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# Guide for Incorporating Bioavailability Adjustments into Human Health and Ecological Risk Assessments at U. S. Navy and Marine Corps Facilities

## Part 2: Technical Background Document for Assessing Metals Bioavailability

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13. ABSTRACT *(Maximum 200 words)*

This guide for incorporating bioavailability into risk assessments has been developed as a resource for use by Navy Remedial Project Managers and others involved in designing studies for remediating Navy sites. This guide includes the most current information on bioavailability of metals and synthesizes it into a practical handbook. The guide explains concepts and identifies data and study needs 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 organic compounds.

The *Guide* consists of two parts. ***Part 1: Overview of Metals Bioavailability*** provides general information on bioavailability and discusses where bioavailability fits in the risk assessment process. In addition, a brief summary of bioavailability information is presented for the following metals: arsenic, cadmium; chromium; lead, mercury; and nickel. ***Part 2: Technical Background Document for Assessing Metals Bioavailability*** provides more in depth technical information for those involved in designing bioavailability studies. Part 2 includes guidelines on the types of studies that need to be performed and methods for collecting the necessary data with specific considerations for individual metals. Standard operating procedures and suggested protocols for the recommended studies are also provided.

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# EXECUTIVE SUMMARY

The *Guide for Incorporating Bioavailability Adjustments into Human Health and Ecological Risk Assessments at U.S. Navy and Marine Corps Facilities, Parts 1 and 2,* has been developed as a resource on the assessment of bioavailability. Specifically, the document is designed for use by Navy Remedial Project Managers (RPMs) and others involved in remediating Navy 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 Navy sites (arsenic, cadmium, chromium, lead, mercury, and nickel for both human health and ecological risk and copper, tin and zinc for ecological risk 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 fraction |
| 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 |
| NOAEL | no observed adverse effect level |
| PTI | PTI Environmental Services, Inc. |
| 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. Navy and Marine Corps Facilities, Parts 1 and 2,* has been developed as a resource on bioavailability studies for use by Navy Remedial Project Managers (RPMs) and others involved in remediating Navy 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 Navy sites (i.e., arsenic, cadmium, chromium, lead, mercury, and nickel for both human health and ecological risk; and copper, tin and zinc for ecological risk 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*. Section 2.0 provides general study design information including a discussion on soil collection and characterization necessary to support bioavailability studies, an overview of in vitro methods for assessing bioavailability, and an overview of in vivo methods for assessing bioavailability. In vitro methods are performed in the laboratory to simulate the amount of metal that dissociates from soil in the stomach and small intestine and thus is available for absorption into the bloodstream. These tests are much less expensive and less time consuming that in vivo animal studies.

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 in vitro and in vivo studies discussed in Sections 2.0 and 3.0 are usually used to determine bioavailability to human receptors for human health risk assessment.

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. 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. 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. More detailed metal-specific information on in vivo and in vitro study design is provided in Section 3.0.

## 2.1 Soil Collection and Characterization

This section discusses general soil collection and characterization issues to consider when obtaining soils for evaluation of mineralogy (i.e., speciation) or metals bioavailability, by either in vitro or in vivo methods. In general, surficial (0-2 in. or 0-2 cm) soils should be collected that represent the material to which exposure is anticipated to occur. 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 composite samples from a large portion of the exposure area in order to capture the average exposure conditions if only one sample can be tested.

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. 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) influences 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). 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. 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, 1993a). 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, 1993a). 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, 1993a).

***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). As discussed below, studies have suggested that cadmium in soil has reduced absorption relative to soluble cadmium.

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, 1997a).

***Design of Previous In Vivo Studies***

Several oral in vivo studies 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***

The oral bioavailability of chromium depends on its valence state, with the hexavalent form (Cr[VI]) being more readily absorbed than the trivalent (Cr[III]). Nondietary trivalent chromium compounds only have very limited bioavailability (approximately 1 percent), while perhaps 10 percent of ingested hexavalent chromium is absorbed. There is evidence that much ingested hexavalent chromium is reduced to the trivalent form in the stomach, which 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, 1993b). 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. 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 data from this study should be available in mid-2000.

### 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, 2000). 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. 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:** 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 1,000 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, 1997b), and that perhaps as little as 0.01-0.1 percent of elemental mercury is absorbed after ingestion (Goyer, 1996; ATSDR, 1997b).

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, 1997b). 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, 1997b). 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, 1997b; 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. 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 vivo studies of oral nickel bioavailability have been performed, nor have any in vitro studies for nickel been reported in the peer-reviewed literature. The SBRC extraction test (see Appendix C) is recommended 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 (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, 1997c). 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, 1997c). 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, 1997c).

**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, 1997c). 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 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 37,000 to 41,000 µg/g in U.S. EPA Regions IX and III, respectively. Consequently, oral bioavailability studies are not likely to be useful unless these soil concentrations are exceeded; an exception to this is California, where the risk-based screening level for residential soils is 150 µg/g. 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

## 4.1 Introduction

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).

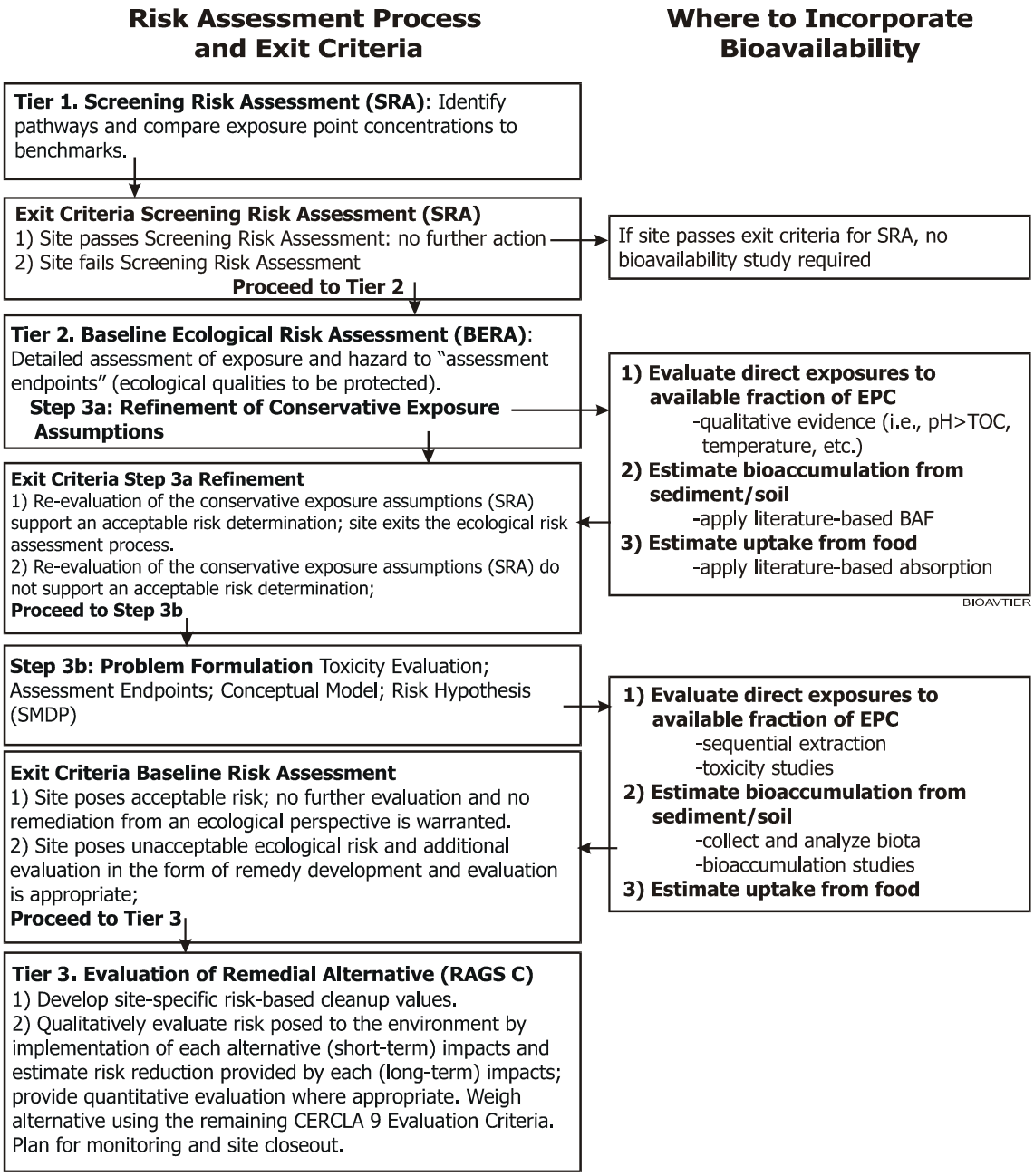
Due to the complexity of this issue, no single methodology exists for incorporating bioavailability into an ecological assessment. Rather, the appropriate means of evaluating the potential bioavailability of chemicals of concern must be determined on a site-by-site basis by considering the associated 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 three different approaches:

- Evaluating direct exposures to the available fraction of metals present in the environmental media (i.e., sediment, soil, or water)
- Estimating or measuring bioaccumulation directly from the environmental media
- 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. For the purpose of clarity, the guidance is based on the ecological risk assessment process outlined by the CNO Policy for conducting Ecological Risk Assessments dated April 5, 1999 (Figure 4-1). Recommendations are provided for each step of the 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.

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

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 to sediments and soils. Therefore, evaluating the total metal concentrations alone as exposure point concentrations (EPCs) does not accurately reflect the fraction biologically available to aquatic and terrestrial organisms. Consideration of qualitative and quantitative evidence related to the physical and chemical conditions can assist in




Note: Modified from the Navy Ecological Risk Assessment Tiered Approach (http:web.ead.anl.gov/ecorisk), which is based on U.S. EPA's 8-Step Ecological Risk Assessment Process.

**Figure 4-1. Incorporating Bioavailability in the Tiered Ecological Risk Assessment Process**

determining whether the total metal concentration measured is actually available to the organisms exposed. Evidence evaluated may include site-specific chemical and physical properties (e.g., salinity, TOC, pH, grain size, temperature, and acid volatile sulfides [AVS]), alternative analytical techniques, or site-specific toxicity tests.

### 4.2.1 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.

### 4.2.2 Sequential Extraction Techniques and Evaluation of Acid Volatile Sulfides

Chemical analytical methods have been developed for metals to better estimate the fraction of the metal that is available for uptake by a receptor. 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 4-2, 4-3, and 4-4.

Figure 4-2 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 4-3) 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 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 the

**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 |

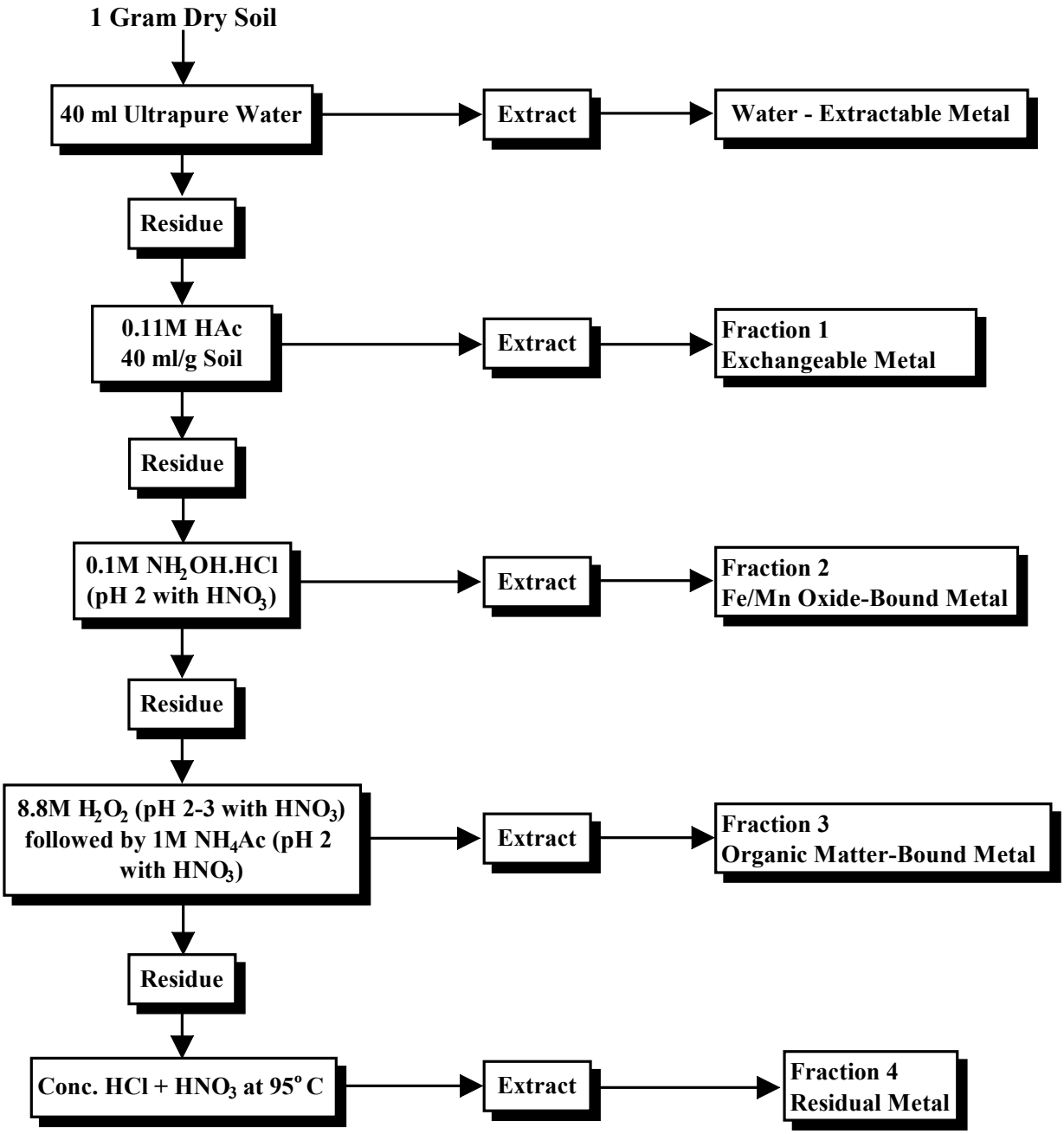



**Figure 4-2. Extraction Scheme Used to Characterize the Distribution of Metals in Geochemical Fractions of Soil**

(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.)

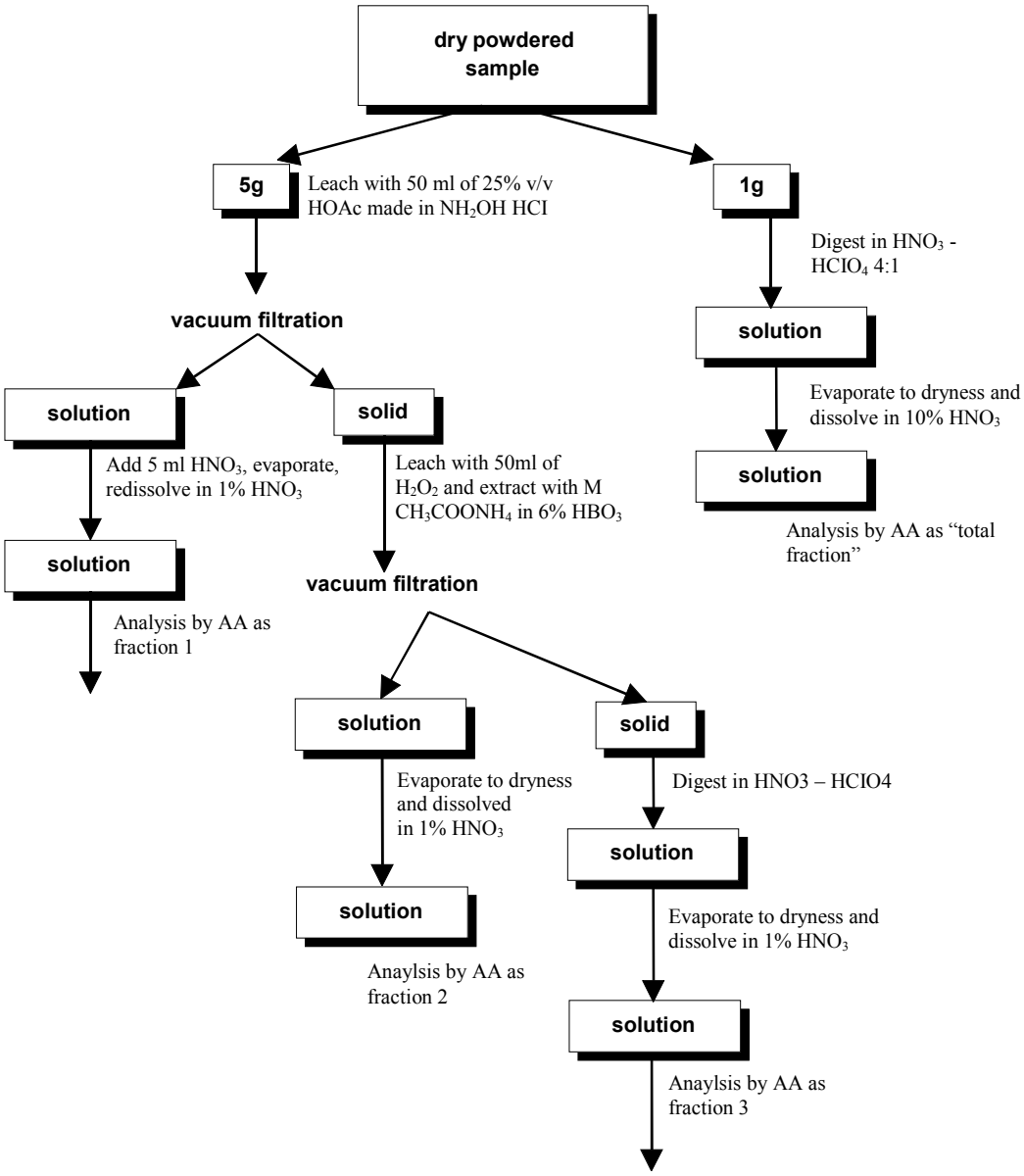



**Figure 4-3. Extraction Scheme Used to Characterize the Distribution of Metals in Different Geochemical Fractions of Coastal Marine Sediments**

(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.)

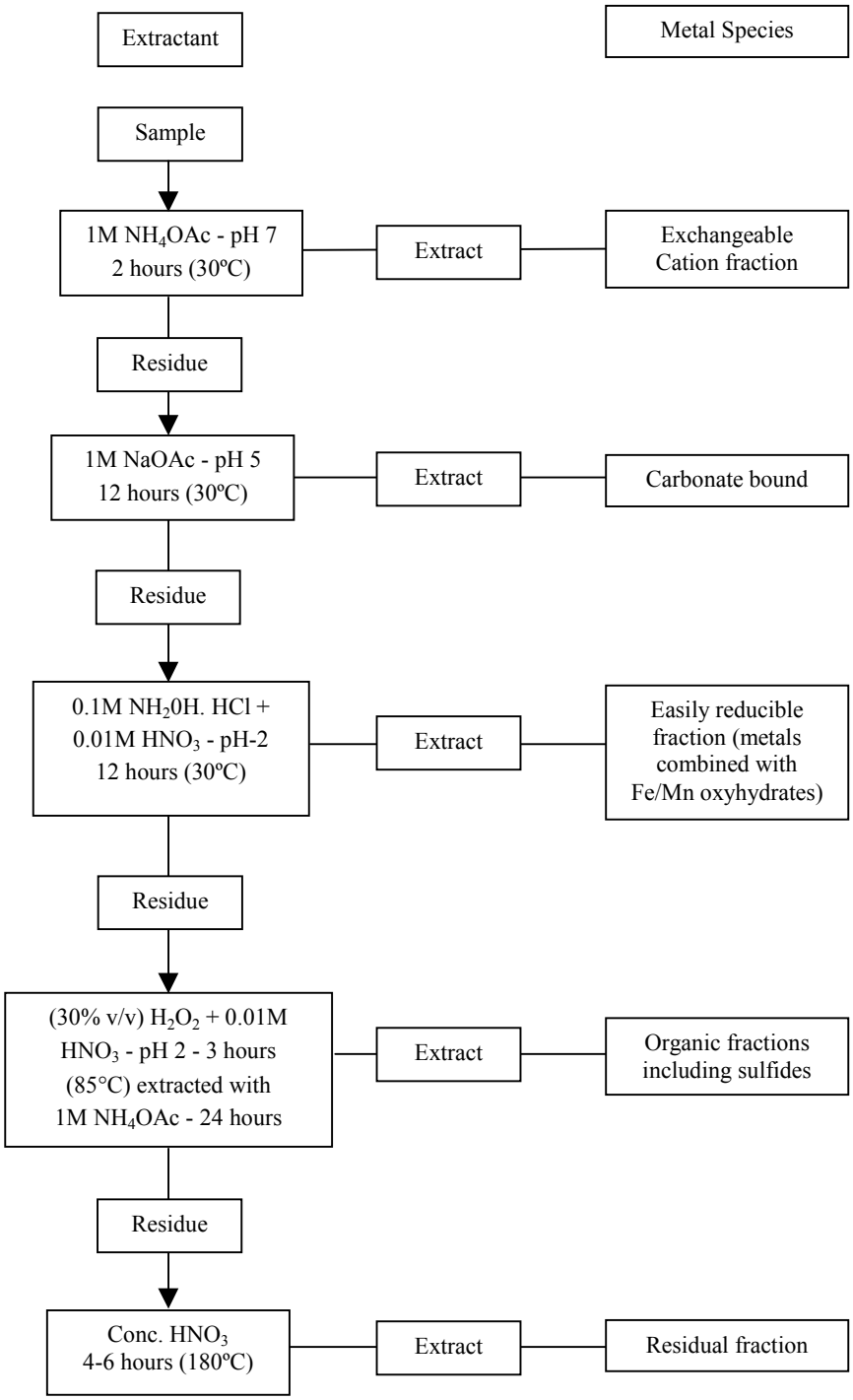



**Figure 4-4. Typical Extraction Sequence for Estimating the Bioavailable Fraction of Metals in Estuarine Sediments**

(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.)

organic 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 4-4) 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.

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 are not bioavailable (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), all of the metal are in nonbioavailable forms in the sediments. This relationship can be summarized in the following manner:

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

SEM:AVS<1, metals are not likely to be bioavailable.

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; 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 considered to be defined 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 determined 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.

### 4.2.3 Toxicity Testing

The use of standard in situ toxicity tests 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 4-2 presents several common methods from the American Society for Testing and Materials (ASTM) and U.S. EPA; however, this list does not represent all available tests. When selecting a test, it is important to consider the key receptors, the environmental media being evaluated, and whether chronic or acute exposures are of primary concern.

## 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.

**Table 4-2. Common Test Methods For Evaluating Site-Specific Toxicity**

| Method | Description |
|---|---|
| **Sediment** | |
| EPA 600/R-94/024 | Methods for Measuring the Toxicity and Bioaccumulation of Sediment-Associated Contaminants with Freshwater Invertebrates |
| EPA 600/R-94/025 | Methods for Measuring the Toxicity and Bioaccumulation of Sediment-Associated Contaminants with Marine Invertebrates |
| ASTM E1367-92 | Standard Guide for Conducting 10-day Static Sediment Toxicity Tests with Marine and Estuarine Amphipods |
| ASTM E1383-94 | Standard Guide for Conducting Sediment Toxicity Tests with Freshwater Invertebrates |
| ASTM E1706-95b | Standard Test Methods for Measuring the Toxicity of Sediment-Associated Contaminants with Freshwater Invertebrates |
| ASTM E1562-94 | Standard Guide for Conducting Acute, Chronic, and Life-Cycle Aquatic Toxicity Tests with Polychaetous Annelids |
| ASTM E1611-94 | Standard Guide for Conducting Sediment Toxicity Tests with Marine and Estuarine Polychaetous Annelids |
| **Soil** | |
| EPA 600/3-88-029 | Measuring Acute Toxicity – Root Elongation and Seed Germination |
| EPA 600/3-88-029 | Methods for Measuring Acute Toxicity and Earthworm Survival at Hazardous Waste Sites |
| ASTM E1676-97 | Standard Guide for Conducting Laboratory Soil Toxicity or Bioaccumulation Tests With the Lumbricid Earthworm *Eisenia fetida* |
| **Water** | |
| ASTM E724-89 | Standard Guide for Conducting Static Acute Toxicity Tests Starting with Embryos of Four Species of Saltwater Bivalve Molluscs |
| ASTM E729-88 | Standard Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians |
| EPA 600/4-90-027R | Methods for Measuring Acute Toxicity for Freshwater and Marine Organisms |
| EPA 600/3-91-063 | Measuring Toxicity of Early Life Stage with Japanese Medaka |

### 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. Typically conducted in situ, 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 test methods for conducting bioaccumulation evaluations are provided in Table 4-3.

**Table 4-3. Common Test Methods for Measuring Bioaccumulation**

| Method | Description |
|---|---|
| **Sediment** | |
| ASTM Method E1525-94a | Designing Biological Tests with Sediments |
| ASTM Method E1688-96 | Determination of the Bioaccumulation of Sediment-Associated Contaminants by Benthic Invertebrates |
| ASTM Method E1706-95b | 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 | Conducting Bioconcentration Tests with Fishes and Saltwater Bivalve Mollusks |

## 4.4 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:

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

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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# APPENDIX A

## GENERALIZED STANDARD OPERATING PROCEDURE FOR METAL SPECIATION BY ELECTRON MICROPROBE ANALYSIS

Date: October 11, 1999 (Rev. #1)

Title: METAL SPECIATION

**SYNOPSIS**: A standardized method for speciating metals in solid samples using an electron microprobe is described. Equipment operating conditions, sample preparation and handling, and statistical equations for data analysis are included.

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## 1.0 OBJECTIVES

The objectives of this Standard Operating Procedure (SOP) are to specify the proper methodologies and protocols to be used during metal speciation of various solid samples, including; soil, house dust, wipes, sediments, tailings, slags, dross, bag house dusts, and paint samples, using an electron microprobe. The metal speciation data generated from this SOP may be used to assess the solid samples, and the provenance and risk associated with the various metal phases. Parameters to be characterized during the speciation analyses include particle size, associations, stoichiometry, frequency of occurrence of metal-bearing forms and relative mass of metal-bearing forms. The sample preparation methods and instrument operation parameters to be used during implementation of this SOP are discussed in the following sections.

## 2.0 BACKGROUND

To date, numerous metal-bearing forms have been identified from various environments within western mining districts (Emmons et al., 1927; Drexler, 1991, per. comm.; Drexler, 1992; Davis et al., 1993; Ruby et al., 1992; CDM, 1994; WESTON, 1995), and industrial or agricultural (Drexler, 1999, per. comm.) settings, Table 2-1. This listing does not preclude the identification of other metal-bearing forms, but only serves as an initial point of reference. Many of these forms are minerals with varying metal concentrations (e.g., lead phosphate, iron-lead oxide, and slag). Since limited thermodynamic information is available for many of these phases and equilibrium conditions are rarely found in soil environments, the identity of the mineral class (e.g., lead phosphate) will generally be sufficient for determination of provenance and risk, and exact stoichiometry is not necessary.

It may be important to know the particle-size distribution of metal-bearing forms to assess potential risk. It is believed that particles less than 250 microns (μm) are most available for human ingestion (Bornschein, et al., 1987). For this study, the largest dimension of any one metal-bearing form will be measured and the frequency of occurrence weighted by that dimension. Although not routinely performed, particle area can be determined; it has been shown (CDM, 1994) that data collected on particle area produces similar results. These measurements add a considerable amount of time to the procedure, introduce new sources of potential error and limit the total number of particles or samples that can be evaluated in a study.

Mineral association may affect the solubility of a metal from a particular sample. For example, if a lead-bearing form in one sample is predominantly found within quartz grains, while in another sample it is free in the sample matrix, the two samples are likely to have different lead solubility, and pose different risks. Therefore, mineral associations are evaluated, and include the following:

1) free or liberated
2) inclusions within a second phase
3) cementing
4) alteration rims.

## 3.0 SAMPLE SELECTION

Samples should be selected and handled according to the procedure described in the Project Plan.

## 4.0 SCHEDULE

A schedule for completion of projects performed under this Metals Speciation SOP will be provided in writing or verbally.

## 5.0 INSTRUMENTATION

Speciation analyses may be conducted at the Laboratory for Environmental and Geological Studies (LEGS) at the University of Colorado, Boulder, or other comparable facilities. Primary equipment used for this work will include:

Electron Microprobe (JEOL 8600) equipped with four wavelength spectrometers, energy dispersive spectrometer (EDS), BEI detector and Geller Microanalytical data processing system. An LEDC spectrometer crystal for carbon and LDE-1 crystal for oxygen analyses are essential.

## 6.0 PRECISION AND ACCURACY

The precision of the EMP speciation and polarized light microscopy (PLM) will be evaluated based on sample duplicates analyzed at a frequency of 10%. The precision of the data generated by the manual PLM particle count and by the "EMP point count" will be evaluated by preparing a graph that compares the original result with the duplicate result. The accuracy of the analyses will be estimated based on a number of methods, depending on the source of the data. Data generated by the "EMP point count" will be evaluated statistically based on the methods of Mosimann (1965) at the 95% confidence level on the frequency data following Equation 1.

$$E_{0.95} = 2P(100-P)/N \qquad \text{(Eq. 1)}$$

Where: $E_{0.95}$ = Probable error at the 95% confidence level

$P$ = Percentage of N of an individual metal-bearing phase based on percent length frequency

$N$ = Total number of metal-bearing grains counted.

In general, site-specific concentrations for these variable, metal-bearing forms will be determined by performing "peak counts" on the appropriate wavelength spectrometer. Average concentrations will then be used for further calculations. Data on specific gravity will be collected from referenced databases or estimated based on similar compounds.

## 7.0 PERSONNEL RESPONSIBILITY

The analysts will carefully read this SOP prior to any sample examination.

It is the responsibility of the laboratory supervisor and designates to ensure that these procedures are followed, to examine quality assurance (QA) samples and replicate standards, and to check EDS and WDS calibrations. The laboratory supervisor will collect results, ensure they are in proper format, and deliver them to the contractor.

Monthly reports summarizing all progress, with a list of samples speciated to date with data analyses sheets (DAS), will be submitted each month.

It is also the responsibility of the laboratory supervisor to notify the contractor representative of any problems encountered in the sample analysis process.

## 8.0 SAMPLE PREPARATION

Grain mounts (1.5 inches in diameter) of each sample will be prepared using air-cured epoxy. This grain mounting technique is appropriate for most speciation projects; however, polished thin-sections, paint chips, dust wipes, or filters may be prepared in a similar manner. The grain mounting is performed as follows:

1) Log the samples for which polished mounts will be prepared.

2) Inspect all disposable plastic cups, making sure each is clean and dry.

3) Label each "mold" with its corresponding sample number.

4) All samples will be split to produce a homogeneous 1-4 gram sample.

5) Mix epoxy resin and hardener according to manufacturer's directions.

6) Pour 1 gram of sample into mold. Double check to make sure sample numbers on mold and the original sample container match. Pour epoxy into mold to just cover sample grains.

7) Use a new wood stirring stick with each sample; carefully blend epoxy and grains so as to coat all grains with epoxy.

8) Set molds to cure at ROOM TEMPERATURE in a clean restricted area. Add labels with sample numbers and cover with more epoxy resin. Leave to cure completely at room temperature.

9) One at a time, remove each sample from its mold and grind flat the back side of the mount.

10) Use 600-grit wet abrasive paper stretched across a grinding wheel to remove the bottom layer and expose as many mineral grains as possible. Follow with 1,000-grit paper.

11) Polish with 15-µm oil-based diamond paste on a polishing paper fixed to a lap. Use of paper instead of cloth minimizes relief.

12) Next use 6-µm diamond polish on a similar lap.

13) Finally polish the sample with 1-µm oil-based diamond paste on polishing paper, followed by 0.05-µm alumina in water suspension. The quality should be checked after each step. Typical polishing times are 30 minutes for 15 µm, 20 minutes for 6 µm, 15 minutes for 1 µm, and 10 minutes for 0.05 µm.

NOTE: use low speed on the polishing laps to avoid "plucking" of sample grains.

14) Samples should be completely cleaned in an ultrasonic cleaner with isopropyl alcohol or similar solvent to remove oil and fingerprints.

15) To ensure that no particles of any metal are being cross-contaminated during sample preparation procedures, a blank (epoxy only) mold will be made every 20$^{th}$ sample (5% of samples) following all of the above procedures. This mold will then be speciated along with the other samples.

16) Each sample must be carbon coated. Once coated, the samples should be stored in a clean, dry environment with the carbon surface protected from scratches or handling.

## 9.0 GEOCHEMICAL SPECIATION USING ELECTRON MICROPROBE

All investigative samples will also be characterized using EMP analysis to determine the chemical speciation, particle size distribution and frequency for several target metals.

### 9.1 Concentration Prescreening

All samples will be initially examined using the electron microprobe to determine if the number of particles are too great to obtain a representative count. The particle counting will be considered representative if the entire sample (puck) has been traversed about the same time in which the counting criteria are achieved.

If this examination reveals that one metal is abundant (> 1% of total metals concentration), clean quartz sand ($SiO_2$) will be mixed with the sample material. The sand should be certified to be free of target analytes. The quartz sand should be added to an aliquot of the investigative sample, then mixed by turning the sample for a minimum of one hour, or until the sample is fully homogenized. The initial mass of the investigative sample aliquot, and the mass of the quartz addition must be recorded on the Data Analysis Sheet (DAS).

### 9.2 Point Counting

Counts are made by traversing each sample from left-to-right and top-to-bottom as illustrated in Figure 9-1. The amount of vertical movement for each traverse depends on magnification and CRT (cathode-ray tube) size. This movement should be minimized so that no portion of the sample is missed when the end of a traverse is reached. Two magnification settings generally are used--one ranging from 40-100X and a second from 300-600X. The last setting will allow one to find the smallest identifiable (1-2 micron) phases.

The portion of the sample examined in the second pass, under the higher magnification, will depend on the time available, the number of metal-bearing particles, and the complexity of metal mineralogy. A maximum of 8 hours will be spent on each analysis.

### 9.3 Data Reduction

Analysts will record data as they are acquired from each sample using the LEGS software, which places all data in a spreadsheet file format. Columns have been established for numbering the metal-bearing phase particles, their identity, size of longest dimension in microns, along with their association (L =

liberated, C= cementing, R = rimming, I = included). The analyst may also summarize his/her observations in the formatted data summary files.

The frequency of occurrence and relative metal mass of each metal-bearing form as it is distributed in each sample will be depicted graphically as a frequency bar graph. The particle size distribution of metal-bearing forms will be depicted in a histogram. Size-histograms of each metal-bearing form can be constructed from data in the file.

Data from EMP will be summarized using two methods. The first method is the determination of FREQUENCY OF OCCURRENCE. This is calculated by summing the longest dimension of all the metal-bearing phases observed and then dividing each phase by the total.

Equation 2 will serve as an example of the calculation.

$$F_M \text{ in phase-1} = \frac{\Sigma\,(PLD)_{\text{phase 1}}}{\Sigma\,(PLD)_{\text{phase-1}} + \Sigma(PLD)_{\text{phase-2}} + \Sigma\,(PLD)_{\text{phase-n}}} \qquad \text{(Eq. 2)}$$

Where:

$F_M$ = Frequency of occurrence of metal in a single phase

PLD = An individual particle's longest dimension

$\%F_M$ in phase-1 = $F_M$ in phase-1 * 100.

These data indicate which metal-bearing phase(s) are the most commonly observed in the sample or relative volume percent.

The second calculation used in this report is the determination of RELATIVE METAL MASS. These data are calculated by substituting the PLD term in the equation above with the value of $M_M$. This term is calculated as defined below:

$$M_M = FM * SG * ppm_M \qquad \text{(Eq. 3)}$$

Where:

$M_M$ = Mass of metal in a phase

SG = Specific Gravity of a phase

$ppm_M$ = Concentration in ppm of metal in a phase.

The advantage in reviewing the RELATIVE METAL MASS determination is that it gives one information as to which metal-bearing phase(s) in a sample are likely to control the total bulk concentration for a metal of interest. For example, PHASE-1 may comprise 98% relative volume of the sample; however, it has a low specific gravity and contains only 1,000 parts per million (ppm) arsenic. PHASE-2 comprised 2% of the sample, has a high specific gravity, and contains 80,000 ppm of arsenic. In this example it is PHASE-2 that is the dominant source of arsenic to the sample.

Finally, a concentration for each phase is calculated. This quantifies the concentration of each metal-bearing phase. This term is calculated as defined below:

$$ppm_M = M_M * \text{Bulk metal concentration in ppm} \qquad \text{(Eq. 4)}$$

### 9.4 Analytical Procedure

A brief visual examination of each sample will be made, prior to EMP examination. This examination may help the operator by noting the occurrence of slag and/or organic matter. Standard operating conditions for quantitative and qualitative analyses of most metal-bearing forms are given in Table 9-1. However, it is the responsibility of the operator to select the appropriate analytical line (crystal/KeV range) to eliminate peak overlaps and ensure proper identification/quantification of each analyte. Quality control will be maintained by analyzing duplicates at regular intervals.

The backscattered electron threshold will be adjusted so that all particles in a sample are seen. This procedure will minimize the possibility that low metal-bearing minerals may be overlooked during the scanning of the polished grain mount. The scanning will be done manually. Typically, the magnification used for scanning all samples except for airborne samples will be 40-100X and 300-600X. The last setting will allow the smallest identifiable (1-2 μm) phases to be found. Once a candidate particle is identified, then the backscatter image will be optimized to discriminate any different phases that may be making up the particle or defining its association. Identification of the metal-bearing phases will be done using both EDS and WDS on an EMP, with spectrometers typically peaked at sulfur, oxygen, carbon and the metal(s) of concern. The size of each metal-bearing phase will be determined by measuring in microns the longest dimension.

As stated previously, a maximum of 8 hours will be spent in scanning and analyzing each mount. For most speciation projects the goal is to count between 100-200 particles. In the event that these goals are achieved in less than 8 hours, particle counting may continue or the analyst may move to another sample in order to increase the sample population.

**Quantitative Analyses**

Quantitative analyses are required to establish the average metal content of the metal-bearing minerals, which have variable metal contents as: iron-(M) sulfate, iron-(M) oxide, manganese-(M) oxide, organic, and slag. These determinations are important, especially in the case of slag, which is expected to have considerable variation in their dissolved metal content.

Results will be analyzed statistically to establish mean values. They may also be depicted as histograms to show the range of metal concentrations measured as well as the presence of one or more populations in terms of metal content. In the later case, non-parametric statistics may have to be used or the median value may have to be established.

**Associations**

The association of the metal-bearing forms will be established from the backscattered electron images. Particular attention will be paid in establishing whether the grains are totally enclosed, encapsulated or liberated. The rinds of metal-bearing grains will be identified. Representative photomicrographs of backscatter electron images establishing the association of the principal metal-bearing forms will be obtained for illustration purposes.

### 9.5 Instrument Calibration and Standardization

The WDS will have spectrometers calibrated for the metal of concern, carbon, oxygen and sulfur on the appropriate crystals using mineral standards. The EDS will have a multi-channel analyzer (MCA)

calibrated for known peak energy centroids. Calibration will be performed so as to have both low (1.0-3.0 KeV) and high (6.0-9.0 KeV) energy peaks fall within 0.05 KeV of its known centroid.

The magnification marker on the instrument will be checked once a week. This will be performed by following manufacturer instructions or by measurement of commercially available grids or leucite spheres. Size measurements must be within 4 microns of certified values.

Initial calibration verification standards (ICVs) must be analyzed at the beginning of each analytical batch or once every 48 hours, whichever is more frequent. A set of mineral or glass standards will be run quantitatively for the metal of concern, sulfur, oxygen and carbon. If elemental quantities of the ICVs do not fall within +/- 5% of certified values for each element, the instrument must be recalibrated prior to analysis of investigative samples.

The metal-bearing forms in these samples will be identified using a combination of EDS, WDS and BEI. Once a particle is isolated with the backscatter detector, a 5-second EDS spectra is collected and peaks identified. The count rates for the metal(s) of concern, sulfur, carbon and oxygen can be either visually observed on the wavelength spectrometers or K-ratios calculated.

### 9.6 Documentation

Photomicrographs must be taken for each sample, at a rate of 5% (1 photograph per 20 particles counted), for a maximum of 10 per sample and submitted with the results. Particles selected for photography must be recorded on the EMP graph, as well as in the DAS. Any additional photographs should be labeled as “opportunistic”.

A positive black-and-white film (Polaroid 52) will be used or a 128x128 (minimum) binary image in “.tif” format may be stored. Recorded on each photomicrograph and negative will be a scale bar, magnification, sample identification, date and phase identification. Abbreviations for the identified phases can be used. Examples are listed in Table 9-2. A final list must be submitted with the laboratory report.

## 10.0 PERSONAL HEALTH AND SAFETY

Each individual operating the electron microprobe instruments will have read the “Radiation Safety Handbook” prepared by the University of Colorado and follow all State of Colorado guidelines for operation of X-ray equipment.

Latex gloves and particulate masks will be worn during preparation of sample cups. All material that comes in contact with the samples or used to clean work surface areas will be placed in poly-bags for disposal.

## 11.0 FINAL REPORT

A final laboratory report will be provided to the Contractor. The report will include all EMP data including summary tables and figures. Individual sample data will be provided on disk.

Speciation results will include: (1) a series of tables summarizing frequency of occurrence for each metal phase identified along with a confidence limit; (2) summary histograms of metal phases identified for each waste type; (3) a summary histogram of particle size distribution in each waste type; and (4) a

summary of metal phase associations. Representative photomicrographs or .tif images also will be included in the final report.

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**Table 2-1**

Common Metal-Bearing Forms Found Within Mining, Smelting, Agricultural, Industrial and Residential Media

OXIDES

Lead oxide
Manganese (metal) oxide
Iron (metal) oxide
Lead molybdenum oxide
Arsenic (metal) oxide
Lead (metal) oxides
Cadmium oxide
Copper oxides
Zinc oxide
Lead arsenate
Arsenic trioxide
Calcium (metal) oxide

SILICATES

Slag
Lead silicate
Arsenic silicate
Zinc silicate
Clays

SULFATES

Iron (metal) sulfate
Lead sulfate
Lead barite
Zinc sulfate
Arsenic sulfate
Copper sulfate

CARBONATES

Lead carbonate
Zinc carbonate

PHOSPHATES

(metal) phosphates

SULFIDES

Lead sulfide
Sulfur-containing salts
Iron-arsenic sulfide
Zinc sulfide
Copper sulfides
Copper-iron sulfide
Cadmium sulfide

OTHER

Native: Lead, Copper, Cadmium, Mercury, Indium, Thallium, Selenium
Lead/Arsenic/Cadmium/ Mercury chlorides
Paint
Solder
Organic lead
Lead vanadate
Minor telluride, and bismuth-lead phases

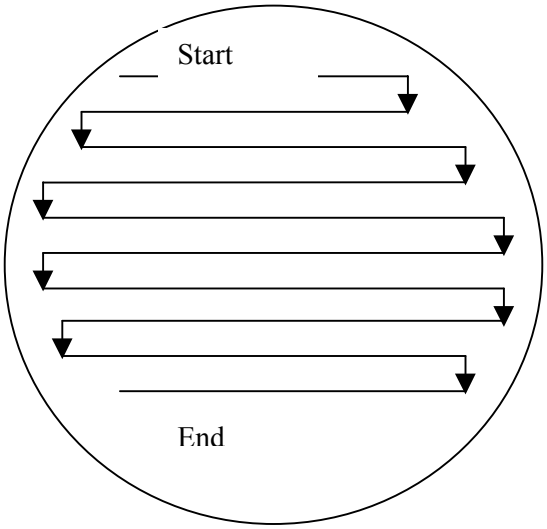


**Figure 9-1**

**Table 9-1**

**EMP Standard Operating Conditions**

| | WDS | EDS |
|---|---|---|
| **Accelerating Voltage** | 15 KV | 15-20 KV |
| Beam Size | 1-2 microns | 1-2 microns |
| Cup Current | 10-30 NanoAmps | 10-30 NanoAmps |
| Ev/Channel | NA | 10 or 20 |
| Stage Tilt | NA | Fixed |
| Working Distance | NA | Fixed |
| MCA time Constant | NA | 7.5-12 microseconds |
| X-ray lines | S K-alpha PET<br>O K-alpha LDE1<br>C K-alpha LDEC<br>Zn K-alpha PET<br>As L-alpha TAP<br>Cu K-alpha LIF<br>Cd L-alpha PET<br>Pb M-alpha PET<br>Pb L-alpha LIF<br>In L-alpha PET<br>Tl L-alpha LIF<br>Hg L-alpha LIF<br>Se L-alpha LIF<br>Sb L-alpha PET | S K-alpha 2.31 KeV<br>O K-alpha 0.52 KeV<br>C K-alpha 0.28 KeV<br>Pb M-alpha 2.34 KeV<br>Pb L-alpha 10.5 KeV<br>Zn K-alpha 8.63 KeV<br>Cu K-alpha 8.04 KeV<br>As K-alpha 10.5 KeV<br>As L-alpha 1.28 KeV<br>Cd L-alpha 3.13 KeV<br>In L-alpha 3.28 KeV<br>Tl M-alpha 2.27 KeV<br>Tl L-alpha 10.26 KeV<br>Hg L-alpha 9.98 KeV<br>Hg M-alpha 2.19 KeV<br>Se L-alpha 1.37 KeV<br>Sb L-alpha 3.60 KeV |

**Table 9-2**

**Suggested Abbreviation for Photomicrographs**

| Metal-bearing Phase | Abbreviation |
|---|---|
| In | In |
| Tl | Tl |
| Hg | Hg |
| Se | Se |
| Sb | Sb |
| Lead Sulfide | Ga |
| Lead Sulfate | Ang |
| Lead Carbonate | Cer |
| Mn-(M) Oxide | Mn(M) |
| Fe-(M) Oxide | Fe(M) |
| (M)Phosphate | (M)Phos |
| Fe-(M) Sulfate | Fe(M)Sul |
| Metal Oxide | (M)O |
| Pb-Mo Oxide | Wulf |
| Slag | Slag |
| Metallic Phase | (M) |
| Metal Silicate | (M)Si |
| Solder | Sold |
| Paint | Pnt |
| Metal-bearing Organic | (M)(Org) |
| (M) barite | (M)Bar |
| Pb arsenate | PbAsO |
| Pb vanadate | PbVan |
| As-Sb Oxide | AsSbO |
| Chalcopyrite | Cp |
| Sphalerite | Sph |
| Arsenopyrite | Apy |

# APPENDIX B

# STANDARD OPERATING PROCEDURES FOR EVALUATING MERCURY SPECIATION IN SOILS

# STANDARD OPERATING PROCEDURE:

# Evaluating Mercury Speciation in Soils

Mercury can occur in soils as elemental mercury in liquid or vapor form, organic mercury compounds, mercuric chloride, or one of several different mineral species, including mercuric oxides, carbonates, and sulfides. In general, organic mercury, mercuric chloride, and elemental mercury in the vapor phase are very soluble and bioavailable; mercuric oxides and carbonates are less soluble; and liquid elemental mercury and mercuric sulfides are relatively insoluble and non-bioavailable.

Recently, several investigators have focused on developing sequential extraction procedures to quantitatively evaluate the speciation of mercury in soils (Revis et al. 1989; Miller 1993; Sakamoto et al. 1992). Application of the procedures of each investigator to mercury-contaminated soils from Oak Ridge, Tennessee, indicated mercury occurring predominantly as elemental mercury and mercuric sulfide minerals (Barnett et al. 1994). However, the relative proportions of the two species did not agree among procedures, indicating that the extractions were either not fully effective in removing specific mercury compounds or not fully specific in extracting individual mercury species. This problem is common to sequential extraction methods (Belzile et al. 1989). All the extraction techniques yielded similar levels of organic mercury in soils. However, the method used by Miller (1993), developed by EPA, generally found much less elemental mercury and mercuric sulfide than did the other two extraction procedures. The method used by Sakamoto et al. (1992) tended to have poor recovery for elemental mercury. The method used by Revis et al. (1993) showed higher recoveries of mercuric sulfide and elemental mercury, but did not include a step for extraction of mercuric oxides and carbonates (acid-soluble mercury). Given these drawbacks to the various methods, a procedure combining the most effective aspects of each was developed. This method has been used to evaluate mercury at a number of sites, and appears to produce reliable results.

## Sample Preparation

Mercury analyses in soils are particularly difficult to reproduce, because elemental mercury commonly occurs as geochemical “nuggets,” where only a small fraction of soil may contain a large proportion of the total mercury in the sample, creating difficulties in obtaining a homogeneous sample. This phenomenon is often evident in field and laboratory duplicates in the form of large relative percent differences (RPDs) between duplicate samples. To minimize this problem as much as possible, all soil samples should be dried thoroughly prior to analysis, and homogenized in a stainless steel bowl. All subsamples should then be prepared using a stainless steel sample splitter.

Soil samples should be air dried beneath a fume hood for 2 days, or until constant weight is achieved. Oven drying should not be used, because it can result in loss of volatile mercury species. The air-dried samples should be desegregated, and sieved through a number 10, 2-mm stainless-steel sieve.

## Sequential Extraction Studies

The intent of this sequential extraction assay is to determine the speciation of mercury in soil for the following mercury species:

- Organic mercury
- Acid-soluble (or bioavailable) mercury, including carbonates, hydroxides, oxides, and chlorides
- Elemental mercury
- Mercuric sulfide.

The sequential extraction of mercury species is outlined in Figure 1. Using air-dried soil samples sieved to less than 2 mm, organic mercury is extracted with chloroform, followed by extraction with a sodium thiosulfate solution. The acid-soluble mercury species are then extracted using sulfuric acid. After extracting these two phases, elemental mercury is determined by the difference between a sample split that has been roasted at 150°C for 5 days and a non-roasted sample split. Mercuric sulfide is assumed to be the mercury that remains after roasting.

## Methods

### Equipment Preparation

Equipment for the organic mercury extraction is prepared by washing four 250-mL glass separatory funnels and four 50-mL Fisher® polypropylene centrifuge tubes (with double-start threads) in acid. The separatory funnels are then pre-rinsed with Fisher Scientific® HPLC-grade chloroform to purify them of any organic mercury. Once the centrifuge tubes are air dried, they are tared on a balance, and 7.5 g of soil sample (less than 2 mm) is weighed into them.

### Organic Mercury Extraction

The organic mercury extraction begins by adding 30 mL of Fisher Scientific® chloroform to the centrifuge tubes and sealing them with the lids. The centrifuge tubes are placed in a wrist-action shaker for 3 minutes and then centrifuged for 3 minutes at 3,000 rpm.

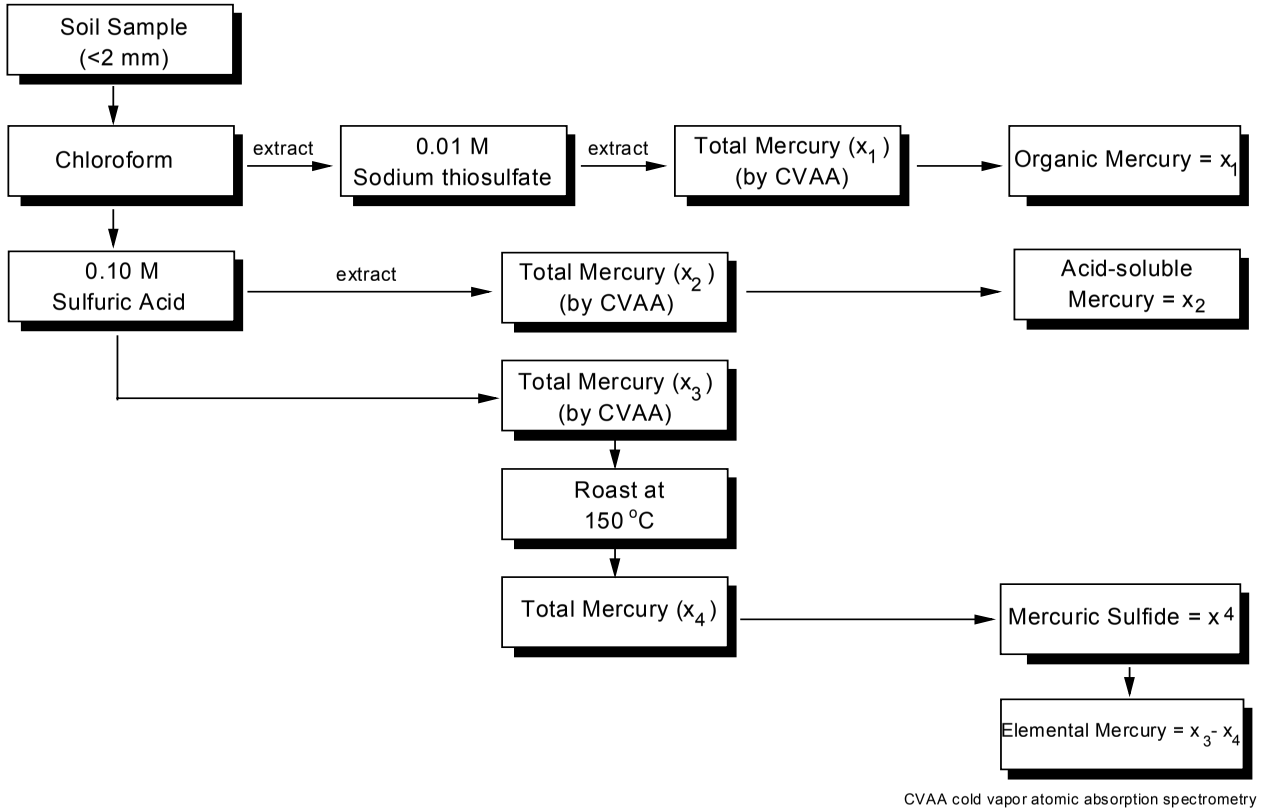


Figure 1. Sequential extraction of mercury phases in soil

After allowing any floating particles to settle, the chloroform phase is decanted into a 250-mL separatory funnel. The extraction is repeated with another 30 mL of chloroform, which is also added to the separatory funnel. Following extraction, 10 mL of 0.01 M $Na_2S_2O_3$ (sodium thiosulfate) is added to the combined chloroform extracts in the separatory funnel to extract the organic mercury from the chloroform. The funnel is hand-shaken for 3 minutes and allowed to settle. Upon separation of the organic and inorganic phases, the chloroform layer is discharged into a large stainless-steel bowl and allowed to evaporate in a hood. The remaining sodium thiosulfate layer and grey layer are filtered though a Whatman® GF/C filter. The filtrate is collected in a 15-mL plastic bottle (or 15-mL Fisher Scientific® centrifuge tube), and preserved with concentrated trace metal nitric acid (20 $\mu$L per 10 mL of sample). This preserved extract should be analyzed for mercury concentration by cold vapor atomic absorption (CVAA) spectroscopy. Using a stainless steel spatula, the solids remaining on the filter paper are returned to the centrifuge tube. The solid remaining in the centrifuge tube is placed under a hood to air dry for the next step of the extraction.

## Acid-Soluble Mercury Extraction

The extraction of acid-soluble mercury consists of adding 15 mL of 0.1 M sulfuric acid to the air-dried residue in the centrifuge tube, shaking the tube in a wrist-action shaker for 3 minutes, and centrifuging for 10 minutes, as specified in Sakamoto et al. (1992). The sulfuric acid solution is then aspirated from the centrifuge tube with a 10-mL plastic syringe, the volume of solution is measured, and the solution is filtered through a Corning® disposable sterile syringe filter (25 mm, 0.45 $\mu$m acrylic, with cellulose acetate membrane). Filtration was added to this step to remove any fine particles that may be suspended in the sulfuric acid solution prior to analysis. The sulfuric acid extract is collected in a 15-mL plastic bottle (or centrifuge tube) and analyzed for total mercury by CVAA. The centrifuge tube is then placed under the hood, and the sample is air dried prior to the next step in the procedure.

## Elemental Mercury Extraction

This step of the procedure begins with removing the sample from the centrifuge tube and splitting the sample using a 2-mm sample splitter. This sub-sample is analyzed for total mercury by CVAA. The remaining sample is weighed in tared stainless-steel pans and placed in the oven. Elemental mercury is removed from the samples by oven roasting for 5 days at 150° C. After 5 days of roasting, the sample is removed from the oven, weighed, and analyzed for total mercury by CVAA. All mercury remaining in the roasted sample is assumed to be mercuric sulfide. Elemental mercury is determined by subtracting the mercury concentration of the roasted sample from the concentration of the unroasted sample.

## Recommended Quality Assurance Samples

It is recommended that a comprehensive set of quality assurance (QA) samples be analyzed when performing the sequential extraction procedure described above. These samples should include method blanks (e.g., sodium thiosulfate and sulfuric acid extraction solutions, sent blind to the laboratory) and mercury spikes made up the sodium thiosulfate and sulfuric acid extraction solutions (to check mercury recovery from these matrices). Duplicate or triplicate analyses of

solid samples should be used to check for homogeneity of mercury in specific samples, and at least one sample should be evaluated in triplicate in the sequential extraction procedure as a check on reproducibility.

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# APPENDIX C

# IN VITRO METHOD FOR DETERMINATION OF LEAD BIOACCESSIBILITY: STANDARD OPERATING PROCEDURE FOR STOMACH PHASE EXTRACTION

# Appendix C

## *In Vitro* Method for Determination of Lead Bioaccessibility:

## Standard Operating Procedure for Stomach Phase Extraction

## Prepared by:

## Solubility/Bioavailability Research Consortium

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# 1. Introduction

## 1.1 Synopsis

This SOP describes an *in vitro* laboratory procedure to determine a bioaccessibility value for lead or arsenic (i.e., the fraction that would be soluble in the gastrointestinal tract) for soils and solid waste materials. A recommended quality assurance program to be followed when performing this extraction procedure is also provided.

## 1.2 Purpose

An increasingly important property of materials/soils found at contaminated sites is the bioavailability of individual contaminants. Bioavailability is the fraction of a contaminant in a particular environmental matrix that is absorbed by an organism via a specific exposure route. Many animal studies have been conducted to experimentally determine the oral bioavailability of individual metals, particularly lead and arsenic. During the period 1989–1997, a juvenile swine model developed by EPA Region VIII was used to predict the relative bioavailability of lead and arsenic in approximately 20 soils/solid materials (Weis and LaVelle 1991; Weis et al. 1994; Casteel et al. 1997a,b). The bioavailability determined was relative to that of a soluble salt (i.e., lead acetate trihydrate or sodium arsenate). The tested materials had a wide range of mineralogy, and produced a range of lead and arsenic bioavailability values. In addition to the swine studies, other animal models (e.g., rats and monkeys) have been used to measure the bioavailability of lead and arsenic from soil.

Several researchers have developed *in vitro* tests to measure the fraction of a chemical solubilized from a soil sample under simulated gastrointestinal conditions. This measurement is referred to as "bioaccessibility" (Ruby et al. 1993). Bioaccessibility is thought to be an important determinant of bioavailability, and several groups have sought to compare bioaccessibility determined in the laboratory to bioavailability determined in animal studies (Imber 1993; Ruby et al. 1996; Medlin 1997; Rodriguez et al. 1999). The *in vitro* tests consist of an aqueous fluid, into which soils containing lead and arsenic are introduced. The solution then solubilizes the soil under simulated gastric conditions. Once this procedure is complete, the solution is analyzed for lead and/or arsenic concentration. The mass of lead and/or arsenic found in the aqueous phase, as defined by filtration at the 0.45-$\mu$m pore size, is compared to the mass introduced into the test. The fraction liberated into the aqueous phase is defined as the bioaccessible fraction of lead or arsenic in that soil. To date, for lead-bearing soils tested in the EPA swine studies, this *in vitro* method has correlated well with relative bioavailability values.

# 2. Procedure

## 2.1 Sample Preparation

All soil/material samples should be prepared for testing by oven drying (<40 °C) and sieving to <250 $\mu$m. The <250-$\mu$m size fraction is used because this particle size is representative of that which adheres to children's hands. Subsamples for testing in this procedure should be obtained using a sample splitter.

## 2.2 Apparatus and Materials

### 2.2.1 Equipment

The main piece of equipment required for this procedure consists of a Toxicity Characteristic Leaching Procedure (TCLP) extractor motor that has been modified to drive a flywheel. This flywheel in turn drives a Plexiglass block situated inside a temperature-controlled water bath. The Plexiglass block contains ten 5-cm holes with stainless steel screw clamps, each of which is designed to hold a 125-mL wide-mouth high-density polyethylene (HDPE) bottle (see Figure 1). The water bath must be filled such that the extraction bottles are immersed. Temperature in the water bath is maintained at 37±2 °C using an immersion circulator heater (for example, Fisher Scientific Model 730). Additional equipment for this method includes typical laboratory supplies and reagents, as described in the following sections.

The 125-mL HDPE bottles must have an air-tight screw-cap seal (for example, Fisher Scientific 125-mL wide-mouth HDPE Cat. No. 02-893-5C), and care must be taken to ensure that the bottles do not leak during the extraction procedure.

### 2.2.2 Standards and Reagents

The leaching procedure for this method uses a buffered extraction fluid at a pH of 1.5. The extraction fluid is prepared as described below.

The extraction fluid should be prepared using ASTM Type II deionized (DI) water. To 1.9 L of DI water, add 60.06 g glycine (free base, Sigma Ultra or equivalent). Place the mixture in a water bath at 37 °C until the extraction fluid reaches 37 °C. Standardize the pH meter using temperature compensation at 37 °C or buffers maintained at 37 °C in the water bath. Add concentrated hydrochloric acid (12.1 N, Trace Metal grade) until the solution pH reaches a value of 1.50 ±0.05 (approximately 120 mL). Bring the solution to a final volume of 2 L (0.4 M glycine).

Cleanliness of all reagents and equipment used to prepare and/or store the extraction fluid is essential. All glassware and equipment used to prepare standards and reagents must be properly cleaned, acid washed, and finally, rinsed with DI water prior to use. All reagents must be free of lead and arsenic, and the final fluid should be tested to confirm that lead and arsenic concentrations are less than 25 and 5 $\mu$g/L, respectively.

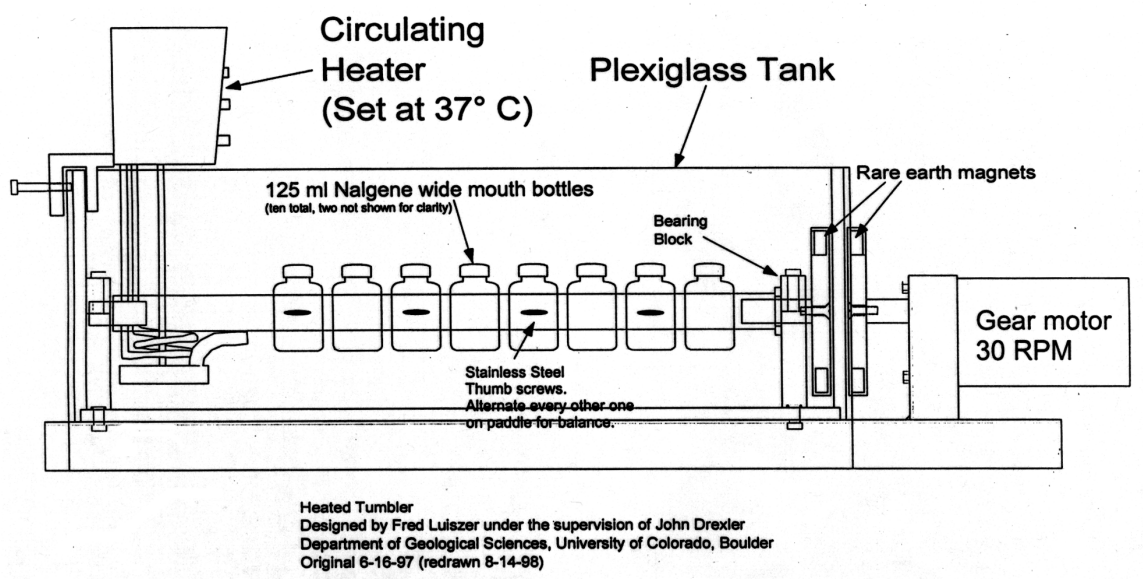


Figure 1. Extraction device for performing the SBRC *in vitro* extraction

## 2.3 Leaching Procedure

Measure 100 ±0.5 mL of the extraction fluid, using a graduated cylinder, and transfer to a 125-mL wide-mouth HDPE bottle. Add 1.00 ±0.05 g of test substrate (<250 $\mu$m) to the bottle, ensuring that static electricity does not cause soil particles to adhere to the lip or outside threads of the bottle. If necessary, use an antistatic brush to eliminate static electricity prior to adding the soil. Record the volume of solution and mass of soil added to the bottle on the extraction test checklist (see Attachment A for example checklists). Hand-tighten each bottle top, and shake/invert to ensure that no leakage occurs, and that no soil is caked on the bottom of the bottle.

Place the bottle into the modified TCLP extractor, making sure each bottle is secure and the lid(s) are tightly fastened. Fill the extractor with 125-mL bottles containing test materials or Quality Control samples.

The temperature of the water bath must be 37±2 °C. Record the temperature of the water bath at the beginning and end of each extraction batch on the appropriate extraction test checklist sheet (see Attachment A).

Rotate the extractor end over end at 30±2 rpm for 1 hour. Record start time of rotation.

When extraction (rotation) is complete, immediately remove bottles, wipe them dry, and place them upright on the bench top.

Draw extract directly from reaction vessel into a disposable 20-cc syringe with a Luer-Lok attachment. Attach a 0.45-$\mu$m cellulose acetate disk filter (25 mm diameter) to the syringe, and filter the extract into a clean 15-mL polypropylene centrifuge tube or other appropriate sample vial for analysis. Store filtered sample(s) in a refrigerator at 4 °C until they are analyzed.

Record the time that the extract is filtered (i.e., extraction is stopped). If the total elapsed time is greater than 1 hour 30 minutes, the test must be repeated.

Measure and record the pH of fluid remaining in the extraction bottle. If the fluid pH is not within ±0.5 pH units of the starting pH, the test must be discarded and the sample reanalyzed as follows.

If the pH has dropped by 0.5 or more pH units, the test will be re-run in an identical fashion. If the second test also results in a decrease in pH of greater than 0.5 s.u., the pH will be recorded, and the extract filtered for analysis. If the pH has increased by 0.5 or more units, the test must be repeated, but the extractor must be stopped at specific intervals and the pH manually adjusted down to pH 1.5 with dropwise addition of HCl (adjustments at 5, 10, 15, and 30 minutes into the extraction, and upon final removal from the water bath [60 minutes]). Samples with rising pH values must be run in a separate extraction, and must not be combined with samples being extracted by the standard method (continuous extraction).

Extracts are to be analyzed for lead and arsenic concentration using analytical procedures taken from the U.S. EPA publication, *Test Methods for Evaluating Solid Waste, Physical/Chemical Methods. SW-846*. (current revisions). Inductively coupled plasma (ICP) analysis, method 6010B (December 1996 revision) will be the method of choice. This method should be adequate for determination of lead concentrations in sample extracts, at a project-required detection limit (PRDL) of 100 $\mu$g/L. The PRDL of 20 $\mu$g/L for arsenic may be too low for ICP analysis for some samples. For extracts that have arsenic concentrations less than five times the PRDL (e.g., <100 $\mu$g/L arsenic), analysis by ICP-hydride generation (method 7061A, July 1992 revision) or ICP-MS (method 6020, September 1994 revision) will be required.

## 2.4 Calculation of the Bioaccessibility Value

A split of each solid material (<250 $\mu$m) that has been subjected to this extraction procedure should be analyzed for total lead and/or arsenic concentration using analytical procedures taken from the U.S. EPA publication, *Test Methods for Evaluating Solid Waste, Physical/Chemical Methods. SW-846*. (current revisions). The solid material should be acid digested according to method 3050A (July 1992 revision) or method 3051 (microwave-assisted digestion, September 1994 revision), and the digestate analyzed for lead and/or arsenic concentration by ICP analysis (method 6010B). For samples that have arsenic concentrations below ICP detection limits, analysis by ICP-hydride generation (method 7061A, July 1992 revision) or ICP-MS (method 6020, September 1994 revision) will be required.

The bioaccessibility of lead or arsenic is calculated in the following manner:

$$Bioaccessibility\ value = \frac{(concentration\ in\ in\ vitro\ extract,\ mg/L)\ (0.1L)}{(concentration\ in\ solid,\ mg/kg)\ (0.001\ kg)} \times 100$$

## 2.5 Chain-of-Custody/Good Laboratory Practices

All laboratories that use this SOP should receive test materials with chain-of-custody documentation. When materials are received, each laboratory will maintain and record custody of samples at all times. All laboratories that perform this procedure should follow good laboratory practices as defined in 40 CFR Part 792 to the extent practical and possible.

## 2.6 Data Handling and Verification

All sample and fluid preparation calculations and operations should be recorded in bound and numbered laboratory notebooks, and on extraction test checklist sheets. Each page must be dated and initialed by the person who performs any operations. Extraction and filtration times must be recorded, along with pH measurements, adjustments, and buffer preparation. Copies of the extraction test checklist sheets should accompany the data package.

# 3. Quality Control Procedures

## 3.1 Elements of Quality Assurance and Quality Control (QA/QC)

A standard method for the *in vitro* extraction of soils/solid materials, and the calculation of an associated bioaccessibility value, are specified above. Associated QC procedures to ensure production of high-quality data are as follows (see Table 1 for summary of QC procedures, frequency, and control limits):

- Reagent blank—Extraction fluid analyzed once per batch.
- Bottle blank—Extraction fluid only run through the complete extraction procedure at a frequency of no less than 1 per 20 samples or one per extraction batch, whichever is more frequent.
- Blank spikes—Extraction fluid spiked at 10 mg/L lead and/or 1 mg/L arsenic and run through the extraction procedure at a frequency of no less than every 20 samples or one per extraction batch, whichever is more frequent. Blank spikes should be prepared using traceable 1,000-mg/L lead and arsenic standards in 2 percent nitric acid.
- Duplicate—duplicate extractions are required at a frequency of 1 for every 10 samples. At least one duplicate must be performed on each day that extractions are conducted.
- Standard Reference Material (SRM)—National Institute of Standards and Technology (NIST) material 2711 (Montana Soil) should be used as a laboratory control sample (LCS).

Control limits for these QC samples are delineated in Table 1, and in the following discussion.

**Table 1. Summary of QC Samples, Frequency of Analysis, and Control Limits**

| QC Sample | Minimum Frequency of Analysis | Control Limits |
|---|---|---|
| Reagent Blank | Once per batch (min. 5%) | <25 $\mu$g/L lead<br><5 $\mu$g/L arsenic |
| Bottle Blank | Once per batch (min. 5%) | <50 $\mu$g/L lead<br><10 $\mu$g/L arsenic |
| Blank Spike | Once per batch (min. 5%) | 85–115% recovery |
| Duplicate | 10% | ±20% RPD |
| SRM (NIST 2711) | 2% | 9.22 ±1.50 mg/L Pb<br>0.59 ±0.09 mg/L As |

## 3.2 QA/QC Procedures

Specific laboratory procedures and QC steps are described in the analytical methods cited in Section 2.3, and should be followed when using this SOP.

### 3.2.1 Laboratory Control Sample (LCS)

The NIST SRM 2711 should be used as a laboratory control sample for the *in vitro* extraction procedure. Analysis of 18 blind splits of NIST SRM 2711 (105 mg/kg arsenic and 1,162 mg/kg lead) in four independent laboratories resulted in arithmetic means ± standard deviations of 9.22 ±1.50 mg/L lead and 0.59 ±0.09 mg/L arsenic. This SRM is available from the National Institute of Standards and Technology, Standard Reference Materials Program, Room 204, Building 202, Gaithersburg, Maryland 20899 (301/975-6776).

### 3.2.2 Reagent Blanks/Bottle Blanks/Blank Spikes

Reagent blanks must not contain more than 5 $\mu$g/L arsenic or 25 $\mu$g/L lead. Bottle blanks must not contain arsenic and/or lead concentrations greater than 10 and 50 $\mu$g/L, respectively. If either the reagent blank or a bottle blank exceeds these values, contamination of reagents, water, or equipment should be suspected. In this case, the laboratory must investigate possible sources of contamination and mitigate the problem before continuing with sample analysis. Blank spikes should be within 15% of their true value. If recovery of any blank spike is outside this range, possible errors in preparation, contamination, or instrument problems should be suspected. In the case of a blank spike outside specified limits, the problems must be investigated and corrected before continuing sample analysis.

# 4. References

Casteel, S.W., R.P. Cowart, C.P. Weis, G.M. Henningsen, E. Hoffman, et al. 1997a. Bioavailability of lead in soil from the Smuggler Mountain site of Aspen, Colorado. Fund. Appl. Toxicol. 36:177–187.

Casteel, S.W., L.D. Brown, M.E. Dunsmore, C.P. Weis, G.M. Henningsen, E. Hoffman, W.J. Brattin, and T.L. Hammon. 1997b. Relative bioavailability of arsenic in mining waste. U.S. Environmental Protection Agency, Region VIII, Denver, CO.

Imber, B.D. 1993. Development of a physiologically relevant extraction procedure. Prepared for BC Ministry of Environment, Lands and Parks, Environmental Protection Division, Victoria, BC. CB Research International Corporation, Sidney, BC.

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Rodriguez, R.R., N.T. Basta, S.W. Casteel, and L.W. Pace. 1999. An in vitro gastrointestinal method to estimate bioavailable arsenic in contaminated soils and solid media. Environ. Sci. Technol. 33(4):642–649.

Ruby, M.W., A. Davis, T.E. Link, R. Schoof, R.L. Chaney, G.B. Freeman, and P. Bergstrom. 1993. Development of an *in vitro* screening test to evaluate the *in vivo* bioaccessibility of ingested mine-waste lead. Environ. Sci. Technol. 27(13):2870–2877.

Ruby, M.W., A. Davis, R. Schoof, S. Eberle, and C.M. Sellstone. 1996. Estimation of lead and arsenic bioavailability using a physiologically based extraction test. Environ. Sci. Technol. 30(2):422–430.

Weis, C.P., and J.M. LaVelle. 1991. Characteristics to consider when choosing an animal model for the study of lead bioavailability. In: Proceedings of the International Symposium on the Bioavailability and Dietary Uptake of Lead. Sci. Technol. Let. 3:113–119.

Weis, C.P., R.H. Poppenga, B.J. Thacker, and G.M. Henningsen. 1994. Design of phamacokinetic and bioavailability studies of lead in an immature swine model. In: Lead in paint, soil, and dust: Health risks, exposure studies, control measures, measurement methods, and quality assurance, ASTM STP 1226, M.E. Beard and S.A. Iske (Eds.). American Society for Testing and Materials, Philadelphia, PA, 19103-1187.

# Attachment A:

# Extraction Test Checklist Sheets

# Extraction Fluid Preparation

Date of Extraction Fluid Preparation:_____ Prepared by:_____

Extraction Fluid Lot #:_____

| Component | Lot Number | Fluid Preparation 1L | Fluid Preparation 2L | Acceptance Range | Actual Quantity | Comments |
|---|---|---|---|---|---|---|
| Deionized Water | | 0.95 L (approx.) | 1.9 L (approx.) | --- | | |
| Glycine | | 30.03±0.05 g | 60.06±0.05g | --- | | |
| HCl [a] | | 60 mL (approx.) | 120 mL (approx.) | --- | | |
| Final Volume | --- | 1 L (Class A, vol.) | 2 L (Class A, vol.) | --- | | |
| Extraction Fluid pH value (@ 37°C) | --- | 1.50±0.05 | 1.50±0.05 | 1.45–1.55 | | |

[a] Concentrated hydrochloric acid (12.1 N)

Required Parameters:

Volume of extraction fluid (V) = 100 ±0.5 mL
Mass of test substrate (M) = 1.00 ±0.05 g
Temperature of water bath = 37 ±2 °C
Extraction time = 60 ±5 min

Extractor rotation speed = 30 ±2 rpm
Maximum elapsed time from extraction to filtration = 90 minutes
Maximum pH difference from start to finish (ΔpH)= 0.5 pH units
Spike solution concentrations: As = 1 mg/L; Pb = 10 mg/L

Date of Extraction:_____
Extraction Fluid Lot #:_____
Extracted by:_____

As Spike Solution Lot #:_____
Pb Spike Solution Lot #:_____

Extraction Log:

| Sample ID | Sample Preparation | | Extraction | | | | | | | | Filtration | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | V (mL) | M (g) | Start Time[a] | End Time[a] | Elapsed Time (min) | Start pH | End pH | ΔpH | Start Temp (°C) | End Temp (°C) | Time[a] | Time Elasped from extraction (min) |
| Acceptance Range | (95.5–100.5) | (0.95–1.05) | --- | --- | (55–65 min) | --- | --- | (Max = 0.5) | (35–39) | (35–39) | | (Max = 90 min) |
| Bottle Blank | | | | | | | | | | | | |
| Duplicate | | | | | | | | | | | | |
| Matrix spike | | | | | | | | | | | | |
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[a] 24-hour time scale

**Analytical Procedures**

QC Requirements:

| QC Sample | Minimum Analysis Frequency | Control Limits | Corrective Action[a] |
|---|---|---|---|
| Reagent blank | once per batch (min. 5%) | < 25 µg/L Pb <5 µg/L As | Investigate possible sources of target analytes. Mitigate contamination problem before continuing analysis. |
| Bottle blank | once per batch (min. 5%) | < 50 µg/L Pb <10 µg/L As | Investigate possible sources of target analytes. Mitigate contamination problem before continuing analysis. |
| Blank spike | once per batch (min. 5%) | 85–115% | Re-extract and reanalyze sample batch |
| Duplicate | 10% (min. once/day) | ±20% RPD | Re-homongenize, re-extract and reanalyze |

RPD – Relative percent difference
a – Action required if control limits are not met

# APPENDIX D

# IN VITRO BIOACCESSIBILITY TEST: STANDARD OPERATING PROCEDURES FOR SEQUENTIAL STOMACH AND SMALL INTESTINAL PHASE EXTRACTION

## *Appendix D*

# *In Vitro* Bioaccessibility Test: Standard Operating Procedures for Sequential Stomach and Small Intestinal Phase Extraction

*The in vitro extraction test presented in this appendix, 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 at the Department of Geological Sciences, University of Colorado at Boulder.*

The *in vitro* test is designed to determine the fraction of an inorganic element that is solubilized and available for absorption in the gastrointestinal tract. Development of the test, and the rationale for selection of representative parameters, are described in detail in the literature cited in Appendix C (In Vitro Method for Determination of Lead Bioaccessibility: Standard Operating Procedure for Stomach Phase Extraction). The *in vitro* method was designed to replicate gastrointestinal-tract parameters for a human child, including stomach and small-intestinal pH and chemistry, soil-to-solution ratio, stomach mixing, and stomach emptying rate. The method is implemented in two phases, simulating the passage of ingested soil from the acidic environment of the stomach to the near-neutral conditions of the small intestine.

The reaction is carried out in a sealed container (Figure 1), to minimize interactions between the reaction fluid and atmospheric oxygen, and the potential for cross contamination. Argon gas is introduced into the reaction vessel at the beginning of the *in vitro* assay to purge it of atmospheric oxygen to simulate the anoxic conditions present in the gastrointestinal tract.

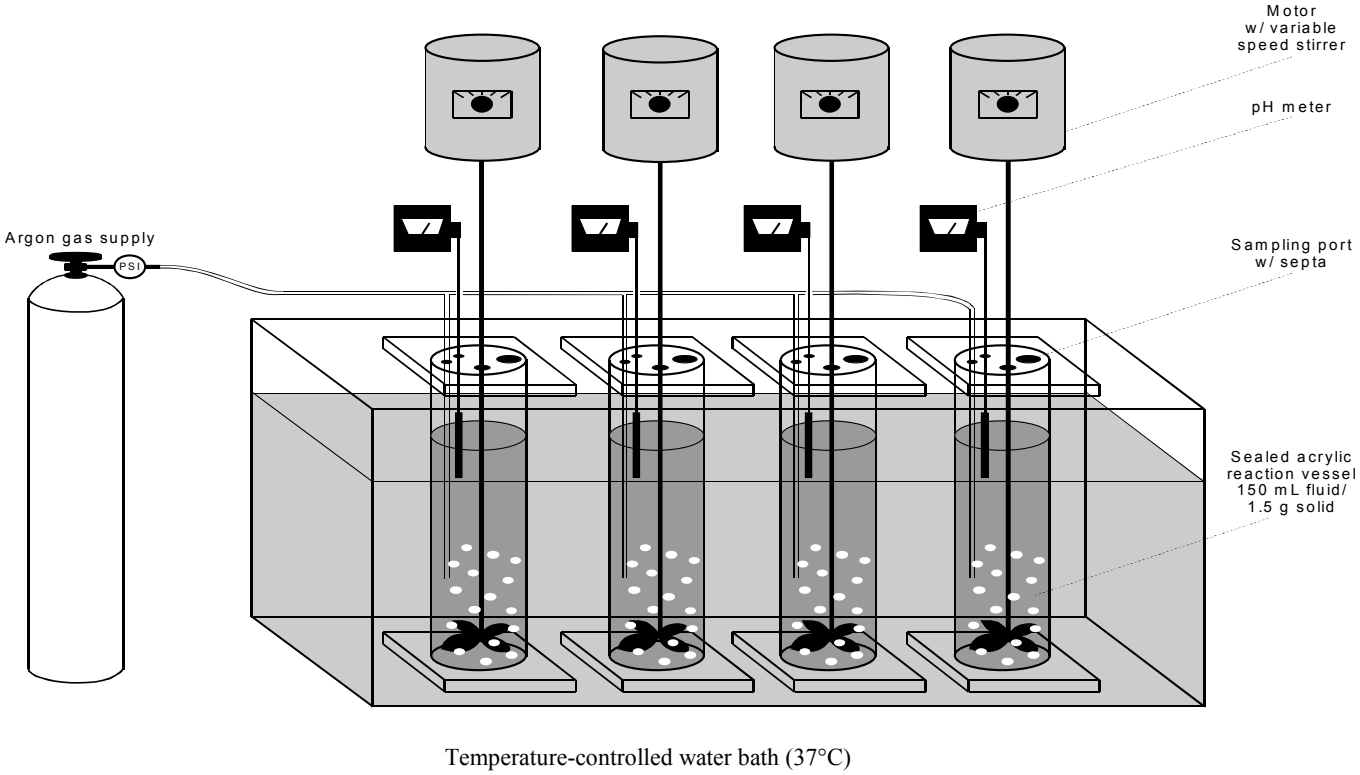


Figure 1. Schematic of *in vitro* experimental system.

The *in vitro* test is conducted according to the following method (all chemicals from Sigma Chemical Company, unless otherwise noted):

- Prepare the stomach solution by adding the following compounds to 1 L of deionized water (stirred continually on a stir plate)
  - 1.25 g pepsin (50 mg, activity of 800–2,500 units/mg)
  - 0.50 g citrate (Fisher Chemical Co.)
  - 0.50 g malate (Aldrich Chemical Co.)
  - 420 $\mu$L lactic acid (synthetic syrup 85 percent w/w)
  - 500 $\mu$L acetic acid (97 percent w/w; Fisher Chemical Co.).
- Adjust the pH of the stomach solution to 2.0 by adding a measured volume of concentrated HCl.
- Add 150 mL of stomach solution to the 200-mL acrylic reaction vessel.
- Sparge the stomach solution with argon for 10 minutes to remove oxygen.
- Add 1.5 g of soil and seal the reaction vessel.
- Submerge the reaction vessel approximately halfway into a temperature-controlled water bath heated to maintain a constant 37 °C in the reaction vessel.
- Allow the soil/stomach solution to stand (no agitation) for 10 minutes.
- Stir the mixture with a plastic propeller stir rod mounted in a rheostat-controlled motor (Arrow Engineering Model 1750 motor on a rheostat setting of 2, resulting in approximately 150 rpm for the stir rod).
- Check the pH at 5-minute intervals, and readjust to pH 2.0 with HCl if necessary.
- Collect 5-mL samples at 30 and 60 minutes, using a stainless-steel hypodermic syringe to pierce the sampling septum. Filter the samples through a 0.45-$\mu$m acetate syringe filter.
- At 1 hour, titrate the solution to pH 7.0 by adding a 5-in. length of dialysis tubing (8000 MWCO, cellulose ester tubing) containing approximately 2 g of $NaHCO_3$ to each reaction vessel.
- Allow the pH of the reaction vessel solution to increase slowly to 7.0 ±0.2 before removing the dialysis bag.
- Dissolve 260 mg of bile salts and 75 mg of pancreatin in 10 mL of deionized water and inject the fluid into the reaction vessel.
- Using a stainless-steel hypodermic syringe, obtain a 5-mL intestinal-phase sample through the septum at 1.0 hour after the reaction fluid reaches equilibrium at pH 7. Filter the sample through a 0.45-$\mu$m filter.
- At 3.0 hours after the reaction fluid reaches pH 7, end the test and collect a final 50-mL sample. Filter the sample through a 0.45-$\mu$m filter.

- After the final sample is collected, measure and record the pH and final volume of the flask contents.
- Preserve the 5-mL stomach-phase samples with 50 $\mu$L concentrated nitric acid.
- Refrigerate the samples, and ship on ice to the laboratory.
- Analyze each of the two stomach-phase and the two small-intestinal-phase samples for chromium and mercury concentrations, by the analytical method described in the work plan.

**Reference**

Ruby, M.V., A. Davis, R. Schoof, S. Eberle, and C.M. Sellstone. 1996. "Estimation of Lead and Arsenic Bioavailability Using a Physiologically Based Extraction Test." *Environ. Sci. Technol., 30*(2): 422-430.

# APPENDIX E

# TEMPLATE PROTOCOL FOR DETERMINATION OF THE BIOAVAILABILITY OF ARSENIC IN SOIL FOLLOWING ORAL ADMINISTRATION IN CYNOMOLGUS MONKEYS

**TEMPLATE PROTOCOL**
**for**
**DETERMINATION OF THE BIOAVAILABILITY OF ARSENIC IN SOIL FOLLOWING ORAL ADMINISTRATION IN CYNOMOLGUS MONKEYS**

## 1.0 PRINCIPALS

Sponsor: *(Specify)*

Sponsor's Project Monitor: *(Specify)*

1.1 Testing Facility: *(Specify)*

Study Director: *(Specify)*

## 2.0 OBJECTIVE

To determine the bioavailability of arsenic in cynomolgus monkeys following oral administration (via capsules) of a test soil containing arsenic. Absolute bioavailability will be determined by comparing the areas under the concentration time curves for the oral and intravenous routes of administration.

## 3.0 TEST/CONTROL ARTICLE INFORMATION

3.1 Test Substance Identification

The test substance for this study will be a composite test soil removed from several areas of test site. Characterization of the identity, concentration, stability and purity of the arsenic in the soil will be done prior to study.

A reference standard of the soil sample or, if such a sample is not available, then a sample from the test substance will be removed upon arrival at the testing laboratory and used as the reference sample. The reference sample will be stored in a Teflon-lined screw cap amber glass vial at approximately -20EC.

For the intravenous and gavage study group animals, sodium arsenate heptahydrate ($Na_2HAsO_4 \cdot 7H_2O$) will be used to administer the appropriate dose of arsenic.

3.2 Dose Analysis

The dosing solution for the intravenous and gavage administration groups will be analyzed for arsenic by graphite furnace atomic absorption spectroscopy or, if the concentration of

arsenic is sufficiently high, the analysis will be conducted by ICP-Atomic Emission Spectroscopy (AES). A sample of the dosing solution will be taken at the time of preparation. Triplicate aliquots of the dosing solution will be analyzed. The actual dosing solution concentration will not differ from the target concentration by more than ± 10 percent.

3.3 Particle Size Determination

For bioavailability studies, only the fraction of the soil sample in the <250-µm size range will be used. Therefore, this fraction will be separated from the remainder of the soil sample by performing a standard sieve analysis using the American Society for Testing and Materials (ASTM) No. 60 Sieve (60 openings/inch of surface area). Soil passing the No. 60 sieve corresponds to the fraction <250 µm. Sample preparation for sieve analysis should be performed in accordance with the procedure outlined in *Methods of Soil Analysis, Volume I: Physical and Mineralogical Analysis* (Gee and Bauder, 1986) or the ASTM Method D422-63 (Standard Test Method for Particle Size Analysis of Soils).

3.4 Dose Preparation

Gelatin capsules will be used to administer test substance (soil). Soil will be transferred into one-half of the gelatin capsule until the desired weight of soil to be placed into a single capsule has been achieved. The capsule will then be reassembled by attaching the other half of the capsule to the soil-filled capsule. Each capsule will be weighed and enough capsules will be prepared for each animal to achieve the desired amount of soil to be administered. The total weight of soil contained in the set of capsules will be within ± 5 percent of the targeted dose. After preparation, the capsules will be stored at room temperature until used.

The sodium arsenate to be used for dosing the intravenous and gavage study groups will be formulated into an aqueous solution. A sufficient quantity of sodium arsenate will be dissolved in deionized water (vehicle) to produce the target concentration of the dosing solution. The solution for intravenous and oral dosing will be prepared at a concentration such that the dosing volumes do not exceed 5 mL/kg. A single batch of sodium arsenate dosing solution will be prepared on one day which will be used to dose all of the intravenous and gavage study group animals.

3.5 Dose Administration

Capsules will be administered to the animals using a small animal capsule applicator. The dose will be based upon individual animal body weights which will be determined just prior to dosing (fasted body weights). On the day of dosing, the interval of time between each capsule administration for a given animal will be long enough to allow the animal to completely swallow each capsule and to minimize the possibility of expulsion of the soil. If this occurs with one of the monkeys, then a washout period will be required before the animal is dosed again.

For the intravenous study group animals, the dosing solution will be administered into the saphenous vein using a butterfly infusion set over approximately a one- to three-minute time period. For the gavage study group animals, the dosing solution will be administered through a rubber feeding tube that has been inserted into the stomach of the animal.

## 4.0 EXPERIMENTAL ANIMALS

### 4.1 Justification of Animal Species

The test systems selected for this study is cynomolgus monkey. This species has been successfully used to estimate the bioavailability of arsenic in humans.

### 4.2 Justification of the Route of Administration

The oral route of administration was selected because this is the most likely route of human exposure to the soil.

### 4.3 Test Systems

Male adult cynomolgus monkeys weighing approximately 3 to 9 kg will be used *(specify vendor here).*

### 4.4 Animal Health and Quarantine

All animals will be quarantined under environmental conditions according to the standard operating procedure of the testing facility. Each monkey will be examined and its health status determined by a laboratory animal veterinarian prior to being released for dosing. During quarantine, blood samples will be collected from all animals, on 3 specified dates for analysis of prestudy arsenic levels.

### 4.5 Animal Housing

Monkeys will be individually housed in stainless steel cages during quarantine period and transferred to metabolic cages during the study. All housing and care will conform to AAALAC and/or ILAR standards and those published in the "Guide for the Care and Use of Laboratory Animals," NIH Publication No. 85-23. The environmental conditions of the animal study room will conform to the standard operating procedures of the testing facility.

### 4.6 Diet and Water

During quarantine, monkeys will be fed *ad libitum* Primate® chow or equivalent, except when fasted prior to dosing. Animals will be fasted for approximately 16 hrs prior to dosing and food will be presented approximately four hrs after dosing. Feed will be analyzed at the testing laboratory. Triplicate samples of feed will be removed from the batch of feed used. The samples will be digested and single aliquots of the digestate removed for arsenic and phosphorous determinations.

Deionized water will be provided to animals *ad libitum* via water bottles fitted with stainless steel sipper tubes. During the in-life period, a sufficient volume of deionized water will be available so that all animals receive water from the same source. A sample of the water will be removed prior to the in-life phase and analyzed for arsenic. Triplicate aliquots of water will be analyzed.

4.7 Animal Identification

All of the animals will be uniquely identified by an indelible ink ear marking or tattoo. Also, each cage will be labeled with the number that corresponds with the ear marking or tattoo of the animal in the cage.

## 5.0 EXPERIMENTAL DESIGN

This study involves using two routes of administration (intravenous and oral) and two dosing formulations (aqueous solution and capsule) for characterizing the bioavailability of arsenic in soil following oral administration. Three monkeys will be used in each treatment group. The intravenous study group will be used to determine the extent of elimination of arsenic in bile.

On Day -1, animals should be weighed in order to determine doses to be used. On Study Day 1, animals will be given the soluble arsenic dosing solution by intravenous injection (intravenous group) or orally using a feeding tube (gavage group). The soil-filled capsules will be administered using a capsule applicator (test soil group). For all groups, samples of whole blood, urine, cage rinse, and feces will be collected from each animal at specified time intervals for 48 hrs (2 days) after administration. In addition, clinical observations will be determined daily for each animal. At the end of the 48 hr in-life period, animals will be removed from the study without additional collection of any biological samples for analysis.

The following table summarizes the study treatment groups:

| **Aqueous Solution (mg of As/kg BW)** | | **Capsule (mg of As/kg BW)** | | | **Number of Animals** |
|---|---|---|---|---|---|
| **IV** | **Oral** | **Low** | **Medium** | **High** | |
| 1.95 | 1.95 | 0.78 | 1.95 | 3.9 | 3 |

## 6.0 SAMPLE COLLECTION

6.1 Blood

Serial samples of whole blood (approximately 1 mL) will be collected from an appropriate blood vessel prior to dosing and at 2, 5, 10, 15, 30, and 60 min and 2, 4, 8, 12, 16, 24, and 48 hr after administration (intravenous dosing) and prior to dosing and at 15, 30, 45, 60, and 90 min and at 2, 4, 6, 8, 12, 16, 24, and 48 hr after administration (oral dosing). Whole blood will be collected into a heparinized container. Serial blood samples will be used to determine arsenic concentrations.
*[Note: Blood analyses are optional and may not be necessary for some studies.]*

6.2 Excreta

Urine and feces will be collected from each animal prior to dosing and thereafter at 24-hr intervals for 120 hrs. The 24-hr samples will be pooled to provide samples for analyses at 0-24, 24-72, and 72-120 hrs after dosing. The total volume (urine) and amount (feces) of each sample will be recorded. The collected samples will be frozen (approximately -20EC) after collection and kept frozen except during preparation for analysis. The pre-dose samples will be saved for possible analysis at a later date.

6.3 Cage Rinse

Each metabolism cage will be rinsed after each 24-hr urine and feces samples have been collected. Approximately 500 ml of deionized water will be used to remove any residual excreta that adheres to the surface of the metabolism cage. These cage rinse samples will be pooled to provide samples for analyses at 0-24, 24-72, and 72-120 hrs after dosing. The total volume of cage rinse used to wash the cage at each interval will be recorded. Cage rinse samples will be stored frozen (approximately -20EC) until removed for preparation.

## 7.0 SAMPLE PREPARATION/STORAGE

The intravenous and gavage dosing solutions will be sampled directly without any preparation prior to analysis. Whole blood, urine, and cage rinse specimens will be acidified using nitric acid to avoid precipitation of any arsenic present in the specimen. Feces samples will be weighed and homogenized in a volume of water equivalent to twice the wet weight of the collected sample to produce a uniform feces mixture.

Whole blood will be stored refrigerated at approximately 5EC until removed for analysis. All other biological samples, will be stored in their original collection container in a freezer at approximately -20EC until removed for preparation for analysis.

## 8.0 ANALYSIS OF SAMPLES

All samples will be analyzed for arsenic using graphite furnace atomic absorption spectrophotometry or ICP-AES if the concentration is sufficiently high to warrant this method. Single analyses of whole blood and duplicate analyses of all other biological samples (urine, cage rinse, and feces) will be conducted. Biological samples will be digested in acid, as necessary, prior to removal of an aliquot for arsenic analysis. When low concentrations of arsenic are found, a sample may have to be concentrated to measure the arsenic levels. Duplicate analyses will be averaged. An additional single analysis will be repeated if values from the original duplicate analyses are disparate (as a general rule, if the duplicate analyses differ by more than 20 percent of the mean) and the concentration is greater than 1 ppm. If the concentration is less than approximately 1 ppm, then the Study Director in conjunction with the Study Chemist will determine whether or not the disparate duplicates warrant additional analyses.

The following list summarizes the approximate numbers of samples per sample type that will be analyzed for arsenic. *[Specify number of samples for each.]*

I. Non-Biological Samples

A. Test Substance
- Intravenous Sodium Arsenate
- Oral Sodium Arsenate
- Oral Soil

B. Dosing Solution
C. Diet
D. Water

II. Biological Samples (for all groups)

A. Urine
B. Whole Blood (prestudy and study samples)
C. Feces
D. Cage Rinse

III. Quality Control Samples

## 9.0 STATISTICS

All individual raw data will be summarized and reported. Absolute bioavailability of arsenic in the soil will be determined in two ways: (a) as the percent of arsenic excreted in the urine of the capsule group animals compared to the intravenous group and (b) by comparing the areas under the plasma concentration time AUC curves for the oral and intravenous routes of administration.

In calculating the AUCs, values will be corrected for background (predose). The equation that will be used to calculate bioavailability values based on blood will be:

$$\frac{\text{AUC for oral treatment}}{\text{AUC for intravenous treatment}} \times \frac{\text{Total administered dose for intravenous treatment (mg/kg)}}{\text{Total administered dose for oral treatment (mg/kg)}} \times 100$$

Bioavailability values based on urine will be determined according to the following equation:

$$\frac{\text{Total amount of As in urine (}\mu\text{g) for oral group}}{\text{Total amount of As in urine (}\mu\text{g) for intravenous group}} \times \frac{\text{Total administered dose for intravenous group (mg/kg)}}{\text{Total administered dose for oral group (mg/kg)}}$$

Relative bioavailability ($Rf_x$) will be calculated using the following equation:

$$Rf_X = \frac{AUC_{X1}}{Dose_{X1}} \times \frac{Dose_{X2}}{AUC_{X2}}$$

where x represents routes of administration or exposure media.

## 10.0 RECORD AND SAMPLE RETENTION

### 10.1 Sample Retention

All samples will be frozen (approximately -20EC) and retained frozen until final report.

### 10.2 Records

All records that would be required to reconstruct the study and to demonstrate adherence to the protocol will be maintained at the test laboratory. The stipulations of this protocol will be implemented in conformance with EPA's Good Laboratory Practice Standards (40 CFR

Part 792). This study will be listed on the test laboratory's Master Study Schedule. All appropriate records will be maintained and will include, but not be limited to, the following:

- Quarantine and acclimation period information pertaining to daily housing and environmental conditions
- Animal body weights at randomization, animal identification, source of animal supply
- Test substance inventory, receipt, and storage conditions
- Original raw and reduced data on arsenic concentrations from all samples
- Dosing and sample collection times
- Food consumption, body weight, and clinical observation data
- Original raw and reduced data from test substance analysis upon receipt, dose preparation, and dose analysis
- Disposition of all samples, including chain-of-custody documentation for transfer of samples between laboratories
- A copy of the signed protocol
- All letters, memos, or notes that pertain to the study
- Original signed final report.

10.3 Report

A written draft final report of this study will be submitted to the Sponsor within (*specify*) days of the sacrifice date of the last animals.

## 11.0 REFERENCES

Gee, G.W., and J.W. Bauder. 1986. *Particle-Size Analysis*, in Methods of Soil Analysis, Volume I: Physical and Mineralogical Analysis. American Society of Agronomy, Inc., and Soil Science Society of America, Inc., Madison, WI. pp. 383-411.

# APPENDIX F

# TEMPLATE PROTOCOL FOR BIOAVAILABILITY STUDY OF ARSENIC AND LEAD IN SOIL FOLLOWING ORAL ADMINISTRATION USING JUVENILE SWINE

# TEMPLATE PROTOCOL
# for
# BIOAVAILABILITY STUDY OF ARSENIC AND LEAD IN SOIL FOLLOWING ORAL ADMINISTRATION USING JUVENILE SWINE

## 1.0 PRINCIPALS

Sponsor: *(Specify)*

Sponsor's Project Monitor: *(Specify)*

1.1 Testing Facility: *(Specify)*

Study Director: *(Specify)*

## 2.0 OBJECTIVES

The objectives of this study will be to use juvenile swine as a test system to determine the bioavailability of arsenic and lead in soil contaminated with arsenic and lead relative to the bioavailability of soluble forms of arsenic and lead. The relative bioavailability of arsenic will be determined based on urinary arsenic excretion after 15 days of daily dosing. The relative bioavailability of lead will be determined based on blood and tissue lead concentrations after 15 days of dosing. Relative bioavailability of arsenic and lead in soil will be estimated by comparison to data from swine dosed with sodium arsenate and lead acetate, respectively, for approximately 15 days.

*Note: This template protocol applies to sites where both arsenic and lead concentrations are elevated in soil. Elements of this protocol may be adapted to test only arsenic or only lead, if only one of these metals is of concern at a site.*

## 3.0 TEST SUBSTANCES

3.1 Test Substance Identification

The test substances for this study will be samples of soil collected from test sites. Before the study, soil arsenic and lead will be characterized and concentrations will be determined. If desired, mineral forms of arsenic and lead also will be determined. *(Note: Methods of determining the speciation of metals are discussed in the text of the main document.)*

For the soluble arsenic-dosed study group animals, sodium arsenate ($Na_2HAsO_4 \cdot 7H_2O$, MW 41.6) will be used to administer appropriate doses of water-soluble forms of arsenic. For the soluble lead-dosed study group animals, lead (II) acetate trihydrate ($[CH_3CO_2]_2Pb \cdot 3H_2O$, MW 379.33) will be used to administer appropriate doses of water-soluble forms of lead.

## 3.2 Test Substance Analysis

### 3.2.1 Concentration

The concentrations of arsenic and lead in the test substances will be determined. Analysis of the test substances will involve extracting arsenic and lead from a sample of each test substance, digesting the extracted material, and measuring the concentrations of arsenic and lead by inductively coupled plasma atomic emission spectroscopy (ICP-AES) or graphite furnace atomic absorption (GFAA). The concentrations of arsenic and lead in the soil will be determined by digesting triplicate aliquots of the sample and analyzing single aliquots of the digestate.

### 3.2.2 Moisture

The percent water in the test substance will be determined by measuring the moisture content. Moisture content will be determined by weighing and drying an approximately 5-g sample at 105-110°C for 2 hours, followed by reweighing. Percent moisture will be determined by calculating the difference between the pre-dried and dried samples.

### 3.2.3 Organic Matter

The organic matter in the test substance will be determined. Organic matter content will be determined using the method of loss-on-ignition at 430°C until sample reaches constant weight or has been heated for 24 hours (Davies, 1974).

### 3.2.4 Total Element Content

Total element content will be determined using EPA SW 846 Method 6010. This method will use ICP-AES for the determination of 25 elements after appropriate sample preparation.

### 3.2.5 pH

The pH of the soil will be determined using EPA SW 846 Method 9045. The soil will be mixed with either Type II water or a calcium chloride solution (depending on whether the soil is calcareous or noncalcareous), and the pH will be measured with a pH meter.

### 3.2.6 Particle Size Determination

For bioavailability studies, only the fraction of the soil sample in the <250-µm size range will be used. Therefore, this fraction will be separated from the remainder of the soil sample by performing a standard sieve analysis using the American Society

for Testing and Materials (ASTM) No. 60 Sieve (60 openings/inch of surface area). Soil passing the No. 60 sieve corresponds to the fraction <250 µm. Sample preparation for sieve analysis should be performed in accordance with the procedure outlined in *Methods of Soil Analysis, Volume I: Physical and Mineralogical Analysis* (Gee and Bauder, 1986) or the ASTM Method D422-63 (Standard Test Method for Particle Size Analysis of Soils).

## 3.3 Dosing Formulation Preparation

### 3.3.1 Soluble Arsenic and Lead Formulations

The appropriate amount of sodium arsenate or lead acetate stock solution is mixed with a 5g (± 1g) mass of moistened feed ("doughball"). The feed is a special low-lead variety (guaranteed less than 0.2 ppm lead by the manufacturer, Zeigler Brothers, Inc., Gardners, PA). Mixture with the doughball is achieved by placing the test material in a small depression in the doughball. After the stock solution has permeated into the doughball and no free liquid remains, the depression is filled by squeezing the dough ball in on itself, and the doughball is administered to the animal by hand feeding.

All animals in each dose group will receive the same volume of sodium arsenate and lead acetate stock solution, based on the mean body weight of all animals in the group. The precise dose to each animal will subsequently be calculated from the individual measured body weights. The volume of the stock solution placed in the dough balls of each dose group (twice each day) will be calculated using the following equation:

$$Vol = 0.5\left(\frac{MBW \times Dose}{Conc}\right)$$

where:

Vol = Volume of stock solution (µL)
MBW = Mean body weight (kg)
Dose = Target dose for the group (µg/kg-d)
Conc = Concentration of arsenic or lead in stock solution (µg/µL)

Three stock solutions of sodium arsenate will be prepared at concentrations that will result in target dose concentrations of 25, 50, and 125 µg/kg when a volume of stock solution between 20 µL and 100 µL is added to the doughball. The concentration of lead acetate stock solutions will be determined based on target doses of 25, 75, and 225 µg/kg.

### 3.3.2 Soil Formulation

The required mass of soil is placed in a small depression in a 5-g (± 1g) doughball. The depression is filled by squeezing the doughball in on itself, trapping the test material in the center. If the mass of soil required is too large to encapsulate into a

single doughball, the soil will be divided into approximately equal portions and placed in the minimum number of doughballs required to contain the soil.

All animals in each dose group will receive the same mass of test material, based on the mean body weight of all animals within the dose group. The precise dose to each animal will subsequently be calculated from the individual measured body weights. The mass of soil placed in the dough balls of each dose group (twice each day) will be calculated using the following equation:

$$\text{Mass} = \tfrac{1}{2}\left(\frac{\text{MBW} \times \text{Dose}}{\text{Conc}}\right)(1{,}000\,\mu\text{g/kg})$$

where:

Mass = Mass of soil (mg)
MBW = Mean body weight (kg)
Dose = Target dose for the group (µg/kg-d)
Conc = Concentration of arsenic or lead in soil (µg/g)

## 3.4 Dose Analysis

### 3.4.1 Dosed Feed Concentration and Stability

At least two extra dough balls (or sets of doughballs if more than one doughball is required to administer the soil) will be prepared for each dose "batch" (a "batch" is a group of doughballs sufficient for three days administration). After all doughballs in the batch are prepared, two will be selected at random, wrapped individually in plastic wrap, and placed together in a plastic bag labeled with the appropriate group/treatment identification number. All dose verification samples will be stored in the freezer until the end of the study. At the end of the study, at least 5% of verification samples will be randomly selected for analysis.

### 3.4.2 Test Article Homogeneity

It is expected that the bulk soil sample will be non-homogeneous with respect to particle size, and the concentration and form of lead and arsenic is expected to vary as a function of particle size. Therefore, it is important that the soil be well-mixed prior to removal of the dose aliquots. This is achieved by placing the bottle containing the bulk soil sample on a roller operating at low speed for about 30 minutes. After rolling, the bottle should be further mixed by inverting five times. It is important that vigorous methods of mixing not be used, since this might lead to a redistribution of the particle size distribution.

## 4.0 EXPERIMENTAL ANIMALS

### 4.1 Justification

The test system for this study will be male juvenile swine. Juvenile swine were selected for use in the study because the gastrointestinal physiology and overall size of young swine are similar to that of young children, who are the population of prime concern for exposure to metals in soil. Additionally, studies of the bioavailability of soil arsenic and lead have previously been conducted in swine. Young swine will be used because lead bioavailability decreases with age after weaning. Use of juveniles should maximize lead absorption. The oral route of administration was selected as the route of exposure since this is the most likely human route of exposure.

### 4.2 Test System

Intact male swine of a genetically defined line, approximately 5-6 weeks of age at initiation of dosing will be obtained from an appropriate vendor in sufficient numbers to provide the required number of healthy animals for testing (approximately 10% more than the number of animals to be tested). The target body weight at purchase will be 7-8 kg. The number of animals to be tested will be 50.

### 4.3 Animal Health and Quarantine

Animals well be held under quarantine to observe their health for one week before beginning exposure to test materials. Swine chosen for each investigation will be monitored throughout the investigation to identify any evidence of disease. The monitoring program will consist of the following elements:

- Daily observation by the Principal Investigator or designated assistant, with consultation as needed by a board-certified food-animal clinician. Observations for each animal will be recorded daily on a health-status chart attached to the cage of each animal. Observations will be generally similar to the "SOAP" (subjective, objective, analysis, plan) process. If any intervention is taken for an animal (e.g., administration of antibiotics), this action shall also be recorded on the chart for that animal.

- Any animal that dies during the study period will have a thorough post-mortem examination conducted to determine the cause of death. The post-mortem examination will include gross and histologic examinations and any ancillary tests, such as microbiology, deemed appropriate by the veterinary pathologist. All observations and findings will be recorded.

- Veterinary records from the swine producer and the producer's veterinarian, including documentation of health status, will be available if needed to assess overall swine herd health, history of vaccinations, and other veterinary data.

- Blood samples will be collected for clinical chemistry and hematological analysis on days –4, 7, and 15 to assist in clinical health assessments. Any animals that do not appear healthy or are not growing at the same rate as the other animals will be

excluded from the investigation. Animals judged to be seriously ill by the attending veterinarian will be removed from the study.

4.4 Animal Housing

Animals will be individually housed in lead-free, stainless steel, metabolic cages. Metabolic cages are designed to collect and separate urine and feces.

4.5 Diet and Water

Animals will be provided with 100% of their recommended daily food requirements. This will be achieved by supplying each animal with food equivalent to 5% of its body weight each day, in two equal portions at 11:00 AM and 5:00 PM. Since the animals are expected to grow significantly (0.3 to 0.8 kg/day) over the investigation period, the food portions must be constantly adjusted upward over time. Two samples of each batch of feed delivered will be analyzed prior to usage to confirm low lead and arsenic concentrations. A swine nutritionist will review the dietary composition. Feed will be purchased from Ziegler, Inc. (Gardners, PA) and detailed analysis of the composition will be provided with each lot purchased.

Food portions will be weighed every three days into disposable paper containers. The total number of portions weighed will be six times the number of animals in the study (two portions per day for each of three days). The mean body weight at each three-day interval will be used to calculate food intake for the following three days, adjusted by expected weight gain between weighings. Specifically, the twice daily food portion will be calculated as follows:

On the day of the weighing,

$$Portion\,(g) = (\tfrac{1}{2})(0.05)(body\ weight\ in\ kg)(1{,}000\,\tfrac{g}{kg})$$

On the day after the weighing, the portion size will increase by 1 kg. On the next day the portion size will increase by 1 kg again.

Water will be provided to animals *ad libitum* via a pipe and nozzle which is activated by the animal. Laboratory technicians will check each day to ensure that all water delivery nozzles are functioning properly. The water source will be a municipal drinking water system. One water sample will be drawn at random from a drinking water nozzle once per week during the study and analyzed for lead and arsenic.

4.6 Animal Identification

All of the animals will be uniquely identified by ear tag and cage card. Each cage will be labeled with the number that corresponds with the ear marking of the animal in the cage.

4.7 Randomization

Animals will be randomly assigned to treatment groups by the following method:

- A list of animals will be prepared by ear tag number order.

- Random numbers will be generated by a computer and these numbers assigned to each animal's ear tag number.
- Animals will be sorted sequentially by assigned random number.
- The first five animals will be assigned to group 1, the next to group 2, etc…
- Animals will be sorted sequentially within assigned group by ear tag number.

## 5.0 EXPERIMENTAL DESIGN

This study involves subchronic oral administration of one soil arsenic source and one soil lead source mixed with diet as a means of characterizing the oral bioavailability of arsenic and lead in soil relative to soluble arsenic and lead. A non-treated group will serve as a control for determining background arsenic and lead levels. Five animals will be used in each treatment group. Specifically, the following groups will be studied.

| Group | Number of Animals | Dose Material Administered | Target Dose (µg/kg-d) |
|---|---|---|---|
| 1 | 5 | Control | 0 |
| 2 | 5 | Lead Acetate | Pb Dose 1 |
| 3 | 5 | Lead Acetate | Pb Dose 2 |
| 4 | 5 | Lead Acetate | Pb Dose 3 |
| 5 | 5 | Sodium Arsenate | As Dose 1 |
| 6 | 5 | Sodium Arsenate | As Dose 2 |
| 7 | 5 | Sodium Arsenate | As Dose 3 |
| 8 | 5 | Soil with As&Pb | As/Pb Dose 1 |
| 9 | 5 | Soil with As&Pb | As/Pb Dose 2 |
| 10 | 5 | Soil with As&Pb | As/Pb Dose 3 |

Doses will be administered in two equal portions given at 9:00 AM and 3:00 PM each day. Doses will be based on the mean weight of the animals in each group, and will be adjusted every three days to account for weight gain.

*The main text describes appropriate target doses for arsenic and lead in the respective sections addressing each metal. It may not be possible to achieve targets for both metals in one soil sample.*

During the in-life phase, clinical observation, food consumption and body weight determinations will be made as described below.

5.1 Clinical Observations

Animals will be examined daily as described in Section 4.3. Clinical observations of possible signs of toxicity will be recorded.

5.2 Food Consumption

Food portions will be calculated based on the mean body weight of all animals in the study. Any fraction of food or doughball not eaten will be recorded and dosage will be corrected to reflect actual intake after completion of the study.

5.3 Body Weights

Animals will be weighed every three days beginning at day –1 of the study. Animals will also be weighed on the day of sacrifice. All body weights will be recorded in the laboratory log book to the nearest 0.1 kg.

5.4 Tissue Collection

Blood samples will be collected at 8:00 AM from each animal four days prior to exposure (day –4), on the first day of exposure (day 0), and on days 1, 2, 3, 5, 7, 9, 12, and 15. All blood samples will be collected into purple-top Vacutainer tubes containing EDTA by vena-puncture of the anterior vena cava. Following euthanization on day 15, samples of liver, kidney, and bone (the right femur) will be removed and stored in lead-free plastic bags for lead analysis. Samples of all biological samples collected will be archived to allow for later reanalysis and verification, if necessary.

Urine and feces samples (48 hour composites) will be collected from each animal on days 6-7, 8-9, and 10-11 of the study, beginning at either 9:00 or 10:00 AM on the first day of the collection period. Urine will be collected by placing a stainless steel pan beneath each cage that drains into a plastic storage bottle. Each collection pan will be fitted with a nylon screen to minimize contamination with feces, spilled food, or other debris. Plastic diverters will be used to minimize urine dilution with drinking water spilled by the animals from the watering nozzle into the collection pan. During the collection period, urine will be removed from the collection pans at least twice daily and stored in a separate container for each animal.

## 6.0 SAMPLE PREPARATION

6.1 Blood

One mL of whole blood is removed from the Vacutainer and added to 9 mL of "matrix modifier", a solution recommended by the Centers for Disease Control and Prevention (CDCP) for analysis of blood samples for lead. The composition of matrix modifier is 0.2% (v/v) ultrapure nitric acid, 0.5% (v/v) Triton X-100, and 0.2% (w/v) dibasic ammonium phosphate in deionized and ultrafiltered water. Samples of the matrix modifier will be analyzed for lead to ensure the absence of lead contamination.

6.2 Liver and kidney

One gram of tissue is placed in a lead-free screw-cap Teflon container with 2 mL of concentrated nitric acid and heated in an oven to 90°C overnight. After cooling, the digestate is transferred to a clean lead-free 10 mL volumetric flask and diluted to volume with deionized and ultrafiltered water.

6.3 Bone

The right femur of each animal is removed, defleshed, and dried overnight at 100°C. The dried bones are dry-ashed in a muffle furnace at 450°C for 48 hours. Following dry ashing, the bone is ground to a fine powder using lead-free mortar and pestle, and 200 mg is removed and dissolved in 10 mL of 1:1 (v:v) concentrated nitric acid:water. After the powdered bone is dissolved and mixed, 1 m of the acid solution is removed and diluted to 10 mL by addition of 0.1% (w/v) lanthanum oxide ($La_2O_3$) in deionized and ultrafiltered water.

6.4 Urine

The 48-hour urine samples will be mixed by swirling in the collection vessels and the volume measured in a graduated cylinder. Three 60-mL aliquots of urine will be retrieved from the samples, placed in capped plastic urine storage bottles and acidified by addition of 0.6 mL of concentrated nitric acid. Two bottles will be archived in the refrigerator, the last sent to the laboratory for analysis.

6.5 Feces

*(Note: It may be advantageous to collect feces and analyze samples for arsenic concentrations. Sample preparation methods would need to be developed.)*

## 7.0 ANALYSIS OF SAMPLES

All urine, blood, tissue, and bone samples will be analyzed for arsenic (urine) or lead (blood, tissue and bone) by graphite furnace atomic absorption spectroscopy (GFAA). Internal quality assurance samples will be run every tenth sample, and the instrument recalibrated every 15th sample. A blank, dupicate and spiked sample will be run every 20th sample.

## 8.0 STATISTICS

All individual raw data will be summarized and reported as the mean and standard deviation for each dose group. The relative bioavailability (RBA) of lead will be calculated for each dose group based on the blood lead results. The following method will be used to calculate an RBA:

1. Plot the biological responses of individual animals exposed to a series of doses of lead acetate. This is done by first calculating the area under the curve (AUC) of the blood lead vs. time response for each animal. Then calculate the dose group mean and standard error

for each dose group. Plot group mean blood lead AUC vs. dose. Fit an equation that gives a smooth line through the observed data points.

2. Plot the biological responses of individual animals exposed to a series of doses of lead in the test material in the same way as for lead acetate. Fit an equation that gives a smooth line through the observed data.

3. Using the best fit equations for lead acetate and the test material, calculate RBA as the ratios of doses of test material and reference material which yield equal biological responses. Depending on the relative shape of the best-fit lines through the lead acetate and test material dose response curves, RBA may either be constant (dose-independent) or variable (dose-dependent). If both curves are linear, RBA equals the ratio of slopes of the test material curve to the lead acetate curve.

The amount of arsenic absorbed will be evaluated by measuring the amount of arsenic which was excreted in urine, the Urinary Excretion Fraction (UEF). UEF is estimated by plotting mass recovered in urine per 48 hours divided by the amount given per 48 hours. The RBA equals the ratio of the test material UEF to the sodium arsenate UEF:

$$RBA = UEF_{test} / UEF_{NaAs}$$

## 9.0 RECORD AND SAMPLE RETENTION

### 9.1 Sample Retention

All samples will be frozen (approximately -20°C) and retained at the test laboratory or its designated facility until acceptance of the final report. At that time, all samples will be disposed of or shipped to the Sponsor as directed by the Sponsor.

### 9.2 Records

All records that would be required to reconstruct the study and to demonstrate adherence to the protocol will be maintained at the test laboratory. The stipulations of this protocol will be implemented in conformance with EPA's Good Laboratory Practice Standards (40 CFR Part 792). This study will be listed on the test laboratory's Master Study Schedule. All appropriate records will be maintained and will include, but not be limited to, the following:

- Quarantine and acclimation period information pertaining to daily housing and environmental conditions
- Animal body weights at randomization, animal identification, source of animal supply
- Test substance inventory, receipt, and storage conditions
- Original raw and reduced data on lead concentrations from all samples
- Dosing and sample collection times

- Food consumption, body weight, and clinical observation data
- Original raw and reduced data from test substance analysis upon receipt, dose preparation, and dose analysis
- Disposition of all samples, including chain-of-custody documentation for transfer of samples between laboratories
- A copy of the signed protocol
- All letters, memos, or notes that pertain to the study
- Original signed final report.

9.3 Report

A written draft final report of this study will be submitted to the Sponsor within a mutually agreed upon timeframe following completion of the dosing experiment.

## 11.0 REFERENCES

Davies, B.W. 1974. Loss-on-ignition as an estimate of soil organic matter. *Soil Sci. Soc. Am. Proc.*, **38**: 150.

Gee, G.W., and J.W. Bauder. 1986. *Particle-Size Analysis*, in Methods of Soil Analysis, Volume I: Physical and Mineralogical Analysis. American Society of Agronomy, Inc., and Soil Science Society of America, Inc., Madison, WI. pp. 383-411.

# APPENDIX G

# TEMPLATE PROTOCOL FOR BIOAVAILABILITY STUDY OF CADMIUM IN SOIL FOLLOWING ORAL ADMINISTRATION USING SPRAGUE-DAWLEY RATS

# TEMPLATE PROTOCOL
# for
# BIOAVAILABILITY STUDY OF CADMIUM IN SOIL FOLLOWING ORAL ADMINISTRATION USING SPRAGUE-DAWLEY RATS

## 1.0 PRINCIPALS

Sponsor: *(Specify)*

Sponsor's Project Monitor: *(Specify)*

1.1 Testing Facility: *(Specify)*

Study Director: *(Specify)*

## 2.0 OBJECTIVE

The objective of this study will be to use blood cadmium concentrations to determine the relative bioavailability of orally administered cadmium (Cd) in Sprague-Dawley rats. Relative bio-availability of cadmium in soil will be estimated by comparison to data from rats administered a single oral (gavage) dose of cadmium chloride.

## 3.0 TEST SUBSTANCES

3.1 Test Substance Identification

The test substances for this study will be capsules of soil samples collected from test sites. Soil cadmium will be characterized, and concentration, stability and purity determined before the study.

An appropriate dose of $CdCl_2$ solution will be used as a reference standard for comparison with the test soil group.

## 3.2 Test Substance Analysis

### 3.2.1 Concentration

The concentrations of cadmium in the test substances will be determined. Analysis of the test substances will involve extracting cadmium from a sample of each test substance, digesting the extracted material, and measuring the concentrations of cadmium by inductively coupled plasma atomic emission spectroscopy (ICP-AES) or graphite furnace atomic absorption (GFAA). The concentrations of cadmium in the soil will be determined by digesting duplicate aliquots of the sample and analyzing single aliquots of the digestate. No attempt to identify the different chemical forms of cadmium will be conducted at the test laboratory.

### 3.2.2 Moisture

The percent water in the test substance will be determined by measuring the moisture content. Moisture content will be determined by weighing and drying an approximately 5-g sample at 105-110°C for 2 hours, followed by reweighing. Percent moisture will be determined by calculating the difference between the pre-dried and dried samples.

### 3.2.3 Organic Matter

The organic matter in the test substance will be determined. Organic matter content will be determined using the method of loss-on-ignition at 430°C until sample reaches constant weight or has been heated for 24 hours (Davies, 1974).

### 3.2.4 Total Element Content

Total element content will be determined using EPA SW 846 Method 6010. This method will use ICP-AES for the determination of 25 elements after appropriate sample preparation.

### 3.2.5 pH

The pH of the soil will be determined using EPA SW 846 Method 9045. The soil will be mixed with either Type II water or a calcium chloride solution (depending on whether the soil is calcareous or noncalcareous), and the pH will be measured with a pH meter.

### 3.2.6 Particle Size Determination

For bioavailability studies, only the fraction of the soil sample in the <250-µm size range will be used. Therefore, this fraction will be separated from the remainder of the soil sample by performing a standard sieve analysis using the American Society for Testing and Materials (ASTM) No. 60 Sieve (60 openings/inch of surface area). Soil passing the No. 60 sieve corresponds to the fraction <250 µm. Sample preparation for sieve analysis should be performed in accordance with the procedure outlined in *Methods of Soil Analysis, Volume I: Physical and Mineralogical Analysis* (Gee and Bauder, 1986) or the ASTM Method D422-63 (Standard Test Method for Particle Size Analysis of Soils).

### 3.3 Dose Preparation

Gelatin capsules will be used to administer test substance (soil). Soil will be transferred into one-half of the gelatin capsule until the desired weight of soil to be placed into a single capsule has been achieved. The capsule will then be reassembled by attaching the other half of the capsule to the soil-filled capsule. Each capsule will be weighed and enough capsules will be prepared for each animal to achieve the desired amount of soil to be administered. The total weight of soil contained in the set of capsules will be within 5 percent of the targeted dose. After preparation, the capsules will be stored at room temperature until used.

The cadmium chloride reference standard to be used for dosing the gavage study groups will be formulated into an aqueous solution. A sufficient quantity of cadmium chloride will be dissolved in deionized water (vehicle) to produce the target concentration of the dosing solution. The solution for oral dosing will be prepared at a concentration such that the dosing volumes do not exceed 5 mL/kg.

### 3.4 Dose Analysis

The dosing solution for the gavage administration groups will be analyzed for cadmium by graphite furnace atomic absorption spectroscopy or, if the concentration of cadmium is sufficiently high, the analysis will be conducted by ICP-Atomic Emission Spectroscopy (AES). A sample of the dosing solution will be taken at the time of preparation. Duplicate aliquots of the dosing solution will be analyzed. The actual dosing solution concentration will not differ from the target concentration by more than $\pm$10 percent.

### 3.5 Dose Administration

Capsules will be administered to the animals using a small animal capsule applicator. The dose will be based upon individual animal body weights which will be determined just prior to dosing (fasted body weights).

For the gavage study group animals, the dosing solution will be administered using a stainless steel gavage needle.

## 4.0 EXPERIMENTAL ANIMALS

### 4.1 Justification

The test system for this study will be Sprague-Dawley (Cd/BR) rats. The rat was selected as the test system because it is recognized by EPA for use in chemical safety evaluation testing and a large historical database exists for this animal. Additionally, studies of the bioavailability of soil cadmium have previously been conducted in rats.

### 4.2 Test System

Male Sprague-Dawley (Cd/BR) rats, 8 weeks of age at initiation of dosing will be obtained from a licensed USDA vendor in sufficient numbers to provide the required number of healthy animals for testing. (*Specify the number of animals here*)

4.3 Animal Health and Quarantine

All animals will be quarantined under environmental conditions simulating those of the study. Each animal will be examined and its health status determined by a laboratory animal veterinarian within 48 hours of receipt. Upon arrival, the rats will be given complete AIN-93G™ diet. At the end of a 48-hour quarantine period, animals of questionable health (as determined by a laboratory animal veterinarian) will be excluded from the study.

4.4 Animal Housing

Animals will be individually housed in polycarbonate cages. All housing and care will conform to ILAR standards and those published in the *Guide for the Care and Use of Laboratory Animals*, NIH Publication No. 86-23. The environmental conditions of the animal study room will conform to the following specifications:

- The light/dark cycle will be set for 12 hours of light and 12 hours of dark each day during the study.
- The room temperature will be 67-77°F and will be recorded twice daily. At least 90 percent of the total recordings will fall within the specified range.
- The relative humidity will be 40-70 percent and will be recorded twice daily. At least 90 percent of the total recordings will be within the specified range.
- Fresh air will be supplied to the room at sufficient rate to provide a minimum of 10 changes of room air per hour.

4.5 Diet and Water

AIN-93G™ meal feed (Zeigler Brothers, Gardners, PA) will be used for this study. Feed will be provided *ad libitum* in glass jars. Feed will be withheld from animals for 16 hours prior to oral dosing. Two hours after dosing, the animals can be allowed free access to food. Analysis of each feed lot will be provided by the vendor. Also, prior to dosing, feed will be analyzed for cadmium, calcium, magnesium, iron, zinc, and phosphorous.

Water will be provided to animals *ad libitum* via glass bottle reservoirs fitted with stainless-steel sipper tubes. Animals will be given deionized water. During the in-life period, a sufficient volume of deionized water will be available so that all animals receive water from the same source. A sample of the water will be removed at the start and after testing, which will be analyzed for cadmium, calcium, magnesium, iron, zinc, and phosphorous.

4.6 Animal Identification

All of the animals will be uniquely identified by ear tag and cage card. Each cage will be labeled with the number that corresponds with the ear marking of the animal in the cage.

4.7 Randomization

Rats will be randomly assigned to treatment groups on the basis of body weights. Body weights will be determined on Day -3. Randomization will be performed using a computer program that places animals in groups to ensure homogeneous group mean body weights at study onset.

## 5.0 EXPERIMENTAL DESIGN

This study involves oral administration of two cadmium sources (soluble cadmium chloride and cadmium in soil), as a means of characterizing the oral bioavailability of cadmium in soil relative to soluble cadmium. A non-treated group will serve as a control for determining background cadmium levels. On Day -3, rats will be weighed in order to determine doses to be used. On Study Day 1, rats will be given the cadmium chloride solution by gavage using a stainless steel gavage needle. The soil-filled capsules will be administered using a capsule applicator (test soil group). Control group will receive orally administered saline only. For all groups, samples of whole blood will be collected from each animal at specified intervals for 6 days after administration of test article or control saline.

The following table summarizes the treatment groups.

| Group | Treatment | Number of Animals[a] |
|---|---|---|
| 1 | Control | 32[b] |
| 2 | $CaCl_2$ | 32[b] |
| 3 | Soil | 32[b] |

[a] One animal may be subjected to as many as 3 bleeds. (Example: one animal may be bled at 10 min, 24 hr and 72 hr.)
[b] Suggested number of animals.

During the in-life phase, clinical observation, food consumption and body weight determinations will be made as described below.

5.1 Blood Collection

Serial samples of whole blood (approximately 1 mL) will be collected by orbital puncture under $CO_2$ anesthesia. Heparinized blood samples will be collected at 0, 10, 20, 30, 60, 120, 240 and 480 min on the first day and at 24, 48, 72, 96, 120 hr and stored at -20°C until analysis.

5.2 Clinical Observations

Clinical observations of any possible signs of toxicity will be done twice daily. Cage checks will be made once a day for moribundity and mortality.

5.3 Food Consumption

Food consumption will be determined once a day at approximately the same time each day for each rat. Known amounts of feed will be provided in cage feeders and at the conclusion of the approximately 24-hour interval, feeders will be reweighed. The net difference

between the original and final feeder weight will serve as a measure of the feed consumed. Procedures for evaluating feed spillage will be specified.

5.4 Body Weights

Body weights will be taken on all rats at the start of the study (Day 1), weekly thereafter, and at termination (Day 6).

## 6.0 SAMPLE PREPARATION

Blood will be digested in concentrated nitric acid. One or more internal standards may be added, and the digestate diluted for analysis.

## 7.0 ANALYSIS OF SAMPLES

All samples will be analyzed for cadmium by GFAA, ICP-AES, or ICP-MS. Single analysis of whole blood will be conducted. Blood samples will be digested in acid, as necessary, prior to removal of the aliquot for cadmium analysis.

Quality control (QC) samples will be analyzed at the beginning and end of each daily analysis. Quality control samples for cadmium in blood will be certified blood standards obtained from a specified commercial laboratory. These will be digested and analyzed along with study samples. Recovery of cadmium from QC samples will be considered adequate if they are within 25 percent of the certified or prepared value.

The following table summarizes the approximate numbers of samples per sample type that will be analyzed for cadmium: *[Specify number of samples for each.]*

| | |
|---|---|
| *I.* | *Non-Biological Samples* |
| | A. Test substance |
| | B. Diet |
| | C. Water |

| | |
|---|---|
| *II.* | *Blood Samples* |
| | Treatment Group: |
| | A. Untreated control |
| | B. Cd-saline |
| | C. Capsule-Cd |

| | |
|---|---|
| *III.* | *Quality Control Samples* |

## 8.0 STATISTICS

All individual raw data will be summarized and reported as the mean and standard deviation. Relative bioavailability ratio will be determined using the following equation:

$$F_{rel} = (AUC_{soil} / AUC_{pure}) \times \frac{\text{dose pure}}{\text{dose soil}}$$

where $AUC_{soil}$ is area under the curve for the capsule-Cd group and $AUC_{pure}$ is the area under the curve for the Cd-saline group.

## 9.0 RECORD AND SAMPLE RETENTION

### 9.1 Sample Retention

All samples will be frozen (approximately -20°C) and retained at the test laboratory or its designated facility until acceptance of the final report. At that time, all samples will be disposed of or shipped to the Sponsor as directed by the Sponsor.

### 9.2 Records

All records that would be required to reconstruct the study and to demonstrate adherence to the protocol will be maintained at the test laboratory. The stipulations of this protocol will be implemented in conformance with EPA's Good Laboratory Practice Standards (40 CFR Part 792). This study will be listed on the test laboratory's Master Study Schedule. All appropriate records will be maintained and will include, but not be limited to, the following:

- Quarantine and acclimation period information pertaining to daily housing and environmental conditions
- Animal body weights at randomization, animal identification, source of animal supply
- Test substance inventory, receipt, and storage conditions
- Original raw and reduced data on cadmium concentrations from all samples
- Dosing and sample collection times
- Food consumption, body weight, and clinical observation data
- Original raw and reduced data from test substance analysis upon receipt, dose preparation, and dose analysis
- Disposition of all samples, including chain-of-custody documentation for transfer of samples between laboratories
- A copy of the signed protocol

- All letters, memos, or notes that pertain to the study
- Original signed final report.

9.3 Report

A written draft final report of this study will be submitted to the Sponsor within __ days *(specify)* of the sacrifice date of the last animals.

## 10.0 REFERENCES

Davies, B.W. 1974. Loss-on-ignition as an estimate of soil organic matter. *Soil Sci. Soc. Am. Proc.*, **38**: 150.

Gee, G.W., and J.W. Bauder. 1986. *Particle-Size Analysis*, in Methods of Soil Analysis, Volume I: Physical and Mineralogical Analysis. American Society of Agronomy, Inc., and Soil Science Society of America, Inc., Madison, WI. pp. 383-411.

# APPENDIX H

## TEMPLATE PROTOCOL FOR PILOT STUDY: BIOAVAILABILITY OF CHROMIUM IN SOIL FOLLOWING ORAL ADMINISTRATION USING SPRAGUE-DAWLEY RATS

*(Note: Because no reliable study design has been developed for assessing bioavailability of chromium in soil, any planned study should begin with a pilot study using a small number of animals.)*

**TEMPLATE PROTOCOL FOR**
**PILOT STUDY:**

**BIOAVAILABILITY OF CHROMIUM IN SOIL FOLLOWING ORAL ADMINISTRATION USING SPRAGUE-DAWLEY RATS**

## 1.0 PRINCIPALS

Sponsor: *(Specify)*

Sponsor's Project Monitor: *(Specify)*

1.1 Testing Facility: *(Specify)*

Study Director: *(Specify)*

## 2.0 OBJECTIVE

Several objectives will be accomplished in this study: a) To determine the half-life of chromium in weanling rats; b) To determine time to reach chromium peak plasma concentration in the weanling rat; c) Estimate the relative bioavailability of chromium in soil.

## 3.0 TEST SUBSTANCES

3.1 Test Substance Identification

The test substances for this study will be capsules of soil samples collected from test sites. Soil chromium will be characterized, and concentration, stability and purity determined before the study.

A mixture of chromium oxide and potassium chromate in the same proportions as $Cr^{+3}$ + $Cr^{+6}$ in soil will be used as the reference standard.

## 3.2 Test Substance Analysis

### 3.2.1 Concentration

The concentrations of chromium in the test substances will be determined. Analysis of the test substances will involve extracting chromium from a sample of each test substance, digesting the extracted material, and measuring the concentrations of chromium by inductively coupled plasma atomic emission spectroscopy (ICP-AES) or graphite furnace atomic absorption (GFAA). The concentrations of chromium in the soil will be determined by digesting duplicate aliquots of the sample and analyzing single aliquots of the digestate. No attempt to identify the different chemical forms of chromium will be conducted at the test laboratory.

### 3.2.2 Moisture

The percent water in the test substance will be determined by measuring the moisture content. Moisture content will be determined by weighing and drying an approximately 5-g sample at 105-110°C for 2 hours, followed by reweighing. Percent moisture will be determined by calculating the difference between the pre-dried and dried samples.

### 3.2.3 Organic Matter

The organic matter in the test substance will be determined. Organic matter content will be determined using the method of loss-on-ignition at 430°C until sample reaches constant weight or has been heated for 24 hours (Davies, 1974).

### 3.2.4 Total Element Content

Total element content will be determined using EPA SW 846 Method 6010. This method will use ICP-AES for the determination of 25 elements after appropriate sample preparation.

### 3.2.5 pH

The pH of the soil will be determined using EPA SW 846 Method 9045. The soil will be mixed with either Type II water or a calcium chloride solution (depending on whether the soil is calcareous or noncalcareous), and the pH will be measured with a pH meter.

### 3.2.6 Particle Size Determination

For bioavailability studies, only the fraction of the soil sample in the <250-µm size range will be used. Therefore, this fraction will be separated from the remainder of the soil sample by performing a standard sieve analysis using the American Society for Testing and Materials (ASTM) No. 60 Sieve (60 openings/inch of surface area). Soil passing the No. 60 sieve corresponds to the fraction <250-µm. Sample preparation for sieve analysis should be performed in accordance with the procedure outlined in *Methods of Soil Analysis, Volume I: Physical and Mineralogical Analysis* (Gee and Bauder, 1986) or the ASTM Method D422-63 (Standard Test Method for Particle Size Analysis of Soils).

Particle size distribution of the test soil will be determined using an electrozone sensor in an Elzone® 280PC System (Particle Data Laboratories, Elmhurst, Illinois) or similar instrument. Briefly, particles will be suspended in an electrolyte solution and drawn through an orifice in which a constant current has been established. As the particle traverses the orifice, it will displace a quantity of suspended electrolyte proportional to the volume of the particle. The resulting change in electrical resistance across the orifice will create a voltage pulse. These pulses will be amplified, scaled, and counted. From these data, particle size distributions will be generated.

3.3 Dose Preparation

Gelatin capsules will be used to administer the test substance (soil). Soil will be transferred into one-half of the gelatin capsule until the desired weight of soil to be placed into a single capsule has been achieved. The capsule will then be reassembled by attaching the other half of the capsule to the soil-filled capsule. Each capsule will be weighed and enough capsules will be prepared for each animal to achieve the desired amount of soil to be administered. The total weight of soil contained in the set of capsules will be within 5 percent of the targeted dose. After preparation, the capsules will be stored at room temperature until used.

The reference standard will be formulated as an aqueous solution and will be administered by gavage to the animals. A sufficient quantity of a mixture of chromium oxide and potassium chromate will be dissolved in deionized water (vehicle) to produce the target concentration of the dosing solution. The solution for oral dosing will be prepared at a concentration such that the dosing volumes do not exceed 5 mL/kg.

3.5 Dose Analysis

The dosing solution for the gavage administration groups will be analyzed for chromium by graphite furnace atomic absorption spectroscopy or, if the concentration of chromium is sufficiently high, the analysis will be conducted by ICP-Atomic Emission Spectroscopy (AES). A sample of the dosing solution will be taken at the time of preparation. Duplicate aliquots of the dosing solution will be analyzed. The actual dosing solution concentration will not differ from the target concentration by more than $\pm$10 percent.

3.6 Dose Administration

Capsules will be administered to the animals using a small animal capsule applicator. The dose will be based upon individual animal body weights which will be determined just prior to dosing (fasted body weights).

For the gavage study group animals, the dosing solution will be administered using a stainless steel gavage needle.

## 4.0 EXPERIMENTAL ANIMALS

### 4.1 Justification

The test system for this study will be weanling Sprague-Dawley (Cd/BR) rats. The rat was selected as the test system because it is recognized by EPA for use in chemical safety evaluation testing and a large historical database exists for this animal. Additionally, studies of the chromium uptake and distribution have previously been conducted in rats.

### 4.2 Test System

Male Sprague-Dawley (Cd/BR) rats, 4 weeks of age at initiation of dosing will be obtained from a licensed USDA vendor in sufficient numbers to provide the required number of healthy animals for testing. A total of 24 rats will be used for this study.

### 4.3 Animal Health and Quarantine

All animals will be quarantined under environmental conditions simulating those of the study. Each animal will be examined and its health status determined by a laboratory animal veterinarian within 48 hours of receipt. Upon arrival, the rats will be given complete AIN-93G™ diet. At the end of a 48-hour quarantine period, animals of questionable health (as determined by a laboratory animal veterinarian) will be excluded from the study.

### 4.4 Animal Housing

Animals will be individually housed in polycarbonate cages. All housing and care will conform to ILAR standards and those published in the *Guide for the Care and Use of Laboratory Animals*, NIH Publication No. 86-23. The environmental conditions of the animal study room will conform to the following specifications:

- The light/dark cycle will be set for 12 hours of light and 12 hours of dark each day during the study.
- The room temperature will be 67-77°F and will be recorded twice daily. At least 90 percent of the total recordings will fall within the specified range.
- The relative humidity will be 40-70 percent and will be recorded twice daily. At least 90 percent of the total recordings will be within the specified range.
- Fresh air will be supplied to the room at sufficient rate to provide a minimum of 10 changes of room air per hour.

### 4.5 Diet and Water

AIN-93G™ meal feed (Zeigler Brothers, Gardners, PA) will be used for this study. Feed will be provided *ad libitum* in glass jars. Feed will be withheld from animals for 16 hours prior to oral dosing. Analysis of each feed lot will be provided by the vendor. Also, prior to dosing, feed will be analyzed for chromium, calcium, magnesium, iron, zinc, and phosphorous.

Water will be provided to animals *ad libitum* via glass bottle reservoirs fitted with stainless-steel sipper tubes. Animals will be given deionized water. During the in-life period, a sufficient volume of deionized water will be available so that all animals receive water from the same source. A sample of the water will be removed at the start and after testing, which will be analyzed for chromium, calcium, magnesium, iron, zinc, and phosphorous.

4.6 Animal Identification

All of the animals will be uniquely identified by ear tag and cage card. Each cage will be labeled with the number that corresponds with the ear marking of the animal in the cage.

4.7 Randomization

Rats will be randomly assigned to treatment groups on the basis of body weights. Body weights will be determined on Day -3. Randomization will be performed using a computer program that places animals in groups to ensure homogeneous group mean body weights at study onset.

## 5.0 EXPERIMENTAL DESIGN

This study involves oral administration of two chromium sources [a mixture of potassium chromate and chromium oxide (the reference standard) and chromium in soil], as a means of characterizing the oral bioavailability of chromium in soil relative to soluble chromium. On Day -3, rats will be weighed in order to determine doses to be used. Rats will be given the potassium chromate/chromium oxide solution by gavage using a stainless steel gavage needle for 35 days. The soil-filled capsules will be administered using a capsule applicator (test soil group). For all groups, samples of whole blood will be collected from each animal at specified intervals for approximately 2 days after the last day of administration of test article or reference standard.

The following table summarizes the treatment groups.

| Group | Treatment | Number of Animals[a] |
|---|---|---|
| 1 | Reference Standard | 12 |
| 2 | Soil | 12 |

[a] One animal may be subjected to as many as 2 bleeds. (Example: one animal may be bled at 0 hr and 24 hr.)

During the in-life phase, clinical observation, food consumption and body weight determinations will be made as described below.

5.1 Blood Collection

Serial samples of whole blood (approximately 1 mL) will be collected by orbital puncture under $CO_2$ anesthesia. Heparinized blood samples will be collected at 2, 4, 8, 16, and 24 hr post-test article administration and stored at -20°C until analysis.

Other tissues that can be collected and saved for later analysis include liver, kidney, spleen and bone (femur).

5.2 Clinical Observations

Clinical observations of any possible signs of toxicity will be done during the study period.

5.3 Food Consumption

No food consumption estimates will be made.

5.4 Body Weights

Body weights will be taken on all rats at the start of the study.

## 6.0 SAMPLE PREPARATION

Blood will be digested in concentrated nitric acid. One or more internal standards may be added, and the digestate diluted for analysis.

## 7.0 ANALYSIS OF SAMPLES

All samples will be analyzed for chromium by GFAA, ICP-AES, or ICP-MS. Single analysis of whole blood will be conducted. Blood samples will be digested in acid, as necessary, prior to removal of the aliquot for chromium analysis.

Quality control samples will be analyzed at the beginning and end of each daily analysis. Quality control samples for chromium in blood will be certified blood standards obtained from a specified commercial laboratory. These will be digested and analyzed along with study samples. Recovery of chromium from QC samples will be considered adequate if they are within 25 percent of the certified or prepared value.

The following table summarizes the approximate numbers of samples per sample type that will be analyzed for chromium: *(Specify number of samples for each)*

| | |
|---|---|
| *I.* | *Non-Biological Samples* |
| | A. Test substance |
| | B. Diet |
| | C. Water |

| | |
|---|---|
| *II.* | *Blood Samples* |
| | Treatment Group |
| | A. Reference standard |
| | B. Chromium (soil-filled capsule) |

*III. Quality Control Samples*

## 8.0 STATISTICS

All individual raw data will be summarized and reported as the mean and standard deviation. Relative bioavailability ratio will be determined using the following equation:

$$Rf = \frac{C_{max\ (soluble\ chromium)} - BKG_{Dose\ (soluble\ chromium)}}{C_{max\ (soil\ chromium)} - BKG_{Dose\ (soil\ chromium)}}$$

where BKG = overall background value from feed and water analysis and $C_{max}$ = peak chromium concentration.

## 9.0 RECORD AND SAMPLE RETENTION

### 9.1 Sample Retention

All samples will be frozen (approximately -20°C) and retained at the test laboratory or its designated facility until acceptance of the final report. At that time, all samples will be disposed of or shipped to the Sponsor as directed by the Sponsor.

### 9.2 Records

All records that would be required to reconstruct the study and to demonstrate adherence to the protocol will be maintained at the test laboratory. The stipulations of this protocol will be implemented in conformance with EPA's Good Laboratory Practice Standards (40 CFR Part 792). This study will be listed on the test laboratory's Master Study Schedule. All appropriate records will be maintained and will include, but not be limited to, the following:

- Quarantine and acclimation period information pertaining to daily housing and environmental conditions
- Animal body weights at randomization, animal identification, source of animal supply
- Test substance inventory, receipt, and storage conditions
- Original raw and reduced data on chromium concentrations from all samples
- Dosing and sample collection times
- Food consumption, body weight, and clinical observation data
- Original raw and reduced data from test substance analysis upon receipt, dose preparation, and dose analysis

- Disposition of all samples, including chain-of-custody documentation for transfer of samples between laboratories
- A copy of the signed protocol
- All letters, memos, or notes that pertain to the study
- Original signed final report.

9.3 Report

A written draft final report of this study will be submitted to the Sponsor within __ days *(specify days)* of the sacrifice date of the last animals.

## 10.0 REFERENCES

Davies, B.W. 1974. Loss-on-ignition as an estimate of soil organic matter. *Soil Sci. Soc. Am. Proc.*, **38**: 150.

Gee, G.W., and J.W. Bauder. 1986. *Particle-Size Analysis*, in Methods of Soil Analysis, Volume I: Physical and Mineralogical Analysis. American Society of Agronomy, Inc., and Soil Science Society of America, Inc., Madison, WI. pp. 383-411.

# APPENDIX I

# TEMPLATE PROTOCOL FOR BIOAVAILABILITY OF LEAD IN SOIL FOLLOWING ORAL ADMINISTRATION USING WEANLING SPRAGUE-DAWLEY RATS

# TEMPLATE PROTOCOL
# for
# BIOAVAILABILITY STUDY OF LEAD IN SOIL FOLLOWING ORAL ADMINISTRATION USING WEANLING SPRAGUE-DAWLEY RATS

## 1.0 PRINCIPALS

Sponsor: *(Specify)*

Sponsor's Project Monitor: *(Specify)*

1.1 Testing Facility: *(Specify)*

Study Director: *(Specify)*

## 2.0 OBJECTIVES

The objectives of this study will be to use blood lead concentrations to determine the bioavailability of lead in weanling Sprague-Dawley rats when administered gelatin capsules containing lead-contaminated soil for approximately 48 days. Relative bioavailability of lead in soil will be estimated by comparison to data from rats administered lead acetate in capsules for approximately 48 days.

## 3.0 TEST SUBSTANCES

3.1 Test Substance Identification

The test substances for this study will be samples of soil collected from test sites. Soil lead will be characterized, and concentration, stability and purity determined before the study.

Reference standard consists of soluble lead (II) acetate trihydrate ($[CH_3CO_2]_2Pb{\cdot}3H_2O$) administrated in gelatin capsules. A non-treated group will be used as a control for determining background lead levels.

## 3.2 Test Substance Analysis

### 3.2.1 Concentration

The concentrations of lead in the test substances will be determined. Analysis of the test substances will involve extracting lead from a sample of each test substance, digesting the extracted material, and measuring the concentrations of lead by inductively coupled plasma atomic emission spectroscopy (ICP-AES) or graphite furnace atomic absorption (GFAA). The concentrations of lead in the soil will be determined by digesting duplicate aliquots of the sample and analyzing single aliquots of the digestate. No attempt to identify the different chemical forms of lead will be conducted at the test laboratory.

### 3.2.2 Moisture

The percent water in the test substance will be determined by measuring the moisture content. Moisture content will be determined by weighing and drying an approximately 5-g sample at 105-110°C for 2 hours, followed by reweighing. Percent moisture will be determined by calculating the difference between the pre-dried and dried samples.

### 3.2.3 Organic Matter

The organic matter in the test substance will be determined. Organic matter content will be determined using the method of loss-on-ignition at 430°C until sample reaches constant weight or has been heated for 24 hours (Davies, 1974).

### 3.2.4 Total Element Content

Total element content will be determined using EPA SW 846 Method 6010. This method will use ICP-AES for the determination of 25 elements after appropriate sample preparation.

### 3.2.5 pH

The pH of the soil will be determined using EPA SW 846 Method 9045. The soil will be mixed with either Type II water or a calcium chloride solution (depending on whether the soil is calcareous or noncalcareous), and the pH will be measured with a pH meter.

### 3.2.6 Particle Size Determination

For bioavailability studies, only the fraction of the soil sample in the <250-µm size range will be used. Therefore, this fraction will be separated from the remainder of the soil sample by performing a standard sieve analysis using the American Society for Testing and Materials (ASTM) No. 60 Sieve (60 openings/inch of surface area). Soil passing the No. 60 sieve corresponds to the fraction <250 µm. Sample preparation for sieve analysis should be performed in accordance with the procedure outlined in *Methods of Soil Analysis, Volume I: Physical and Mineralogical Analysis* (Gee and Bauder, 1986) or the ASTM Method D422-63 (Standard Test Method for Particle Size Analysis of Soils).

### 3.3 Dose Preparation

Gelatin capsules will be used to administer test substance (soil). Soil will be transferred into one-half of the gelatin capsule until the desired weight of soil to be placed into a single capsule has been achieved. The capsule will then be reassembled by attaching the other half of the capsule to the soil-filled capsule. Each capsule will be weighed and enough capsules will be prepared for each animal to achieve the desired amount of soil to be administered. The total weight of soil contained in the set of capsules will be within 5 percent of the targeted dose. After preparation, the capsules will be stored at room temperature until used.

A specified amount of the reference standard will be transferred into gelatin capsules and assembled as described above.

### 3.4 Dose Administration

Capsules will be administered to the animals using a small animal capsule applicator. The dose will be based upon individual animal body weights, which will be determined just prior to dosing (fasted body weights).

## 4.0 EXPERIMENTAL ANIMALS

### 4.1 Justification

The test system for this study will be weanling Sprague-Dawley (CD/BR) rats. The rat was selected as the test system because it is recognized by EPA for use in chemical safety evaluation testing and a large historical database exists for this animal. Additionally, studies of the bioavailability of soil lead have previously been conducted in rats. Weanlings (approximately 4 weeks old) will be used because lead bioavailability decreases with age after weaning. Use of weanlings should maximize lead absorption. The oral route of administration was selected as the route of exposure since this is the most likely human route of exposure.

### 4.2 Test System

Male and female Sprague-Dawley (CD/BR) rats, approximately 4 weeks of age at initiation of dosing will be obtained from a licensed USDA vendor in sufficient numbers to provide the required number of healthy animals for testing. The number of animals on test will be 40 males and 40 females.

### 4.3 Animal Health and Quarantine

All animals will be quarantined under environmental conditions simulating those of the study. Each animal will be examined and its health status determined by a laboratory animal veterinarian within 48 hours of receipt. Upon arrival, the rats will be given complete AIN-93G™ diet. At the end of a 48-hour quarantine period, animals of questionable health (as determined by a laboratory animal veterinarian) will be excluded from the study.

## 4.4 Animal Housing

Animals will be individually housed in polycarbonate cages. All housing and care will conform to ILAR standards and those published in the *Guide for the Care and Use of Laboratory Animals*, NIH Publication No. 86-23. The environmental conditions of the animal study room will conform to the following specifications:

- The light/dark cycle will be set for 12 hours of light and 12 hours of dark each day during the study.
- The room temperature will be 64-79°F and will be recorded twice daily. At least 90 percent of the total recordings will fall within the specified range.
- The relative humidity will be 40-70 percent and will be recorded twice daily. At least 90 percent of the total recordings will be within the specified range.
- Fresh air will be supplied to the room at sufficient rate to provide a minimum of 10 changes of room air per hour.

## 4.5 Diet and Water

AIN-93G™ meal feed (Zeigler Brothers, Gardners, PA) will be used for this study. The complete AIN-93G™ meal feed will be refrigerated at approximately 4°C and will have expiration dates of approximately 4 months after milling. Feed will be provided *ad libitum* in glass jars. Feeders with fresh feed will be provided at least biweekly. Analysis of each feed lot will be provided by the vendor. Also, prior to dosing, feed will be analyzed at a designated laboratory for lead, calcium, magnesium, iron, zinc, and phosphorous.

Water will be provided to animals *ad libitum* via glass bottle reservoirs fitted with stainless-steel sipper tubes. Animals will be given deionized water. During the in-life period, a sufficient volume of deionized water will be available so that all animals receive water from the same source. A sample of the water will be removed at the start and after testing, which will be analyzed for lead, calcium, magnesium, iron, zinc, and phosphorous.

## 4.6 Animal Identification

All of the animals will be uniquely identified by ear tag and cage card. Each cage will be labeled with the number that corresponds with the ear marking of the animal in the cage.

## 4.7 Randomization

Rats will be randomly assigned to treatment groups on the basis of body weights. Body weights will be determined the day before dosing. Randomization will be performed using a computer program that places animals in groups to ensure similar group mean body weights at study onset.

## 5.0 EXPERIMENTAL DESIGN

This study involves subchronic daily administration of lead (soluble lead acetate) and test soil (soil lead) administered in gelatin capsules, as a means of characterizing the relative bioavailability of lead. A non-treated group will serve as a control for determining background lead levels. Forty rats per sex will be used in each treatment group. Animals will be administered test soil or soluble level for 48 days.

| Treatment Group | Dose Levels[a] | |
|---|---|---|
| | Pb (µg) | mg Pb/kg BW |
| Untreated (control) | NA | NA |
| Soluble lead in capsule | Low | TBD |
| | Medium | TBD |
| | High | TBD |
| Test soil in capsule | Low | TBD |
| | Medium | TBD |
| | High | TBD |

[a] The estimated doses of lead will be based on estimated soil lead concentrations.
NA = Not applicable.
TBD = To be determined.

During the in-life phase, clinical observation, food consumption and body weight determinations will be made as described below.

### 5.1 Clinical Observations

Clinical observations of any possible signs of toxicity will be recorded. Otherwise, cage checks will be made once a day for moribundity and mortality.

### 5.2 Food Consumption

Food consumption will be determined weekly for each rat. Known amounts of feed will be provided in cage feeders, and at the conclusion of the approximately 7-day interval, feeders will be reweighed. The net difference between the original and final feeder weight will serve as a measure of the feed consumed. Procedures for evaluating feed spillage will be specified.

### 5.3 Body Weights

Body weights will be taken on all rats at the start of the study (Day 1), weekly thereafter, and at termination.

### 5.4 Tissue Collection

At termination, and prior to cessation of heart contractions following an injection of sodium pentobarbital, a whole blood sample will be collected from each rat by cardiac puncture. The blood will be transferred to an appropriate container and stored frozen approximately (-20°C) until prepared for analysis. The kidneys will be removed and stored in polyethylene tissue bags or liquid scintillation vials with plastic tops at approximately

-20°C until removed for preparation and analysis. In addition, the femur and liver will be removed and stored in polyethylene tissue bags or scintillation vials at approximately -20°C until removed for possible preparation and analysis at a later date if needed. A wet weight of the tissues upon removal will be collected. The residual carcass will be saved and stored frozen for possible additional tissue sample collection and analysis. Samples will be shipped to *(specify name of test facility)* for analysis.

## 6.0 SAMPLE PREPARATION

Blood will be digested in concentrated nitric acid. One or more internal standards may be added, and the digestate diluted for analysis.

## 7.0 ANALYSIS OF SAMPLES

All samples will be analyzed for lead by GFAA, ICP-AES, or ICP-MS. Single analysis of whole blood will be conducted. Blood samples will be digested in acid, as necessary, prior to removal of the aliquot for lead analysis.

Quality control samples will be analyzed at the beginning and end of each daily analysis. Quality control samples for lead in blood will be certified blood standards obtained from a specified commercial laboratory. These will be digested and analyzed along with study samples. Recovery of lead from QC samples will be considered adequate if they are within 25 percent of the certified or prepared value.

The following table summarizes the approximate numbers of samples per sample type that will be analyzed for lead: *(Specify number of samples for each.)*

| | |
|---|---|
| *I.* | *Non-Biological Samples* |
| | A. Test substance (soil lead) |
| | B. Soluble lead |
| | C. Diet |
| | D. Water |

| | |
|---|---|
| *II.* | *Biological Samples* |
| | Treatment Group: |
| | A. Untreated control |
| | B. Soluble lead |
| | C. Test soil |

| | |
|---|---|
| *III.* | *Quality Control Samples* |

## 8.0 STATISTICS

All individual raw data will be summarized and reported as the mean and standard deviation.

Relative bioavailability can be calculated by the following equation:

$$Rf = \frac{C_{min\ (soluble\ lead)} - BKG_{Dose\ (soluble\ lead)}}{C_{min\ (soil\ lead)} - BKG_{Dose\ (soil\ lead)}}$$

where: $C_{min}$ = mean minimum concentration of lead
BKG = overall background value from feed and water analysis.

## 9.0 RECORD AND SAMPLE RETENTION

### 9.1 Sample Retention

All samples will be frozen (approximately -20°C) and retained at the test laboratory or its designated facility until acceptance of the final report. At that time, all samples will be disposed of or shipped to the Sponsor as directed by the Sponsor.

### 9.2 Records

All records that would be required to reconstruct the study and to demonstrate adherence to the protocol will be maintained at the test laboratory. The stipulations of this protocol will be implemented in conformance with EPA's Good Laboratory Practice Standards (40 CFR Part 792). This study will be listed on the test laboratory's Master Study Schedule. All appropriate records will be maintained and will include, but not be limited to, the following:

- Quarantine and acclimation period information pertaining to daily housing and environmental conditions
- Animal body weights at randomization, animal identification, source of animal supply
- Test substance inventory, receipt, and storage conditions
- Original raw and reduced data on lead concentrations from all samples
- Dosing and sample collection times
- Food consumption, body weight, and clinical observation data
- Original raw and reduced data from test substance analysis upon receipt, dose preparation, and dose analysis
- Disposition of all samples, including chain-of-custody documentation for transfer of samples between laboratories

- A copy of the signed protocol
- All letters, memos, or notes that pertain to the study
- Original signed final report.

9.3 Report

A written draft final report of this study will be submitted to the Sponsor within days (specify) of the sacrifice date of the last animals.

## 10.0 REFERENCES

Davies, B.W. 1974. Loss-on-ignition as an estimate of soil organic matter. *Soil Sci. Soc. Am. Proc.*, **38**: 150.

Gee, G.W., and J.W. Bauder. 1986. *Particle-Size Analysis*, in Methods of Soil Analysis, Volume I: Physical and Mineralogical Analysis. American Society of Agronomy, Inc., and Soil Science Society of America, Inc., Madison, WI. pp. 383-411.

# APPENDIX J

# TEMPLATE PROTOCOL FOR BIOAVAILABILITY STUDY OF MERCURY IN SOIL FOLLOWING DOSED FEED ADMINISTRATION USING WEANLING SPRAGUE-DAWLEY RATS

**TEMPLATE PROTOCOL**
**for**
**BIOAVAILABILITY STUDY OF MERCURY IN SOIL FOLLOWING DOSED FEED ADMINISTRATION USING WEANLING SPRAGUE-DAWLEY RATS**

## 1.0 PRINCIPALS

Sponsor: *(Specify)*

Sponsor's Project Monitor: *(Specify)*

1.1 Testing Facility: *(Specify)*

Study Director: *(Specify)*

## 2.0 OBJECTIVES

The objectives of this mercury study will be to use blood mercury concentrations to determine the relative bioavailability of mercury in weanling Sprague-Dawley rats when fed soil contaminated with mercury for approximately 30 days. Relative bioavailability of mercury in soil will be estimated by comparison to data from rats fed mercuric chloride-dosed feed for approximately 70 days.

## 3.0 TEST SUBSTANCES

3.1 Test Substance Identification

The test substances for this study will be samples of soil collected from test sites. Soil mercury will be characterized and concentration, stability and purity determined before the study.

For the soluble mercury-dosed feed group, $HgCl_2$ will be used to administer appropriate doses of water-soluble forms of mercury.

3.2 Test Substance Analysis

3.2.1 Concentration

The concentrations of mercury in the test substances will be determined. Analysis of the test substances will involve extracting mercury from a sample of each test substance, digesting the extracted material, and measuring the concentrations of mercury

by inductively coupled plasma atomic emission spectroscopy (ICP-AES) or graphite furnace atomic absorption (GFAA). The concentrations of mercury in the soil will be determined by digesting duplicate aliquots of the sample and analyzing single aliquots of the digestate. No attempt to identify the different chemical forms of mercury will be conducted at the test laboratory.

3.2.2 Moisture

The percent water in the test substance will be determined by measuring the moisture content. Moisture content will be determined by weighing and drying an approximately 5-g sample at 105-110°C for 2 hours, followed by reweighing. Percent moisture will be determined by calculating the difference between the pre-dried and dried samples.

3.2.3 Organic Matter

The organic matter in the test substance will be determined. Organic matter content will be determined using the method of loss-on-ignition at 430°C until constant weight or after heating for 24 hours (Davies, 1974).

3.2.4 Total Element Content

Total element content will be determined using EPA SW 846 Method 6010. This method will use ICP-AES for the determination of 25 elements after appropriate sample preparation.

3.2.5 pH

The pH of the soil will be determined using EPA SW 846 Method 9045. The soil will be mixed with either Type II water or a calcium chloride solution (depending on whether the soil is calcareous or noncalcareous), and the pH will be measured with a pH meter.

3.2.6 Particle Size Determination

For bioavailability studies, only the fraction of the soil sample in the <250-µm size range will be used. Therefore, this fraction will be separated from the remainder of the soil sample by performing a standard sieve analysis using the American Society for Testing and Materials (ASTM) No. 60 Sieve (60 openings/inch of surface area). Soil passing the No. 60 sieve corresponds to the fraction <250 µm. Sample preparation for sieve analysis should be performed in accordance with the procedure outlined in *Methods of Soil Analysis, Volume I: Physical and Mineralogical Analysis* (Gee and Bauder, 1986) or the ASTM Method D422-63 (Standard Test Method for Particle Size Analysis of Soils).

3.3 Dosing Formulation Preparation

The appropriate amount of test soil (dosed feed test soil group) or $HgCl_2$ to be mixed with AIN-93G™ complete meal to make approximately 20-25 kg of diet will be determined for each dose group. Approximately 2 kg premix will be prepared by adding the calculated

amount of test soil or $HgCl_2$ to an equal amount of AIN-93G™ complete meal feed. A sufficient amount of the $HgCl_2$ to be used for the dosed feed soluble mercury group may be dissolved in deionized water. The mixture will be stirred with a spatula, and more of the AIN-93G™ feed will be added until the premix totals nearly 2 kg. Approximately 10 kg of the AIN-93G™ feed will be placed in the bottom of a Patterson-Kelley blender (or equivalent), the premix will be layered roughly equally between the two blender ports, and approximately 12 kg more of the AIN-93G™ feed will be added to the blender. The blender will be operated for 5 minutes with the intensifier bar on, and an additional 10 minutes with the intensifier bar off. The corners of the blender will be tapped during preparation to minimize the possibility that feed will be compacted in the corners and prevent proper mixing. An archive sample (approximately 150 g) will be taken from each dosed-feed batch at the time of preparation and will be stored in individually labeled, sealed containers at approximately -20°C.

Mixed feed preparations will be refrigerated in sealed containers at approximately 4°C and protected from light.

### 3.4 Dose Analysis

#### 3.4.1 Dosed Feed Concentration and Stability

The stability of mercury and $HgCl_2$ in feed will be determined prior to study initiation. For each mixed dosed feed preparation, a sample of each dose level will be removed at the time of preparation for analysis of mercury concentrations. At the conclusion of the in-life phase, one dosed feed preparation from each treatment group will be sampled and analyzed for mercury. The actual dosed feed concentrations of mercury as determined at the time of preparation will not differ from the target concentration by more than ±20 percent. A comparison between the sample removed at preparation and the sample removed at the conclusion of the in-life phase will serve to evaluate the stability of the dosed feed preparation for mercury. Duplicate samples of the dosed feed preparation will be digested and single aliquots of the digestate will be analyzed for mercury by GFAA or ICP-AES.

#### 3.4.2 Dosed Feed Homogeneity

An analysis of homogeneity of mixing will be performed prior to study initiation for the high and low dose levels of each treatment group. Samples will be taken from the top right, top left, and bottom part of the twin-shell blender. Triplicate samples will be digested and analyzed for mercury by GFAA or ICP-AES. The preparation will be considered to be homogeneous if the relative standard deviation (RSD) is less than 15 percent. The mean value from the three areas of the blender will be used as the concentration of these specific dosed feed samples.

## 4.0 EXPERIMENTAL ANIMALS

### 4.1 Justification

The test system for this study will be weanling Sprague-Dawley (CD/BR) rats. The rat was selected as the test system because it is recognized by the EPA for use in chemical safety

evaluation testing and a large historical database exists for this animal. Additionally, mercury absorption studies have previously been conducted in rats. The oral route of administration was selected as the route of exposure since this is the most likely human route of exposure.

4.2 Test System

Male and female Sprague-Dawley (CD/BR) rats, approximately 4 weeks of age at initiation of dosing will be obtained from a licensed USDA vendor in sufficient numbers to provide the required number of healthy animals for testing.

4.3 Animal Health and Quarantine

All animals will be quarantined under environmental conditions simulating those of the study. Each animal will be examined and its health status determined by a laboratory animal veterinarian within 48 hours of receipt. Upon arrival, the rats will be given complete AIN-93G™ diet. At the end of a 48-hour quarantine period, animals of questionable health (as determined by a laboratory animal veterinarian) will be excluded from the study.

4.4 Animal Housing

Animals will be individually housed in polycarbonate cages. All housing and care will conform to ILAR standards and those published in the *Guide for the Care and Use of Laboratory Animals*, NIH Publication No. 86-23. The environmental conditions of the animal study room will conform to the following specifications:

- The light/dark cycle will be set for 12 hours of light and 12 hours of dark each day during the study.
- The room temperature will be 64-79°F and will be recorded twice daily. At least 90 percent of the total recordings will fall within the specified range.
- The relative humidity will be 40-70 percent and will be recorded twice daily. At least 90 percent of the total recordings will be within the specified range.
- Fresh air will be supplied to the room at sufficient rate to provide a minimum of 10 changes of room air per hour.

4.5 Diet and Water

AIN-93G™ meal feed (Zeigler Brothers, Gardners, PA) to which the appropriate amount of soil, or $HgCl_2$ has been added will be used for this study. Complete AIN-93G™ meal feed will be given to the control group. The complete AIN-93G™ meal feed will be refrigerated at approximately 4°C and will have expiration dates of approximately 4 months after milling. Feed will be provided *ad libitum* in glass jars. Feeders with fresh feed will be provided at least biweekly. Analysis of each feed lot will be provided by the vendor. Also, prior to dosing, feed will be analyzed at a designated laboratory for acetate, calcium, magnesium, iron, zinc, and phosphorous.

Water will be provided to animals *ad libitum* via glass bottle reservoirs fitted with stainless-steel sipper tubes. Animals will be given deionized water. During the in-life period, a sufficient volume of deionized water will be available so that all animals receive water from the same source. A sample of the water will be removed at the start and after testing, which will be analyzed for acetate, calcium, magnesium, iron, zinc, and phosphorous.

4.6 Animal Identification

All of the animals will be uniquely identified by ear tag and cage card. Each cage will be labeled with the number that corresponds with the ear marking of the animal in the cage.

4.7 Randomization

Rats will be randomly assigned to treatment groups on the basis of body weights. Body weights will be determined the day before dosing. Randomization will be performed using a computer program that places animals in groups to ensure similar group mean body weights at study onset.

## 5.0 EXPERIMENTAL DESIGN

This study involves subchronic daily oral administration of mercury (soluble $HgCl_2$ mixed in feed) as a means of characterizing the oral relative bioavailability of mercury in soil relative to soluble mercury. A non-treated group will serve as a control for determining background mercury levels. Five rats per sex will be used in each treatment group. Specifically, the following groups (five per sex per group) will be studied. Soluble $HgCl_2$ will be given by intravenous administration using the tail vein for 30 days in a group of rats. A group of rats will receive daily oral administration of dosed feed-soluble mercury for 30 days. A second group will receive daily dosed feed soil from a contaminated site for 30 days. Untreated controls will be fed complete AIN-93G™ meal feed for 30 days.

| Treatment Group | Dose Levels[a] | | Soil Concentration in the feed |
|---|---|---|---|
| | µg Hg/g feed | mg Hg/kg BW | g soil/kg feed |
| Untreated (control) | NA | NA | NA |
| Dosed feed-soluble $HgCl_2$ | Low | TBD | NA |
| | Medium | TBD | NA |
| | High | TBD | NA |
| Dosed feed-soil | Low | TBD | TBD |
| | Medium | TBD | TBD |
| | High | TBD | TBD |

[a] The estimated doses of mercury for the dosed feed soil groups will be based on estimated soil mercury concentrations.
NA = Not applicable.
TBD = To be determined.

During the in-life phase, clinical observation, food consumption and body weight determinations will be made as described below.

5.1 Clinical Observations

Clinical observations of any possible signs of toxicity will be recorded. Otherwise, cage checks will be made once a day for moribundity and mortality.

5.2 Food Consumption

Food consumption will be determined once a day at approximately the same time each day for each rat. Known amounts of feed will be provided in cage feeders and at the conclusion of the approximately 24-hour interval, feeders will be reweighed. The net difference between the original and final feeder weight will serve as a measure of the feed consumed. Procedures for evaluating feed spillage will be specified.

5.3 Body Weights

Body weights will be taken on all rats at the start of the study (Day 1), weekly thereafter, and at termination.

5.4 Tissue Collection

At termination, and prior to cessation of heart contractions following an injection of sodium pentobarbital, a whole blood sample will be collected from each rat by cardiac puncture. The kidneys will be removed and stored in polyethylene tissue bags or liquid scintillation vials with plastic tops at approximately -20°C until removed for preparation and analysis. A wet weight of the tissues upon removal will be collected. The residual carcass will be saved and stored frozen for possible additional tissue sample collection and analysis. Samples will be shipped to (*specify name of test facility here*) for analysis.

## 6.0 SAMPLE PREPARATION

The kidney will be digested and prepared according to the testing facility standard operating procedure.

## 7.0 ANALYSIS OF SAMPLES

All samples will be analyzed for mercury by GFAA, ICP-AES, or ICP-MS.

Quality control samples will be analyzed at the beginning and end of each daily analysis. These will be digested and analyzed along with study samples. Recovery of mercury from QC samples will be considered adequate if they are within 25 percent of the certified or prepared value.

The following table summarizes the approximate numbers of samples per sample type that will be analyzed for mercury: *(specify number of samples for each.)*

*I. Non-Biological Samples*

A. Test substance
B. Mixed feed
   1. Homogeneity
   2. Concentration
C. Diet
D. Water

*II. Biological Samples*

Treatment Group

A. Untreated control
B. Dosed feed soluble mercury
C. Dosed feed soil
D. Water

*III. Quality Control Samples*

## 8.0 STATISTICS

All individual raw data will be summarized and reported as the mean and standard deviation. The test laboratory will also calculate actual administered doses based on actual feed mercury concentrations, food consumption, and body weight data. The relative bioavailability (Rf) ratio for each dose level will be determined using the following equation:

$$Rf = \frac{C_{\text{(soluble mercury)}} - BKG_{\text{Dose (soil mercury)}}}{C_{\text{(soil mercury)}} - BKG_{\text{Dose (soluble mercury)}}}$$

where
BKG = overall background value from feed and water analysis and
C = mean concentration of mercury in kidney.

## 9.0 RECORD AND SAMPLE RETENTION

### 9.1 Sample Retention

All samples will be frozen (approximately -20°C) and retained at the test laboratory or its designated facility until acceptance of the final report. At that time, all samples will be disposed of or shipped to the Sponsor as directed by the Sponsor.

### 9.2 Records

All records that would be required to reconstruct the study and to demonstrate adherence to the protocol will be maintained at the test laboratory. The stipulations of this protocol will be implemented in conformance with EPA's Good Laboratory Practice Standards (40 CFR Part 792). This study will be listed on the test laboratory's Master Study Schedule. All

appropriate records will be maintained and will include, but not be limited to, the following:

- Quarantine and acclimation period information pertaining to daily housing and environmental conditions
- Animal body weights at randomization, animal identification, source of animal supply
- Test substance inventory, receipt, and storage conditions
- Original raw and reduced data on mercury concentrations from all samples
- Dosing and sample collection times
- Food consumption, body weight, and clinical observation data
- Original raw and reduced data from test substance analysis upon receipt, dose preparation, and dose analysis
- Disposition of all samples, including chain-of-custody documentation for transfer of samples between laboratories
- A copy of the signed protocol
- All letters, memos, or notes that pertain to the study
- Original signed final report.

9.3 Report

A written draft final report of this study will be submitted to the Sponsor within __ days *(specify)* of the sacrifice date of the last animals.

## 10.0 REFERENCES

Davies, B.W. 1974. Loss-on-ignition as an estimate of soil organic matter. *Soil Sci. Soc. Am. Proc.*, **38**: 150.

Gee, G.W., and J.W. Bauder. 1986. *Particle-Size Analysis*, in Methods of Soil Analysis, Volume I: Physical and Mineralogical Analysis. American Society of Agronomy, Inc., and Soil Science Society of America, Inc., Madison, WI. pp. 383-411.

# APPENDIX K
# TEMPLATE PROTOCOL FOR BIOAVAILABILITY STUDY OF NICKEL IN SOIL FOLLOWING ORAL ADMINISTRATION USING SPRAGUE-DAWLEY RATS

# TEMPLATE PROTOCOL
# for
# BIOAVAILABILITY STUDY OF NICKEL IN SOIL FOLLOWING ORAL ADMINISTRATION USING SPRAGUE-DAWLEY RATS

## 1.0 PRINCIPALS

Sponsor: *(Specify)*

Sponsor's Project Monitor: *(Specify)*

1.1 Testing Facility: *(Specify)*

Study Director: *(Specify)*

## 2.0 OBJECTIVES

The objectives of this study will be to use blood nickel concentrations to determine the bioavailability of nickel in Sprague-Dawley rats after oral administration of soil contaminated with nickel. Relative bioavailability of nickel in soil will be estimated by comparison to data from rats administered nickel sulfate hexahydrate by gavage.

## 3.0 TEST SUBSTANCES

3.1 Test Substance Identification

The test substances for this study will be samples of soil collected from test sites. Soil nickel will be characterized, and concentration, stability and purity determined before the study.

For the soluble nickel group animals, nickel sulfate hexahydrate will be used to administer appropriate doses of water soluble nickel.

3.2 Test Substance Analysis

3.2.1 Concentration

The concentrations of nickel in the test substances will be determined. Analysis of the test substances will involve extracting nickel from a sample of each test substance, digesting the extracted material, and measuring the concentrations of nickel by

inductively coupled plasma atomic emission spectroscopy (ICP-AES) or graphite furnace atomic absorption (GFAA). The concentrations of nickel in the soil will be determined by digesting duplicate aliquots of the sample and analyzing single aliquots of the digestate. No attempt to identify the different chemical forms of nickel will be conducted at the test laboratory.

3.2.2 Moisture

The percent water in the test substance will be determined by measuring the moisture content. Moisture content will be determined by weighing and drying an approximately 5-g sample at 105-110°C for 2 hours, followed by reweighing. Percent moisture will be determined by calculating the difference between the pre-dried and dried samples.

3.2.3 Organic Matter

The organic matter in the test substance will be determined. Organic matter content will be determined using the method of loss-on-ignition at 430°C until sample reaches constant weight or has been heated for 24 hours (Davies, 1974).

3.2.4 Total Element Content

Total element content will be determined using EPA SW 846 Method 6010. This method will use ICP-AES for the determination of 25 elements after appropriate sample preparation.

3.2.5 pH

The pH of the soil will be determined using EPA SW 846 Method 9045. The soil will be mixed with either Type II water or a calcium chloride solution (depending on whether the soil is calcareous or noncalcareous), and the pH will be measured with a pH meter.

3.2.6 Particle Size Determination

For bioavailability studies, only the fraction of the soil sample in the <250-µm size range will be used. Therefore, this fraction will be separated from the remainder of the soil sample by performing a standard sieve analysis using the American Society for Testing and Materials (ASTM) No. 60 Sieve (60 openings/inch of surface area). Soil passing the No. 60 sieve corresponds to the fraction <250 µm. Sample preparation for sieve analysis should be performed in accordance with the procedure outlined in *Methods of Soil Analysis, Volume I: Physical and Mineralogical Analysis* (Gee and Bauder, 1986) or the ASTM Method D422-63 (Standard Test Method for Particle Size Analysis of Soils).

3.3 Dose Preparation

Gelatin capsules will be used to administer test substance (soil). Soil will be transferred into one-half of the gelatin capsule until the desired weight of soil to be placed into a single capsule has been achieved. The capsule will then be reassembled by attaching the other half of the capsule to the soil-filled capsule. Each capsule will be weighed and enough

capsules will be prepared for each animal to achieve the desired amount of soil to be administered. The total weight of soil contained in the set of capsules will be within 5 percent of the targeted dose. After preparation, the capsules will be stored at room temperature until used.

The nickel sulfate hexahydrate to be used for dosing the gavage study groups will be formulated into an aqueous solution. A sufficient quantity of nickel sulfate hexahydrate will be dissolved in deionized water (vehicle) to produce the target concentration of the dosing solution. The solution for oral dosing will be prepared at a concentration such that the dosing volumes do not exceed 5 mL/kg.

### 3.4 Dose Analysis

The dosing solution for the gavage administration groups will be analyzed for nickel by graphite furnace atomic absorption spectroscopy or, if the concentration of nickel is sufficiently high, the analysis will be conducted by ICP-Atomic Emission Spectroscopy (AES). A sample of the dosing solution will be taken at the time of preparation. Duplicate aliquots of the dosing solution will be analyzed. The actual dosing solution concentration will not differ from the target concentration by more than $\pm$10 percent.

### 3.5 Dose Administration

Capsules will be administered to the animals using a small animal capsule applicator. The dose will be based upon individual animal body weights which will be determined just prior to dosing (fasted body weights).

For the gavage study group animals, the dosing solution will be administered using a stainless steel gavage needle.

## 4.0 EXPERIMENTAL ANIMALS

### 4.1 Justification

The test system for this study will be Sprague-Dawley (CD/BR) rats. The rat was selected as the test system because it is recognized by EPA for use in chemical safety evaluation testing and a large historical database exists for this animal. Additionally, studies of the bioavailability of soil nickel have previously been conducted in rats.

### 4.2 Test System

Male Sprague-Dawley (CD/BR) rats, 8 weeks of age at initiation of dosing will be obtained from a licensed USDA vendor in sufficient numbers to provide the required number of healthy animals for testing. *(Specify the number of animals here.)*

### 4.3 Animal Health and Quarantine

All animals will be quarantined under environmental conditions simulating those of the study. Each animal will be examined and its health status determined by a laboratory animal veterinarian within 48 hours of receipt. Upon arrival, the rats will be given complete AIN-93G™ diet. At the end of a 48-hour quarantine period, animals of

questionable health (as determined by a laboratory animal veterinarian) will be excluded from the study.

4.4 Animal Housing

Animals will be individually housed in polycarbonate cages. All housing and care will conform to ILAR standards and those published in the *Guide for the Care and Use of Laboratory Animals, NIH Publication No. 86-23*. The environmental conditions of the animal study room will conform to the following specifications:

- The light/dark cycle will be set for 12 hours of light and 12 hours of dark each day during the study.
- The room temperature will be 67-77°F and will be recorded twice daily. At least 90 percent of the total recordings will fall within the specified range.
- The relative humidity will be 40-70 percent and will be recorded twice daily. At least 90 percent of the total recordings will be within the specified range.
- Fresh air will be supplied to the room at sufficient rate to provide a minimum of 10 changes of room air per hour.

4.5 Diet and Water

AIN-93G™ meal feed (Zeigler Brothers, Gardners, PA) will be used for this study. Complete AIN-93G™ meal feed will be given to the control group. Feed will be held from animals for 16 hours prior to oral dosing. Analysis of each feed lot will be provided by the vendor. Also, prior to dosing, feed will be analyzed at (insert name of laboratory here) for nickel, calcium, magnesium, iron, zinc, and phosphorous.

Water will be provided to animals ad libitum via glass bottle reservoirs fitted with stainless-steel sipper tubes. Animals will be given deionized water. During the in-life period, a sufficient volume of deionized water will be available so that all animals receive water from the same source. A sample of the water will be removed at the start and after testing, which will be analyzed for nickel, calcium, magnesium, iron, zinc, and phosphorous.

4.6 Animal Identification

All of the animals will be uniquely identified by ear tag and cage card. Each cage will be labeled with the number that corresponds with the ear marking of the animal in the cage.

4.7 Randomization

Rats will be randomly assigned to treatment groups on the basis of body weights. Body weights will be determined the day before dosing. Randomization will be performed using a computer program that places animals in groups to ensure similar group mean body weights at study onset.

## 5.0 EXPERIMENTAL DESIGN

This study involves oral administration of nickel from two sources (soluble nickel sulfate hexahydrate and nickel in soil) as a means of characterizing the oral bioavailability of nickel in soil relative to soluble nickel. A non-treated group will serve as a control for determining background nickel levels. On Day -1, rats will be weighed in order to determine doses to be used. On Study Day 1, rats will be given the nickel-saline solution by gavage using a stainless steel gavage needle. The soil-filled capsules will be administered using a capsule applicator (test soil group). Control group will receive orally administered saline only. For all groups, samples of whole blood will be collected from each animal at specified intervals, for 6 days after administration of test article or control saline.

The following table summarizes the treatment groups.

| Group | Treatment | Number of Animals[a] |
|---|---|---|
| 1 | Control | 32[b] |
| 2 | $NiSO_4 \cdot 6H_2O$ | 32[b] |
| 3 | Soil | 32[b] |

[a] One animal may be subjected to as many as 3 bleeds. (Example: one animal may be bled at 10 min, 24 hr and 72 hr.)
[b] Suggested number of animals.

During the in-life phase, clinical observation, food consumption and body weight determinations will be made as described below.

### 5.1 Blood Collection

Serial samples of whole blood (approximately 1 mL) will be collected by orbital puncture under $CO_2$ anesthesia. Heparinized blood samples will be collected at 0, 10, 20, 30, 60, 120, 240 and 480 min on the first day and at 24, 48, 72, 96 and 120 hr and stored at -20°C until analysis.

### 5.2 Clinical Observations

Clinical observations of any possible signs of toxicity will be done twice daily. Cage checks will be made once a day for moribundity and mortality.

### 5.3 Food Consumption

Food consumption will be determined once a day at approximately the same time each day for each rat. Known amounts of feed will be provided in cage feeders and at the conclusion of the approximately 24-hour interval, feeders will be reweighed. The net difference between the original and final feeder weight will serve as a measure of the feed consumed. Procedures for evaluating feed spillage will be specified.

### 5.4 Body Weights

Body weights will be taken on all rats at the start of the study (Day 1) and at termination.

## 6.0 SAMPLE PREPARATION

Blood will be digested in concentrated nitric acid. One or more internal standards may be added, and the digestate diluted for analysis.

## 7.0 ANALYSIS OF SAMPLES

All samples will be analyzed for nickel by GFAA, ICP-AES, or ICP-MS. Single analysis of whole blood will be conducted. Blood samples will be digested in acid, as necessary, prior to removal of the aliquot for nickel analysis.

Quality control (QC) samples will be analyzed at the beginning and end of each daily analysis. Quality control samples for nickel in blood will be certified blood standards obtained from a specified commercial laboratory. These will be digested and analyzed along with study samples. Recovery of nickel from QC samples will be considered adequate if they are within 25 percent of the certified or prepared value.

The following table summarizes the approximate numbers of samples per sample type that will be analyzed for nickel: *(Specify numbers of samples for each)*

| | |
|---|---|
| *I.* | *Non-Biological Samples* |
| | A. Test substance |
| | B. Mixed feed |
| | 1. Homogeneity |
| | 2. Concentration |
| | C. Water |

| | |
|---|---|
| *II.* | *Biological Samples* |
| | Treatment Group: |
| | A. Untreated control |
| | B. Nickel-saline |
| | C. Capsule (soil nickel) |

| | |
|---|---|
| *III.* | *Quality Control Samples* |

## 8.0 STATISTICS

All individual raw data will be summarized and reported as the mean and standard deviation. Relative bioavailability ratio will be determined using the following equation:

$$F_{rel} = (AUC_{soil} / AUC_{pure}) \times \frac{dose_{pure}}{dose_{soil}}$$

where $AUC_{soil}$ is area under the curve for the capsule-Ni group and $AUC_{pure}$ is the area under the curve for the Ni-saline group.

## 9.0 RECORD AND SAMPLE RETENTION

### 9.1 Sample Retention

All samples will be frozen (approximately -20°C) and retained at the test laboratory or its designated facility until acceptance of the final report. At that time, all samples will be disposed of or shipped to the Sponsor as directed by the Sponsor.

### 9.2 Records

All records that would be required to reconstruct the study and to demonstrate adherence to the protocol will be maintained at the test laboratory. The stipulations of this protocol will be implemented in conformance with EPA's Good Laboratory Practice Standards (40 CFR Part 792). This study will be listed on the test laboratory's Master Study Schedule. All appropriate records will be maintained and will include, but not be limited to, the following:

- Quarantine and acclimation period information pertaining to daily housing and environmental conditions
- Animal body weights at randomization, animal identification, source of animal supply
- Test substance inventory, receipt, and storage conditions
- Original raw and reduced data on nickel concentrations from all samples
- Dosing and sample collection times
- Food consumption, body weight, and clinical observation data
- Original raw and reduced data from test substance analysis upon receipt, dose preparation, and dose analysis
- Disposition of all samples, including chain-of-custody documentation for transfer of samples between laboratories
- A copy of the signed protocol
- All letters, memos, or notes that pertain to the study
- Original signed final report.

### 9.3 Report

A written draft final report of this study will be submitted to the Sponsor within *(specify)* days of the sacrifice date of the last animals.

## 10.0 REFERENCES

Davies, B.W. 1974. Loss-on-ignition as an estimate of soil organic matter. *Soil Sci. Soc. Am. Proc.*, **38**: 150.

Gee, G.W., and J.W. Bauder. 1986. *Particle-Size Analysis*, in Methods of Soil Analysis, Volume I: Physical and Mineralogical Analysis. American Society of Agronomy, Inc., and Soil Science Society of America, Inc., Madison, WI. pp. 383-411.