Guide for Incorporating Bioavailability Adjustments into Human Health and Ecological Risk Assessments at US Department of Defense Facilities

Part 2: Technical Background Document for Assessing Metals Bioavailability

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Guide for Incorporating Bioavailability Adjustments into Human Health and Ecological Risk Assessments at Department of Defense Facilities

Part 2: Technical Background Document for Assessing Metals Bioavailability

Update Prepared for

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EXECUTIVE SUMMARY

The *Guide for Incorporating Bioavailability Adjustments into Human Health and Ecological Risk Assessments at U.S. Department of Defense (DoD) Facilities, Parts 1 and 2,* has been developed as a resource on the assessment of bioavailability. Specifically, the document is designed for use by DoD Remedial Project Managers (RPMs) and others involved in remediating DoD sites and designing studies to support remediation. The guide brings together the most current information on bioavailability of metals, and synthesizes this information into a practical handbook that explains concepts and identifies types of data that need to be collected to assess bioavailability and incorporate it into risk assessment. Although the guide focuses on bioavailability of metals, many of the basic principles described herein also can be applied to assessing bioavailability of organic compounds.

Part 1: Overview of Metals Bioavailability, contained in the previous volume, provides a definition of bioavailability and discusses where bioavailability fits in the risk assessment process for both human health and ecological receptors. The *Overview* provides general information on the types of situations where it may be beneficial to perform additional studies to assess bioavailability and outlines key steps in conducting bioavailability studies. In addition, a brief summary of bioavailability information is presented for those metals that are most often found as contaminants at DoD sites (arsenic, cadmium, chromium, lead, mercury, and nickel for both terrestrial and aquatic settings; and copper, tin and zinc for aquatic settings only).

Part 2: Technical Background Document for Assessing Metals Bioavailability, contained in this volume, provides more in depth technical information for professionals involved in designing and performing bioavailability studies. This volume includes general study design considerations for assessing bioavailability, including information on soil collection and characterization necessary to support bioavailability studies, a general discussion of *in vitro* methods for assessing bioavailability, and a general discussion of *in vivo* methods for assessing bioavailability. Following the general information, a discussion of more specific considerations that must be addressed in designing human health bioavailability studies for individual metals is presented. Metals addressed in this section include arsenic, cadmium, chromium, lead, mercury, and nickel.

Standard operating procedures (SOPs) for soil speciation and for *in vitro* tests are provided in the appendices. The appendices also include a suggested template protocol for an *in vivo* bioavailability study for each of the six metals. The template protocols are provided as a starting point and include information (such as the recommended animal model, numbers of animals, and dosing methods) that is most often appropriate for a particular metal. A study director then can adjust the protocol to address any site-specific conditions.

Bioavailability to ecological receptors can be assessed by evaluating direct exposure to the available fraction of the metals in the environmental media, estimating bioaccumulation from the environmental media, or estimating uptake from ingestion of food. A discussion of study design considerations and methods for each of these three routes is presented. Because ecological risk assessment can cover a diverse set of receptors, a list of published methods that may be useful is provided rather than the actual protocols.

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ACRONYMS AND ABBREVIATIONS

ABS	absorption fraction
ASTM	American Society for Testing and Materials
ATSDR	Agency for Toxic Substances and Disease Registry
AVS	acid volatile sulfides
BAF	bioaccumulation factor
BW	body weight
CEC	cation exchange capacity
CEN	Comite European de Normalization
CFR	Code of Federal Regulations
CNO	Office of the Chief of Naval Operations
CSF	cancer slope factor
DoD	Department of Defense
Eh	redox potential
EPA	(United States) Environmental Protection Agency
EPC	exposure point concentration
GLP	Good Laboratory Practices
HCl	hydrochloric acid
Hg^{0}	mercury, elemental form
Hg^{+1}	mercury, mercurous form
Hg^{+2}	mercury, mercuric form
ICP-AES	inductively coupled plasma atomic emission spectroscopy
ISO	International Stadardization Organization
NOAEL	no observed adverse effect level
RAF	relative absorption factor
RfD	reference dose
RPM	Remedial Project Manager
SBRC	Solubility/Bioavailability Research Consortium
SEM	simultaneously extracted metals
SOP	standard operating procedure
TOC	total organic carbon
TRV	toxicity reference value
U.S. EPA	United States Environmental Protection Agency
UV-VIS	ultraviolet-visible spectrophotometry

1.0 INTRODUCTION

The *Guide for Incorporating Bioavailability Adjustments into Human Health and Ecological Risk Assessments at U.S. Department of Defense (DoD) Facilities, Parts 1 and 2,* has been developed as a resource on bioavailability studies for use by DoD Remedial Project Managers (RPMs) and others involved in remediating DoD sites and designing studies to support remediation. The guide brings together the most current information on bioavailability of metals, and synthesizes this information into a practical handbook that explains concepts and identifies types of data that need to be collected to assess bioavailability and incorporate it into risk assessment. Although the guide focuses on bioavailability of metals, many of the basic principles described herein also can be applied to assessing bioavailability of organic compounds.

Part 1: Overview of Metals Bioavailability, contained in the previous volume, provides a definition of bioavailability and discusses where bioavailability fits in the risk assessment process for both human health and ecological receptors. The *Overview* provides general information on the types of situations where it may be beneficial to perform additional studies to assess bioavailability and outlines key steps in determining when it is feasible to conduct a bioavailability study for a particular site. In addition, a brief summary of chemical-specific bioavailability information is presented for those metals that are most often found as contaminants at DoD sites (i.e., arsenic, cadmium, chromium, lead, mercury, and nickel for both terrestrial and aquatic settings; and copper, tin and zinc for aquatic settings only).

Part 2: Technical Background Document for Assessing Metals Bioavailability, contained in this volume, is designed to provide more in-depth technical information for professionals involved in designing and performing bioavailability studies. The *Technical Background Document* includes both general study design considerations applicable to bioavailability studies for all metals as well as considerations specific to a particular metal. Section 1.1 reviews the definitions that were presented in more detail in the *Overview*.

Sections 2.0 and 3.0 address issues for bioavailability studies conducted in support of human health risk assessments. Section 2.0 provides general study design information including a discussion of soil collection and characterization necessary to support bioavailability studies, and an overview of *in vitro* (i.e., laboratory benchtop) and *in vivo* (i.e., whole animal) methods for assessing bioavailability. Following the general study design information, Section 3.0 presents a discussion of metal-specific considerations that must be addressed in designing both *in vitro* and *in vivo* bioavailability studies for individual metals. Metals addressed in this section include arsenic, cadmium, chromium, lead, mercury, and nickel.

The standard operating procedures (SOPs) for soil speciation and for *in vitro* tests discussed in Sections 2.0 and 3.0 are provided in the appendices to this document. Also, for each of the six metals, a suggested template protocol for an *in vivo* bioavailability study is provided. The template protocols are provided as a starting point for designing the *in vivo* bioavailability study and include information (such as the recommended animal model, numbers of animals, and dosing methods) that is most often appropriate for a particular metal. A study director then can adjust the protocol to address any site-specific conditions.

Section 4.0 provides information on bioavailability studies for ecological receptors. The bioavailability of metals to ecological receptors can be assessed by evaluating direct exposure to the available fraction of the metals in the environmental media, estimating bioaccumulation from the environmental media, or estimating uptake from ingestion of food. A discussion of study design considerations and methods for each of these three evaluations is presented. Because ecological risk assessments can address a diverse

set of receptors, a list of published methods that are readily available and that potentially may be useful is provided.

1.1 Definitions and Concepts

Bioavailability is the extent to which a substance can be absorbed by a living organism and can cause an adverse physiological or toxicological response. For environmental risk assessments involving soil and sediments, this definition implicitly includes the extent to which a substance can desorb, dissolve, or otherwise dissociate from the environmental medium in which it occurs to become available for absorption. For incorporation into a risk assessment, bioavailability must be quantified much like any other parameter in a risk calculation. Thus, it is also useful to define bioavailability in the context of how it is measured.

For human health risk assessment, absolute bioavailability and relative bioavailability are two important and separate measures. ***Absolute bioavailability*** is the fraction or percentage of a compound which is ingested, inhaled, or applied on the skin surface that is actually absorbed and reaches the systemic circulation (Hrudey *et al.*, 1996). Absolute bioavailability can be defined as the ratio of an absorbed dose to an administered dose:

$$\text{Absolute Bioavailability} = \frac{\text{absorbed dose}}{\text{administered dose}} \times 100 \qquad (1\text{-}1)$$

For studies of absolute bioavailability, the absorbed dose often is determined by measuring the concentration of the compound in blood over time or by measuring the mass of the compound in such excreta as urine, feces, or exhaled air. Internal (i.e., absorbed) doses are useful for characterizing risk if toxicity factors describing the dose-response relationship (i.e., reference dose [RfD], or cancer slope factor [CSF]) are based on an absorbed dose. However, because toxicity parameters generally are based on an administered dose rather than an absorbed dose, it is usually not necessary to determine the absolute bioavailability of a contaminant for use in human health risk assessments.

Relative bioavailability is a measure of the extent of absorption among two or more forms of the same chemical (e.g., lead carbonate vs. lead acetate), different vehicles (e.g., food, soil, and/or water), or different doses. Relative bioavailability is important for environmental studies because matrix effects can substantially decrease the bioavailability of a soil- or sediment-bound metal compared to the form of the metal and dosing medium used in the critical toxicity study. In the context of environmental risk assessment, relative bioavailability is the ratio of the absorbed fraction from the exposure medium in the risk assessment (e.g., soil) to the absorbed fraction from the dosing medium used in the critical toxicity study:

$$\text{Relative Bioavailability} = \frac{\text{absorbed fraction from soil}}{\text{absorbed fraction from dosing medium used in toxicity study}} \times 100 \qquad (1\text{-}2)$$

Relative bioavailability expressed in this manner has been termed the relative absorption fraction (RAF). Incorporation of relative bioavailability (i.e., the RAF) into an exposure assessment results in an improved estimate of the external (i.e., administered) dose. ***Bioaccessibility*** is a term used to describe the fractional dissolution of a metal from soil in an *in vitro* study. Measures of bioaccessibility are used tgo estimate relative bioavailability. When characterizing risk, it is appropriate to combine the adjusted external dose with toxicity parameters based on an administered dose in order to achieve a more representative estimate of risk. The following sections of this document will focus on the methods used in measuring relative bioavailability of contaminants in soils.

2.0 GENERAL STUDY DESIGN CONSIDERATIONS FOR ASSESSING BIOAVAILABILITY

Section 2.0 provides information on the general aspects of study design that should be considered when a bioavailability study is being planned for use in human health risk assessments. The section first discusses general soil collection and characterization issues to consider when obtaining soils for evaluation of mineralogy (i.e., speciation) or metals bioavailability. Next, this section includes an overview of considerations for designing *in vitro* and *in vivo* bioavailability studies.

2.1 Soil Collection and Characterization

Soil collection and characterization for evaluation of mineralogy (i.e., speciation) or metals bioavailability should be designed based on the expected nature of exposures to the soil. In a residential setting humans will generally have contact primarily with surface soils. Specific activities such as gardening or putting in fences may lead to exposure to deeper soils as well. In general, surficial (0-2 in. or 0-2 cm) soils should be collected to represent the material to which most human exposure is anticipated to occur. In contrast, studies for ecological risk assessment should generally use soils from the 0-6 inch horizon. Samples should be representative of the different soil or waste material types believed to be present at the site. For mineralogical and *in vitro* studies, 5 to 10 soil samples (either grab or composite) are likely adequate for characterization of mineralogy and bioaccessibility in a given exposure area. However, for *in vivo* studies, evaluation of one or two soil samples is more realistic due to the greater cost of testing and analysis. If a site is large and heterogeneous, it may be desirable to conduct an *in vivo* study using a few soil samples from the areas where exposure is most likely, and couple those with additional *in vitro* studies of other areas.

Soil samples should be disaggregated (i.e., soil clods should be gently broken up; samples should never be crushed) in the laboratory, and oven dried at ≤45°C (temperatures higher than this may cause changes to soil structure and organic material). Soils should initially be sieved to the <2-mm size fraction generally accepted as "soil", and a portion retained for testing for the soil parameters described below, so that the characterization data are comparable to literature values. For studies to support human health risk assessment, the remainder of the sample then should be sieved to <250-µm (60 mesh). The <250-µm size fraction is used for the bioavailability studies because this size fraction is the upper limit on particle sizes that are likely to adhere to hands and may be ingested during hand-to-mouth activity (Duggan and Inskip, 1985). Also, this size fraction has become the industry standard for conducting *in vivo* studies of relative arsenic and lead bioavailability from soil (Casteel *et al.*, 1997a and 1997b; Freeman *et al.*, 1992, 1993, 1994, 1995, and 1996; Maddaloni *et al.*, 1998; Schoof *et al.*, 1995; Weis *et al.*, 1994).

Soils used in bioavailability studies should be characterized for a consistent set of soil parameters, to aid in future data interpretation. These parameters should be measured on the <2-mm soil fraction, and include the following: pH, total organic carbon (TOC), cation exchange capacity (CEC), particle size (sand, silt, clay), and moisture content. In addition to analysis for the metals of concern, soil characterization should include analysis for elements that are particularly important in soil alteration reactions. At a minimum, this should include analysis for iron, manganese, calcium, and phosphorous concentrations (<2-mm soil fraction). Given that the forms of metals in soil (i.e., their speciation) influence the extent to which they will be bioavailable, speciation can provide valuable supporting information to help explain the results of the bioavailability studies. However, a speciation study is required only when it is necessary to distinguish the form of the metal present in order to calculate risk and cleanup goals correctly, as discussed below (i.e., for mercury and chromium). Note that it has proven quite difficult to develop defensible bioavailability estimates solely from speciation data because of the

complexity of metal speciation in soils and the difficulty in fully evaluating this parameter. One exception to this is the case of simple systems that contain only one or two different mineral forms of the metal (this is often the case with mercury); because of this, *in vitro* and *in vivo* methods are the primary methods for quantifying bioavailability.

Arsenic

Trivalent (III) and pentavalent (V) inorganic arsenic compounds predominate in soils, occurring as discrete mineral phases of widely varying solubility and as ionic forms that may be sorbed to soil constituents. However, as discussed in Part 1 of this Bioavailability Guide, all inorganic arsenic compounds induce toxic effects by the same mechanism regardless of their valence state. Therefore, all forms of arsenic may be considered together when assessing bioavailability, and speciation studies aimed at identifying specific forms of arsenic present at a site are not a critical requirement for a bioavailability study. However, if speciation data are desired, a generalized Microprobe SOP is presented in Appendix A that can be used to evaluate forms of arsenic in soil.

Chromium

Chromium occurs in soil in the trivalent (III) and hexavalent (VI) oxidation states. Speciation is required in order to determine if chromium is present in the trivalent or hexavalent form. This is necessary data to support any risk assessment because Cr(III) and Cr(VI) have different reference doses.

Speciation is also useful for determining if a bioavailability study has merit. As pointed out in Section 3.3.2, default risk-based cleanup levels based on ingestion of Cr(III)-containing soils are typically quite high (e.g., 120,000 mg/kg in residential soil), so it is unlikely that any remedial actions would be driven by this exposure pathway. Therefore, when Cr(III) is the only form of this element present at a site, an oral bioavailability study generally will not be useful unless levels far exceed the default risk-based value. Default cleanup levels for ingestion of Cr(VI) are much lower (e.g., 390 mg/kg for residential soil); therefore, a bioavailability study generally will be useful when Cr(VI) is present at a site.

EPA Method SW-846 3060A is useful for quantifying hexavalent chromium in soil samples. This method uses a hot alkaline extraction to solubilize Cr(VI), in conjunction with such methods as EPA SW-846 7196 (ion chromotography by UV-VIS spectrophotometry) to quantify the Cr(VI) in the extract. Trivalent chromium can be determined by analyzing for total chromium, using common analytical methods such as EPA SW-846 6010 (ICP-AES), and subtracting the concentration of hexavalent chromium. In addition, the generalized Microprobe SOP presented in Appendix A can be used to evaluate forms of chromium in soil.

Mercury

Mercury usually is present in soils as inorganic mercury, either as elemental mercury (Hg^0), or as one of two nonelemental ionic forms: mercurous (Hg^{+1}) or mercuric (Hg^{+2}). A speciation study will be needed to determine the form of mercury present at a site prior to conducting any bioavailability studies. Speciation is necessary because elemental mercury has different toxic endpoints from the other inorganic compounds of mercury. Organic mercury compounds usually are not present in significant quantities in soil in the absence of a specific manufacturing process that generated such compounds, and are not considered further in this document. When evaluating sediments, of course, methylmercury must be considered.

Recently, sequential extraction procedures have been developed to quantitatively evaluate forms of mercury in soil. Sequential extraction methods are advantageous because they are relatively easy to perform compared to other highly specialized analytical techniques. Appendix B presents one such sequential extraction procedure that has been used to evaluate mercury at several sites and that appears to provide highly reliable results. The method is useful for distinguishing elemental mercury from various other inorganic forms (i.e., mercuric sulfide, carbonates, hydroxides, oxides, and chlorides) as well as

quantifying the amount of organic mercury in the soil. This procedure is recommended prior to designing and conducting *in vitro* or *in vivo* bioavailability studies for mercury. In addition, the generalized Microprobe SOP presented in Appendix A can be used to evaluate nonelemental inorganic forms of mercury in soil.

Lead

Inorganic lead occurs in numerous mineral forms that vary widely in solubility; however, all of the inorganic forms that occur in soil have the same toxic endpoint. Therefore, speciation studies are not needed to distinguish the specific forms of lead present in soil at a site, and all forms may be considered together when assessing bioavailability. However, if speciation data is desired, the generalized Microprobe SOP presented in Appendix A can be used to evaluate forms of lead in soil.

Cadmium

Cadmium occurs in soil in discrete mineral phases that range in solubility from sparingly soluble (e.g., sulfides) to highly soluble (e.g., carbonates) and in ionic forms sorbed to soil constituents. However, all inorganic forms of cadmium found in soils induce chronic toxic effects after ingestion by the same mechanism. Consequently, speciation studies are not needed to distinguish the specific cadmium compounds present at a site, and all forms may be considered together when assessing bioavailability. However, if speciation data is desired, the generalized Microprobe SOP presented in Appendix A can be used to evaluate forms of cadmium in soil.

Nickel

Nickel occurs in soil sorbed to soil constituents and as discrete mineral phases that range in solubility from poorly soluble (e.g., sulfides and sulfates) to moderately soluble (e.g., carbonates). However, the nature of the oral toxicity of nickel does not vary among the different forms expected to be present in soil. Therefore, speciation studies are not needed to distinguish the specific nickel compounds present at a site, and all forms of the metal may be considered together when assessing bioavailability. However, if speciation data is desired, the generalized Microprobe SOP presented in Appendix A can be used to evaluate forms of nickel in soil.

2.2 Development and Application of *In vitro* Methods for Assessing Oral Bioavailability From Soil

Simple extraction tests have been used for a number of years to measure the degree of metals dissolution in a simulated gastrointestinal-tract environment as a means of predicting the relative bioavailability of metals ingested in soil (Ruby *et al.*, 1993, 1996, and 1999). ***Bioaccessibility*** is a term used to describe the fractional dissolution of a metal from soil in an *in vitro* study. Measures of bioaccessibility are used to estimate relative bioavailability. SOPs for specific extraction methods are provided in Appendices C and D. The *in vitro* method for lead (stomach phase extraction, see Appendix C) also is recommended for evaluation of arsenic, cadmium, and nickel bioavailability from soil. The *in vitro* method for stomach and small-intestine extraction (see Appendix D) is recommended for assessment of chromium and mercury. The *in vitro* extraction test presented in Appendix D, which involves sequential simulated stomach and small intestinal phases, is based on the method of Ruby *et al.* (1996), but incorporates the test cell and mixing method developed by Dr. John Drexler (University of Colorado at Boulder).

The predecessor of these systems was developed originally to assess the bioavailability of iron from food, for studies of nutrition (Miller *et al.*, 1981; Miller and Schricker, 1982). In these systems, various metal salts or soils containing metals are incubated in a low-pH solution for a period intended to mimic residence time in the stomach. The pH is then increased to near neutral, and incubation continues for a period intended to mimic residence time in the small intestine. Enzymes and organic acids are added to simulate gastric and small-intestinal fluids. The fraction of lead, arsenic, or other metals that dissolve

during the stomach and small-intestinal incubations represents the fraction that is bioaccessible (i.e., is soluble and available for absorption). For example, the European Standard for Safety of Toys (CEN, 1994) provides for an extraction test to evaluate the bioaccessibility of eight metals (including arsenic and lead) from children's toys. The European method involves extraction of the particular metal (toy material reduced to <500 µm in size, at a liquid-to-solid ratio of 50:1) in pH 1.5 (HCl) fluid at 37±2°C for two hours. This method has been in used since 1994 by the 18 member countries of the Comite European de Normalization (CEN) to regulate the safety of toys.

Variation in the bioaccessibility of arsenic, chromium, nickel, cadmium, and lead, as a function of liquid to solid ratio, was evaluated by Hamel *et al.* (1998). These authors determined that bioaccessibility in synthetic gastric juice was affected only slightly by changes in the liquid to solid ratios in the range of 100:1 to 5,000:1 (mL/g). Ruby *et al.* (1996) demonstrated that, for a set of seven soils that had been evaluated for relative lead bioavailability in a weanling rat model, the stomach phase of the *in vitro* test at a pH value of either 1.3 or 2.5 correlated with relative bioavailability estimates from the *in vivo* model (r^2 = 0.93 at both pH values, $p < 0.01$). More recently, a revised version of the extraction test (different test cell and stirring method) developed in the laboratory of Dr. John Drexler (University of Colorado at Boulder) has indicated that data from the stomach phase of the test correlates well with *in vivo* data for samples used in a series of young swine studies conducted by United States Environmental Protection Agency (U.S. EPA) Region VIII and the University of Missouri (r^2 = 0.85, n = 15; Medlin, 1997). These results indicate that the extent of lead dissolution in the acidic stomach environment of the extraction test is predictive of relative lead bioavailability in two animal models (weanling rats and young swine).

The Solubility/Bioavailability Research Consortium (SBRC), a collaborative group of regulators, academics, and industry members, has developed a streamlined extraction test for estimating relative lead bioavailability: one-hour extraction (mixing by end-over-end rotation at 37°C) of 1 g of soil (<250-µm size fraction) in 100 mL of buffered (HCl and 0.4M glycine) pH 1.5 solution (Ruby *et al.*, 1999). Preliminary results for this test appear to correlate well with relative lead bioavailability values determined from the U.S. EPA Region VIII swine studies. A formal validation of this extraction test in three independent laboratories has been conducted, and data will be available for release in the near future.

For arsenic, the correlation between *in vitro* and *in vivo* estimates of relative arsenic bioavailability is less clear, primarily because the *in vivo* database for arsenic is less comprehensive and reliable than that for lead. Preliminary comparisons between the SBRC extraction text and relative arsenic bioavailability results from the U.S. EPA Region VIII swine studies have been inconclusive due to a lack of sufficient data. However, recent research in the laboratory of Dr. Nick Basta (Oklahoma State University) indicates that results from both stomach-phase (pH 1.8, 60 min. in a stirred beaker at 37 °C) and small-intestinal-phase (pH 5.5, bile acids, pancreatic enzymes, 60 min. in a stirred beaker at 37°C) extractions correlated equally well with relative bioavailability estimates from the U.S. EPA Region VIII young swine model for 13 mining-related samples (r^2 = 0.69 and 0.67, respectively, $p < 0.01$; Rodriguez *et al.*, 1999). As with lead, these data suggest that the extent of arsenic dissolution during an acidic gastric-like extraction is predictive of relative bioavailability estimates in the young swine model.

2.3 Development and Application of *In vivo* Methods for Assessing Oral Bioavailability From Soil

An overview of the kinds of approaches or methods that may be used to assess the oral bioavailability of chemicals in soil was provided in Part 1 of this Bioavailability Guide. These methods include:

- Estimates based on comparison of the area under the curve of blood concentrations over time for different dosage forms or routes
- Determination of the fraction of the administered dose that is excreted in urine
- Comparison of tissue concentrations for different dosage forms or routes
- Estimates of absorption based on subtraction from the administered dose of the unabsorbed fraction excreted in feces.

A determination of the most appropriate approach to use for a specific metal should begin with a review of what is known about how completely the most soluble forms of the metal are absorbed, with identification of the primary routes of excretion, and with identification of any tissues that the metal might accumulate in. For example, soluble forms of arsenic are almost completely absorbed (> 80 percent), and most of the absorbed arsenic is excreted in the urine (ATSDR, 2000a). In contrast, only a small fraction of an oral dose of soluble forms of cadmium is absorbed, and the absorbed cadmium is accumulated in liver and kidney. Thus for these two metals, different *in vivo* methods or protocols are needed to measure bioavailability.

Once the general approach or method for assessing bioavailability has been identified, a detailed study design needs to be developed, and documented in a study protocol. The protocol should include all of the study elements specified in the Good Laboratory Practice (GLP) Standards (40 CFR 792). Some critical study design elements include:

- Animal model, including species, age, and sex, and number of animals per group
- Diet and feeding frequency
- Animal husbandry and quarantine
- Test substance specifications, including source of soil and soil characteristics, such as desired metal concentration range, particle size (<250 μm has frequently been used for oral studies), and control substance specifications
- Dosing regimen (e.g., single vs. repeated doses, or dosing by gavage vs. by mixing with feed)
- Dose levels for test and control substances
- Target tissues and sample collection time points and procedures
- Analytical methods and detection limits
- Statistical methods of data analysis
- Quality assurance procedures.

It is important to share this study protocol with all interested stakeholders prior to initiating the study in order to ensure that there is general agreement regarding study design. If the proposed study design is a new approach, it is advisable to conduct an initial "pilot" study with a small number of animals and dose levels to test the approach and ensure that analytical methods are sufficiently sensitive.

Rats are frequently used for bioavailability studies, and may be most appropriate when the toxicity value for a metal is based on studies conducted in rats, as is the case for chromium, inorganic mercury compounds, and nickel. However, it should be noted that the goal of these studies is to assess potential differences in bioavailability of different forms of metals in humans, especially in children. Although no animal model is identical to humans, and although there are substantial differences in gastrointestinal

physiology and anatomy between rats and humans, rats may still give an accurate estimate of the relative bioavailability of metals in soil vs. soluble metal forms. Animals with gastrointestinal physiologies and anatomies more similar to humans, such as monkeys and swine, have also been used successfully in bioavailability studies. A swine model developed by U.S. EPA Region VIII has been used in studies of lead and arsenic bioavailability (Casteel *et al.*, 1996, 1997a, and 1997b). Monkeys and dogs also have been used to study arsenic (Freeman *et al.*, 1995; Gröen *et al.*, 1994). The use of dogs should be carefully considered due to their high fasting pH, which will affect results for forms of metals that dissolve more readily in acid environments. It is generally advisable to avoid the use of ruminants, and if animals that exhibit coprophagy are used (e.g., rats or rabbits), metabolism cages may be needed to reduce the extent of this behavior.

In vivo studies also may be used to generate estimates of relative bioavailability for ecological risk assessments in cases where literature-based toxicity values are applied. In such cases, the selection of an animal model will be driven by similarities to the ecological receptors of concern. For ecological assessment of terrestrial receptors, ruminants and avian species may frequently be of concern. Very little is known about the relative bioavailability of metals in soils in these species.

Further specification of the exact animal model to be used should be based on consideration of other metal-specific characteristics, such as variations in absorption with age or gender. For example, this is a particularly important consideration for lead, with lead absorption being much higher in sucklings than in older animals.

Most of the rat bioavailability studies of metals in soil conducted to date have been dietary feeding studies. It is currently recommended that the soil be administered to rats in gelatin capsules if soil volumes are sufficiently small. Capsules allow for a much more precise administration of the desired dose. If a dietary feeding study is conducted, care must be exercised to verify the homogeneity of the soil-feed mixture. The animals must be housed individually and food consumption estimates must be made daily, with the quantity of any spillage estimated.

The diet to be fed to the animals should be specified because in many cases a special diet will be needed. Many metals, including chromium and lead, bind to phytates and other fibers that are high in commercial laboratory chow. For rodent studies, a purified diet such as AIN-93G, with documented concentrations of metals should be used. For rodent feeding studies the presence of soil in the diet will affect palatability, so no more than 5 percent soil should be mixed with the rat chow. Another consideration is the need to include a period of fasting prior to dosing the animals. Chromium, lead, and nickel are absorbed more completely after a fast, so the soil dose should be administered after a fast if an estimate of maximum absorption is desired. Drinking water also should be tested for metals concentrations prior to beginning a study.

Dose levels that are feasible will be determined by concentrations of the test metal in soil. Unless the metal concentrations are very high in the soil, the highest dose may be limited by the amount of soil that the animal can tolerate. It also is advisable to try to test soils with metal concentrations in a range where remediation decisions could be affected by the study outcome. For example, soils with very high metal concentrations may be remediated regardless of the outcome of a bioavailability study. Conversely, there is no point in testing soils with metal concentrations below risk-based screening levels that do not trigger any requirements for remediation. The lowest dose also should be several times (e.g., 5 times) the background dose the animals receive in their diet and drinking water. These constraints may lead to dose levels that yield very low metal concentrations in the target tissues (i.e., blood and solid tissues) and excreta (i.e., urine and feces). These low concentrations may make it necessary to use the most sensitive analytical techniques available.

The selection of specific samples to be collected and the timing of collection should be based on a review of the pharmacokinetic behavior of each metal. For example, urinary arsenic excretion might be monitored throughout the study period, whereas liver or kidney samples might be collected at the end of a study of cadmium absorption. This issue is addressed in greater detail for each of six metals in Section 3.0.

3.0 BIOAVAILABILITY OF METALS IN SOILS IN HUMAN HEALTH RISK ASSESSMENT: STUDY DESIGN CONSIDERATIONS AND TEST PROTOCOLS

The following section discusses factors that should be considered when designing a study to assess the bioavailability of a particular metal. These considerations have been developed based on previous experience gained in conducting bioavailability studies and knowledge of the behavior of the specific metals in the environment. Information is provided on both *in vitro* and *in vivo* test methods. In addition, recommendations are made for various study design parameters such as animal model, dosing regimen, and target tissues for sampling, among others. The individual metals addressed are arsenic, cadmium, chromium, lead, mercury, and nickel.

In addition to the discussion and recommendations provided in this section, SOPs for the *in vitro* studies and suggested protocols for *in vivo* studies are provided in the appendices at the end of this document. For the *in vivo* studies, template protocols are provided for each metal. The purpose of the template protocols is to provide a starting point for those involved in designing site-specific bioavailability studies, not to specify a required protocol that must be followed. For each study, the protocol will need to be reviewed and tailored to address the specific conditions at a particular site.

3.1 Arsenic

3.1.1 Arsenic *In Vitro* Methods

There are currently several *in vitro* methods that are used routinely to determine arsenic bioaccessibility, (defined in Part 1) each of which has advantages and limitations. The two most frequently used methods are the SBRC extraction test (developed for lead), and the Rodriguez *et al.* (1999) extraction test, which are both discussed in Section 2.2. Validation of these methods is incomplete due to the lack of sufficient *in vivo* data. Studies currently are being performed to develop an adequate *in vivo* data set for validation of the *in vitro* test for arsenic.

Despite the uncertainties associated with the arsenic *in vivo* data collected in swine, the SBRC extraction test has been demonstrated to be highly reproducible in several different laboratories. An SOP for this method is provided in Appendix C. The Rodriguez *et al.* (1999) extraction test has the advantage that a validation against the young swine model has been published in the peer-reviewed literature. Since the correlation between results from this test and the *in vivo* data were best for the stomach phase extraction, only the stomach phase of the test should be used for establishing arsenic bioaccessibility. In addition, the swine feed used in the Rodriguez *et al.* method should not be added to the *in vitro* test, because it does not appear to increase the predictive ability of the test but does add considerable complexity.

3.1.2 Arsenic *In Vivo* Methods

Most arsenic in soils is present as inorganic compounds that all have the same chronic toxicity endpoints in humans, regardless of valence state. Therefore, one set of toxicity values applies to all inorganic arsenic compounds typically present in soils. The U.S. EPA and Agency for Toxic Substances and Disease Registry (ATSDR) oral toxicity values for inorganic arsenic are based on studies of human populations exposed to dissolved arsenic naturally present in drinking water. The critical effects for the CSF (skin cancer) and RfD (skin lesions) are due to the effects of absorbed arsenic.

Absorption, Distribution, Excretion

After ingestion, water-soluble forms of inorganic arsenic are almost completely absorbed from the gastrointestinal tract of humans and many laboratory animals. Estimates for humans, mice, dogs, and monkeys indicate greater than 80 percent oral absorption of soluble forms of arsenic. Several species (e.g., rabbits, hamsters) may have lower absorption of soluble arsenic via the oral route. Also, many laboratory animal studies have demonstrated that ingestion of less soluble forms of arsenic, such as forms that may exist in soil, leads to reduced absorption. In those studies, soil arsenic was typically one-half to one-tenth as bioavailable as soluble forms of arsenic.

After oral absorption, arsenic appears to be distributed to most tissues of the body with little tendency to accumulate preferentially in any internal organ (ATSDR, 2000a). Most absorbed arsenic is rapidly cleared from blood and excreted in urine. Studies in cynomolgus monkeys indicate that approximately 70 percent of gavaged doses of soluble arsenic were excreted in urine, most within the first 24 hours (Freeman *et al.*, 1995). Urinary arsenic excretion was virtually complete within 72 hours. Only a small amount of absorbed arsenic was excreted in feces.

The data indicate that the distribution and excretion of arsenic in cynomolgus monkeys and dogs is similar to that in humans (e.g., Charbonneau *et al.*, 1979; ATSDR, 2000a). However, arsenic may behave differently in several other species, which should be considered before they are selected as models of arsenic bioavailability in humans. In the rat, a large amount of absorbed arsenic is bound to the red blood cells, so very little reaches other tissues. Consequently, rats are not good models of arsenic disposition in humans (ATSDR, 2000a).

Design of Previous In vivo Studies

Various animal models have been used in the past to assess the bioavailability of soil arsenic. These include New Zealand White rabbits, cynomolgus monkeys, dogs, and swine. In one of the first studies of the relative bioavailability of arsenic in weathered soil, New Zealand White rabbits were used to study the oral absorption of arsenic in a soil sample from Anaconda, MT (Freeman *et al.*, 1993). The rabbits were given a single oral capsule containing arsenic in soil, as well as receiving soluble sodium arsenate by gavage and by intravenous injection. Based on the results of this study, the relative bioavailability of smelter-site soil arsenic was estimated to be 47 percent when compared to the soluble arsenate compound.

Relative arsenic bioavailability from a composite residential soil sample from the Anaconda Smelter site was also determined in a study of monkeys. Three female cynomolgus monkeys were used in a random cross-over design in which each animal received each treatment in random order with a suitable washout period between doses. Treatments included a single oral dose of soil (0.62 mg As/kg BW), house dust (0.26 mg As/kg BW), and soluble sodium arsenate by gavage or intravenous injection (0.62 mg As/kg BW). Based on urinary arsenic data, the relative bioavailability of arsenic in soil was 20 percent compared to the soluble arsenic compound. The relative bioavailability estimate for arsenic in house dust was 28 percent. Serial blood samples also were collected during the study and used to estimate bioavailability. These data resulted in estimates for both soil and house dust of 10-12 percent relative arsenic bioavailability.

Arsenic bioavailability from soil has been evaluated in female beagles (Gröen *et al.*, 1994). Six beagles were used in a two-way crossover design, in which each dog received, in random order, arsenic as an intravenous solution or as an oral dose of arsenic-containing soil. Urinary arsenic data indicated that about 8 percent of the soil arsenic dose was absorbed. No dose group for ingestion of soluble arsenic was included in the study. Relative bioavailability of soil arsenic compared to ingested soluble arsenic is estimated to be 12 percent, assuming the absorption of ingested soluble arsenic is about 70 percent in beagles.

The bioavailability of soil arsenic has also been evaluated in a weanling swine assay that was initially designed to estimate lead bioavailability (Casteel *et al.*, 1997a). Groups of five swine were orally dosed twice daily with varying concentrations of arsenic in soil or slag for 15 days. Urinary arsenic data for the 14 substrates evaluated indicate that relative arsenic uptake in these studies varied from near 0 to 50 percent and depended on the form of arsenic present in the sample (Casteel *et al.*, 1997a). Initially, the data indicated low overall recovery of arsenic in urine, feces, and tissues. However, the low recovery was determined to be due to an analytical error and reanalyses are expected to support the utility of this model.

Study Design Recommendations

Approach: Because of the relatively rapid uptake and excretion of arsenic compounds, bioavailability may be estimated using a one-time oral dosing regimen. Using this approach, relative arsenic bioavailability has been successfully estimated from blood or urine data.

Animal model: Because the monkey is a nonhuman primate, closely related to man both physiologically and anatomically, this species is favored for bioavailability studies. Juvenile swine also may be an appropriate animal for these studies. The use of rats as test animals should be avoided, as they are known to have different distribution patterns from humans for arsenic. Similarly, although rabbits may provide useful data, they are less favorable for bioavailability studies because of the occurrence of coprophagy.

Dosing regimen and dose levels: A one-time dosing regimen should provide data to successfully estimate relative arsenic bioavailability. After site soils are characterized for physical parameters and arsenic speciation (if desired), and sieved to <250-μm particle size, the soil can be administered in gelatin capsules. Delivery of several capsules may be necessary to obtain the target dose.

The risk-based screening levels for arsenic are less than 0.5 μg/g for residential soil and less than 4 μg/g for industrial soil, which are lower than expected background values for much of the United States (range of 0.1-97 μg/g [Shacklette and Boerngen, 1984]). In general, oral bioavailability study test soils should be in the range of 200-2,000 μg As/g soil. Assuming delivery of 1.5 g soil/kg BW for each animal, this value would correspond with arsenic doses of 0.3 to 3.0 mg As/kg BW. The lower value is above the lowest dose used in the Freeman *et al.* (1995) monkey study (for house dust, estimated 28 percent relative bioavailability) and therefore should provide data useful for estimation of bioavailability.

Target tissues and sample collection: Arsenic should be measured in urine and feces or in blood. Although blood and urine collection are sufficient for estimation of relative bioavailability, the feces data are useful for calculation of mass balance and for characterization (if desired) of absolute bioavailability. In the latter case, the fecal elimination data from animals dosed intravenously allows for correction for the fraction of absorbed arsenic that is excreted via bile. Animals should be housed in individual metabolism cages, to allow for the separate collection of urine and feces. To adequately quantitate arsenic excretion, cage rinses should be conducted during the study. It should be noted that it is not necessary to sacrifice the animals after collection of these samples, and that the animals may be reused after a washout period. This consideration may be important in the use of nonhuman primates.

Based on interpretation of the previous *in vivo* studies, in particular Freeman *et al.* (1995), the following sampling specifics are proposed. Samples of whole blood, urine, cage rinse, and feces should be collected prior to dosing, and for a period of 48 hours after administration. Samples collected 48 hours post-administration provide little additional data. Excreta samples can be pooled into 24-hour intervals.

After oral dosing, suggested blood sampling times are predose; 15, 30, 45, 60, and 90 minutes; and 2, 4, 6, 8, 12, 16, 24, and 48 hours. This schedule is based on the Freeman *et al.* (1995) monkey data that showed a triphasic concentration time curve with a much faster absorption than distribution or elimination

phases. If monkeys are dosed intravenously, proposed blood sampling times are predose; 2, 5, 10, 15, 30, and 60 minutes; 2, 4, 8, 12, 16, 24, and 48 hours.

Feeding and diet: Animals must be quarantined prior to dosing. This quarantine allows for a period of washout and for the collection of samples to correct for background levels of arsenic. Pre-study arsenic levels are assessed from a minimum of three blood samples collected on separate days.

During quarantine, monkeys may be fed Primate® chow or equivalent (which is provided ad libitum), except when fasted prior to dosing. Animals should be fasted for approximately 16 hours prior to dosing. They may be given free access to food approximately four hours after dosing. Food and water should be characterized for concentrations of arsenic.

Controls and reference standards: The reference standards include animals gavaged with soluble arsenic, typically sodium arsenate heptahydrate ($Na_2HAsO_4 \cdot 7H_2O$). If it is desired to evaluate absolute arsenic bioavailability, then animals intravenously dosed with soluble arsenic also may be included. Each animal serves as its own negative control, in that background exposures to arsenic are assessed prior to dosing.

Template protocol: A template study protocol for assessing the oral bioavailability of arsenic in cynomolgus monkeys using a one-time dosing regimen administered in capsules is provided in Appendix E. In addition, a template protocol for assessing arsenic and lead bioavailability in young swine is provided in Appendix F. The protocol given in Appendix F includes assessment of both lead and arsenic in the same study but it can be modified to assess only arsenic, as appropriate to the site.

3.2 Cadmium

3.2.1 Cadmium *In Vitro* Methods

Only one *in vitro* study of cadmium bioaccessibility from soil has been conducted for which companion *in vivo* data on the same soil are available. This study was conducted on residential soils collected in the vicinity of the National Zinc Smelter in Bartlesville, OK. *In vitro* testing, using the procedure presented in Appendix C (stomach phase only at a pH value of 1.3), on a composite soil sample indicated a bioaccessibility of 70 percent. A companion *in vivo* study was conducted in young rats that were given either soil containing cadmium (174 mg/kg cadmium) or cadmium chloride mixed in the purified diet. A relative bioavailability estimate of 33 percent was obtained based on liver and kidney tissue concentrations in animals receiving soil relative to soluble cadmium (Schoof and Freeman, 1995). Based on this comparison, it appears that *in vitro* results may overpredict *in vivo* measures of relative cadmium bioavailability.

Given that cadmium behaves similarly to lead under environmental conditions, the SBRC *in vitro* test (see Appendix C), which was developed specifically for lead, should be used for determining cadmium bioaccessibility. Keep in mind that results from this *in vitro* test may overpredict cadmium bioavailability determined using *in vivo* methods, and that only a very limited *in vivo* evaluation of soil cadmium bioavailability has been performed.

3.2.2 Cadmium *In Vivo* Methods

All inorganic cadmium forms commonly present in soils induce toxicity by the same mechanism, so these forms may be considered together when assessing bioavailability. The oral toxicity reference values for cadmium are based on a number of chronic studies in humans. A toxicokinetic model was used to estimate the no observed adverse effect level (NOAEL) from cumulative exposures.

Traditionally, the U.S. EPA has differentiated between exposures to cadmium in food (less available) and water (more available), and provided individual toxicity and risk-based numbers for each of these forms of exposure. Recently, the U.S. EPA has argued that there is no basis for differentiating between these exposures (U.S. EPA, 1999). Nonetheless, cadmium in soil (and in food) may have bioavailability that is reduced relative to cadmium in water.

Absorption, Distribution, Excretion

The oral absorption of soluble cadmium in humans and several laboratory animals is generally reported to be very low (1-8 percent) (Friberg *et al.*, 1985; U.S. EPA, 1999). However, most estimates are based on fecal excretion data and are only approximations because there is evidence of both biliary excretion and the trapping of cadmium in the intestinal wall (similar to mercury). It has been suggested that what appeared to be a slightly smaller absorption in laboratory animals than in humans is more related to differences in diet than to differences in physiology (U.S. EPA, 1999). Cadmium absorption is increased by low intakes of iron and calcium, and high levels of zinc may affect cadmium absorption, distribution, or elimination. As with several other metals, younger animals may have greater absorption of cadmium than older animals (Hrudey *et al.*, 1996).

Absorbed cadmium is widely distributed in the body, but the majority is located in liver and kidney tissue. The distribution pattern in both animals and humans is similar and appears to be unrelated to the route of exposure, but may vary depending on the duration of exposure. Absorbed cadmium is excreted very slowly from the body, with urinary and fecal excretion being approximately equal (Kjellstrom and Nordberg, 1985). Body half-lives for cadmium have been estimated to vary from several months to several years for mice, rats, rabbits, and monkeys (ATSDR, 1999a).

Design of Previous In vivo Studies

Several oral *in vivo* studies of cadmium in soil are reported in the literature, two which assess the bioavailability of soluble cadmium added to soil mixtures and one which evaluates the absorption of cadmium from residential soil samples collected near a historic zinc smelter. All three of these studies use rats as their test animal.

Griffin *et al.* (1990) administered gavage doses of radiolabeled soluble cadmium chloride to rats, including two samples where soluble cadmium had been absorbed onto soil (either clay loam or sandy loam). Relative bioavailability was estimated from the radioactivity in serial blood samples collected over a 48-hour period. A reduction in relative bioavailability was noted with the clay loam, with more modest reductions (not statistically significant) with the sandy loam. However, this method of sample preparation is not likely to yield results indicative of cadmium in environmental samples (see Section 3.2.1).

Schoof and Freeman (1995) evaluated the relative bioavailability of cadmium in a composite soil sample from a residential area near a former zinc smelter site, using a dosed-feed approach (Schoof and Freeman 1995; PTI, 1994). Approximately four-week-old weanling Sprague-Dawley rats were fed diets containing either soil cadmium (four dose levels; 0.06–0.98 mg Cd/kg BW) or soluble cadmium chloride (four dose levels; approximately 0.03–0.54 mg Cd/kg BW) for a period of 30 days. At the end of the dosing period, blood, liver, and kidney were analyzed for tissue concentrations of cadmium. Based on a comparison of liver and kidney data, cadmium in soil was estimated to be 33 percent bioavailable relative to soluble cadmium.

Schilderman *et al.* (1997) presents the results of a bioavailability study on an artificial soil that had been spiked with cadmium chloride and mixed on a mechanical rotator for a two-week period (final concentration of 4,400 mg/kg). This soil was administered with 5 percent gum acacia to 8-week-old male Lewis rats in a single gavage dose (0.15 mg Cd/rat, equivalent to 0.75 mg Cd/kg BW assuming 0.2 kg

BW). A relative bioavailability of 43 percent was calculated for the two-week-aged cadmium in soil relative to cadmium in saline based on the area under the curve of blood concentrations versus time. The majority of cadmium was cleared from blood within six days. In addition, cadmium concentrations in the liver and kidneys of the soil cadmium-treated rats were significantly lower than in those of the saline cadmium dosed group, at six days posttreatment. This suggests that for cadmium exposures approximating 0.75 mg/kg BW, cadmium bioavailability can be estimated from blood, liver, and kidney tissue data collected within six days of a single oral administration.

Study Design Recommendations

Approach: Although cadmium has a relatively long half-life in the body, the Schilderman *et al.* (1997) study demonstrated that bioavailability can be successfully estimated from rat tissue data after a one-time exposure if there is a sufficient concentration of cadmium in the soil. If lower soil concentrations are to be tested, then it may be more appropriate to use a subchronic dosed-feed approach.

Animal model: The rat has been successfully utilized in studies of the relative bioavailability of cadmium in soil; it is recommended as a relatively inexpensive, easy to use surrogate for evaluations of human exposures to cadmium. Young animals should be used to maximize the uptake of the metal. It is only necessary to use one sex of the animal.

Dosing regimen and dose levels: Risk-based screening concentrations for cadmium in residential soil generally vary from 37-78 μg/g, and up to 2,000 μg/g for industrial soil. The California-EPA modified residential soil risk-based screening value is 9 μg/g. These screening concentrations suggest that, in general, bioavailability studies will be conducted using soils with concentrations of cadmium ranging from 50 to 2,000 μg/g. However, for sites where lower risk-based values apply, such as sites in California, bioavailability studies may be conducted using soils with concentrations less than 50 μg/g.

Soils with concentrations above 200 μg Cd/g probably can be successfully assessed using a one-time dose regimen. This regimen assumes dosing a 200-g weanling rat with 0.25 grams of soil in a gelatin capsule, resulting in dosage of 0.25 mg Cd/kg BW; which likely will result in detectable tissue concentrations of cadmium. However, for soils with concentrations much lower than 200 μg/g, it is suggested that bioavailability be assessed using a subchronic feeding study where the test soil is mixed with the diet, similar to the study design presented in Schoof and Freeman (1995). For example, using soils containing 100 μg Cd/g, the Schoof and Freeman method would result in rats dosed with 0.50 mg Cd/kg BW per day for 30 days. This assumes that rats consume 20 g of feed per day, that there is 5 percent soil in the feed, and that rat body weight is 200 g. The 30-day feeding period would assure that concentrations of cadmium in animal tissues are above the analytical limit of detection.

Finally, there may be sites where it is important to assess the bioavailability of lead, as well as cadmium. It that case, the subchronic methodology may be more appropriate, so that the bioavailability of both metals can be assessed in a single animal study.

Target tissues and sample collection: Target tissues include blood, liver and kidney samples. If a subchronic dosed feed design is used, all tissues may be collected at study termination. If a one-time dosing regimen is used, the following sampling information should be considered. Due to the temporal nature of the blood sampling, sufficient number of animals must be used per time point to obtain enough blood without compromising homeostatic mechanisms or triggering hypovolemia. As a general rule, no more than 25 percent of an animal's blood volume should be drawn in a 24-hour period. After dosing, serial samples of whole blood should be collected at 0, 10, 20, 30, 60, 120, 240, and 480 minutes; at 24, 48, 72, 96, and 120 hours; and at study termination (approximately 144 hours). Kidney and liver tissues will be harvested and stored at the end of the study for further analysis, if necessary at a later time.

Feeding and diet: The animals should be fed a purified diet such as AIN-93G. This diet will be mixed with the test substrate if a subchronic feeding study design is utilized. If a one-time dosing regimen is used, the animals should have feed withheld for 16 hours prior to oral dosing. Two hours after dosing, the animals may be allowed free access to food. Because of the interactions of cadmium with other metals, each feed lot should be analyzed for calcium, magnesium, iron, zinc, and phosphorous, as well as cadmium.

Controls and reference standards: For a subchronic feeding study, reference standards include animals given rat chow mixed with soluble cadmium chloride, and negative controls would be used to assess background exposures in the diet and water. For a one-time dosing regimen, reference standards include animals dosed with cadmium chloride in an aqueous solution. The negative control groups should include rats gavaged with the aqueous carrier, again to assess background levels of cadmium in the water and diet.

Template protocol: A template study protocol for assessing oral bioavailability of cadmium in rats using a one-time dosing regimen administered in capsules is provided in Appendix G.

3.3 Chromium

3.3.1 Chromium *In Vitro* Methods

The oral absorption of chromium depends on its valence state (present either as hexavalent [Cr(VI)] or trivalent [Cr(III)] species), with Cr(VI) being more readily absorbed than Cr(III). However, this difference may be limited by the conversion of Cr(VI) to Cr(III) in the acid environment of the stomach. A number of studies indicate that ingested soluble Cr(VI) will be reduced in the acidic stomach fluid (Chute *et al.*, 1996; DeFlora *et al.*, 1987; Stollenwerk and Grove, 1985), but it is not clear if Cr(VI) in soil would be similarly reduced. No *in vitro* studies of chromium bioavailability from soil have been published. Given this situation, it is recommended that chromium bioaccessibility from soil be determined using the *in vitro* method provided in Appendix D (sequential stomach and small intestinal phase extraction), and that all of the extracts be analyzed for both hexavalent and trivalent chromium concentrations. Concentrations of hexavalent and trivalent chromium also should be evaluated in test soils, so that chromium redox reactions during the *in vitro* extraction can be evaluated.

3.3.2 Chromium *In Vivo* Methods

Oral RfDs exist for both hexavalent (Cr[VI]) and trivalent (Cr[III]) chromium. The oral RfD for hexavalent chromium applies to the soluble salts of Cr(VI) and is based on a toxicity study in rats given potassium chromate in drinking water. Most salts of Cr(III) have low water solubility. The oral RfD for trivalent chromium applies to these insoluble salts, and is based on administration of chromium (III) oxide in diet to rats. The RfD for the trivalent form is 500 times greater than that for the hexavalent form; this difference in toxicity has been suggested to be the result of differences in absorption among forms of chromium (U.S. EPA, 1998a and 1998b).

Absorption, Distribution, Excretion

As described above, the hexavalent form of chromium (Cr[VI]) is more readily absorbed than the trivalent form (Cr[III]). Nondietary trivalent chromium compounds only have very limited bioavailability (approximately 1 percent), while perhaps 10 percent of ingested hexavalent chromium is absorbed. As described above, the reduction of much ingested hexavalent chromium to the trivalent form in the stomach would limit the oral bioavailability of hexavalent chromium (O'Flaherty, 1996).

Both Cr(VI) and Cr(III) are better absorbed from the gastrointestinal tract in the fasted than in the fed state, and there is some evidence that absorption increases with dietary deficiency (O'Flaherty, 1996; Hrudey *et al.*, 1996). Chelating agents naturally present in food may affect chromium uptake; phytate has been shown to decrease absorption, whereas oxalate may increase it (ATSDR, 2000b). As with many metals, younger animals appear to absorb more ingested chromium than older animals (Hrudey *et al.*, 1996).

Once absorbed, trivalent chromium is cleared relatively rapidly from blood, but more slowly from the tissues. Chromium has been measured in blood, liver, kidney, spleen, lung, bone, testes, and muscles. There is evidence that the relative distribution between several of these organs (e.g., blood, liver, and kidney) may vary with the form of chromium and the type of exposure (e.g., oral vs. intravenous) (Witmer *et al.*, 1991).

Most absorbed chromium is excreted in urine (e.g., Hrudey *et al.*, 1996). Several authors report little (<5 percent) or no chromium excretion via bile or the gastrointestinal tract (e.g., Witmer *et al.*, 1991; Manzo *et al.*, 1983). Also, an assumption of no biliary or gastrointestinal excretion was the best fit for several sets of data to a physiologically based model of chromium kinetics in the rat (O'Flaherty, 1996). Contrary to this assumption, though, several authors report fecal excretion percentages in the range of 10-30 percent, for parenteral administration of chromium, which represents biliary excretion (e.g., Nieboer and Jusys, 1988; Sayato *et al.*, 1980). Several authors expressed the opinion that, in many cases, tissue and excreta data are contradictory and suspect, particularly from older studies (e.g., O'Flaherty, 1996; Hrudey *et al.*, 1996; Nieboer and Jusys, 1988).

Design of Previous In vivo Studies

Two oral *in vivo* studies using environmental soil chromium samples are reported in the literature, one performed in humans and one in laboratory animals. Both studies used soils containing chromite ore processing residues. In the human study, volunteers consumed a single daily bolus of a mixture of soil and chromite ore-processing residue for three consecutive days, with chromium excretion monitored in the urine (Gargas *et al.*, 1994). The soil contained 103 mg total Cr/kg soil (81 percent as Cr[III] and 9 percent as Cr[VI]), and was sieved to ≤500-µm particle size. No significant increases in urinary chromium were found when comparing the individual baseline values with the post-dose samples. Because no positive control (i.e., pure chromium compounds without soil) was included in the study, relative bioavailability cannot be estimated from this study. Although not a formal bioavailablity study, this study does provide evidence of very limited absorption of chromium from these samples.

Witmer *et al.* (1989 and 1991) performed several experiments in rats dosed with chromium-containing soil. Tissue distribution of chromium and excretion in urine and feces was compared after rats were gavaged with solutions of chromate salts, chromite ore-processing residues in soil (described as 30-35 percent hexavalent chromium), and an equimolar mixture of the soil chromium and a chromate salt. Gavage dosing regimens included: aqueous solutions and corn oil suspensions. Oral absorption of the chromium compounds was less than 2 percent as indicated by urinary excretion data in one case, and total chromium recovered from body organs in another case.

The authors reported greater uptake of the soil chromium than the calcium chromate based on greater urinary excretion (1.8 vs. <0.5 percent after 2 days) and tissue concentrations when gavaged in a corn oil medium. Conversely, when administered in an aqueous solution, the authors reported that tissue data generally indicated greater absorption of the sodium chromate than the soil chromium, calcium chromate, or soil and calcium chromate mixture (Witmer *et al.*, 1989). Corn oil is not an appropriate dosing vehicle for studies of metals in soil, so the studies using an aqueous solution are likely to be more representative of the absorption of chromium in soil relative to the chromate salts.

Study Design Recommendations

Approach: Designing a study of the relative bioavailability of chromium in soil is greatly complicated by the possible presence of both Cr(III) and Cr(VI). When both forms of chromium are present, as in the studies described above, careful thought must be given to identification of appropriate positive control test substances. A mixture of chromium oxide and potassium chromate in the same proportions as Cr(III) and Cr(VI) in the soil may be appropriate.

Another complication relates to the reduction of Cr(VI) to Cr(III) in the stomach. It has been estimated that 85 percent of ingested Cr(VI) is reduced to Cr(III) prior to absorption (O'Flaherty, 1996). Because animal data indicate that the distribution of Cr(VI) in the body differs from the distribution of Cr(III), it is inappropriate to use intravenously dosed Cr(VI) to estimate absolute bioavailability of orally administered Cr(VI). Consequently, studies of the bioavailability of chromium in soil should focus on directly measuring relative bioavailability. Until a reliable study design has been developed, any planned study of soil chromium bioavailability should begin with a pilot study with a small number of animals.

Animal model: Rats or swine are appropriate animal models to consider. Because the reduction of Cr(VI) to Cr(III) in the stomach is expected to be a controlling factor in the relative absorption of Cr(VI), it may be useful to monitor the valence state of chromium in the stomach of test animals.

Dosing regimen and dose levels: The soil dose should preferably be administered in gelatin capsules for one to two weeks. For a rat feeding study, a purified diet such as AIN-93G should be used, and the animals should be housed individually so that daily measurements of food consumption can be made. Food consumption data should be used to estimate the actual dose received by each animal. If a swine study is performed, soil and other test substances may be administered once or twice daily in a solid vehicle such as cookie dough.

Risk-based soil screening levels for ingested Cr(III) are generally so high that it is unlikely any remedial actions would be driven by this exposure pathway (e.g., 120,000 μg Cr(III)/g residential soil for U.S. EPA's soil screening levels). Consequently, bioavailability studies are not likely to be useful for soils containing only Cr(III). For Cr(VI), risk-based soil cleanup levels based on ingestion are much lower (e.g., 390 μg/g residential soil for U.S. EPA's soil screening levels), but risk-based cleanup levels based on inhalation of resuspended soil may be even lower. In general, oral bioavailability study test soils should be in the range of 200 to 1,000 μg Cr(VI)/g soil for residential soils, and in the range of 5,000 to 10,000 μg Cr(VI)/g soil for industrial soils. Risk-based screening levels in U.S. EPA Region IX, and in California, are even lower. Thus, in California it may be appropriate to conduct bioavailability studies using test soils with much lower Cr(VI) concentrations.

Target tissues and sample collection: Until a reliable study design is developed, it will be necessary to collect excreta (both urine and feces) and samples from a number of tissues. Metabolism cages should be used to collect urine separately from feces. Tissues collected should initially include liver, kidney, spleen, blood and bone. If a large animal such as swine is used, it may be helpful to collect serial blood samples during the study. Although it is critical to account for the forms of chromium present in soil, there is no need to differentiate between oxidation states while monitoring chromium in tissue or excreta for *in vivo* estimates of relative bioavailability. In fact, there is evidence that most excreted chromium is in a reduced form (De Flora and Wetterhahn, 1989).

Feeding and diet: Because it is known that dietary chelating agents (e.g., oxalate and phytate) can affect chromium uptake, only purified diets low in phytates and other chelating agents should be used (ATSDR, 2000b). Because chromium absorption is higher in fasted animals, it may be advisable to dose animals after an overnight fast.

Controls and reference standards: As described above, chromium oxide should be used as a reference standard for Cr(III) in soil, whereas potassium chromate should be used as a positive control for Cr(VI). A mixture of the two in the same proportions as Cr(III) and Cr(VI) in the soil may be used as a reference for soils containing a mixture of chromium valence states.

It is particularly important to include a negative control group in chromium studies to detect possible inadvertent sources of chromium (although for the pilot study, the test groups may serve as their own negative controls by taking a pretreatment blood sample). Chromium (like nickel) is present in stainless steel, and may be inadvertently introduced as a contaminant into tissue and excreta samples during *in vivo* studies (e.g., from scalpels, syringes, or cages). Because of the limited bioavailability of most forms of chromium, this possible source of contamination of samples is of concern and may compromise the results of an otherwise carefully designed study (Nieboer and Jusys, 1988). Therefore, the use of chromium-free materials is recommended for *in vivo* studies of relative chromium bioavailability.

Template protocol: As noted above, there is no established protocol for assessing the bioavailability of chromium in soil. Therefore, it is recommended that a pilot study be conducted first using a fairly small number of animals before a full-scale study is undertaken. A template study protocol for performing a pilot study of oral chromium bioavailability in rats is provided in Appendix H.

3.4 Lead

3.4.1 Lead *In Vitro* Methods

As described at the beginning of Section 3.0, *in vitro* methods for assessing lead bioavailability have been extensively developed, and validated by comparison to *in vivo* data. The SBRC *in vitro* extraction procedures (see Appendix C) were developed specifically for predicting the relative bioavailability of lead from soil and solid waste samples. To date, studies demonstrate that the SBRC extraction yields data that are equivalent to results from the young swine *in vivo* model (e.g., bioaccessibility data is equivalent to the bioavailability estimates) (Ruby, 2000). A comprehensive validation study has been conducted for this method (i.e., all lead substrates tested in the swine and rat *in vivo* lead models have been analyzed by the SBRC *in vitro* method in three independent laboratories), and a publication is in preparation.

3.4.2 Lead *In Vivo* Methods

Inorganic forms of lead in soil all have the same toxic endpoints, so they may be considered together when assessing bioavailability. The U.S. EPA has deemed it inappropriate to develop a RfD for inorganic lead compounds (U.S. EPA, 2002). In contrast to risk assessment techniques for most other chemicals, the toxic effects of lead are usually correlated with observed or predicted blood lead concentrations rather than with calculated intake levels or doses. Consequently, exposures to lead are typically assessed using models that incorporate specific assumptions for lead absorption from water, diet, and soil.

Absorption, Distribution, Excretion

The gastrointestinal absorption of lead varies with the age, diet, and nutritional status of the subject, as well as with the chemical species and the particle size of lead that is administered. Age is a well-established determinant of lead absorption; adults typically absorb 7-15 percent of lead ingested from dietary sources, and estimates of lead absorption from dietary sources in infants and children range from 40-53 percent (Ziegler *et al.*, 1978; Alexander *et al.*, 1973; U.S. EPA, 1990). Most absorbed lead partitions to bone, with lesser amounts present in blood and soft tissue (ATSDR, 1999b). Because lead is a bone-seeking element, complete excretion of absorbed lead requires an extended period of time. Therefore, oral absorption of lead has commonly been estimated by comparing the fraction of an orally

administered dose that is present in blood, bone, and soft tissues with the fraction of an intravenously administered dose that is present in these compartments.

Design of Previous In vivo Studies

The oral bioavailability of lead in soil has been more extensively studied than any other metal. Soil lead absorption has been studied in rats, swine, and humans.

Several studies of relative lead bioavailability from soil at mining sites have been conducted in a weanling rat model (Dieter *et al.*, 1993; Freeman *et al.*, 1992 and 1996; Schoof *et al.*, 1995). These studies involved dosing groups of five weanling rats for 30 to 45 days with varying concentrations of lead-bearing soil or lead acetate in the diet. At the end of the studies, lead concentrations were measured in blood and bone (femur), and various soft tissues (liver, kidney, and brain), depending on the study. Estimates of relative lead bioavailability developed from these studies in rats ranged from 0.087 to 0.41, depending on the origin of the various materials studied.

U.S. EPA Region VIII has developed an oral lead bioavailability assay in a weanling swine model and has used this model to evaluate relative lead bioavailability from hazardous waste sites across the country (e.g., Casteel *et al.*, 1997b). In the weanling swine model, groups of five swine were dosed with varying concentrations of lead in soil or lead acetate for 15 days. The swine were dosed twice daily in a temporal pattern, which is conservatively designed to mimic childhood lead exposure, with the first dose delivered after an overnight fast, and the second dose delivered in the afternoon after a four-hour fast. The swine were fed two hours after each dosing. Serial blood samples were collected during the study and analyzed for lead concentration. At the completion of the study, samples of blood, bone (femur), liver, and kidney were collected and analyzed for lead concentration. The resulting data were used to estimate relative lead bioavailability from the test substrates. Relative lead bioavailability estimates for 19 different substrates ranged from less than 0.01 to 0.90, based on measurement of lead in blood, bone, liver, and kidney (values are recommended point estimates based on a combination of these data, with blood data weighted most heavily).

Both the weanling rat and swine models described above were designed to evaluate oral lead absorption in an animal model that, to the extent possible, mimics children. However, at some sites (e.g., industrial sites), it is adult exposure that determines risk from lead in soil. To evaluate lead uptake in adults, Maddaloni *et al.* (1998) performed a study using stable lead-isotope dilution in blood following ingestion of soil from Bunker Hill, ID, to determine absolute lead bioavailability in adult human volunteers. Six adults were dosed with the soil (2,924 mg/kg lead, <250-µm fraction) in gelatin capsules (250 µg lead/70 kg BW), following an overnight fast. Serial blood samples were obtained at 14 time points through 30 hours and analyzed for total lead and ratios. Results indicated that, on average, 26.2 ± 8.1 percent of the administered dose was absorbed (Maddaloni *et al.*, 1998). In a follow-up study, six adult volunteers were dosed with Bunker Hill, ID soil following ingestion of a meal designed to simulate a standard breakfast. These results indicate that when the test subject has been fed, absolute lead bioavailability is reduced to approximately 2.5 ± 1.7 percent (Maddaloni *et al.*, 1998). These values can be compared to an assumption in U.S. EPA's adult lead model that 20 percent of soluble lead forms are absorbed from water and food, and that 12 percent is absorbed from soil. This study demonstrates the importance of the feeding regimen in the design of lead bioavailability studies.

Study Design Recommendations

Approach: A number of studies have demonstrated that the relative bioavailability of lead in soil can be successfully determined from tissue concentration data obtained during subchronic feeding studies in weanling rats or swine. The concentrations of lead in blood, bone, liver, and kidney from the soil-dosed animals are compared to those treated with soluble lead acetate.

Animal model: As described above, the two animal models used consistently in the study of lead bioavailability are the weanling rat and weanling swine. The weanling swine model presents many advantages. First, at this stage of development, the pig is similar in weight to children. Its omnivorous behavior is more like that of humans than that of rodents or lagomorphs. The pig also remains in its prepubertal state throughout the study period, which makes it a good surrogate for study of bioavailability in children. Finally, extensive blood samples can be drawn for pharmacokinetic modeling without the risk of anemia or exsanguination.

Arguments in favor of the weanling rat include the fact that lead uptake determinations can be made at a time of rapid growth and active bone formation. This time approximates the period in children in which they are most vulnerable to lead. Additionally, more toxicology laboratories are able to conduct rat studies. However, rat studies also present some disadvantages, primarily related to the low absolute bioavailability of lead in rats compared to humans. Evidence from published reports show that both these animal species have been used successfully for bioavailability studies when relative bioavailability estimates are used.

Dosing regimen and dose levels: The most commonly applied risk-based screening levels for lead in residential soil is 400 μg/g. As with several other metals, there is a lower value (130 μg/g) that may be applied to sites in California. The risk-based concentration of lead in soils that is acceptable for industrial sites is generally 750 μg/g. A wide range of concentrations of soil lead has been assessed in bioavailability assays, but tested substrates often range between 1,000 and 10,000 μg Pb/g soil. These soil lead concentrations are within the range that is appropriate for dosed-feed animal studies. Additionally, some chronic feeding studies have been performed using concentrations of soil lead less than 1,000 μg/g.

Previous studies in rats have been dietary feeding studies. As described above, it is recommended that soil be administered in gelatin capsules if the volume of soil is small enough. If a dietary study is conducted, the test soil is administered after mixing with a purified diet such as AIN-93G, which is provided ad libitum. The animals must be housed individually, so that daily measurements of food consumption may be performed. If swine are used as the test animals, then the soil and other test substances are administered twice daily, as described above in the subsection *Design of Previous In vivo Studies* for lead. It is important to characterize site soils for lead mineralogy because this is an important determinant of bioavailability.

Target tissues and sample collection: Biological samples necessary for determination of lead bioavailability include blood, bone, liver, and kidney. In swine, serial blood samples can be drawn easily during the course of the study, and the other tissues collected at study termination. This procedure has been successfully employed to estimate lead bioavailability in the U.S. EPA Region VIII swine studies, as discussed above.

In rats, blood samples (and other tissues) are often collected at the end of the study and used to evaluate lead bioavailability (e.g., Freeman *et al.*, 1992). Because steady-state is often not reached until after 4-5 half-lives [the half-life of lead in rats has been reported as 12 days (280 hrs) (Morgan *et al.*, 1977)], it is recommended that chronic feeding studies in rats be conducted for 48 days (12 days × 4). This 48-day period balances the need for exposure during a period of rapid growth, while providing sufficient time for accumulation of lead in blood and bone. In addition, because it is desired to estimate relative bioavailability using the most constant blood data, it is recommended that the blood be collected when lead concentrations are at their daily minimum. Therefore, at study termination, rats should be bled just prior to lights out, in order to sample prior to a feeding cycle (because rats are generally nocturnal, they feed at lights out).

Feeding and diet: Low dietary calcium increases lead absorption because calcium and lead are absorbed competitively in the gastrointestinal tract. Therefore, diets low in calcium and fiber should be used to maximize lead absorption. For example, a purified diet such as AIN-93G should be utilized for rats. A similarly formulated diet is available for swine. Samples of food and water should be analyzed (by the supplier or conductor of the study) for cadmium, lead, calcium, magnesium, iron, zinc, and phosphorous.

Controls and reference standards: Reference standards include animals dosed with soluble lead (lead (II) acetate trihydrate ($[CH_3CO_2]_2Pb{\cdot}3H_2O$)) added to their diet. A nontreated group will serve as a control for determining background lead levels. Animals should be housed in polycarbonate cages to reduce the risk of inadvertent exposures to lead.

Template protocol: A template study protocol for assessing oral bioavailability of lead in soil using rats administered soil in capsules is provided in Appendix I. In addition, a template protocol for lead bioavailability using young swine is provided in Appendix F. The protocol provided in Appendix F includes assessment of both arsenic and lead but can be modified to assess only lead, as appropriate to the site.

3.5 Mercury

3.5.1 Mercury *In Vitro* Methods

A review of *in vitro* studies that have been conducted on mercury in soil are provided in Schoof and Nielsen (1997) and in Davis *et al.* (1997). All of these studies involve extraction in an acidic stomach phase followed by a neutral small intestinal phase, and determination of the fraction of mercury liberated by the extraction fluids. The *in vitro* method presented in Appendix D, which follows this format, has been used to assess mercury bioaccessibility from soil at two sites, and the results were consistent with those that would have been expected based on the mercury speciation determined in soil at those two sites (unpublished data). Therefore, this method is recommended for evaluating mercury bioaccessibility.

3.5.2 Mercury *In Vivo* Methods

As discussed in Part 1 of the Bioavailability Guide, because of differences in pharmacokinetics and toxicity, elemental mecury and other inorganic mercury compounds (i.e., mercury in the Hg^{+1} [mercurous] or Hg^{+2} [mercuric] ionic state) of mercury must be addressed separately. Therefore, the dominant forms of mercury in soil should be determined prior to the design of an *in vivo* mercury bioavailability study. If elemental mercury predominates, then the primary concern is for inhalation exposures, as there is no oral RfD for elemental mercury because of its very limited oral absorption. If most soil mercury is present as a nonelemental inorganic form (Hg^{+1} or Hg^{+2}), then oral exposures may drive risk-based cleanups. Oral exposures to mercurous and mercuric compounds are typically evaluated using the RfD for mercuric chloride, a water-soluble mercury compound. This RfD is based on a study in which rats were dosed with mercuric chloride via gavage and subcutaneous injection.

Absorption, Distribution and Excretion

Based on studies in humans and in mice, soluble forms of inorganic mercury, such as mercuric chloride or mercuric nitrate, are 15 to 25 percent absorbed across the gastrointestinal tract (Rahola *et al.*, 1973; Nielsen and Anderson, 1990). Relatively insoluble mercury compounds, such as mercuric sulfide, appear to be absorbed to a much smaller extent. Several authors have interpreted animal data and calculated the oral absorption of mercuric sulfide to be 1-4 percent that of mercuric chloride (Schoof and Nielsen, 1997; Pastenbach *et al.*, 1997). There is evidence that mercurous compounds have more limited absorption than

the divalent forms of inorganic mercuric (ATSDR, 1999c), and that perhaps as little as 0.01-0.1 percent of elemental mercury is absorbed after ingestion (Goyer, 1996; ATSDR, 1999c).

The excretion of both elemental and inorganic mercury occurs primarily through urine and feces (via bile), whereas expiration from the lung may contribute to excretion for some exposures to elemental mercury (ATSDR, 1999c). Some of an ingested mercury dose forms insoluble deposits in epithelial cells lining the intestine and is slowly eliminated as intestinal epithelial cells are shed in feces. As a result, this mercury is not absorbed into the body. This delayed elimination effect may vary with different forms of mercury. For example, while less than 1 percent of a mercuric chloride dose remained in the intestine 96 hours after dosing, more than 11 percent of a mercuric sulfide dose was still in the intestine after that time period (Revis *et al.*, 1989 and 1990). These studies suggest that it took more than 10 days for complete clearance of unabsorbed mercuric sulfide from the intestine. If soil mercury behaves more like mercuric sulfide, intestinal retention would be an important factor to consider in the design of bioavailability studies.

Because elemental mercury is oxidized to the mercuric ion in the body, the distribution of the majority of absorbed elemental and inorganic mercury appears to be similar in the body (ATSDR, 1999c). After exposures to both elemental (via inhalation) and inorganic mercury, the highest concentrations of mercury are typically measured in kidney tissue, with smaller amounts in the spleen, liver, and brain (ATSDR, 1999c; Sin *et al.*, 1983; Yeoh *et al.*, 1989).

Design of Previous In vivo Studies

One animal study was identified in the literature that attempts to estimate the bioavailability of environmental soil mercury (Revis *et al.*, 1989 and 1990). The study has design limitations, including the lack of appropriate control groups and an insufficient time-scale for the duration of the study. The study duration is crucial, because the researchers were estimating soil mercury bioavailability from percent mercury recovered in feces, and some forms of mercury are cleared from the intestines more slowly than others.

A study evaluating relative absorption of mercuric chloride and mercuric sulfide may offer the best animal model for studies of mercury absorption from soil. Sin *et al.* (1983) compared mercury concentrations in kidney, spleen, and brain in groups of mice gavaged with the two mercury compounds for two weeks and 8 weeks. This study found that mercury accumulates in the greatest concentrations in kidney, even when it is not detectable in other tissues. These, and other data, suggest that kidney tissue is an appropriate measurement endpoint for the study of relative mercury bioavailability in laboratory animals (Schoof and Nielsen, 1997).

Study Design Recommendations

Approach: Based on the studies of Sin *et al.* (1983), the comparison of kidney tissue concentrations in rats after a two- to four-week exposure is likely to yield reliable estimates of soil mercury bioavailability relative to soluble mercury. Rat feeding studies of cadmium and lead in soil (Freeman *et al.*, 1992 and 1994; Schoof and Freeman, 1995) provide a model for similar studies with mercury. In these studies rats were fed diets mixed with soil and soluble salts of the metal, and tissue levels were then assessed, typically after an exposure period of 2 to 4 weeks.

Animal model: For mercury, rats are a likely choice of experimental animal because of their ease of use, cost, and because they are the animal used in the toxicity assessment for mercuric chloride.

Dosing regimen and dose levels: The animal studies performed using mercuric sulfide (Sin *et al.*, 1983) suggest that an exposure period of approximately 30 days should be sufficient to yield tissue concentration data high enough to reliably estimate relative mercury bioavailability. The highest dose

should be below the limits of toxicity for the animal, because mercury toxicity can affect both mercury absorption and excretion. In the soil bioavailability study discussed above, no overt signs of toxicity were observed in positive control mice administered up to 2,000 μg/g soluble mercuric chloride in soil (diet mixed with 5 percent soil) for periods of 6 months or more (Revis *et al.*, 1989). It is unlikely that it will be necessary to test soils with higher mercury concentrations than 2,000 μg/g. As described above, it is generally preferable to administer the soil to rats in capsules rather than mixed with feed to ensure the reliability of administering the planned doses.

Residential risk-based soil screening levels for inorganic mercury compounds are generally between 20 and 25 μg/g of soil (U.S. EPA, 1996, U.S. EPA, 2002b, U.S. EPA, 2002c). If a rat were to eat 20 g of chow per day containing 5 percent soil, it would ingest a 1-g dose of soil, then a dose equivalent to the risk-based screening level would be about 20 μg per rat, or 100 μg/kg BW for a 200-g rat. As stated earlier, it is not necessary to test any dose lower than this dose. At the high end of the range, risk-based mercury soil cleanup levels for industrial land are about 600 μg/g. If a soil sample to be tested were to contain as much as 2,000 μg/g of mercury, the mercury dose a rat would receive would be about 10 mg/kg BW. Thus, the ideal range of doses for a study of mercury in soil would be 0.1 to 10 mg/kg BW.

Target tissues and sample collection: It may be appropriate to collect only samples of kidneys for evaluation.

Feeding and diet: If a rat feeding study is performed, rat chow should be available ad libitum. A purified rat chow such as AIN-93G should be used. Food consumption will need to be measured daily for each animal (i.e., animals must be housed individually).

Controls and reference standards: The reference standard group should include animals dosed with mercuric chloride mixed with the rat chow. Negative control animals are important to provide a baseline to correct for background mercury exposures in food or drinking water.

Template protocol: A template study protocol for assessing oral bioavailability of mercury in soil is provided in Appendix J.

3.6 Nickel

3.6.1 Nickel *In Vitro* Methods

No *in vitro* studies for nickel bioavailability in soil have been reported in the peer-reviewed literature; however, OME (2002) includes a report of an *in vitro* study that included both stomach and intestinal phases. This study used weathered soil from a former nickel refinery site in which nickel was predominatntly in the form of nickel oxide. Relative bioavailability estimates for the ten samples tested ranged from 11-28 percent, with an average of 19 percent. Results from the stomach and intestinal phases were similar, consequently the single phase SBRC extraction test (see Appendix C) may be used for determining nickel bioaccessibility from soil or solid waste.

3.6.2 Nickel *In Vivo* Methods

The oral toxicity of nickel does not vary among the forms of nickel expected to be found in soils. The oral RfD for nickel is based on reduced body and organ weights, in rats administered nickel sulfate hexahydrate in the diet. That research was corroborated by a study of nickel chloride administered to rats in drinking water.

Absorption, Distribution, Excretion

In general, nickel is not well absorbed from the gastrointestinal tract of either animals or humans. Studies show that typical exposures result in less than 5 percent of soluble nickel salts being absorbed (e.g., Christensen and Lagesson, 1981; Ho and Furst, 1973; Griffin *et al.*, 1990). However, this value appears to increase when nickel is administered during a fast (Nielsen, *et al.* 1999, Sunderman *et al.*, 1989). In an *in vivo* study in rats, the gastrointestinal absorption of nickel correlated with the solubility of the nickel compound, with less than 1 percent of the least soluble forms (e.g., sulfides, oxides) being absorbed.

Absorbed nickel is excreted almost completely in the urine, with excretion in bile being minimal (Sunderman *et al.*, 1989; ATSDR, 1997). Rat data indicate that only 1-2 percent of absorbed nickel, administered intraperitoneally, was excreted in feces (Ho and Furst, 1973). In humans, the maximal elimination of nickel occurs in urine within the first 12 hours, and returns to near baseline within 72 hours after treatment (Christensen and Lagesson, 1981; Sunderman *et al.*, 1989). Rats completed their urinary excretion of absorbed nickel chloride within 48 hours, reaching a peak elimination in 4 hours or less (Ho and Furst, 1973). Similarly, other data from rats indicated that absorbed nickel in organ tissues was almost entirely eliminated within 72 hours postoral administration (Ishimatsu *et al.*, 1995).

Studies have variously utilized urine, blood, and body tissues to measure the uptake of nickel. In animals, nickel has been reported to be found primarily in kidneys after absorption; however, it is also measured in other organs and adipose tissue (ATSDR, 1997). Ishimatsu *et al.* (1995) determined the uptake of different nickel compounds in rats by assessing the sum of the amount of nickel in lungs, liver, kidneys, spleen, pancreas, heart, and brain, as well as in blood and urine. When examining the data for individual organs, the authors noted that the greatest amounts of nickel were measured in kidneys for most types of nickel tested, but in at least one experimental group (dosed with relatively insoluble green nickel oxide), more nickel was found in liver than in kidney. The authors concluded that the ratio of nickel in kidney, relative to other organs, varied by the solubility of the administered nickel compound (Ishimatsu *et al.*, 1995). Therefore, the measurement of individual organ tissue concentrations to assess nickel absorption, appears to be appropriate only if the form of nickel is known to be identical for all dose groups.

Although data are limited, it appears that both urine and blood samples provide data that is reflective of ingested soluble nickel (e.g., Griffin *et al.*, 1990; Christensen and Lagesson, 1981). However, because of the low absorption expected for nickel forms in soil, as well as limits on feasible dose levels, the limited volume of blood available for collection from small laboratory animals (e.g., rats) is not likely to yield an adequate sample to detect nickel in the blood. In the experiments of Ishimatsu *et al.* (1995), data for nickel in urine cumulatively collected over a 24-hour period correlated very well with absorption values calculated by summing the total amount measured in rat organs, blood, and urine after 24 hours. In contrast, the blood data presented in the article, apparently estimated from a one-time sample collected at 24 hours, do not appear to agree as well with the absorption values calculated from the sum of all tissue and urine data.

Design of Previous In vivo Studies

No studies were located in the literature of the relative bioavailability of nickel in environmental soil samples. Griffin *et al.* (1990) measured the oral bioavailability of a soluble form of nickel, radiolabeled nickel chloride, that was mixed with two kinds of soil and administered to rats by gavage, as an aqueous slurry. Bioavailability was evaluated by measuring nickel concentrations in serial blood samples. In this study, the aqueous nickel chloride soil slurries had reduced bioavailability relative to nickel chloride administered to the rats in water.

Study Design Recommendations

Approach: Because of the relatively rapid uptake and excretion of nickel compounds, a one-time dose regimen may be considered, with bioavailability estimated from urinary excretion data.

Animal model: Rats are a likely choice for experimental animal, because of cost, ease of use, and because the RfD for nickel is based on data from rats studies. Larger animals such as swine can be used, if it is desired to more closely mimic human gastrointestinal anatomy and physiology. There are no data to suggest that nickel absorption differs among animals. However, the use of dogs should be avoided if it is desired to extrapolate results to humans, because dogs lack a major nickel binding site on blood serum albumin that is found in humans (ATSDR, 1997).

Dosing regimen and dose levels: After site soils are characterized for physical parameters and mineralogy, and also are sieved to <250-µm particle size, the samples could be administered via gelatin capsules (preferred) or by gavage in an aqueous slurry. If swine are used, it may be possible to enclose the soil sample in a solid vehicle such as cookie dough.

Dose levels will be determined by concentrations of nickel in site soils, but should be several times (e.g., 5 times) above the background nickel concentration present in the diet and drinking water. Doses should be below levels that are toxic or affect elimination. There is a reported LD_{50} in rats for nickel sulfate of 39 mg nickel/kg body weight (Mastromatteo, 1986; as cited in ATSDR, 1997). Nonetheless, several authors report administering doses of soluble nickel up to 50 or 64 mg Ni/kg BW to rats that were apparently well tolerated (Ishimatsu *et al.*, 1995; Ho and Furst, 1973).

U.S. EPA's (1996) risk-based soil screening level for ingested nickel is 1,600 µg Ni/g residential soil. Risk-based screening levels for industrial soils range from 20,000 (11,000 for nickel subsulfide) to 41,000 µg/g in U.S. EPA Regions IX and III, respectively (U.S. EPA, 2002c, U.S. EPA 2002b). Consequently, oral bioavailability studies are not likely to be useful unless these soil concentrations are exceeded. Test soils for oral bioavailability studies should span a range of concentrations from the relevant risk-based screening level to 3 to 10 times greater than the screening level.

Target tissues and sample collection: Nickel absorption should be assessed by collecting continuous urine samples for a minimum of 24 hours, but preferably for 48 hours. Collection can be accomplished through the use of catheters or metabolic cages that collect urine and feces separately.

Feeding and diet: Prior to dosing, animals should be fasted overnight to minimize differential nickel absorption that could be caused by the presence of food. Fasting likely will increase nickel absorption, but the effect should be similar across all dose groups. Two hours after dosing, animals can be allowed free access to food.

Controls and reference standards: Reference standards should include animals dosed with one of the more soluble nickel salts, preferably nickel sulfate hexahydrate (the form of nickel used in the RfD toxicity study). The negative control group should include animals gavaged with the aqueous carrier, to assess background levels of nickel in the drinking water and in the diet. As was described for chromium, nickel is a component of stainless steel, and may be introduced into animals or tissue samples by stainless steel cages or instruments. Nickel-free materials should be considered where feasible.

Template protocol: A template study protocol for assessing oral bioavailability of nickel in soil is provided in Appendix K.

4.0 BIOAVAILABILITY OF METALS IN ECOLOGICAL RISK ASSESSMENT: STUDY DESIGN CONSIDERATIONS AND TEST PROTOCOLS

Ecological risk assessment can involve a wide range of receptors and a wide range of exposure pathways. Thus, determination of bioavailability in ecological risk assessment is less straightforward than in human health risk assessment. Plants and animals can take up bioavailable metals from soils, sediments, and water through contact with external surfaces, ingestion of contaminated soil, sediment or water, and by inhalation of vapor-phase metals or airborne particles (Brown and Neff, 1993). In addition, the manner in which a chemical is absorbed may vary for each identified receptor species. A fish, for example, can take up a metal directly from environmental media through its gills, its skin, or through incidental ingestion of sediment; however, it may also ingest and ultimately absorb contaminants through consumption of food (Campbell *et al.*, 1988). Conversely, a piscivorous bird's primary route of exposure would be the absorption of contaminants through the consumption of food (i.e., fish).

Due to the complexity of this issue, no single methodology exists for incorporating bioavailability into an ecological assessment. Rather, the appropriate means of evaluating the potential bioavailability of chemicals of concern must be determined on a site-by-site basis by considering the associated issues with respect to site-specific conditions. These conditions include the types of species being evaluated (e.g., aquatic vs. terrestrial, or primary producers vs. tertiary consumers), the types of exposure that primarily affect those organisms (e.g., direct contact with sediment or soil versus exposures through the food web), as well as the media being evaluated (i.e., soil, sediment or water). In general, bioavailability can be addressed using different levels of approaches:

- Evaluation of chemical and physical parameters of soil and sediment
- Measurement of the available fraction of metals present in the environmental media (i.e., sediment, soil)
- Site-specific studies of tissue concentrations or bioaccumulation directly from the environmental media
- Site-specific toxicity tests
- Estimating uptake from ingestion of food.

This section provides guidance on how to assess the conditions at a site to determine whether consideration of bioavailability will help to reduce the uncertainty. Recommendations are provided for each step of the ERA process regarding the types of data to collect and evaluate depending on site-specific factors, and possible bioassays are suggested for further evaluation. It is important to note that the bioassays listed are examples only; there may be other standard tests that would apply.

4.1 Evaluation of Chemical and Physical Properties

Metals present in sediments or soils can result in toxicity to organisms directly exposed to them. However, site-specific chemical and physical conditions greatly influence the form in which metals occur in the environment and thus the degree to which they are sorbed and ultimately "available" to ecological receptors. Metals that are soluble tend to be more bioavailable than metals that are insoluble. Metal cation species can preferentially bind to available anions (e.g., chlorides, sulfides, and hydroxides) and form soluble or insoluble salts. Metals also may bind to other particulate compounds (e.g., clay particules), thereby rendering them less available for uptake. Therefore, evaluating just the total metal

concentrations measured at the site does not accurately reflect the fraction biologically available to aquatic and terrestrial organisms.

Predictions about the potential bioavailability of a metal can be made by evaluating the form of metal present, as well as various chemical and physical conditions that affect the solubility and mobility of metals. A summary of key chemical and physical parameters is provided in Table 4-1. As described in Section 2.0, it is important that soil and sediment samples be representative of likely exposues for the receptor of interest. For most ecological receptors, soil from 0-6 inches deep, sieved to <2 mm will be appropriate.

4.2 Evaluating Direct Exposures to the Available Fraction of Metals Present in the Environmental Media (i.e., Sediment, Soil, or Water)

The available fraction of metals in soil or sediment can be assessed by a variety of active extraction techniques that have been developed to mimic conditions for specific receptors, e.g., plant roots or benthic macroinvertebrates (section 4.2.1). These techniques typically modify the solid phase being extracted. Generally, these methods do not yet have broad acceptance or application in risk assessment (NRC, 2002). Passive extracts and pore water analyses have also been developed, and have been widely and successfully used to measure the available fraction of metals in sediments (section 4.2.2). Both approaches are described below.

4.2.1 Sequential Extraction Techniques

Chemical analytical methods have been developed for metals to better estimate the fraction of the metal that is available for uptake by a receptor; however, such techniques do not yet have broad acceptance in the context of ecological risk assessment (NRC, 2002). Sequential extraction, or leaching, schemes have been used extensively to partially characterize the phase associations of metals in soils and sediments to identify the fraction or fractions of total metal that are or could become bioavailable (Tessier and Campbell, 1987; Campbell *et al.*, 1988). A few examples of extraction schemes developed for identifying the mobile, bioavailable fractions of total metals in soils and sediments are given schematically in Figures 4-1, 4-2, and 4-3.

Figure 4-1 presents an extraction scheme for soils (Wasay *et al.*, 1998). Typical extractants for dissolving each of four metals fractions are shown; many alternative extractants have been used for isolating each fraction. Most surface soils are oxidized and so do not contain geochemically significant concentrations of labile sulfides. Metal sulfides, if present, would appear in fraction 3. This scheme was intended to aid in identifying soil metal fractions that are bioavailable to plants (via root uptake) because most organically bound metal in soil is not considered bioavailable. Concentrations of exchangeable metal cations (fraction 1) and Fe/Mn oxide-bound metals (fraction 2) in soil generally are believed to provide the best correlation to bioaccumulation by rooted plants. However, Lebourg *et al.* (1996) did not find a good correlation between the metal uptake by radish plants and the easily exchangeable fractions of cadmium, copper, lead, and zinc (extracted with calcium chloride, sodium nitrate, or ammonium nitrate). The chosen extractants released little or none of the metals to soil water.

The second extraction scheme (Figure 4-2) was developed to characterize the distribution of metals in nearshore marine sediments (Rosental *et al.*, 1986). Fraction 1, extracted with hydroxylamine hydrochloride in acetic acid, contains the exchangeable, carbonate, and easily reducible metal fractions. The solid residue from the first extraction was digested with hydrogen peroxide in dilute nitric acid, followed by extraction with ammonium acetate in nitric acid. This fraction contains mainly metals

Table 4-1. Key Chemical and Physical Parameters Affecting the Bioavailability of Metals

Chemical/ Physical Parameter	Description	Example	Applicability
Metal speciation	Metals occur in the environment in a variety of forms. The specific form of a metal that is present can determine its mobility and solubility, ultimately affecting its bioavailability.	Trivalent chromium (i.e., chromic chromium) has a very low aqueous solubility and is practically non-toxic to aquatic species. In contrast, hexavalent chromium (i.e., chromate chromium) is much more soluble, and is associated with a higher potential for adverse effects.	Terrestrial and aquatic
Salinity/ conductivity	The salinity and conductivity of the aquatic system being evaluated can have a substantial impact on the form and behavior of metals present at the site.		Aquatic
Dissolved oxygen (DO)	The presence or absence of oxygen in an aquatic system influences the potential for oxidation and reduction and, therefore, the form of the metal present.	Chromium in oxidized sediments often is adsorbed primarily to amorphous iron oxide and organic/sulfide fractions of the sediment. Copper in anoxic sediments may undergo a variety of reactions with different inorganic and organic sulfur species to form a variety of soluble and insoluble complexes.	Aquatic
Redox potential (Eh)	The Eh affects the dissolution or precipation of various metals, providing another indication of the likely form in which the metal exists at the site as well as its potential solubility.	In reducing sediments, much of the zinc present is associated primarily with the organic/sulfide fraction and is therefore is not bioavailable.	Terrestrial and aquatic
pH	The pH of the system can affect the form of the metal present at the site in freshwater systems.	In freshwater systems, aluminum bioavailable at low pHs, but less so at high pH.	Terrestrial and aquatic
TOC/AVS	Metals can form complexes with organic material and with sulfides, thus rendering them unavailable for uptake by biological organisms. Measuring total organic carbon (TOC) and acid-volatile sulfides (AVS) thus provides an indication of the degree to which metals may be bioavailable.	In general, metals will be less bioavailable at higher concentrations of TOC and AVS.	Terrestrial (TOC) and aquatic (TOC and AVS)
Grain size and type	The amount of organic material present, and thus the bioavailability of metals, can vary depending on the grain size and type of soil/sediment. Parameters such as crystalline lattice structure, porosity and permeability, surface area, surface coatings/films, mineralogy, and chemical composition of the soil/sediment along with the form of the metal will render some metals more bioavailable than others.	In general, metals are more bioavailable in coarser soils and sediments (Breteler and Neff, 1983; Luoma, 1989). Fine soil/sediments have a much greater surface area which provides greater adsorption for organic material.	Terrestrial and aquatic

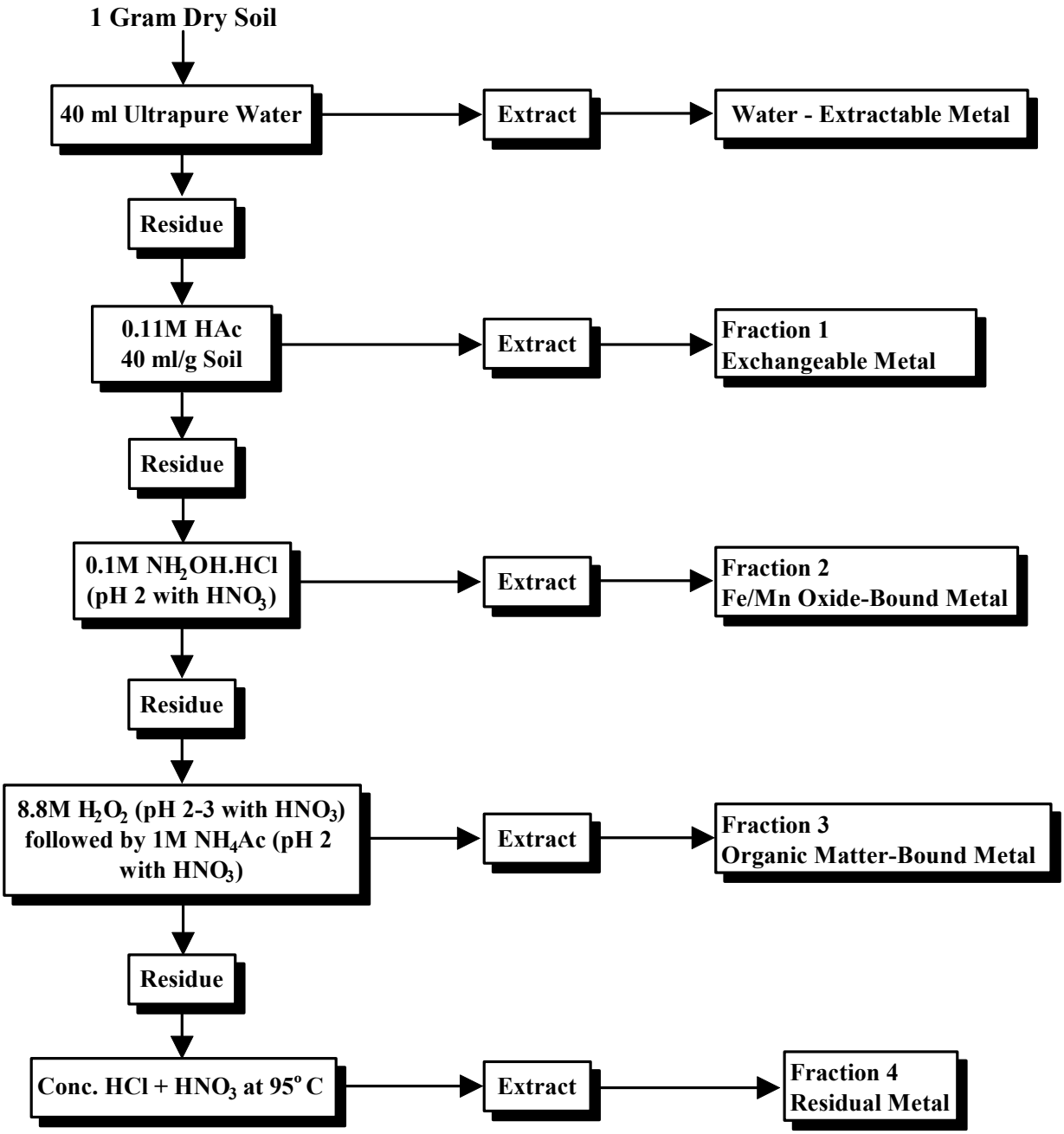

Figure 4-1. Extraction Scheme Used to Characterize the Distribution of Metals in Geochemical Fractions of Soil

(Reprinted from Wasay *et al.*, "Retention Form of Heavy Metals in Three Polluted Soils," Journal of Soil Contamination, 1998. Printed with permission from CRC Press, Boca Raton, FL.)

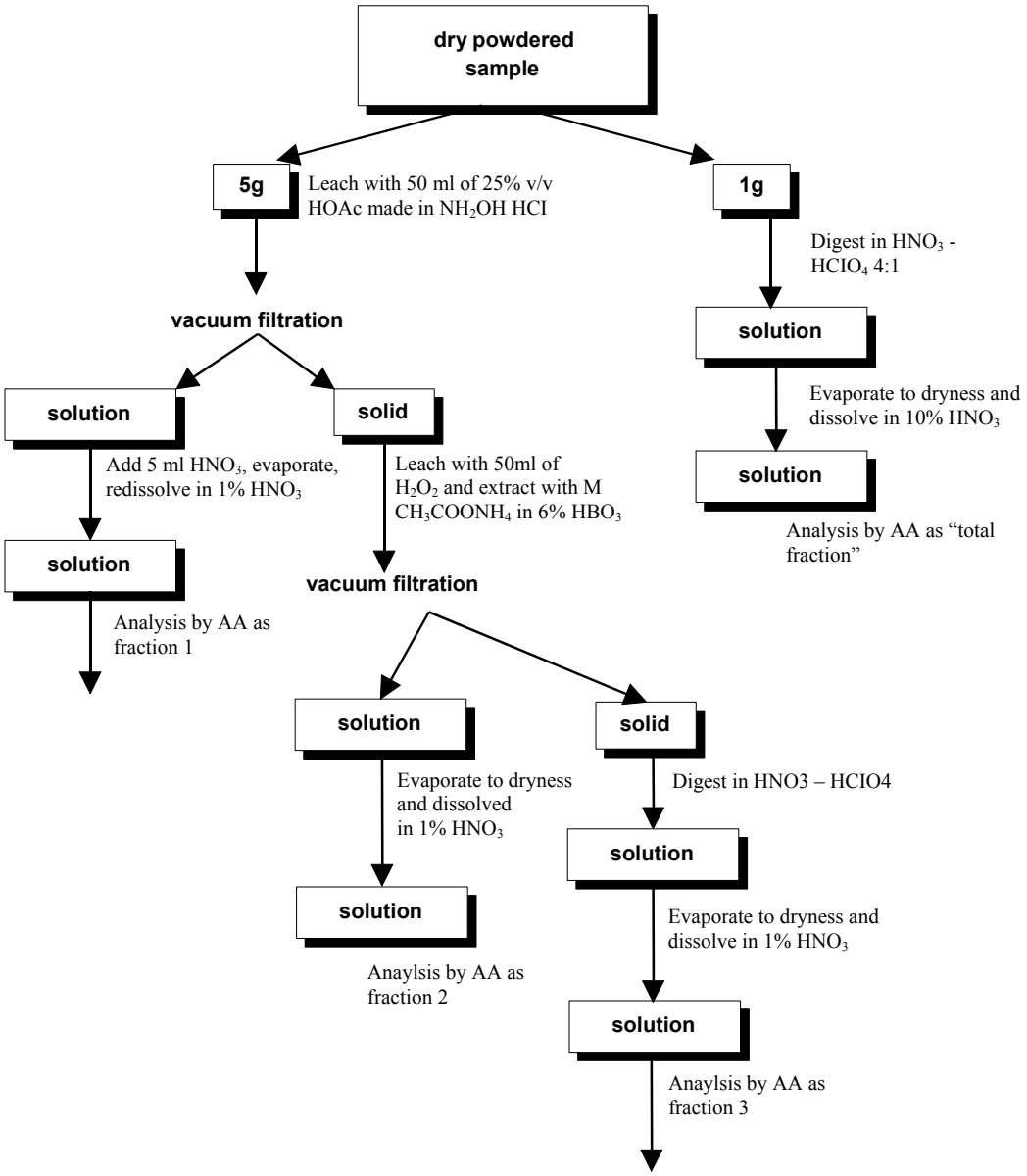

Figure 4-2. Extraction Scheme Used to Characterize the Distribution of Metals in Different Geochemical Fractions of Coastal Marine Sediments

(From R. Rosental, *et al.*, "Trace Metal Distribution in Different Chemical Fractions of Nearshore Marine Sediments," Estuar. Cstl. Shelf Sci., 1986. Printed with permission from Academic Press.)

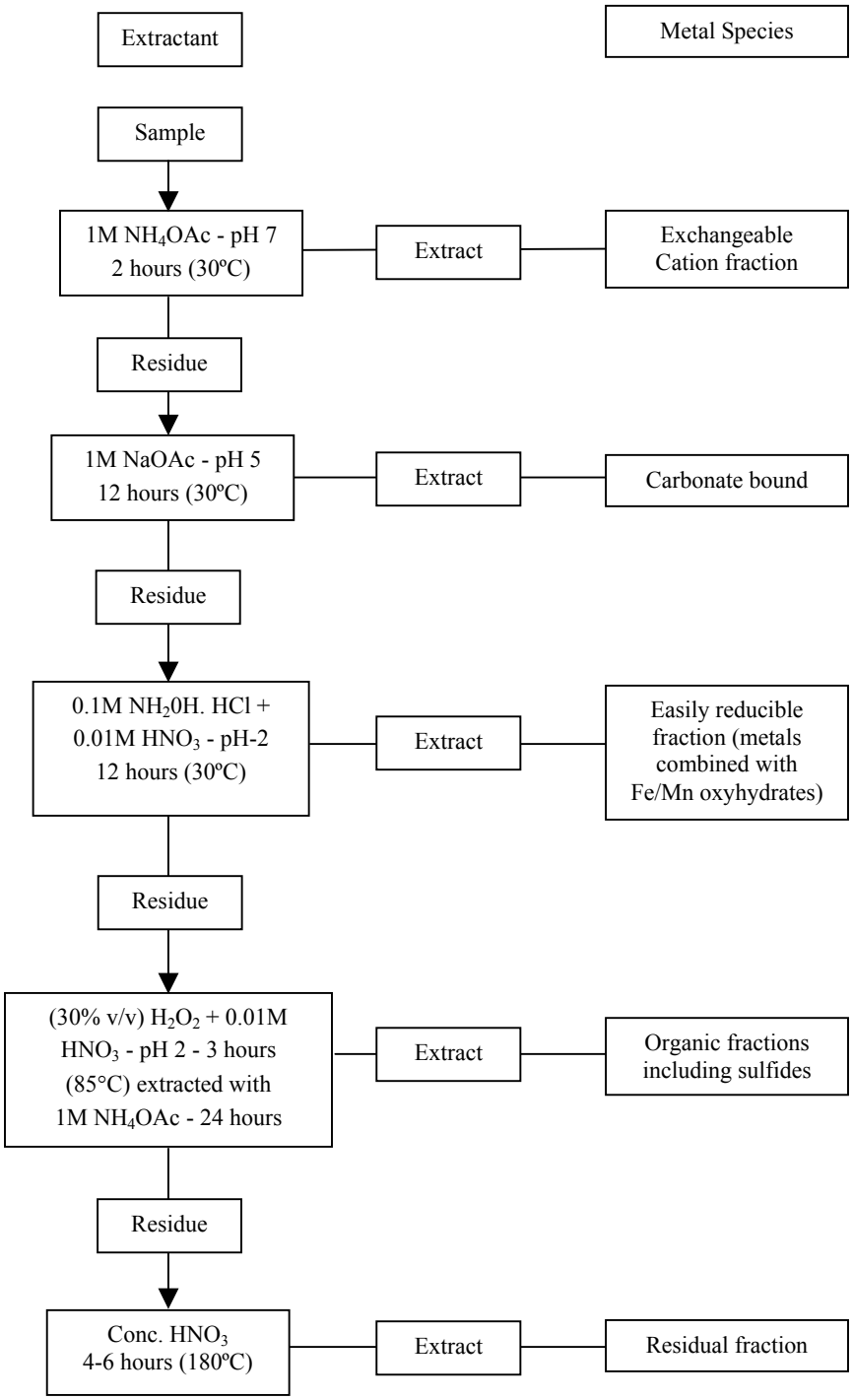

Figure 4-3. Typical Extraction Sequence for Estimating the Bioavailable Fraction of Metals in Estuarine Sediments

(Reprinted with permission from Environmental Toxicology and Chemistry, 1995. "Chemical partioning and bioavailability of lead and nickel in an estuarine sediment," Y. Babukutty and J. Chacko, 14:427-434. Copyright Society of Environmental Toxicology and Chemistry (SETAC), Pensacola, FL, 1995.)

associated with easily oxidized organic matter and labile sulfides. The solid residue from the second fraction was extracted with hot nitric and perchloric acid, yielding the residual fraction. A total metal fraction (fraction 3) was obtained by extracting the bulk sediment with the nitric-perchloric acid mixture. Copper, nickel, and zinc in fine sediments from False Bay, South Africa, are associated primarily with theorganic fraction (fraction 2). About 60 percent of the cadmium is found in the first fraction, associated primarily with reducible and carbonate phases of the sediments. About 80 percent of the chromium in the sediments is associated with the organic and residual fractions. About 45 percent of the lead is found in the residual fraction, with most of the remainder in fraction 1. These results give an indication of the complexity of metals distributions in soils and sediments.

The third extraction scheme (Figure 4-3) is a more typical sequence for estuarine sediments (Babukutty and Chacko, 1995). For instance, most of the lead (54 to 92 percent) and nickel (74 to 97 percent) in surficial sediments from the Cochin Estuary, India, is associated with the residual fraction (non-bioavailable). Most of the remainder of lead and nickel are associated with the organic/sulfide fraction. This distribution is typical for relatively uncontaminated fine-grained sediments (Loring, 1982). Bourgoin *et al.* (1991) used a similar extraction sequence to determine the bioavailable fractions of lead in marine sediments near a Canadian lead/zinc smelter. The best correlation to lead concentrations in mussels (*Mytilus edulis*) is the lead concentration in fraction 4 (the organic/sulfide fraction) normalized to the concentration of extractable sulfide in the fraction.

The extraction sequences roughly approximate the sequence of decreasing bioavailability of different bound forms of metals in soils and sediments. At least part of the metals in the first five fractions may be or become bioavailable under some natural conditions, including changes in soil/sediment pH and redox, and digestion in the digestive tracts of sediment-ingesting animals. The metals in the residual fraction are considered inert and nonbioavailable. Although no single extraction sequence can adequately describe the bioavailable fraction of metals in soils and sediments, dilute hydrochloric or nitric acid (1 to 3 N) is the most widely accepted extractant for estimating this fraction (Luoma and Bryan, 1982). The best correlations with the bioavailable fraction of metals in soils and sediments usually are for 1-N HCl-extractable metals (Luoma, 1989; U.S. EPA, 1991). This acid extractant tends to remove at least a portion of the metals from the first five fractions discussed above.

Use of the metal concentration derived from a 1-N HCl extraction technique analytical technique as the EPC can provide a more accurate estimate of the actual exposures to ecological receptors than the total metal concentrations.

4.2.2 Evaluation of Acid Volatile Sulfides

For sediments, the estimates of the bioavailable concentration can be further modified based on evaluation of acid volatile sulfides (AVS). In the presence of AVS in sediments, certain metals, including copper, cadmium, lead, nickel, zinc (Ankley, 1996; Ankley *et al.*, 1996) and possibly arsenic and mercury (Luoma, 1989; Allen *et al.*, 1993; Ankley *et al.*, 1996; Neff, 1997; Berry *et al.*, 1999) precipitate as their respective metal sulfides which have very limited bioavailability (Di Toro *et al.*, 1990). If the molar concentration of AVS in sediments is higher than the sum of the molar concentrations of these metals in the 1 N HCl extract (i.e, the simultaneously extracted metals [SEM] of the sediment), the metals will be predominantly in nonbioavailable forms in the sediments. This relationship can be summarized in the following manner:

SEM:AVS >1, metals are present in more bioavailable forms

SEM:AVS<1, metals are generally present in forms with only limited bioavailability.

If the SEM:AVS>1, then these data can be used to calculate the available fraction of metals for use as an EPC. It is important to note that each of the metals evaluated has a different binding affinity for sulfides (U.S. EPA, 1994). Currently there is considerable debate regarding the relative affinities of each of the metals (NRC, 2002); however, typically it is assumed that at equilibrium copper will preferentially react with AVS, displacing all other metals. If the available AVS is not completely saturated by copper, then the remaining metals will react in the following order: lead, cadmium, zinc, and nickel. In this model, the fraction of copper in the sediment that is potentially bioavailable and toxic is estimated as follows:

$$Cu_b = ([Cu_{SEM}] - [AVS]) \times (MW_{Cu}) \quad (4\text{-}1)$$

where,

Cu_b = fraction of copper that is bioavailable
Cu_{SEM} = molar concentration of Cu as defined by simultaneous extraction
AVS = molar concentration of AVS
MW_{Cu} = molecular weight of copper (mg/moles).

The bioavailable fraction of the other metals in sediment may be estimated in the same manner, following the order described above. For each successive metal, the molar concentration of AVS applied should be decreased according to the molar concentration of the preceding chemical; when the concentration of AVS is zero, all remaining metals are assumed to be bioavailable. It should be noted that there are considerable uncertainties associated with this approach, and that these relations continue to be evaluated (NRC, 2002).

4.3 Estimating or Measuring Bioaccumulation Directly from the Environmental Media

Bioavailability also may be considered by either estimating or directly measuring bioaccumulation of specific metals in tissues of organisms potentially exposed to those metals. If a metal is not bioavailable, then it will not be taken up by an organism and will not accumulate in the tissues. The amount of chemical bioaccumulated in the tissues of an organism is not an accurate indicator of the total bioavailable fraction, however, because many metals may be metabolized or excreted. Therefore, bioaccumulation only measures that portion of the bioavailable fraction that is sequestered in the tissues. For the purpose of screening-level assessments, bioaccumulation may be estimated through the application of literature-derived bioaccumulation factors (BAFs). However, as the assessment is refined more site-specific data will be required as discussed below.

4.3.1 Chemical Analysis of Tissue Data

Perhaps the simplest method of evaluating bioaccumulation is to collect site-specific biota and determine the concentration of metals in their tissues. Elevated tissue concentrations indicate that the organism has been exposed to bioavailable metals. It is important to note, however, that the origin of metals measured in field-collected tissue samples is uncertain. If the home range of the organism evaluated extends beyond the boundaries of the site, there is no way to accurately determine the fraction of metal present that is associated with the site and the fraction that is attributable to other sources. As a result, field collection of biota is typically limited to those species with relatively limited mobility. Common examples of terrestrial organisms collected to evaluate bioaccumulation from soil are earthworms, insects, plant tissue, and small rodents like meadow voles or field mice. In the aquatic environment, organisms

that are most often collected for tissue analysis include benthic invertebrates (e.g., aquatic insect larvae, molluscs, and various aquatic worms), and small forage fish.

4.3.2 Bioaccumulation Tests

An alternative to field collection of biota tissues is to conduct a bioaccumulation bioassay. These tests evaluate the uptake of specific metals from site-specific media. The benefit of these tests is that uptake occurs in a controlled setting, with a known exposure concentration and period. In addition, unlike with toxicity tests, it can clearly be determined which chemicals are bioavailable based on which are found in the tissues. However, as with any laboratory bioassay, care must be taken when extrapolating these results to the field. Many metals are regulated by terrestrial and aquatic organisms and there is growing evidence that tissue concentrations may not reach steady-state during the duration of a standard bioaccumulation test (i.e., typically 28 days) (Amiard-Triqet *et al.*, 1986; Coleman *et al.*, 1986; Coimbra and Carraça, 1990; and Swaileh and Adelung, 1994). As a result, concentrations observed in a laboratory setting may underestimate actual field conditions.

Several common American Society for Testing and Materials (ASTM, www.astm.org) test methods for conducting bioaccumulation evaluations are provided in Table 4-2. U.S. EPA (2000) has also provided detailed guidance for assessing bioaccumulation and toxicity of sediment-associated chemicals with freshwater invertebrates.

Table 4-2. Common Test Methods for Measuring Bioaccumulation

Method	Description
Sediment	
ASTM Method E1525-02	Designing Biological Tests with Sediments
ASTM Method E1688-00a	Determination of the Bioaccumulation of Sediment-Associated Contaminants by Benthic Invertebrates
ASTM Method E1706-00e1	Test Methods for Measuring the Toxicity of Sediment-Associated Contaminants with Freshwater Invertebrates
Soil	
ASTM Method E1676-97	Conducting Laboratory Soil Toxicity or Bioaccumulation Tests With the Lumbricid Earthworm *Eisenia fetida*
Water	
ASTM Method E1022-94(2002)	Conducting Bioconcentration Tests with Fishes and Saltwater Bivalve Mollusks

4.4 Toxicity Testing

The use of standard toxicity tests using contaminated media from a site provides information regarding the bioavailability of contaminants at the site. Although toxicity tests cannot provide a quantitative estimate of the bioavailable fraction of metals in sediments or soil, the observance of adverse effects indicates that a given metal is likely available to the exposed organisms. This information is especially compelling if combined with chemical and physical data confirming that specific metals are likely present in bioavailable forms as concentrations associated with toxic responses. It is important to note that toxicity tests do not provide information regarding the source of the toxicity. Therefore, it is important to consider all other chemical parameters that may be present, as well as confounding factors (e.g., ammonia

or changes in test conditions) that could contribute to an observed toxic response before drawing the conclusion that measured metals concentrations are bioavailable.

Many toxicity tests methods are available for evaluating toxicity to various organisms from metals in sediments and soil. Table 4-3 presents some common methods from ASTM, the International Standardization Organization (ISO, www.iso.ch/iso/en/ISOOnline.openpage) and U.S. EPA; however, this list does not represent all available tests and updates of individual tests are issued frequently. When selecting a test, it is important to consider the key receptors, the environmental media being evaluated, and relevant exposure periods.

Table 4-3. Common Test Methods for Evaluating Site-Specific Toxicity

Method	Description
Sediment	
EPA 600/R-99/064	Methods for Measuring the Toxicity and Bioaccumulation of Sediment-Associated Contaminants with Freshwater Invertebrates. Second Edition.
EPA 600/R-01/020	Methods for Assesing the Chronic Toxicity of Marine and Estuarine Sediment-Associated Contaminants with the Amphipod *Leptocheirus plumosus*.
ASTM E1367-99	Standard Guide for Conducting 10-day Static Sediment Toxicity Tests with Marine and Estuarine Amphipods
ASTM E1706-00e1	Standard Test Methods for Measuring the Toxicity of Sediment-Associated Contaminants with Freshwater Invertebrates
ASTM E1562-00	Standard Guide for Conducting Acute, Chronic, and Life-Cycle Aquatic Toxicity Tests with Polychaetous Annelids
ASTM E1611-00	Standard Guide for Conducting Sediment Toxicity Tests with Marine and Estuarine Polychaetous Annelids
Soil	
ASTM E1676-97	Standard Guide for Conducting Laboratory Soil Toxicity or Bioaccumulation Tests With the Lumbricid Earthworm *Eisenia fetida*
ASTM E1963-98	Conducting Terrestrial Plant Toxicity Tests
ISO/16387:2001	Soil Quality – Effects of Soil Pollutants on *Enchytraeidae* (*Enchytraeus sp.*) – Determinations of Effects on Reproduction and Survival.
ISO/11267:1998	Soil Quality - Inhibition of Reproduction of *Collembola* (*Folsomia candida*) by Soil Pollutants.
ISO/11268-2:1998	Soil Quality - Effects of Pollutants on Earthworms (Eisenia fetida) - Part 2: Determination of Effects on Reproduction.
Water	
ASTM E724-98	Standard Guide for Conducting Static Acute Toxicity Tests Starting with Embryos of Four Species of Saltwater Bivalve Molluscs
ASTM E729-96(2002)	Standard Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians
EPA 600/4-90-027R	Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms

4.5 Estimating Uptake From Ingestion of Food

Terrestrial, freshwater, and marine animals are able to accumulate most bioavailable forms of metals from their food. When an animal consumes a lower trophic-level organism, any metals that have accumulated in the tissues of that organism can be transferred to the animal (i.e., through trophic transfer). This process occurs primarily or exclusively in the unique environment of the gut of the consumer. Metals that are sorbed or bound to the tissues of a food item and are introduced into the gut of the consumer may be

desorbed from the food, dissolved in the gut fluids during digestion, and then partitioned from the gut fluids across the gut lining into the tissues of the consumer. As with uptake directly from soils or sediment, the amount of metal desorbed from the food (i.e., the bioavailable fraction) may be dependent on a number of chemical factors (e.g., chemical form or pH). Consideration of qualitative and quantitative evidence related to the physical and chemical conditions associated with ingestion and absorption can assist in determining what portion of the total measured concentration is actually available to the organisms exposed.

In general, however, the most efficient means of incorporating this estimate of the bioavailable fraction would be as described for the noncarcinogenic human health risk assessment. For example, when evaluating exposures resulting from the ingestion of contaminated prey items, the following simplified equation may be used to determine the risk from food ingested by the ecological receptor:

$$\text{Risk} = (\text{Intake} \times \text{ABS}) / \text{TRV} \tag{4-2}$$

where,

Intake = ingested dose (mg/kg/day)
ABS = absorption factor (unitless)
TRV = toxicity reference value (mg/kg/day).

For screening-level evaluations, the ABS is typically assumed to be one (i.e., absorption is 100 percent). However, as the investigation progresses through the ecological risk assessment process, it may be possible to refine this value to reflect actual conditions through either a review of the relevant literature, or through bioassays as described for human health exposures in Sections 2.0 and 3.0.

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