty State Park Soils: Pb

Parameter	
Table Analyzed	Data 8
Kruskal-Wallis test	
P value	0.0569
Exact or approximate P value?	Gaussian Approximation
P value summary	ns
Do the medians vary signif. (P < 0.05)	No
Number of groups	3
Kruskal-Wallis statistic	5.733

Dunn's Multiple Comparison Test	Difference in rank sum	Significant? P < 0.05?	Summary
LSP 125-500 vs LSP 45-125	-2.333	No	ns
LSP 125-500 vs LSP >45	-8.167	No	ns
LSP 45-125 vs LSP >45	-5.833	No	ns

Table C.2 Comparison of Means Liberty State Park Soils: Arsenic

Parameter	
Table Analyzed	Data 8
Kruskal-Wallis test	
P value	0.023
Exact or approximate P value?	Gaussian Approximation
P value summary	*
Do the medians vary signif. (P < 0.05)	Yes
Number of groups	3
Kruskal-Wallis statistic	7.543

Dunn's Multiple Comparison Test	Difference in rank sum	Significant? P < 0.05?	Summary
LSP 125-500 vs LSP 45-125	-4	No	ns
LSP 125-500 vs LSP >45	-9	Yes	*
LSP 45-125 vs LSP >45	-5	No	ns

Table C.3 Comparison of Means Liberty State Park Soils: Cadmium (Cd)

Parameter	
Table Analyzed	Data 8
Kruskal-Wallis test	
P value	0.1341
Exact or approximate P value?	Gaussian Approximation
P value summary	ns
Do the medians vary signif. (P < 0.05)	No
Number of groups	3
Kruskal-Wallis statistic	4.019

Dunn's Multiple Comparison Test	Difference in rank sum	Significant? P < 0.05?	Summary
LSP 125-500 vs LSP 45-125	-2	No	ns
LSP 125-500 vs LSP >45	-6.833	No	ns
LSP 45-125 vs LSP >45	-4.833	No	ns

Table C.4 Comparison of Means Liberty State Park Soils: Nickel (Ni)

Parameter	
Table Analyzed	Data 8
Kruskal-Wallis test	
P value	0.2307
Exact or approximate P value?	Gaussian Approximation
P value summary	ns
Do the medians vary signif. (P < 0.05)	No
Number of groups	3
Kruskal-Wallis statistic	2.933

Dunn's Multiple Comparison Test	Difference in rank sum	Significant? P < 0.05?	Summary
LSP 125-500 vs LSP 45-125	-3.5	No	ns
LSP 125-500 vs LSP >45	-4.667	No	ns
LSP 45-125 vs LSP >45	-1.167	No	ns

Table C.5 Comparison of Means Liberty State Park Soils: Chromium (Cr)

Parameter	
Table Analyzed	Data 8
Kruskal-Wallis test	
P value	0.5144
Exact or approximate P value?	Gaussian Approximation
P value summary	ns
Do the medians vary signif. (P < 0.05)	No
Number of groups	3
Kruskal-Wallis statistic	1.33

Dunn's Multiple Comparison Test	Difference in rank sum	Significant? P < 0.05?	Summary
LSP 125-500 vs LSP 45-125	-2.333	No	ns
LSP 125-500 vs LSP >45	-2.833	No	ns
LSP 45-125 vs LSP >45	-0.5	No	ns

Appendix D

Summary Statistics of EPA 3051A Charts in Chapter 3 For Unknown Soil Samples Received from the EPA Analyzed in GraphPad Prism

Appendix D: 3051A extraction Summary Statistics by Soil Sample

Table D.1 Summary Statistics for unknown EPA sample 1

	Pb	As	Cd	Ni	Cr
Number of values	3	3	3	3	3
Minimum	1171	553.6	27.05	5.36	6.108
25% Percentile	1171	553.6	27.05	5.36	6.108
Median	1257	657.6	28.99	6.304	7.26
75% Percentile	1310	663.6	29.04	6.508	7.596
Maximum	1310	663.6	29.04	6.508	7.596
Mean	1246	624.9	28.36	6.057	6.988
Std. Deviation	70.06	61.85	1.135	0.6125	0.7804
Std. Error	40.45	35.71	0.6555	0.3536	0.4506
Lower 95% CI of mean	1072	471.3	25.54	4.536	5.049
Upper 95% CI of mean	1420	778.6	31.18	7.579	8.927
Sum	3737	1875	85.08	18.17	20.96

Table D.2 Summary Statistics for unknown EPA sample 2

	Pb	As	Cd	Ni	Cr
Number of values	3	3	3	3	3
Minimum	2008	1215	30.38	7.704	7.812
25% Percentile	2008	1215	30.38	7.704	7.812
Median	2013	1216	30.39	7.976	7.948
75% Percentile	2083	1230	32.63	8.148	8.124
Maximum	2083	1230	32.63	8.148	8.124
Mean	2035	1220	31.13	7.943	7.961
Std. Deviation	41.97	8.663	1.298	0.2239	0.1564
Std. Error	24.23	5.001	0.7493	0.1293	0.09031
Lower 95% CI of mean	1931	1199	27.91	7.387	7.573
Upper 95% CI of mean	2139	1242	34.36	8.499	8.35
Sum	6104	3661	93.4	23.83	23.88

Table D.3 Summary Statistics for unknown EPA sample 3

	Pb	As	Cd	Ni	Cr
Number of values	3	3	3	3	3
Minimum	1371	1174	9.972	4.628	8.024
25% Percentile	1371	1174	9.972	4.628	8.024
Median	1439	1294	10.6	4.8	8.584
75% Percentile	1494	1340	10.95	7.42	8.76
Maximum	1494	1340	10.95	7.42	8.76
Mean	1435	1269	10.51	5.616	8.456
Std. Deviation	61.72	85.7	0.4964	1.565	0.3843
Std. Error	35.64	49.48	0.2866	0.9034	0.2219
Lower 95% CI of mean	1281	1056	9.275	1.729	7.501
Upper 95% CI of mean	1588	1482	11.74	9.503	9.411
Sum	4304	3808	31.52	16.85	25.37

Table D.4 Summary Statistics for unknown EPA sample 4

	Pb	As	Cd	Ni	Cr
Number of values	3	3	3	3	3
Minimum	334.3	308.2	0.2208	24.79	3.108
25% Percentile	334.3	308.2	0.2208	24.79	3.108
Median	345.8	316	0.2292	25.37	3.178
75% Percentile	363.7	320.7	0.2452	25.93	3.692
Maximum	363.7	320.7	0.2452	25.93	3.692
Mean	347.9	314.9	0.2317	25.37	3.326
Std. Deviation	14.82	6.301	0.0124	0.57	0.3192
Std. Error	8.556	3.638	0.007157	0.3291	0.1843
Lower 95% CI of mean	311.1	299.3	0.2009	23.95	2.533
Upper 95% CI of mean	384.7	330.6	0.2625	26.78	4.119
Sum	1044	944.8	0.6952	76.1	9.978

Table D.5 Summary Statistics for unknown EPA sample 5

	Pb	As	Cd	Ni	Cr
Number of values	3	3	3	3	3
Minimum	1699	544.4	53.96	5.136	54.88
25% Percentile	1699	544.4	53.96	5.136	54.88
Median	1786	607.2	55.08	6.3	55.84
75% Percentile	1806	646.4	57.76	6.32	56.48
Maximum	1806	646.4	57.76	6.32	56.48
Mean	1764	599.3	55.6	5.919	55.73
Std. Deviation	56.63	51.45	1.953	0.6779	0.8053
Std. Error	32.69	29.71	1.127	0.3914	0.4649
Lower 95% CI of mean	1623	471.5	50.75	4.235	53.73
Upper 95% CI of mean	1904	727.1	60.45	7.603	57.73
Sum	5291	1798	166.8	17.76	167.2

Table D.6 Summary Statistics for unknown EPA sample 6

	Pb	As	Cd	Ni	Cr
Number of values	3	3	3	3	3
Minimum	756.4	1783	34.51	9.88	14.33
25% Percentile	756.4	1783	34.51	9.88	14.33
Median	759.2	1838	35	11.14	16.63
75% Percentile	810.4	2022	37.71	12.07	18.08
Maximum	810.4	2022	37.71	12.07	18.08
Mean	775.3	1881	35.74	11.03	16.35
Std. Deviation	30.4	125.2	1.725	1.098	1.892
Std. Error	17.55	72.31	0.9959	0.6339	1.092
Lower 95% CI of mean	699.8	1570	31.46	8.3	11.65
Upper 95% CI of mean	850.9	2192	40.02	13.76	21.05
Sum	2326	5643	107.2	33.08	49.04

Appendix E

Summary Statistics of Biofluid Extractions Presented Graphically Analyzed in GraphPad Prism

Appendix E: Biofluid extraction summary statistics by soil

Table E.1 NIST 2710 Gastric

	Pb	As	Cd	Ni	Cr
Number of values	7	10	10	8	9
Minimum	936.4	298.5	217.8	16.47	6.76
25% Percentile	938.9	349.5	265.1	16.68	7.155
Median	956.6	370.6	293.6	17.53	7.843
75% Percentile	991.1	380.6	321.4	18.68	8.158
Maximum	1075	454.9	358.7	20.19	10.43
Mean	975.7	368.4	292.5	17.82	7.932
Std. Deviation	48.84	39.62	42.67	1.273	1.063
Std. Error	18.46	12.53	13.49	0.4502	0.3545
Lower 95% CI of mean	930.6	340.1	262	16.76	7.115
Upper 95% CI of mean	1021	396.8	323	18.89	8.75
Sum	6830	3684	2925	142.6	71.39

Table E.2 NIST 2710 Intestinal

	Pb	As	Cd	Ni	Cr
Number of values	7	10	10	9	10
Minimum	493.9	217.8	5.739	15.78	5.678
25% Percentile	498.9	265.1	6.111	16.01	7.385
Median	511	293.6	6.36	17.81	9.176
75% Percentile	582.7	321.4	6.817	21.27	18.38
Maximum	708.1	358.7	7.298	25.49	24.97
Mean	551.5	292.5	6.471	18.75	12.51
Std. Deviation	76.99	42.67	0.4849	3.546	6.769

Std. Error	29.1	13.49	0.1533	1.182	2.141
Lower 95% CI of mean	480.3	262	6.124	16.02	7.67
Upper 95% CI of mean	622.8	323	6.818	21.47	17.35
Sum	3861	2925	64.71	168.7	125.1

Appendix F

Summary Statistics of Biofluid Extractions Presented Graphically Analyzed in GraphPad Prism

Appendix F: Selected in vitro bioaccessibility results

Table F.1. In vitro bioaccessibility results using the HMBSR method for NIST 2710

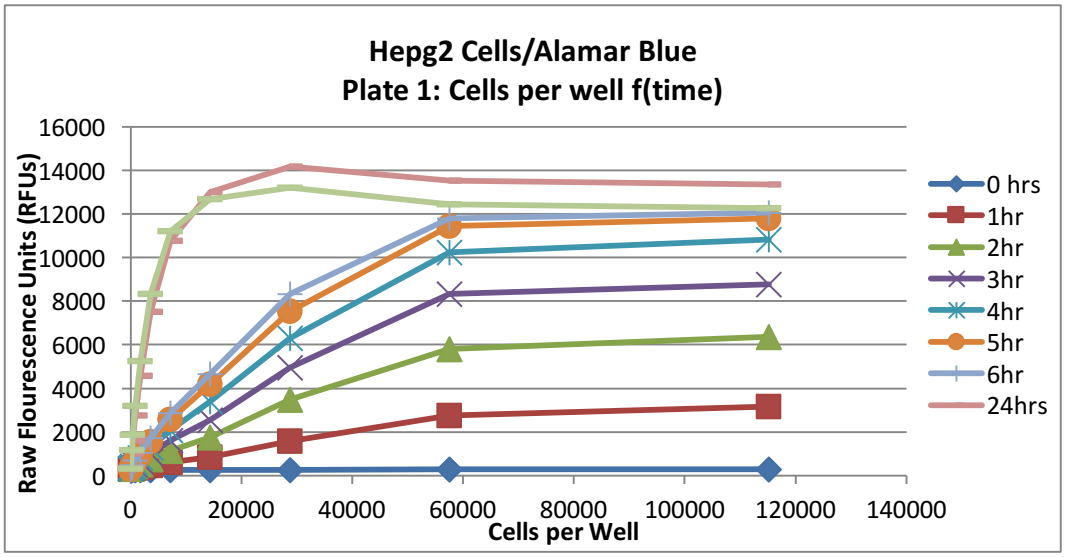
Table 1. Total Metal Mass in artificial gastrointestinal fluids and soil post-extraction (µg)			
ug	**Soluble Metal in *Saliva-Gastric***	**Soluble Metal in *Saliva-Gastric+ Intestinal/Soil***	**Mass Recovered from Residual Soil***
As	15.9 ± 1.9	7.5 ± 1.1	9.5 ± 0.1
Pb	46.4 ± 2.3	14.3 ± 2.0	269 ± 4.4
Cd	0.6 ± 0.05	0.2 ± .01	0.5 ±.004
Ni	0.8 ± 0.06	0.4 ± 0.03	9.8 ± 0.5
Cr	0.34 ±0.04	0.15 ± .01	2.5 ± 0.6
Table 2.*In vitro* bioaccessibility of selected metals			
	%Soluble Metal in *Saliva-Gastric*	**% Soluble Metal in *Saliva-Gastric+ Intestinal/Soil***	**Recovery %**
As	50.9 ± 6.8	24.2 ± 3.8	30.4 ± 1.9
Pb	16.8 ± 0.9	5.2 ± 0.7	97.5 ± 2.1
Cd	58.4 ± 5.3	15.4 ± 1.2	42.6 ± 0.5
Ni	118 ± 11.9	52.1 ± 5.9	1368.9 ± 114.8
Cr	17.8 ± 1.9	8.0 ± 0.6	129.3 ± 32.8

Appendix G

Determination of Cell Plating Density and Time Function

Figure G.1. Determination of Cell Plating Density for a Cell Line as a function of Time

a)



b)

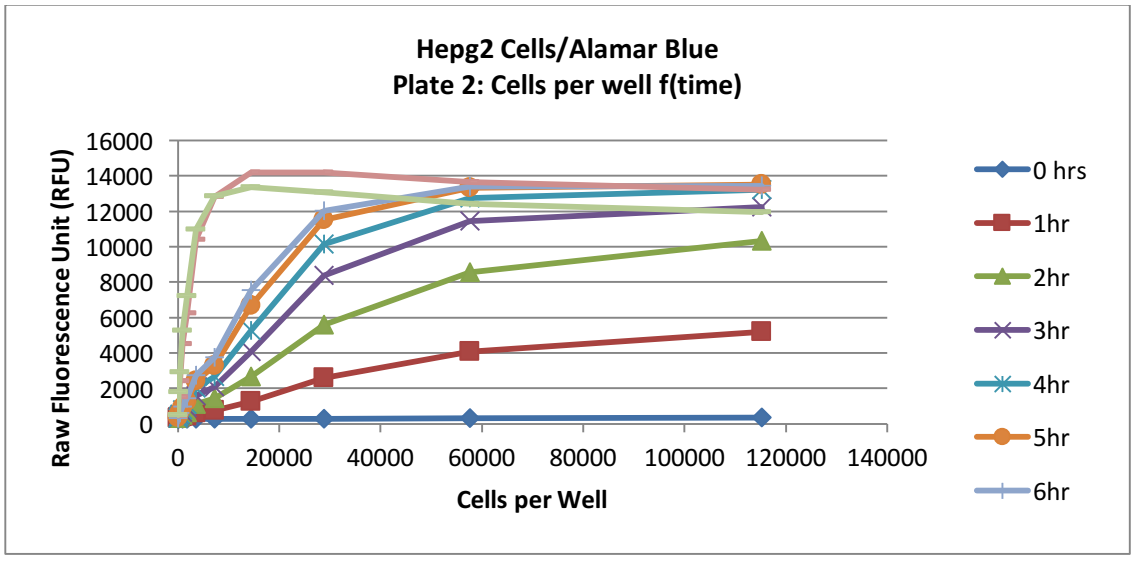
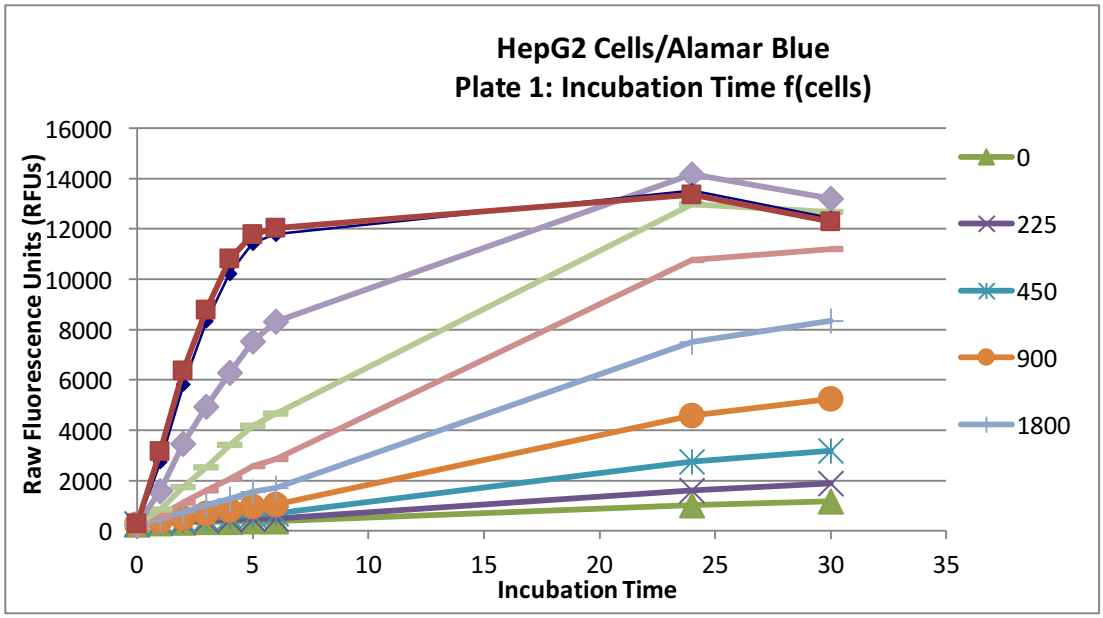
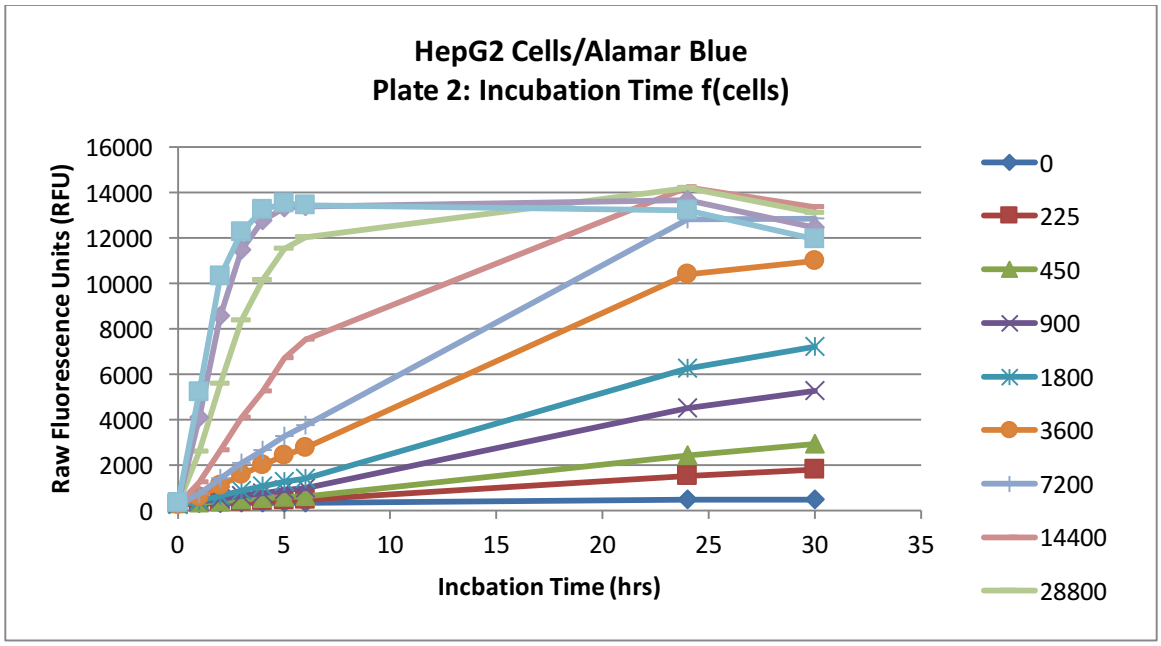


Figure G.2 Determination of Incubation Time for a Cell Line as a Function of Cell Density

a)



b)



Appendix H

Data Used for Comparison of Risk using Hazard Quotients

Appendix H: Comparison of IVBA results and HQ.

1=represents no GSH depletion

2= GSH reduction

Table H.1 HQ scatter plot for 100% Bioaccessible

		NIST 2710	**NIST 2709**	**LSP 125-500**	**LSP 45-125**	**Unknown 1**	**Unknown 2**	**Unknown 3**	**Unknown 4**	**Unknown 5**	**Unknown 6**
2	As	2176.4	61.5	428	543.4	2172.6	4242.9	4412.9	1094.8	2083.6	6539.3
1	Cd	2.3	0.04	0.2	0.1	3	3.2	1.1	2.6	5.8	3.4
1	Cr III	0.03	0.1	1.7	1.8	0.005	0.01	0.01	0.002	0.04	0.01

Table H.2 HQ scatter plot for IVBA

		NIST 2710	**NIST 2709**	**LSP 125-500**	**LSP 45-125**	**Unknown 1**	**Unknown 2**	**Unknown 3**	**Unknown 4**	**Unknown 5**	**Unknown 6**
2	As	1108.1	2	21.9	55.8	975.5	1777.5	1804.9	389.7	452.1	2720.3
1	Cd	1.3	0.02	0.04	0.03	1.1	0.9	0.2	0.037	0.9	0.3
1	Cr III	0.01	0.004	0.02	0.03	0.005	0.004	0.01	0.005	0.005	0.01

Table H.3 HQ scatter plot for Viability

	NIST 2710	**NIST 2709**	**LSP 125-500**	**LSP 45-125**	**Unknown 1**	**Unknown 2**	**Unknown 3**	**Unknown 4**	**Unknown 5**	**Unknown 6**	
2	55.4	2	40.5	16.5	217.3	1777.8	4.4	547.4	208.4	653.9	As
1	0.1	0.02	0.02	0.003	0.3	0.3	0.001	1.862	0.6	0.3	Cd
1	0.0003	0.004	0.01	0.003	0.0005	0.001	0	0.001	0.004	0.001	Cr III

Appendix I

GSH Data

Interpolations Calculated Using Graphpad Prisim

Appendix I: GSH interpolations

Figure I.1 GSH Standard Curve

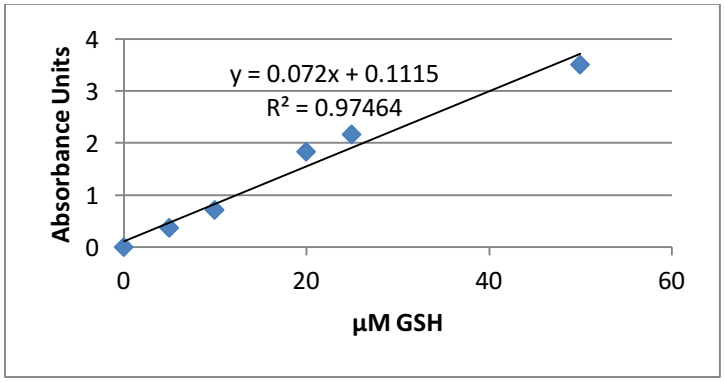


Table I.2 Interpolations from Standard Curve and Cell Counts

	GSH	cell count	x200	per 10^6 cells	GSH/10^6 cells
Control	2.136641	372	74400	0.0744	28.71829301
Control	4.197853	349	69800	0.0698	60.14116046
Control	2.467302	283	56600	0.0566	43.59190813
Control	2.891047	388	77600	0.0776	37.25576031
Control	2.322256	330	66000	0.066	35.18569697
Control	2.02466	540	108000	0.108	18.74685185
Control	1.421135	450	90000	0.09	15.79038889
Control	1.959639	486	97200	0.0972	20.16089506
5 Pb	2.152201	668	133600	0.1336	16.10928892
50 Pb	4.942535	245	49000	0.049	100.8680612
100 Pb	21.2294	391	78200	0.0782	271.4757033
200 Pb	26.71281	370	74000	0.074	360.9839189
5 Pb	1.845714	491	98200	0.0982	18.79545825
50 Pb	1.734012	542	108400	0.1084	15.99642066
100 Pb	1.602581	369	73800	0.0738	21.7151897
200 Pb	1.752907	549	109800	0.1098	15.96454463
5 Cr III	2.706822	705	141000	0.141	19.19731915
50 Cr III	8.781261	268	53600	0.0536	163.8294963
100 Cr III	1.807924	544	108800	0.1088	16.61694853
200 Cr III	1.684552	404	80800	0.0808	20.84841584
5 Cr III	2.53121	709	141800	0.1418	17.85056417
50 Cr III	1.952138	439	87800	0.0878	22.233918
100 Cr III	1.725676	771	154200	0.1542	11.19115435
200 Cr III	1.654542	225	45000	0.045	36.7676
5 Cd	1.835989	18	3600	0.0036	509.9969444
50 Cd	1.419746	92	18400	0.0184	77.1601087
100 Cd	8.264429	259	51800	0.0518	159.5449614
200 Cd	1.375009	597	119400	0.1194	11.51598827
5 Cd	1.865999	74	14800	0.0148	126.0810135
50 Cd	1.22774	150	30000	0.03	40.92466667
100 Cd	1.134376	195	39000	0.039	29.0865641
200 Cd	1.493102	219	43800	0.0438	34.08908676
5 As III	4.265931	581	116200	0.1162	36.71197074
50 As III	2.097184	804	160800	0.1608	13.04218905
100 As III	1.343054	569	113800	0.1138	11.80188049

200 As III	1.466705	904	180800	0.1808	8.112306416
5 As III	1.991872	566	113200	0.1132	17.5960424
50 As III	2.223335	1117	223400	0.2234	9.952260519
100 As III	1.605082	1245	249000	0.249	6.44611245
200 As III	1.363894	1277	255400	0.2554	5.340227095
5 As V	1.899343	543	108600	0.1086	17.48934622
50 As V	2.249176	653	130600	0.1306	17.2218683
100 As V	13.91095	633	126600	0.1266	109.8811216
200 As V	2.153312	395	79000	0.079	27.25711392
5 As V	1.688998	400	80000	0.08	21.112475
50 As V	1.965753	819	163800	0.1638	12.00093407
100 As V	1.599802	523	104600	0.1046	15.29447419
200 As V	1.381122	295	59000	0.059	23.40884746