# Relative Bioavailability Tests for Application to Environmental Risk Assessment of Lead in Birds

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## Executive Summary

Accurate assessment of relative bioavailability of lead is important because underestimation may lead to ecological damage and overestimation may lead to unnecessary and expensive cleanup causing site disturbance and also ecological damage. *In vitro* methods of determining relative bioavailability of lead are preferable over *in vivo* methods as they are less expensive and do not use live animals causing ethical issues. Of the various *in vitro* methods available for determining the relative bioavailability of lead, the Waterfowl – Physiologically Based Extraction Test (W-PBET) shows the most promise for use in birds. The W-PBET was designed specifically to meet physiological parameters of waterfowl, and has been positively correlated with *in vivo* feeding studies to gain validity. Additionally, the W-PBET was designed for use in temperate soils increasing its relevance for use at many North American mining sites.

Correlation of environmental determinants with bioaccessibility of lead shows promise as an emerging method of initial site estimation of the relative bioavailability of lead. Environmental determinants that can affect the bioaccessibility of lead range from soil particle size, to soil composition (e.g., sand, silt, clay), to concentrations of iron, manganese and aluminum. Additionally, different speciations of lead vary in levels of bioaccessibility. Site assessment using X-ray techniques can be used as a tool to provide initial estimates of the specific bioaccessibility at a site.

Recommendations resulting from this report include use and/or adaptation of the W-PBET to meet specific site and target species needs, and development of regression analysis for specific site condition variables for use as a framework for initial site analysis and estimation of risk. The solutions discussed within this report are recently developed and will become more streamlined and accurate as research in this field progresses.

## Introduction

This paper provides a review of current and emerging methods to determine the relative bioavailability of lead in birds, specific to environments contaminated with mine waste in temperate regions. This topic has relevance to the conduct of environmental risk assessments. Different methods of testing relative bioavailability of lead are compared with respect to strengths and weaknesses. The preferred method is cost-effective, time efficient, accurate, environmentally relevant, and simple to perform. A strong method for determining relative bioavailability of lead is an important aspect of risk assessment because: (1) overestimation of relative bioavailability in the field leads to unnecessary and expensive cleanup causing disturbance to the environment; and (2) underestimation leads to a lack of remediation and increases ecological risk (Suedel, Nicholson, Day & Spicer II, 2006).

Informed management of lead at contaminated sites is important for the welfare of birds. According to Furman et al. (2006) potential consequences to waterfowl from elevated exposures associated with high relative bioavailability of lead are "severe pectoral muscle atrophy, bile-stained feces, greenish diarrhea, excessive bile in the gall bladder, impaction of the gastrointestinal tract with food leading to starvation, up to 40% loss in original body weight, erosion of the gizzard lining, loss of vision, convulsions, coma, and death" (p. 450). Therefore, accurate testing of relative bioavailability in the field plays an important role in risk assessment, and links strongly to appropriate environmental remediation upon completion of a project.

In the field, chemicals are found in a variety of forms and conditions in contrast to the highly soluble chemicals used in laboratory testing. Bioavailability refers to "the extent to which a chemical can be absorbed by a living organism" (Kelley et al., 2002, p. xi). The measure of the difference of the quantity of the chemical that is absorbed into an organism between two or more

different forms of the same chemical is defined as the relative bioavailability (Kelley et al., 2002). Therefore, if lead is in a different form in the field from the form that is used in the laboratory to determine the bioavailability, the relative bioavailability will typically be less than 100%. The relative bioavailability is usually determined through *in vivo* or *in vitro* tests (Kelley et al., 2002). Consequently, this paper will examine the strengths and weaknesses of different methods of *in vivo* and *in vitro* tests. Another term that is common in literature on relative bioavailability is bioaccessibility. Bioaccessibility is the fraction of the total potential chemical bioavailability that is absorbed into an organism and determined through *in vitro* test methods (Kelley et al., 2002; Beak et al., 2006).

## Current and Emerging Methods of Bioavailability Testing

### *In vivo*

The traditional method for testing the relative bioavailability of lead is the *in vivo* method. *In vivo* refers to studies within a living organism, and *in vivo* methods for bioavailability studies are usually conducted using live animals (Kelley et al., 2002). The advantage of *in vivo* methods is that they are highly representative of the biological endpoint of interest; they can be performed on the same type of animal that is of interest, except in the case of studies on humans where young swine have been successfully used as a substitute for children (Beak et al., 2006). Despite the historic use and advantages of *in vivo* methods, disadvantages include the long length of time required to conduct trials, expense, dosing issues, and test animal welfare considerations. These drawbacks may culminate in such tests being impractical for the environmental assessment and management of contaminated sites (Beak et al., 2006; Drexler and Brattin, 2007; Furman et al., 2006).

### *In vitro*

*In vitro* methods of measuring bioavailability of lead have been emerging and undergoing much scrutiny and interest in recent decades; several authors have attempted to validate them as a preferred bioavailability measure over *in vivo* methods. *In vitro* literally, in glass, refers to studies conducted outside of a living organism in an artificial environment inside a laboratory, and does not require the use of live animals (Kelley et al., 2002). Physiologically based extraction tests (PBET), waterfowl physiologically based extraction tests (W-PBET), the relative bioaccessibility leaching procedure (RBALP), and *in vitro* gastrointestinal methods (IVG) are all examples of methods of *in vitro* bioavailability measures discussed in this paper. Furman et al. (2006) claim that the PBET is particularly representative for toxicity assessment as it incorporates physiological parameters of the target species (Furman et al., 2006). The other *in vitro* methods also incorporate physiological parameters, but are currently designed for tests on humans. To assess the validity of an *in vitro* method, it must show a strong positive correlation with an *in vivo* test that uses similar parameters. As the gastrointestinal tract is extremely complex and impossible to replicate in detail, an *in vitro* test can only provide an estimate of relative bioavailability (Drexler and Brattin, 2007).

### Discussion of *in vivo* and *in vitro* methods

*In vitro* bioavailability methods use a two-phase procedure to imitate the gastrointestinal tract and the stomach phases of digestion. The IVG method and the RBALP method were specifically developed to simulate bioavailability of humans and correspond to *in vivo* bioavailability in young swine, which have similar gastrointestinal characteristics to human children (Beak et al., 2006; Drexler and Brattin, 2007). Therefore, the IVG and RBALP methods are likely to be weak methods for bioavailability testing in birds. The W-PBET is an adaptation

of the PBET that was developed by Furman et al. (2006) specifically for use in waterfowl. As the W-PBET incorporates the physiological parameters of the PBET along with specific characteristics of waterfowl gastrointestinal and stomach traits, it is likely a strong method for measuring bioavailability in birds.

To adapt an *in vitro* method to a particular species, certain parameters are changed to imitate the target species. Furman et al. (2006) state that the PBET can be adapted to simulate the gastrointestinal tract of different types of species. They adapt the model of the traditional PBET to waterfowl to create the W-PBET, acknowledging the important of several factors that strongly mediate bioavailability. Drexler and Brattin (2007) list pH, temperature, and degree of agitation as key controlling factors for *in vitro* methods. These factors are designed to simulate the target species as much as possible. For example, Drexler and Brattin (2007) conducted a validation study of *in vitro* versus *in vivo* methods in which parameter values were compared between in vitro simulations and in vivo results. Even when they did not find statistically significant differences, such as in the case of temperature, Drexler and Brattin (2007) recommended adoption of the temperature most representative of the target species to maintain biological consistency.

For an *in vitro* test to establish validity it must demonstrate a strong positive correlation with the adjacent *in vivo* study. The most significant parameter in lead bioavailability studies appears to be pH (Drexler and Brattin, 2007); this is consistent with the toxicological literature that demonstrates higher bioavailability and toxicity at low levels of pH. Levels of pH, both in soil and in the gastrointestinal and stomach phases of *in vitro* methods, arise in several other studies as well (Furman et al., 2006; Kelley et al., 2002; Ruby, 2004; Schroder et al., 2004; Suedel et al., 2006; United States Environmental Protection Agency, 2008). Current research

illustrates the significance of pH levels, but fails to clarify which direction to proceed in order to use this knowledge towards increased understanding of bioavailability. Drexler and Brattin (2007) opt for a lower-bound (conservative) pH level of 1.5 in their validation study to limit the risk of under-estimating bioavailability. Their work indicates a relationship of increased bioavailability at lower pH levels, but further research is required to validate their work.

Before and after *in vitro* tests are performed, the soil must be evaluated to determine lead concentrations. This is commonly done through the use of different types of X-ray analysis tools. Beak et al. (2006) report that X-ray diffraction (XRD) and energy dispersive X-ray analysis (EDXA) have been used to determine the changes in the simulated gastrointestinal system. Drexler and Brattin (2007) add that X-ray fluorescence (XRF), inductively coupled atomic emission spectrometry (ICP-AES), and inductively coupled mass spectrometry (ICP-MS) are other chemical analysis methods used. Ruby (2004) mentions electron microbeam methods complement XRD as other tools to determine mineral forms in soil before and after *in vitro* experiments.

Although there are many benefits to the use of *in vitro* methods, such as the PBET, compared to *in vivo* methods, there are some limitations as well. Ruby (2004) argues that the PBET is only reliable if the only limiting step in oral bioavailability of a particular compound is the liberation from the soil in the gastrointestinal tract as the PBET does not measure the actual absorption into tissue. This argument is supported by Furman et al. (2006) who add that lead speciation affects the solubility, adsorption, complexation, redox reactions and biological uptake. Therefore, factors other than gastrointestinal processes are important considerations before relative bioavailability can be reliably estimated. This demonstrates why it is important to understand both chemical and biological factors related to lead bioavailability. Schroder et al.

(2004) conclude that it is unlikely that an *in vitro* method can be developed to completely replace *in vivo* animal studies, but also that it may be possible to develop an *in vitro* method appropriate for efficient screening of approximate levels of relative bioavailability of lead at contaminated sites.

**W-PBET: Strongest Method of *in Vitro* for use in Birds**

In my review of the literature, the W-PBET *in vitro* method emerged as the strongest method currently available to estimate relative bioavailability of lead in birds. It has been developed recently, published in 2006, particularly for use in waterfowl, and has high environmental relevance to birds evaluated in risk assessments. The W-PBET was developed by Furman et al. (2006), and is based on the previously developed PBET *in vitro* method. According to Furman et al. (2006), the gastric phase pH level is particularly influential to test results. Consequently, Furman et al. (2006) have adjusted the stomach phase to pH 2.6, which is their best estimate of conditions in the bird gizzard, which range between pH 2.0 and 3.2 depending on the presence of food. They have adjusted the intestine phase to pH 6.2 to simulate intestinal pH ranges of 5.2 to 7.2 in birds. Furman et al. (2006) note that bioaccessibility of lead increases as pH is lowered from pH 3.0 to 2.0. This provides a suggestion of a possible useful regression analysis that could be applied to other species that may have variations in pH in the gastrointestinal tract. By knowing whether the intestinal pH of a species is lower or higher than the target species for which the *in vitro* test was designed for, we can estimate whether the species will be more or less conducive to high relative lead bioavailability. The W-PBET also adjusted the temperature of the *in vitro* test to 42°C to mimic that of waterfowl (Furman et al., 2006).

An additional parameter that significantly influences bioavailability of lead is the particle size of the soil. As soil particle size decreases, the surface area to volume ratio increases, resulting in higher levels of relative lead bioavailability in soils with smaller particle sizes. Smaller particle sizes are solubilised much more rapidly within the gastrointestinal system (Furman et al., 2006). Lead compounds that demonstrate the lowest solubility in the gastrointestinal tract will be the least harmful to waterfowl (Furman et al., 2006). The W-PBET method developed by Furman et al. (2006) uses a particle size of less than 1mm to replicate the accompanying *in vivo* study that was used to validate the results of the *in vitro* W-PBET method. When used in the field, accurate measure of the particle size of soil used in the *in vitro* method is particularly important as performing the *in vitro* method based on a non-representative particle size leads to overestimation or underestimation of the relative bioavailability of lead in the field.

The W-PBET has shown positive correlation when validated through feeding studies using *in vivo* methods. This indicates a potential for the W-PBET as an *in vitro* method for determining relative bioavailability of lead (Furman et al., 2006). However, Furman et al. (2006) caution that further validation studies on different types of soils are needed before this method can be universally adopted.

In a later study, Furman et al. (2007) assesses the effect that drying of soil samples from the field has on the relative bioavailability of lead; this response was determined through examination of changes in physiochemical properties. According to Furman et al. (2007), current practice is to air-dry soil samples before performing tests on them which increases oxygenation. The results showed that lead bioaccessibility is affected by the moisture in the soil. Average lead bioaccessibility was 15% less in wet soils than air-dried soils, and 10% greater in freeze-dried soils than wet soils (Furman et al., 2007). This demonstrates that air-drying of soils

can lead to overestimation of lead bioaccessibility as shown in Figure 1. This provides an example of just one of many variables that influence bioavailability; further research and validation of the W-PBET and other *in vitro* methods is required before they can be trusted as a universal method.

Figure 1. Soil treatment drying effect on lead bioaccessibility

**Lead bioaccessibility = wet soil < freeze-dried soil < air-dried soil**

**Environmental determinants**

The previous discussion of *in vitro* and *in vivo* methods has emphasized the importance of knowing the chemicals conditions (e.g., speciation of lead) relevant to environmental conditions of the site. This section further examines environmental determinants, and assesses whether specific environmental determinants can be used as an initial method to estimate relative lead bioavailability prior to the use of a more detailed *in vitro* or *in vivo* test method. Ruby (2004) emphasizes the importance of understanding the site history and soil chemistry in evaluating the relative bioavailability of lead in soils. Soils with basic or alkaline pH and soil components and/or with high total organic carbon have high relative bioavailability for lead. In contrast, sulphide-producing soils have moderate relative bioavailability for lead (Rudy, 2004). By understanding some basic information about the types of soil present at the site, an initial prediction can be made of the level of risk of relative bioavailability of lead at the site.

The particular speciation of lead-mineral compound at the site also acts as a determinant of the level of relative bioavailability of lead at the site. Figure 2 shows the increase of bioaccessibility of lead from galena to cerussite. This indicates that knowledge of the speciation of lead, which can be determined through X-ray techniques mentioned above, also provides initial information that can be used to assess the risk level of relative lead availability at the site

prior to use of *in vitro* or *in vivo* test methods. According to Furman et al. (2006), even within one particular site, a great deal of variability of lead bioavailability can be found within the soils, thus emphasizing the importance of lead speciation studies as a tool to be used in conjunction with *in vitro* and *in vivo* methods.

Figure 2. Bioaccessibility of lead minerals (adapted from Furman et al., 2006, p. 451)

**Galena < pyromorphite < Fe-Pb oxides < lead jarosite < Mn-Pb oxides < Pb oxides<cerussite**

Based on environmental determinants, Houle (2008) demonstrates how stepwise regression of minerals in the soil at a contaminated site can be used to predict bioavailability of lead. Houle (2008) shows how soil properties such as iron, manganese, aluminum, clay content, and particle size can be used to predict lead bioaccessibility by relating bioaccessibility as a function of the levels of these particular minerals in the soil. Ruby (2004) suggests that this technique might become a way of predicting bioaccessibility in the future, but Houle (2008) demonstrates this method in more practical terms, using a graphical approach. Although this method is not currently a routine practice, it has potential as an initial cost-effective measure to determine whether further bioavailability testing is required for a site. Further validation of this method in conjunction with lead speciation studies could lead to acceptance of this method as a preliminary measure for bioavailability testing.

## Site Remediation

The research on bioavailability of lead is useful not only for assessing risks, but also for influencing management actions associated with unacceptable risk levels. Phosphorus amendments currently are the most common form of cost-effective remediation at lead contaminated sites (Heinz et al., 2004). Other methods of remediation include ferrihydrite and corundum amendments. Beak et al. (2006) found ferrihydrite to have a higher affinity and

sorption capacity for lead, thus concluding that ferrihydrite would be the more useful of these two for lead contaminated site remediation. Aluminum and iron oxides are also remedial treatments which can be used to decrease bioavailability of lead in contaminated sites, however they tend to release a large quantity of lead during gastric conditions with pH of 1.8 (Beak et al., 2006). Further research is required to determine the cost and ecological risks that may be associated with all of these methods of remediation. Although phosphorus amendments do appear to be the most cost-effective form of remediation, further research is necessary to refine uncertainty and evaluate potential ecological side effects.

## Conclusions, Recommendations and Future Research

This literature review of current and emerging methods to determine relative bioavailability of lead suggests that the W-PBET *in vitro* method developed by Furman et al. (2006) is the strongest method currently available for use in birds. The utility of *in vitro* methods as opposed to the more expensive *in vivo* methods is validated through positive correlation with *in vivo* studies by Drexler and Brattin (2007), Furman et al. (2006), Schroder et al. (2004), and the United States Environmental Protection Agency (2008). Although positive correlation support the potential value of *in vitro* methods, further research and validation is required of the *in vitro* method to reduce uncertainties, and provide refinements that increase environmental relevance. This is demonstrated by Furman et al. (2007) through their study of the effects of soil drying on lead relative bioavailability prior to testing. This provides just one example of a variable, moisture content in this case, that alters the results of the test when manipulated; other modifying factors can be explored in future research. As validation of *in vitro* methods progresses, the process will become increasingly streamlined as knowledge of potential variables increases.

In support of the recommendation of the W-PBET as a strong method for determining the relative bioavailability of lead at a site, Furman et al. (2006) provide analysis of a case study in the Coeur d'Alene River Basin, Idaho. This case study, which assisted in the development of the W-PBET *in vitro* method, is believed to be representative of lead contaminated soils in many other global environments, many of which require development of a simple and inexpensive method to determine risks to waterfowl. The Coeur d'Alene River Basin site provides a useful example of an *in vitro* experiment conducted in a temperate region.

In my research, the importance of lead speciation analysis of contaminated soils and the influence of pH conditions in soil and gastrointestinal tract have been emphasized. Prior to applying any method of bioavailability testing, soil analysis must be performed. Initial analysis can provide important screening-level information for estimation of bioaccessible levels of lead contamination at a specific site. Further research, focussing on mathematical relationships between lead bioaccessibility and a range of different soil variables, shows great promise as a means of refining our initial evaluations of the potential harm at a specific site.

In summary, there have been considerable recent advances in the understanding of how lead accumulates in birds. Whereas a common default assumption in ecological risk assessments is that relative bioavailability in the field is 100% of laboratory bioavailability, research has indicated several key parameters that modify the uptake of lead in birds and other organisms. Risks assessments can make better use of this knowledge, first by using simple screening methods to evaluate bioavailability potential, and second by selectively applying more detailed approaches such as the W-PBET to reduce uncertainties. The improvements in risk assessment methods will, in turn, support more informed management of lead-contaminated sites.

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