Incorporating Diet into *In Vitro* Bioaccessibility Assays to Improve Prediction of Bioavailability of Soil Pb in Birds and Humans

THESIS

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Abstract

Anthropogenic use of lead has resulted in widespread soil contamination. Exposure to lead when contaminated soil is incidentally ingested with food poses a risk to both humans and wildlife. When soil is ingested, only a fraction of total Pb is bioavailable, or absorbed into the bloodstream. Conservative soil bioavailability defaults are used to calculate exposure in the absence of bioavailability data. This often overestimates exposure and risk. Generating bioavailability data allows a more accurate representation of risk, reducing cleanup costs or making sites appropriate for a wider variety of uses. However, the animal feeding studies traditionally used to generate these data are time-consuming and expensive.

Extensive research has led to development and adoption of in vitro bioaccessibility assays that simulate gastrointestinal conditions to determine in vitro bioaccessible Pb (IVBA Pb), which is then used to predict relative soil Pb bioavailability (RBA Pb). These methods simulate fasting conditions and it is unclear whether they can predict bioavailability of soil Pb consumed with food. This study sought to incorporate diet into soil Pb in vitro bioaccessibility assays to simulate exposure in wildlife incidentally consuming soil while feeding and in humans eating produce contaminated with soil.

To determine whether in vitro bioaccessibility assays were predictive of RBA Pb of eight soils dosed to Japanese quail (Coturnix japonica), the soils were combined with the feeding study diet and IVBA Pb determined with three in vitro bioaccessibility methods:
USEPA Method 1340 at pH 1.5, USEPA Method 1340 at pH 2.5, and a newly developed Avian Ohio State University In Vitro Gastrointestinal evaluation. All methods were predictive of RBA Pb in Japanese quail (Coturnix japonica), and diet+soil methods produced somewhat stronger relationships than soil-alone methods. Seven wildlife diets were then evaluated with a single soil in the 3 previously mentioned methods to explore the impact of different diets on IVBA Pb. IVBA Pb was lower in the more nutrient-dense stock diets compared to natural diets, suggesting risk may be underestimated when using stock diets in in vitro or in vivo toxicological assays.

A new in vitro bioaccessibility assay was developed to simulate human exposure to Pb from soil-contaminated vegetables. Gastric and intestinal phase IVBA Pb was determined in eight vegetables contaminated with a smelter soil or a residential soil. Diet+soil IVBA Pb differed from soil-alone IVBA Pb and the effects of diet were dependent on phase. Future work is needed to determine which is more appropriate for simulating IVBA Pb in soil-contaminated vegetables.

These first steps towards developing diet+soil in vitro bioaccessibility assays have demonstrated that diet influence IVBA Pb and should be considered when evaluating exposure. With further research, these methods will facilitate the use of RBA Pb when calculating exposure for risk assessment.
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AOSU IVG: Avian Ohio State University In Vitro Gastrointestinal extraction

ERA: Ecological risk assessment

GI: Gastrointestinal

HHRA: Human health risk assessment

IVBA: In vitro bioaccessible

OSU IVG: Ohio State University In Vitro Gastrointestinal extraction

RBA: Relative bioavailable

TRV: Toxicity reference value

USEPA: United States Environmental Protection Agency
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Chapter 1. Impacts of Diet on Soil *In Vitro* Bioaccessible Pb (IVBA Pb) and Ability of Diet+Soil IVBA Pb to Predict Soil Pb Bioavailability in Japanese Quail

Introduction

Human activities including mining, leaded gasoline combustion and use of lead-based paint have introduced lead (Pb) into the environment. While soils naturally contain small amounts of Pb, addition of Pb from anthropogenic sources raises Pb concentrations above background levels. A soil with elevated Pb, or a contaminated soil, may or may not pose a risk to the organisms that interact with that soil. This depends upon many factors, including the site use, Pb concentration, and availability of the soil Pb. A polluted soil is a contaminated soil which poses a risk of adverse health effects. Lead negatively impacts all major organ systems, and chronic exposure to Pb in soil and sediment can lead to muscle atrophy, gastrointestinal damage and weight loss, reduced fertility, vision problems, and death in wildlife (Furman, Strawn, et al., 2006, Levengood and Skowron, 2001).

Ecological risk assessment (ERA) is used in the United States Environmental Protection Agency’s (USEPA) Superfund program and in other land cleanup programs to identify polluted soils and determine remediation strategies that will minimize risk to wildlife. The first step of risk assessment is to compare contaminant concentrations with ecological soil screening limits (Eco-SSLs). These are soil contaminant concentrations above which sensitive ecological receptors may be at risk of adverse health effects. When a site exceeds these limits, a full ERA is conducted. The avian Eco-SSL for Pb is only 11
mg kg\(^{-1}\). This concentration is lower than background Pb content of most soils in the United States (Smith, Cannon et al., 2013). This means that almost every screening-level risk assessment triggers a full investigation to characterize the Pb risks to birds. Risk assessors need techniques to accurately and efficiently characterize the exposure risk soil Pb poses to birds.

The exposure risk of Pb is determined by comparing the Pb dose each receptor of interest absorbs from the environment to a toxicity reference value (TRV), or the dose of Pb above which an organism experiences adverse health effects. The soluble Pb forms used in toxicological assays to generate TRVs are maximally bioavailable (absorbed into the bloodstream, see Figure 1). Soil Pb is less readily absorbed than soluble Pb forms (relative bioavailability (RBA)<100%). The default RBA in exposure calculations is 100%, however, so exposure and risk from soil Pb tends to be overestimated. Using soil-specific Pb bioavailability data to adjust exposure calculations provides a more accurate assessment of risk and in many cases could reduce cleanup costs (Suedel, Nicholson, et al., 2006).

Traditionally, soil RBA Pb has been determined through animal feeding studies, which are time-consuming and expensive. Extensive research in the past two decades has led to development and adoption of \textit{in vitro} bioaccessibility assays to predict RBA Pb to adjust exposure for human health risk assessment (Drexler and Brattin, 2007, Intawongse and Dean, 2006, Juhasz, Smith, et al., 2007, Ruby, Davis, et al., 1993, Scheckel, Chaney, et al., 2009, Schroder, Basta, et al., 2004). These methods consist of a gastric and occasionally an intestinal phase where simulated digestive fluid is combined with soil and
mixed at body temperature to mimic digestion. In these stimulated gastrointestinal
conditions, a portion of the total soil Pb dissolves. This \textit{in vitro} soluble, or bioaccessible,
Pb (IVBA Pb) represents the fraction of Pb that is made available for absorption in the
gut, which can be used to predict RBA (Figure 1). Less expensive and time-consuming,
\textit{in vitro} bioaccessibility assays could be adopted for use in ERA to adjust exposure for the
multiple species, or receptors, that must be assessed at each site to represent the major
ecologic guilds and sensitive, economically important, and endangered species (Cura,

Most soil Pb \textit{in vitro} bioaccessibility assays were developed for human health risk
assessment (HHRA) and aim to simulate fasting conditions. In HHRA, the receptor of
concern is a child consuming soil on an empty stomach. Key parameters that simulate
fasting gastric conditions include a low gastric pH (pH 1.3-2.5) with a small
solid:solution ratio (typically <1:100), to represent the small mass of soil that is
incidentally ingested by humans (Drexler and Brattin, 2007, Rodriguez and Basta, 1999,
Ruby, Davis, et al., 1996). ERA also seeks to protect the most sensitive receptors, but
under typical circumstances. Many animals are exposed to Pb when they consume diet
materials low in Pb, such as plants, but incidentally ingest small amounts of contaminated
soil while feeding (i.e., incidental ingestion). In these cases, soil is the Pb source but diet
is the primary media, and it is the interaction between these two that determines exposure
(Beyer, Chen, et al., 2014). Previous studies have shown that diet can reduce
bioavailability of Pb (Flanagan, Chamberlain, et al., 1982, James, Hilburn, et al., 1985,
Mylroie, Moore, et al., 1978, Ragan, 1983). Diet also reduces IVBA Pb (Martinez-Haro,
Dietary components such as phosphates, calcium, phytate, fiber, oxalate, and tannins have been shown to reduce bioavailability of metals (Chaney, Mielke, et al., 1989, Dendougui and Schwedt, 2004). Proposed mechanisms include increased synthesis of transport proteins in the intestine, competitive absorption with Pb, changing the affinity of target tissues for Pb, adsorption of Pb, and precipitation of Pb compounds (Aungst and Fung, 1985, Barltrop and Khoo, 1975, Gulson, Mizon, et al., 2006). Different diets have varying impacts on Pb bioavailability (Anders, Bagnell Jr, et al., 1982, Barltrop and Khoo, 1975, Crews, Burrell, et al., 1983, James, Hilburn, et al., 1985, Mylroie, Moore, et al., 1978). The bioavailability of soil Pb, therefore, depends on the diet with which soil is consumed. Using *in vitro* bioaccessibility assays to evaluated the IVBA Pb of soil without the influence of diet, therefore, may not be appropriate for assessing the risk posed by incidental soil in animals.

*In vitro* bioaccessibility assays for predicting metal bioavailability were adopted from *in vitro* assays used to determine bioaccessibility of Fe and other metals in food (Crews, Burrell, et al., 1983). Thus, incorporating diet into existing soil *in vitro* bioaccessibility assays may be an appropriate means to evaluate the RBA of soil Pb in diet for wildlife. The objectives of our study were to develop and evaluate *in vitro* bioaccessibility assays to predict soil RBA Pb with diet in Japanese quail (*Coturnix japonica*), a model species commonly used to study Pb toxicology in birds.


Materials and Methods

Soils, Diet, and Quail

Soils in this study were used in previous studies, and their collection and treatment are detailed in Beyer, Basta, et al. (2016). Briefly, eight soils contaminated with Pb from mining waste were sampled at five locations: 1) the Coeur d’Alene River basin in Idaho; 2) East Helena in Montana; and 3) Joplin, 4) the Viburnum Trend, and 5) Big River, all located in Missouri. The Coeur d’Alene River and Big River soils were contaminated via riverine transport of mining-related wastes. Soils from the other sites were contaminated primarily by smelter emissions. Two of the soils were treated with phosphate-containing soil amendments to reduce RBA Pb: Big River soil (from here on, Big River) was treated with 0.75% P as triple superphosphate (Big River 0.75% P); and Joplin soil (from here on, Joplin) was treated with 1% P as phosphoric acid, KCl, and lime (Joplin 1% P). Both soils were treated several years prior to being sampled. Soil was air dried and sieved to ≤250um as recommended by USEPA for use in human in vitro bioaccessibility assays. This is the size fraction which adheres to hands, and hand-to-mouth play is a primary way children are exposed to soil (USEPA, 2007). There is no recommendation on the size fraction appropriate for ecological in vitro bioaccessibility assays, though animals likely ingest a larger size fraction. Using the ≤250um fraction is a conservative approach as Pb concentration tends to increase with decreasing particle size (Chaney, Mielke, et al., 1989). X-ray fluorescence (Thermo Fisher Scientific Niton FXL) was used to determine total Pb content of each soil.
RBA Pb in the study soils was determined in a Japanese quail feeding study detailed in Beyer, Basta, et al. (2016). Briefly, groups of five male four-week-old quail from a Patuxent Wildlife Research Center breeding colony were housed individually in stainless steel cages and exposed to 16 hours of light. After acclamation, quail were randomly assigned to treatment groups, which were fed ad libitum the basal diet (63% oven-dried Purina ® Game Bird Maintenance Chow, 27% oven-dried corn, and 10% water as a binder) spiked with 4% soil or Pb acetate for 15 days. Eight quail were included in the control group and fed only the basal diet. Blood was drawn from the jugular vein at the end of the trial and analyzed for Pb via heated acid and peroxide digestion (USEPA 3050B) and analysis by ICP-MS (USEPA 6020A) (USEPA 1996, 1998). Total blood Pb concentration was divided by dietary Pb concentration to give absolute bioavailability (ABA). Relative bioavailable Pb (RBA Pb) was determined by dividing ABA of soil by the ABA of the Pb acetate-spiked diet and is expressed as a percentage.

The diet (basal diet) used in the in vitro experiments was nearly identical to that used in feeding study: Purina ® Game Bird Maintenance Chow (70%) was combined with 30% corn. Moisture content was 8.5%, determined gravimetrically after oven drying at 60°C. Differences in diet processing and moisture content were not expected to influence the study results. The basal diet was ground until fine in a grain mill (NutriMill Classic Grain Mill, St. George, Utah) and mixed until homogenized. Total elemental content for the basal diet was determined with acid digestion. Dried diet (0.1g) was added to 0.6mL trace metal grade hydrochloric acid (TMG HCl) and 2.0mL TMG nitric acid (HNO₃), allowed to digest for 24 hours, and brought to 10mL with deionized water (DI) (Barnstead
Thermolyne Nanopure Ultrapure Water System D4751 6152s). Reagents were sourced from Thermo Fisher Scientific (Waltham, Massachusetts, USA). Plant tissue digests were analyzed with inductively coupled plasma optical emission spectroscopy (ICP-OES) (USEPA Method 6010D, USEPA, 2000).

**Development of Avian Ohio State University In Vitro Gastrointestinal Extraction**

A method was developed for evaluating soil IVBA Pb with diet specifically for birds. Although several *in vitro* bioaccessibility assays have been developed for avian ecological risk assessment (Bean, Arnold, et al., 2016, Kaufman, Bennett, et al., 2007, Kimball and Munir, 1971, Levengood and Skowron, 2001, Martinez-Haro, Taggart, et al., 2009, Turner and Hambling, 2012), their ability to predict RBA Pb has not been reported. Beyer, Basta, et al. (2016) reported The Ohio State University *in vitro* gastrointestinal method (OSU IVG) was able to predict RBA Pb of contaminated soil for Japanese quail, so this method was modified to better simulate avian physiology. Parameters for the resulting method (Avian OSU IVG, or AOSU IVG) were chosen after reviewing *in vitro* bioaccessibility assays developed for assessing poultry diets and for evaluating heavy metal IVBA Pb in soil for humans and birds. The parameters are summarized in Table 3. Denbow (2015) reported that retention time in the proventriculus and gizzard, which make up the gastric phase in birds, ranged from 33-71 minutes in chickens. In the pH adjustment trial, IVBA Pb did not vary more than 15% at 20, 40, and 70 min. A 60-minute extraction time was chosen for the AOSU IVG gastric phase, in line with other human and ecological *in vitro* bioaccessibility assays (Table 3). Extractant was held at 42°C which represents body temperature of most bird species (Denbow, 2015).
As amylase is present in the saliva of some bird species, the crop phase included 1U/mL amylase (Denbow, 2015). Two preliminary experiments were conducted to determine 1) whether pH should be continually adjusted or buffered by diet and 2) whether to include an intestinal phase.

**pH Adjustment**

Purina ® Game Bird Maintenance diet was spiked with Pb acetate to 85 mg kg⁻¹ Pb, within the range of Pb concentrations in soil-contaminated bird diet (Beyer, Connor, et al., 1994, Turner and Hambling, 2012). Spiked diet was aged for one year. In each of six 200mL polyethylene bottles, 37.5g spiked feed was added to an equal mass of 42°C 1U/mL amylase in DI. After 2 minutes, each mixture received 75 mL of 42°C gastric solution (0.1M NaCl, 1% w/v pepsin, DI) that had been acidified with TMG HCl to pH 2.5 (Thermo Scientific Orion 3-Star Benchtop pH Meter equipped with a double junction Accumet electrode. The gastric solution used here was that of the OSU IVG method (Schroder, Basta, et al., 2004) adjusted to pH 2.5 rather than 1.8 and was similar to the methods used in the remainder of the study.

After gastric solution was added to the spiked diet, half of the samples were immediately returned to pH 2.5 with 6M TMG HCl, while the remaining samples were not adjusted. Samples rotated at 30rpm on an end-over-end shaker in an incubator at 42°C, which represents body temperature of most bird species (Denbow, 2015). The pH was corrected with TMG HCl at 20 and 40 minutes. At 20, 40, and 70 minutes 10mL of supernatant solution was extracted and syringe filtered. The pH of the remaining suspension was then
recorded. Samples were refrigerated at 4°C until analysis with ICP-OES to determine IVBA Pb. Amylase and pepsin were sourced from Sigma-Aldrich (St. Louis, Missouri, USA) while NaCl was sourced from Thermo Fisher Scientific (Waltham, Massachusetts, USA).

**Intestinal Phase**

Wildlife *in vitro* bioaccessibility assays may include only a gastric phase (Kimball and Munir, 1971, Levengood and Skowron, 2001, Thompson and Sparks, 1978) or both gastric and intestinal phases (Bean, Arnold, et al., 2016, Furman, Strawn, et al., 2006, Martinez-Haro, Taggart, et al., 2009), To determine whether an intestinal phase would be appropriate for a variety of diets, six diets were analyzed in an intestinal *in vitro*. bird diets were analyzed: Purina ® Game Bird Maintenance Chow (avian granivore), Purina ® Game Bird Layena ETTS (avian granivore), horsemeat (avian carnivore). Including three rodent diets allowed a greater variety of diets to be analyzed: rodent chow (small mammal), oats (large mammal), and corn (small mammal). These diets are commonly fed during toxicological assays that determine TRVs and soil Pb bioavailability used in ERA. Joplin soil (1.0g) and 25.0g diet were placed in a 150 mL polyethylene bottle and run through the gastric and intestinal phases of the Ohio State University *in vitro* bioaccessibility assay (OSU IVG), detailed by Schroder, Basta, et al. (2004). Each diet was analyzed in triplicate. IVBA Pb was determined with ICP-OES.
Determination of In Vitro Bioaccessible Pb

Three *in vitro* bioaccessibility assays were used to evaluate IVBA Pb: the relative bioavailability leaching procedure (RBALP) (Drexler and Brattin, 2007), which is also known as USEPA Method 1340 (USEPA, 2017); a modified USEPA 1340 where an extraction solution of pH 2.5 was used instead of pH 1.5; and Avian Ohio State University *in vitro* gastrointestinal method (AOSU IVG). USEPA Method 1340 is the accepted method for determining soil RBA to humans. A modified USEPA 1340 at pH 2.5 is also predictive of RBA, is a less aggressive method that is better able to predict RBA Pb in treated soils (Beyer, Basta, et al., 2016), and was shown in Beyer, Basta, et al. (2016) to predict soil Pb RBA in Japanese quail (*Coturnix japonica*). AOSU IVG is based on OSU IVG, which was predictive of *C. japonica* RBA in Beyer, Basta, et al. (2016), with the parameters modified to more closely match avian physiology. IVBA Pb was assessed for diet+soil blends with USEPA 1340 at pH 1.5 and 2.5 and AOSU IVG. Soil-alone IVBA Pb was evaluated with AOSU IVG, while soil-alone IVBA Pb data for USEPA 1340 at pH 1.5 and 2.5 was obtained from Beyer, Basta, et al. (2016). A short description of each method follows.

**USEPA 1340** (USEPA, 2017). Soil (1.0 g, ≤250um) and 25.0g homogenized diet was placed in an open beaker with gastric solution (100mL). Gastric solution was 0.40M glycine in DI, adjusted with TMG HCl to pH 1.5. All solutions were preheated (42°C, which represents body temperature of most bird species (Denbow, 2015)) and the temperature maintained in a water bath (Figure 2). Beakers were continuously mixed using a paddle stirrer to maintain a homogenous suspension. Solution pH was
continuously monitored and adjusted to 1.5±0.05 with dropwise addition of 2.3 M Na$_2$CO$_3$ and/or 6M TMG HCl. After 1 hour, 10mL of gastric solution was immediately centrifuged (3800 rpm; 3374 X $g$ for 15 min) and the supernatant was refrigerated at 4ºC prior to analysis with ICP-OES. Glycine and Na$_2$CO$_3$ were sourced from Thermo Fisher Scientific (Waltham, Massachusetts, USA).

*USEPA 1340 with pH modification.* This modified procedure follows the previously described method, except the solution pH was adjusted to 2.5±0.05 instead of 1.5 using dropwise addition of 2.3 M Na$_2$CO$_3$ and/or 6M trace metal HCl.

*Avian OSU IVG.* The final method is detailed below, and references for each parameter are given in Table 3. Soil (1.0 g, ≤250µm) and ground basal diet (25.0g) were stirred into 25 mL of salivary solution (1U/mL amylase) in an open beaker. After 2 minutes, 75 mL of concentrated gastric solution (0.133M NaCl and 0.133% [w/w] porcine pepsin in DI adjusted with TMG HCl to pH 2.0) was added, which resulted in a final gastric solution of 0.10M NaCl and 0.10% [w/w] porcine pepsin. All solutions were preheated to 42ºC (bird body temperature) and the temperature maintained in a water bath. Beakers were continuously mixed using a paddle stirrer to maintain a homogenous suspension. Solution pH was continuously monitored and adjusted to 2.0±0.05 with dropwise addition of 2.3 M Na$_2$CO$_3$ and/or 6M trace metal HCl. After 1 hour, 10mL of gastric solution was immediately centrifuged (3800 rpm; 3374 X $g$ for 15 min) and the supernatant was refrigerated at 4ºC prior to analysis with ICP-OES.
Calculation of in Vitro Bioaccessible Pb.

The *in vitro* bioaccessible Pb (IVBA Pb) was determined and expressed as a percentage of total Pb as follows:

\[
\%\text{IVBA Pb} = \frac{\text{IVBA extractable Pb} \ [\text{mg kg}^{-1}]}{\text{total soil Pb} \ [\text{mg kg}^{-1}]} \times 100\%
\]

Statistics and Quality Assurance

All analyses were conducted in duplicate. A blank and reference material were included with each batch for quality assurance and quality control. The relative percent difference (RPD) of in duplicate analyses did not exceed 15%. Blanks did not exceed 10% of the reporting limit for Pb (0.05mg/L) for both the *in vitro* assays and total elemental content acid digestions. Pb recovery for a SiO$_2$ blank obtained with XRF was below the instrument detection limit (<3 mg kg$^{-1}$).

Although no published values exist for the bioaccessibility of the reference soil (National Institute of Standards and Technology (NIST) SRM 2711a Montana II Soil) in any of the *in vitro* bioaccessibility assays trialed, the reference soil was used to track interbatch variability. Variability between batches was low, with %RSD averaging 11% or less. Accuracy of the plant tissue digest was verified using NIST SRM 1573a Tomato Leaves. Total elemental recoveries in % in parentheses were: Ca(81%); K(82%); Mg(77%); Mn(81%); Na(80%) P(90%); and S(111%). Accuracy of the soil total Pb analysis with XRF was verified with a RCRA multielement standard and NIST SRM 2709a (San Joaquin Soil). Total Pb recoveries were between 80-101%.
Statistical tests were conducted with SigmaPlot 10 software (Systat Software) and R 3.5.0 (R core Team, 2018). The average IVBA Pb of \( n = 2 \) subsamples were used in the analyses. Differences in IVBA Pb between the three diet+soil methods were evaluated with a one-way analysis of variance (ANOVA, R function “aov”). The assumptions of ANOVA were met: Bartlett’s test confirmed homogeneity of variances, no outliers were observed, samples were independent, and normality and homogeneity of residuals were verified using Normal Q-Q and residual plots, respectively. A Tukey multiple comparison of means test was then used to detect significant differences between the three methods and Japanese quail RBA. This test was also used to compare the IVBA Pb of the Big River and Joplin treated soils with their untreated counterparts. A paired t-test was used to compare the IVBA Pb of the diet+soil methods with the corresponding soil-alone method. The quail RBA for the 8 contaminated soils was linearly regressed against IVBA Pb for each method. These regressions are referred to as in vitro-in vivo correlations (IVIVCs). Significance of the regressions was assessed at \( \alpha = 0.05 \).

**Results and Discussion**

**Soil and Diet Composition**

Total calcium and phosphorus contents in basal diet (70% Purina ® Game Bird Maintenance Chow, 30% corn) were 6600 mg kg\(^{-1}\) Ca and 4630 mg kg\(^{-1}\) P (wet mass). Moisture content was 8.5%. Phosphorus and Ca were particularly high in the basal diet, which was fortified with CaCO\(_3\). These elements have been shown to impact absorption of heavy metals, including Pb (Bullock, Duffin, et al., 1995). The basal diet contained
0.27 mg kg\(^{-1}\) Pb. Content of other elements, in mg kg\(^{-1}\) wet mass in parentheses, were: K (5100); Mg (1310); Mn (82), Na (948); and S(1550). The Pb concentrations for the study soils ranged from 2070 to 4984 mg kg\(^{-1}\) dry mass (Table 1).

*Gastric Solution pH Adjustment*

IVBA Pb of the adjusted *in vitro* bioaccessibility assays was 22% but only 1% in the unadjusted assays. The pH of the unadjusted assays rose from 2.5 to 5.3. This is much higher than fed gastric pH values reported in birds, which range from 2.1-3.8 (Denbow, 2015). Gastric pH rises as high as pH when food is digested. Buffering effects of diet may reduce RBA Pb (Martinez-Haro, Taggart, et al., 2009). However, gastric secretion lowers digesta pH over time, and the pH of chyme released in the small intestine is similar to that of gastric secretions (around pH 2-3 in birds) (Denbow, 2015). Maintaining pH by adding acid or gastric solution may better simulate gastric digestion and also ensures detectable IVBA Pb. Gastric pH was therefore continuously maintained for all three methods.

*Intestinal Phase*

IVBA Pb of spiked diets in simulated avian small intestinal conditions was below detection limit (<0.16%) in the Purina ® Game Bird Maintenance Chow and rodent chow; 0.27% in Purina ® Game Bird Layena ETTS; and 10% in horsemeat and corn. The near complete binding of Pb in the commercially processed stock diets is not reflective of what occurs *in vivo*. Additionally, gastric IVBA Pb often shows stronger correlations
with RBA Pb than intestinal IVBA Pb in human \textit{in vitro} bioaccessibility assays, (Schroder, Basta, et al., 2004). Better correlation with gastric IVBA Pb may be due to rapid absorption of digesta Pb soon after it is released into the small intestine. Absorption occurs before pH of the digesta reaches equilibrium (Rodriguez, 1998). In contrast, intestinal extractions are several hours and IVBA Pb is measured after pH has risen to a steady state. As intestinal IVBA Pb was undetectable for some diets, only a gastric phase was included in AOSU IVG.

\textit{In Vitro Bioaccessible Pb}

Compared to soil-alone IVBA Pb, the addition of diet significantly reduced IVBA Pb for each method (p<0.001). IVBA Pb varied with the \textit{in vitro} bioaccessibility assay used (Table 4). USEPA 1340 at pH 1.5 produced higher IVBA Pb than USEPA 1340 at pH 2.5 (p=0.001) while AOSU IVG was intermediate (p=0.11 and 0.21 versus USEPA 1340 at pH 1.5 and 2.5, respectively). This differs somewhat from the soil-alone \textit{in vitro} bioaccessibility assays: IVBA Pb in AOSU IVG was not different than in USEPA 1340 at pH 2.5 (p=0.25), while soils analyzed with USEPA 1340 at pH 1.5 had significantly greater IVBA Pb than AOSU IVG (p<0.0001) and USEPA 1340 at pH 2.5 (p<0.0001) (Table 4).

\textit{In Vitro-In Vivo Correlation}

The IVBA Pb for all methods were significantly different from RBA Pb (p<0.05). According to USEPA guidance for human risk assessment, IVBA Pb need not be
identical to RBA Pb in order for the method to be predictive, but a regression with in vitro values should accurately predict in vivo values (USEPA, 2007). An in vitro-in vivo regression (IVIVC) was used to determine the ability of each in vitro bioaccessibility assay to predict RBA Pb (Fig. 2). Figure 2A gives the IVIVC with diet and all soils, while 2B includes diet but excludes the Joplin Treated soil. Figure 2C shows the IVIVCs for all soils without diet.

Two soils in the dataset had been treated with phosphorus to reduce Pb bioavailability: Joplin 1% P and Big River 0.75% P. Big River 0.75% P responded poorly to treatment: RBA Pb decreased by 25%, little pyromorphite formed (Beyer, Basta, et al., 2016), and the IVBA Pb was not significantly different between the treated and untreated soil in any method trialed in this study. This treated soil behaved similarly to the untreated soils. Joplin 1% P responded well to treatment: RBA Pb decreased by 58%, pyromorphite formed (Beyer, Basta, et al., 2016), and IVBA Pb was significantly lower than that of the untreated Joplin soil in every method (p<0.05). This soil had a higher IVBA Pb than its RBA Pb would suggest. Thus it had an effect on the slopes of the IVIVCs (Figures 1A and 1B). Despite this, predicted RBA did not differ strongly between the IVIVCs for soils with IVBA Pb>20%. Additionally, an extended range of IVBA Pb values improved the coefficient of determinations, and a robust method that is predictive for both P-treated and untreated soils is highly desirable. All of this supports the inclusion of Joplin (Figure 2A).

Diet affected the predictive regression in the IVIVCs in all methods trialed here (Fig. 1A, 1C). Diet increased the RBA Pb vs. IVBA Pb slope because it decreased IVBA Pb. This
suggests separate IVIVCs are needed when assessing soil-alone and diet+soil Pb availability rather than using the same regression equation. Strong relationships were present between RBA Pb and IVBA Pb in all in vitro bioaccessibility assays. The diet+soil methods were somewhat improved over the soil alone methods ($r^2=0.70$-$0.92$ vs $0.58$-$0.83$, respectively). Quail were dosed Pb with diet, rather than while fasting. Diet+soil assays may better simulate fed conditions compared to soil-alone methods. These results agree with Schroder, Basta, et al. (2004) who found an equally good correlation between the OSU IVG method with soil alone and with diet added.

Conclusions

While official guidance does not exist for ecological IVIVCs, in general, the regression should have a high coefficient of determination that demonstrates the data fit the statistical model and an intercept close to zero to accurately predict RBA for soils with low IVBA Pb. The $r^2$ for all methods suggested predictive relationships. Intercepts for all methods were low aside from USEPA 1340 at pH 1.5 with diet. This method would not be appropriate for assessing soils with low IVBA Pb. USEPA 1340 at pH 1.5 may be too aggressive for evaluating soil with diet and cannot assess soils with low IVBA Pb. AOSU IVG was the strongest relationship ($r^2=0.92$). Evaluating additional treated soils and soil types would ensure these IVIVCs are applicable to a broader range of soils, however.

These data suggest that an IVIVC derived from either a diet+soil or soil-alone method would yield a predictive equation to estimate RBA Pb. Using an in vitro bioaccessibility assay to predict soil Pb RBA would allow risk assessors to adjust soil RBA Pb from the
default 100% bioavailability (Equation 1). The RBA Pb in these soils, for example, ranged from 17-63%, meaning calculated exposure would be reduced 37-83% compared to the default assumption. Cleanup soil Pb concentrations would be substantially increased, likely decreasing the amount of soil that needs to be remediated.

The diet+soil in vitro bioaccessibility assays had somewhat better relationships with quail RBA compared to the the soil-alone methods, yet the latter are easier to conduct. However, diet+soil methods may prove useful over soil-alone methods if they can predict the impact of different diets on bioavailability. This would require multiple diets to be trialed and validated with feeding studies, as IVBA Pb cannot be used directly when assessing exposure. Using IVBA Pb data generated in this experiment as RBA would underestimate exposure as diet reduced IVBA Pb.

These first steps towards developing diet+soil ecological in vitro bioaccessibility assays have demonstrated that diet does influence IVBA Pb and therefore should be considered when evaluating exposure for risk assessment. In vitro bioaccessibility assays can accurately predict soil Pb RBA in Japanese quail when diet is included directly in the in vitro. With further research, these methods will facilitate the use of RBA Pb to adjust exposure for ecological risk assessments.
Figure 1.1. Schematic of Pb uptake. Soil is ingested and a portion of Pb solubilizes in the gastrointestinal (GI) solution. The soluble, or bioaccessible, Pb is able to be absorbed. A portion of bioaccessible Pb is absorbed into the bloodstream (bioavailable Pb). (Modified from White, 2005)
Figure 1.2. Experimental setup. Top: diet+soil blends in open beakers alongside simulated saliva and gastric solutions; Bottom: hot water bath, stirrers, and pH meters used to simulate gastric conditions during the analysis.
Table 1.1. Total elemental concentrations of study soils as determined with X-ray fluorescence.

<table>
<thead>
<tr>
<th>Soil</th>
<th>Ca</th>
<th>Cd</th>
<th>Fe</th>
<th>Pb</th>
<th>S</th>
<th>Zn</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDA</td>
<td>2540 ±36</td>
<td>4 ±0.25</td>
<td>63600 ±398</td>
<td>3160 ±14</td>
<td>497 ±29</td>
<td>1340 ±12</td>
</tr>
<tr>
<td>Helena</td>
<td>13200 ±151</td>
<td>99 ±3</td>
<td>28300 ±469</td>
<td>3810 ±199</td>
<td>1040 ±88</td>
<td>3090 ±184</td>
</tr>
<tr>
<td>Viburnum</td>
<td>3230 ±195</td>
<td>&lt;8</td>
<td>6180 ±98</td>
<td>3200 ±54</td>
<td>629 ±56</td>
<td>231 ±16</td>
</tr>
<tr>
<td>Big River</td>
<td>32500 ±1680</td>
<td>3 ±2.02</td>
<td>19600 ±275</td>
<td>2150 ±13</td>
<td>441 ±39</td>
<td>597 ±4</td>
</tr>
<tr>
<td>Big River 0.75% P</td>
<td>50500 ±1260</td>
<td>8 ±0.96</td>
<td>18300 ±510</td>
<td>2000 ±76</td>
<td>520 ±24</td>
<td>632 ±28</td>
</tr>
<tr>
<td>Joplin</td>
<td>9380 ±885</td>
<td>8 ±1.62</td>
<td>15600 ±569</td>
<td>5120 ±84</td>
<td>1410 ±72</td>
<td>3980 ±162</td>
</tr>
<tr>
<td>Joplin 1% P</td>
<td>19400 ±983</td>
<td>9 ±0.46</td>
<td>15800 ±639</td>
<td>4270 ±56</td>
<td>1220 ±121</td>
<td>2980 ±157</td>
</tr>
<tr>
<td>Joplin 10% Compost</td>
<td>33000 ±1470</td>
<td>9 ±1.20</td>
<td>22890 ±799</td>
<td>3310 ±108</td>
<td>1010 ±66</td>
<td>2540 ±146</td>
</tr>
</tbody>
</table>

Background soil Pb (95th percentile) in a) Idaho=33 mg kg⁻¹, b) Montana=40 mg kg⁻¹ and c) Missouri=47 mg kg⁻¹ (Smith, Cannon et al., 2013)

*Mean of n=3 subsamples
Table 1.2. Properties of study soils as reported in Beyer, Basta, et al. (2016).

<table>
<thead>
<tr>
<th>Soil</th>
<th>Pb (mg kg(^{-1}) dry wt)</th>
<th>Ca (mg kg(^{-1}) dry wt)</th>
<th>P (mg kg(^{-1}) dry wt)</th>
<th>pH</th>
<th>Cation exchange capacity (meq/100g)</th>
<th>Organic matter (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDA</td>
<td>960</td>
<td>398</td>
<td>6</td>
<td>5.4</td>
<td>4</td>
<td>10.9</td>
</tr>
<tr>
<td>Helena</td>
<td>900</td>
<td>3330</td>
<td>110</td>
<td>6.5</td>
<td>11</td>
<td>21.7</td>
</tr>
<tr>
<td>Viburnum</td>
<td>1600</td>
<td>1150</td>
<td>19</td>
<td>5.1</td>
<td>12</td>
<td>14.2</td>
</tr>
<tr>
<td>Big River</td>
<td>1100</td>
<td>3030</td>
<td>42</td>
<td>7.6</td>
<td>3.1</td>
<td>20.4</td>
</tr>
<tr>
<td>Big River 0.75% P</td>
<td>570</td>
<td>8350</td>
<td>3300</td>
<td>7.2</td>
<td>3</td>
<td>21.2</td>
</tr>
<tr>
<td>Joplin Control</td>
<td>1100</td>
<td>3800</td>
<td>74</td>
<td>7</td>
<td>7.3</td>
<td>17.5</td>
</tr>
<tr>
<td>Joplin 1% P</td>
<td>130</td>
<td>7840</td>
<td>2900</td>
<td>6.3</td>
<td>8</td>
<td>23.7</td>
</tr>
<tr>
<td>Joplin 10% Compost</td>
<td>6 190</td>
<td>13 600</td>
<td>320</td>
<td>7.7</td>
<td>8.6</td>
<td>17.8</td>
</tr>
</tbody>
</table>
Table 1.3. Parameters of Ohio State University Gastrointestinal extraction (OSU IVG) and the modified method, Avian OSU IVG (AOSU IVG) which simulates avian physiology. References justifying each parameter are given.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>OSU IVG</th>
<th>AOSU IVG</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Crop Phase</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diet-soil:solution (w/v)</td>
<td>n/a</td>
<td>1:1</td>
<td>Minekus, Alminger, et al., 2014, Philips and Fuller, 1983</td>
</tr>
<tr>
<td>Amylase</td>
<td></td>
<td>1U/mL</td>
<td>Minekus, Alminger, et al., 2014, Philips and Fuller, 1983</td>
</tr>
<tr>
<td>Extraction time (min)</td>
<td></td>
<td>2</td>
<td>Minekus, Alminger, et al., 2014, Philips and Fuller, 1983</td>
</tr>
<tr>
<td><strong>Gastric Phase</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>1.8</td>
<td>2</td>
<td>Bean, Arnold, et al., 2016, Scanes, 2015, Zyla, Ledoux, et al., 1995</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.15M</td>
<td>0.1M</td>
<td>Crews, Burrell, et al., 1983, Rodriguez and Basta, 1999</td>
</tr>
<tr>
<td>Pepsin</td>
<td>1%</td>
<td>0.1%</td>
<td>Kimball and Munir, 1971, Scanes, 2015</td>
</tr>
<tr>
<td>Soil: solution (w/v)</td>
<td>1:150</td>
<td>1:100</td>
<td>Drexler and Brattin, 2007, Hamel, Buckley, et al., 1998</td>
</tr>
<tr>
<td>Diet:solution (w/v)</td>
<td>n/a</td>
<td>1:4</td>
<td>Ao, Cantor, et al., 2010, TerviLaWilo, Parkkonen, et al., 1996, Zyla and Koreleski, 1993</td>
</tr>
<tr>
<td>Extraction time (min)</td>
<td>60</td>
<td>60</td>
<td>Furman, Strawn, et al., 2006, Levengood and Skowron, 2001, Scanes, 2015</td>
</tr>
</tbody>
</table>
Table 1.4. *In vitro* bioaccessible Pb (IVBA Pb), without and with basal data (70% Purina® game bird maintenance chow, 30% corn) for soils fed to Japanese quail (Coturnix japonica) as assessed with 3 in vitro bioaccessibility assays.

Methods with different letters are significantly different according to a Tukey multiple comparison of means test.

*Average of \( n = 2 \) subsamples. Relative percent difference of subsamples (RPD) did not exceed 15%.

**Reported in Beyer et al., 2016

<table>
<thead>
<tr>
<th>Soil</th>
<th>Bioaccessible Pb (%)*</th>
<th>Quail RBA Pb (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1340 pH 1.5 no diet(^a)</td>
<td>1340 pH 1.5 with diet(^c,)**</td>
</tr>
<tr>
<td>Coeur d'Alene</td>
<td>67</td>
<td>24</td>
</tr>
<tr>
<td>Helena</td>
<td>89</td>
<td>42</td>
</tr>
<tr>
<td>Viburnum Trend</td>
<td>86</td>
<td>34</td>
</tr>
<tr>
<td>Big River 0.75% P</td>
<td>97</td>
<td>42</td>
</tr>
<tr>
<td>Big River control</td>
<td>101</td>
<td>37</td>
</tr>
<tr>
<td>Joplin control</td>
<td>91</td>
<td>36</td>
</tr>
<tr>
<td>Joplin 1% P</td>
<td>63</td>
<td>38</td>
</tr>
<tr>
<td>Joplin 10% compost</td>
<td>81</td>
<td>33</td>
</tr>
</tbody>
</table>
Figure 1.3. Percent relative bioavailable Pb (RBA) vs percent bioaccessible Pb (IVBA) for: (A) all soils with diet, including Joplin Treated (JT); (B) soils with diet, excluding JT; and (C) all soils without diet, including JT. Significance is *0.05 and **0.01.
References


Chapter 2. Comparison of Diet Impact on *In Vitro* Bioaccessibility of Soil Lead

*Introduction*

While soils naturally contain small amounts of Pb, redistribution of Pb from anthropogenic use has raised concentrations above background levels in many soils (i.e. contaminated soils). Animals incidentally ingest soil while feeding and while ingesting soil with background levels of soil Pb do not appear to pose a risk to animals, ingestion of contaminated soil increases Pb exposure. Ecological risk assessment (ERA) determines whether a contaminated soil is polluted, or poses a risk of adverse biological impacts. Cleanup standards for Pb-polluted sites are determined by animals’ Pb exposure, or the dose of Pb absorbed from the environment. Exposure estimates for each receptor of interest are compared to a toxicity reference value (TRV), or the Pb above which adverse health effects are likely. The soluble Pb forms used in toxicological assays to generate TRVs are maximally bioavailable (Figure 1). Soil Pb is less readily absorbed than soluble Pb forms (relative bioavailability (RBA) <100%). Furthermore, there is a large body of research suggesting that diet reduces soil Pb bioavailability and that these effects vary between diets (Ragan, 1983). Dietary components of plant matter such as minerals and phytate have been shown to reduce bioavailability of metals (Chaney, Mielke, et al., 1989, Dendougui and Schwedt, 2004, Schroder, Basta, et al., 2004). Exposure calculations in ecological risk assessment (ERA) can be adjusted using diet-soil RBA Pb. As RBA Pb would be less than the default 100% RBA in most cases, this would reduce
soil cleanup values and therefore remediation costs. (Sample, Schlekat, et al., 2014, Suedel, Nicholson, et al., 2006).

Animal dosing studies currently used to gather soil RBA data are expensive and time-consuming. In an effort to reduce costs of obtaining human bioavailability data, extensive research in the past two decades has led to development and adoption of *in vitro* bioaccessibility assays to predict RBA Pb through *in vitro* bioaccessibility (IVBA) (Basta and Juhasz, 2014, Drexler and Brattin, 2007, Intawongse and Dean, 2006, Rodriguez and Basta, 1999, Ruby, Davis, et al., 1993). These soil *in vitro* bioaccessibility assays simulate gastrointestinal conditions to measure the amount of soluble, or *in vitro* bioaccessible Pb (IVBA), which is able to be absorbed in the gut (Figure 1). IVBA Pb can be used to predict RBA Pb. Though human methods simulate fasting conditions, these methods were originally developed from diet *in vitro* bioaccessibility assays that aimed to measure metal availability from food. This approach may be appropriate for assessing the Pb availability in soil consumed with different diets. This diet+soil RBA data could be used to adjust exposure calculations in ERA. This study seeks (1) to assess the impact of eight diets used in ERA on soil Pb IVBA, (2) to relate diet characteristics to reductions in IVBA Pb, and (3) to assess whether reductions in IVBA Pb from diet were dependent on soil type.
Materials and Methods

Experimental Design

The study consisted of three experiments detailed in Table 1. The diagram comparison experiment, diagramed in Table 1A, examined the impact of different diets on soil IVBA Pb. Seven diets used to assess animals Pb toxicity were analyzed with three \textit{in vitro} bioaccessibility assays with a single soil (Joplin) (7x3 factorial). Mineral and phytate concentrations of diets were compared with principal component analysis and related to IVBA Pb with linear regression. The soil comparison experiment (Table 1B) compared the impacts of diet on IVBA Pb of different soils. Eight soils were combined with a single diet (basal diet, 70\% Purina \textregistered Game Bird Maintenance Chow, 30\% corn) and analyzed with three \textit{in vitro} bioaccessibility assays (8x3 factorial). Soil IVBA Pb with and without diet were compared with a linear regression. The source comparison experiment (Table 1C) examined the impact of different diets on two Pb sources. Six ERA diets were combined with either Joplin soil or a Pb(NO$_3$)$_2$ spike and analyzed with one \textit{in vitro} bioaccessibility assay, AOSU IVG (6x2 factorial). IVBA Pb in soil- and spike-contaminated diets were compared with a linear regression.

Diet Preparation and Characterization

Six diets used in ecological risk assessment toxicological assays used in toxicological assays used to generate TRVs and soil Pb bioavailability data for different species were
analyzed along with basal diet fed to Japanese quail (*Coturnix japonica*) in a Pb dosing trial conducted by Beyer, Basta, et al. (2016). Three bird diets were analyzed: Purina® Game Bird Maintenance Chow (avian granivore), Purina® Game Bird Layena ETTS (avian granivore), and horsemeat (avian carnivore). So that a greater variety of diets could be explored, three mammal diets were also analyzed: rodent chow (small mammal), oats (large mammal), and corn (small mammal). The basal diet was 70% Purina® Game Bird Maintenance Chow and 30% corn. Purina® Game Bird Maintenance Chow, Purina® Game Bird Layena ETTS, and rodent chow were commercially prepared stock diets; oats, corn, and horsemeat were natural diets; and the basal diet was a blend of stock and natural diets.

All diets aside from horsemeat were ground until homogenous with a grain mill (NutriMill Classic Grain Mill). Horsemeat was ground twice in a manual meat grinder. All *in vitro* analyses were conducted with undried diet and data is reported in wet mass. Subsamples of diets were dried at 60°C for 24 hours obtain moisture content. Total elemental content for each diet was determined through acid digestion. Dried diet (0.1g) was added to 0.6mL trace metal grade hydrochloric acid (TMG HCl) and 2.0mL TMG nitric acid (HNO₃), allowed to digest for 24 hours, and brought to 10mL with deionized water (DI) (Barnstead Thermolyne Nanopure Ultrapure Water System D4751 6152s). Reagents were sourced from Thermo Fisher Scientific (Waltham, Massachusetts, USA).

Plant tissue digests were analyzed with inductively coupled plasma optical emission spectroscopy (ICP-OES) (USEPA Method 6010D, USEPA, 2000). Phytate was
estimated in each diet by measuring the total phosphorus (free P and phytate P, as released by phytase and alkaline phosphatase) and subtracting the free phosphorus. These were measured using the method described by McKie and McCleary (2016). Alkaline phosphatase (80 U/mL), phytase (12,000 U/mL), alkaline phosphatase assay buffer (400 mM glycine, 4 mM magnesium chloride, 0.4 mM zinc sulfate and 0.02% s/v sodium azide, pH 10.4), and phytase assay buffer (200 mM sodium acetate and 0.02% s/v sodium azide, pH 5.5), phosphate standard solution (50ug/mL) and an oat flour control powder (0.535% P) were supplied as part of the Total Phosphorus and Phytic Acid Assay Kit obtained from Megazyme International (Bray, County Wicklow, Ireland). Ascorbic acid sulfuric acid, ammonium molybdate, hydrochloric acid and sodium hydroxide pellets were all reagent grade and obtained from Thermo Fisher Scientific (Waltham, Massachusetts, USA). Trichloroacetic acid was obtained from Sigma-Aldrich (St. Louis, Missouri, USA).

Each diet (1.0g) was added to 20mL 0.66M trace metal grade (TMG) HCl and shaken overnight to extract free P and phytic acid (total P). The extract was transferred to a 1.5 mL microfuge tube and centrifuged at 13,000 rpm (13,800 X g) for 10 minutes. Supernatant (0.5mL) was transferred to a fresh microfuge tube and neutralized with 0.75M sodium hydroxide (0.5mL). In a fresh microfuge tube the neutralized sample extract (0.05mL) was added to 0.20 mL phytase assay buffer in each of two fresh microfuge tubes: free P was measured in one subsample while total P was evaluated in
the other. Phytase (0.02mL) was added only to the total P subsamples. Deionized water
was then added to the free P (0.062mL) and total P (0.60mL) samples.

Samples were vortexed and incubated in a 40°C water bath for 10 minutes. Following
incubation, samples received 0.20mL alkaline phosphatase buffer. Alkaline phosphatase
(0.02mL) was added to the total P subsamples, while the same volume of DI was added
to the free P subsamples. The solutions were vortexed and incubated at 40° C for 15
minutes. Addition of 0.30mL trichloroacetic acid to the samples terminated further
reaction. Samples were centrifuged at 13,000 rpm (13,800 X g) for 10 minutes and 1.0mL
of supernatant was transferred to a microfuge tube along with color reagent (0.5mL).
After incubating at 40° C for 1 hour, absorbance at 655nm was recorded using a
spectrophotometer (Evolution 60S UV-Visible Spectrophotometer) calibrated with 0, 0.5,
2.5, 5, and 7.5 ug P/L standards prepared in deionized water.

Soil Processing and Characterization

Soils in this study were used in previous studies, and their collection and treatment are
detailed in Beyer, Basta, et al. (2016). Briefly, eight soils contaminated with Pb from
mining waste were sampled at five locations: 1) the Coeur d’Alene River basin in Idaho;
2) East Helena in Montana; and 3) Joplin, 4) the Viburnum Trend, and 5) Big River, all
located in Missouri. The Coeur d’Alene River and Big River soils were contaminated via
riverine transport of mining-related wastes. Soils from the other sites were contaminated
primarily by smelter emissions. Two of the soils were treated with phosphate-containing
soil amendments to reduce RBA Pb: Big River soil (from here on, Big River) was treated with 0.75% P as triple superphosphate (Big River 0.75% P); and Joplin soil (from here on, Joplin) was treated with 1% P as phosphoric acid, KCl, and lime (Joplin 1% P). Both soils were treated several years prior to being sampled. Soil was air dried and sieved to ≤250μm as recommended by USEPA for use in human in vitro bioaccessibility assays. This is the size fraction which adheres to hands, and hand-to-mouth play is a primary way children are exposed to soil (USEPA, 2007). There is no recommendation on the size fraction appropriate for ecological in vitro bioaccessibility assays, though animals likely ingest a larger size fraction. Using the ≤250μm fraction is a conservative approach as Pb concentration tends to increase with decreasing particle size (Chaney, Mielke, et al., 1989). X-ray fluorescence (Thermo Fisher Scientific Niton FXL) was used to determine total Pb content of each soil.

Determination of In Vitro Bioaccessible Pb

Three in vitro bioaccessibility assays were used to evaluate IVBA Pb: the relative bioavailability leaching procedure (RBALP) (Drexler and Brattin, 2007), which is also known as USEPA Method 1340 (USEPA 2017); a modified USEPA 1340 where an extraction solution of pH 2.5 was used instead of pH 1.5; and Avian Ohio State University in vitro gastrointestinal method (AOSU IVG). USEPA 1340 is the accepted method for predicting soil RBA in humans. USEPA 1340 at pH 2.5 is a less aggressive method that is better able to predict RBA Pb in treated soils and was shown in Beyer, Basta, et al. (2016) to predict soil Pb RBA in Japanese quail (Coturnix japonica). AOSU
IVG is based on OSU IVG, which was predictive of *C. japonica* RBA in Beyer, Basta, et al. (2016), with parameters modified to more closely match avian physiology. IVBA Pb was assessed for diet+soil blends with USEPA 1340 and AOSU IVG. AOSU IVG was also used to assess diet spiked with PbNO₃ (See *Diet and Soil Interactions*). IVBA Pb of soil-alone was evaluated with AOSU IVG here, while IVBA Pb of soil-alone using USEPA 1340 at pH 1.5 and 2.5 was obtained from Beyer, Basta, et al. (2016). A short description of each method follows.

**USEPA 1340** (USEPA, 2017). Soil (1.0 g, ≤250um) and 25.0g homogenized diet was placed in an open beaker with 100mL of gastric solution (0.40M glycine in DI adjusted with TMG HCl to pH 1.5). All solutions were preheated to 42°C, which represents body temperature of most bird species (Denbow, 2015). This is a modification from the official method, where human body temperature (37°C) is used. The gastric suspension temperature was maintained throughout the extraction by holding the beakers in a hot water bath. Beakers were continuously mixed using a paddle stirrer to maintain a homogenous suspension. Solution pH was continuously monitored and adjusted to 1.5±0.05 with dropwise addition of 2.3 M Na₂CO₃ and/or 6M TMG HCl. After 1 hour, 10mL of gastric solution was immediately centrifuged (3800 rpm; 3374 X g) for 15 min) and the supernatant was refrigerated at 4°C prior to analysis with ICP-OES. Glycine was sourced from Thermo Fisher Scientific (Waltham, Massachusetts, USA) and Na₂CO₃ from Sigma-Aldrich (St. Louis, Missouri, USA).
USEPA 1340 with pH modification. This modified procedure follows the previously
described method, except that the solution pH was adjusted to 2.5±0.05 instead of 1.5
using dropwise addition of 2.3 M Na₂CO₃ and/or 6M trace metal HCl.

Avian OSU IVG. Soil (1.0 g, ≤250um) and ground diet (25.0g) were stirred into 25 mL of
salivary solution (1U/mL amylase) in an open beaker. After 2 minutes, 75 mL of gastric
solution (0.133M NaCl and 0.133% [w/w] porcine pepsin in DI adjusted with TMG HCl
to pH 2.0) was added. The resulting extractant solution was 0.10M NaCl and 0.10%
[w/w] porcine pepsin. All solutions were preheated (42°C) and the temperature
maintained in a water bath. Beakers were continuously mixed using a paddle stirrer to
maintain a homogenous suspension. Solution pH was continuously monitored and
adjusted to 2.0±0.05 with dropwise addition of 2.3 M Na₂CO₃ and/or 6M trace metal
HCl. After 1 hour, 10mL of gastric solution was immediately centrifuged (3800 rpm;
3374 x g for 15 min) and the supernatant was refrigerated at 4°C prior to analysis with
ICP OES. Pepsin and amylase were sourced from Sigma-Aldrich (St. Louis, Missouri,
USA) and NaCl from Thermo Fisher Scientific.

Calculation of in vitro bioaccessible Pb.

The in vitro bioaccessible Pb (IVBA Pb) was determined and expressed as a percentage
of total Pb as follows:

%IVBA Pb= (IVBA extractable Pb [mg kg⁻¹]/(total soil Pb [mg kg⁻¹])) x 100%
Statistics and Quality Assurance

Statistical analysis was conducted with SigmaPlot 10 software (Systat Software) and R and R 3.5.0 (R core Team, 2018). The average IVBA Pb of \( n=2 \) subsamples were used in the analyses. Differences in IVBA Pb between methods were evaluated with a one-way analysis of variance (R function “aov”). The assumptions of ANOVA were met: Bartlett’s test confirmed homogeneity of variances, no outliers were observed, samples were independent, and normality and homogeneity of residuals were verified using Normal Q-Q and residual plots, respectively. A Tukey multiple comparison of means test (R function “TukeyHSD”) was used to detect significant differences between the three methods. Relative mineral and phytate concentrations of diets were compared with a multivariate data reduction technique, standardized principal component analysis (PCA, R function “prcomp”) and related to IVBA Pb with linear regression. A Royson test confirmed multivariate normality (R package “MVN”, function “mvn”). Linear regressions were used to relate diet+soil and soil-alone IVBA Pb in the soil comparison (Table 1B) experiment and soil and spike IVBA Pb in the source comparison experiment (Table 1C).

A blank and reference material were included with each batch for quality assurance and control. The percent relative percent difference (RPD) of in duplicate analyses did not exceed 15%. Blanks did not exceed 10% of the reporting limit for Pb (0.05mg/L) for both the \textit{in vitro} assays and tissue digests. The relative percent difference (RPD) of replicate \textit{in vitro} bioaccessibility analyses averaged 8% and did not exceed 32%.
Although no published values exist for the bioaccessibility of the reference soil (National Institute of Standards and Technology (NIST) SRM 2711a Montana II Soil) in any of the in vitro bioaccessibility assays trialed, the reference soil was used to track interbatch variability. Variability between batches was low (4% RSD). Accuracy of the plant tissue digest was verified using NIST SRM 1573a (Tomato Leaves). Total elemental recoveries in % in parentheses were: Ca(81%); K(82%); Mg(77%); Mn(81%); Na(80%); P(90%); and S(111%).

Results and Discussion

Diet Components and Bioaccessibility

Total elemental and phytate contents of diets are shown in Table 2. The selected elements have been previously investigated for their impact on absorption of Pb and other cationic heavy metals (Barltrop and Khoo, 1975, Gulson, Mizon, et al., 2006, Mylroie, Moore, et al., 1978). The natural diets contained primarily phytate-P, while the stock diets had considerable free P (Table 6), likely from processing and P supplementation. All diets had < 1 mg kg⁻¹ Pb. Soil heavy metal content and chemical properties are given in Tables 3 and 4, respectively.

Diet Comparison: In Vitro Bioaccessible Pb in Ecological Risk Assessment Diets

Table 2 shows diet+soil IVBA Pb. USEPA 1340 at pH 2.5 and AOSU IVG (pH 2.0) had similar IVBA Pb (p=0.96), while IVBA Pb was greater in USEPA 1340 at pH 1.5.
Either AOSU IVG (pH 2.0) or USEPA 1340 at pH 2.5 would be appropriate for most bird species, where fasting gizzard pH ranges from 2.0-2.5 (Denbow, 2015). Some diets were not appropriate for the evaluated methods. Without the addition of pepsin, meat did not dissolve. The low pH in USEPA 1340 at pH 1.5 coagulated the proteins in the meat and it could not be analyzed using this method. In AOSU IVG and USEPA 1340 at pH 1.5, oats produced a thick solution that could not be accurately analyzed on the ICP-OES instrument.


Diet Comparison: Diet Composition and Impacts on IVBA Pb

Results of the diet principal component analysis are shown in Table 6. PC1 explained 72% of variance and PC2 13%. Highly weighted loading factors were those within 10% of the greatest factor (Andrews, Karlen, et al., 2002). These were K, Mn, Mg, Na, and P in PC1 and Phytate in PC2. Minerals had similar factor loadings in PC1, suggesting this
component describes nutrient density. Plotting PC1 and PC2 clusters diets into stock diets (Purina ® Game bird Maintenance Chow, Purina ® Game Bird Layena ETTS; and rodent chow) and natural diets (corn, oats, horsemeat), with the blended basal diet intermediate. Compared to the natural diets, processed diets had higher mineral concentrations. Phytate had the highest weighted factor loading in PC2, suggesting diets differed within categories by their phytate concentrations. PC2 did not correlate with IVBA Pb in any method.

PC1 was significantly correlated with IVBA Pb in AOSU IVG (p=0.01), USEPA Method 1340 at pH 1.5 (p=0.02), and nearly correlated in USEPA Method 1340 at pH 2.5 (p=0.051). This suggests nutrient density of diets influenced IVBA Pb. In general, stock diets suppressed IVBA Pb more than meat or corn. These results agree with in vivo trials, where high-mineral diets reduce Pb uptake more than low-mineral diets (Barltrop and Khoo, 1975, Chaney, Mielke, et al., 1989, Mylroie, Moore, et al., 1978). Rodent chow produced the lowest IVBA Pb. Though rodents are often used as test animals in lead toxicity and absorption studies, it has been noted that they are able to withstand very high Pb doses (O’Flaherty, Adams, et al., 1986, Quarterman, Morrison, et al., 1978). Diet may be partially responsible for this. Rats dosed with Pb while fed a semipurified diet have much greater tissue Pb and toxic effects than their counterparts fed stock diets (Aungst and Fung, 1985, O’Flaherty, Adams, et al., 1986). A similar effect was observed in humans dosed with Pb when fed diets similar in composition to stock and semi-purified diets (James, Hilburn, et al., 1985). Hoffman, Heinz, et al. (2000) found that mallard
ducklings fed contaminated sediments in a corn and stock diet blend had greater tissue Pb and toxic effects than those fed sediments in a pure stock diet. Reduced nutrition was faulted here and in other studies, yet this work suggests that some diet items such as corn have a smaller effect on Pb bioavailability relative to other foods. These results support using natural diets over stock diets for gathering bioavailability data, both in vitro and in vivo, as exposure risk may be underestimated when using stock diets.

Highly weighted factor loadings in PC1 were K, Mg, Mn, and P. A mechanistic explanation of the ability of K and Mn to affect Pb bonding to soil and IVBA Pb has not been reported, nor have these elements been shown to reduce RBA Pb (Barltrop and Khoo, 1975). Protective effects of Mg have been observed in vivo (Barltrop and Khoo, 1975, Mendez, Liu, et al., 2017, Singh, Thind, et al., 1979). Fine, Barth, et al. (1976) demonstrated that fortifying a semipurified, low mineral diet with Mg reduced Pb uptake in dogs. These studies suggested Mg reduces Pb uptake by interfering with its absorption, rather than complexing with or reducing the solubility of Pb. Absorption is not simulated during an in vitro bioaccessibility assay and it is unlikely that reductions in IVBA Pb noted here were related directly to Mg concentration.

Phosphorus has been shown to reduce Pb RBA and IVBA Pb (Barltrop and Khoo, 1975, Morrison, Quarterman, et al., 1974, Quarterman, Morrison, et al., 1978). The formation of insoluble P-Pb complexes is considered the main mechanism behind P-induced reductions in RBA Pb (Barton and Conrad, 1981, Juhasz, Gancarz, et al., 2014, Mykkanen, Fullmer, et al., 1984). Phosphorus in corn and oats exists primarily as phytate
(90-92%), while the stock diets have a smaller portion of phytate-P (36-62%). More free P was available to precipitate Pb in stock diets. Stock diets were more effective at reducing IVBA Pb than corn and meat, which had low P contents. However, in USEPA 1340 at pH 2.5, the oats had similar IVBA Pb despite low P content.

Studies of diet and Pb uptake commonly find that Ca reduces RBA, yet Ca concentration appears unrelated to IVBA Pb in these diets (Aungst and Fung, 1985, Barltrop and Khoo, 1975, James, Hilburn, et al., 1985). Proposed mechanisms include increased synthesis of transport proteins in the intestine, competitive absorption with Pb, changing the affinity of target tissues for Pb, and precipitation (Aungst and Fung, 1985, Barltrop and Khoo, 1975, Gulson, Mizon, et al., 2006). Of these mechanisms, only precipitation would be captured in an in vitro, which may explain why Ca did not correlate with IVBA Pb here. Another explanation is that Ca combines with other dietary components to form products that precipitate Pb. This could explain why some studies fail to find a protective effect of Ca in simplified diets (James, Hilburn, et al., 1985, Kello and Kostial, 1973, O’Flaherty, Adams, et al., 1986). Coprecipitation of Pb with Ca-P complexes is a suggested mechanism behind Ca-induced reductions in Pb RBA. These occur above pH 5, however, and would not be captured in the gastric-phase in vitro bioaccessibility assays trialed here (Chaney, Mielke, et al., 1989). Phytate is a compound that is thought to interact with Ca to reduce Pb availability. Unlike Ca-P complexes, Ca-phytate complexes can precipitate at low pH (Bullock, Duffin, et al., 1995). Phytate is a P compound found in seeds that has been shown to bind to Pb both in vitro (Bullock, Duffin, et al., 1995, Wise and Gilburt,
1981) and *in vivo* (Wise, 1981). Heavy metal concentrations in diet are typically too low to precipitate phytate, but the concentrations of other metals in diet such as Ca may be sufficient to do this. Other metals, including Pb, are then coprecipitated with Ca-phytate complexes (Wise and Gilburt, 1981). Though phytate was not correlated with IVBA Pb here, it still may have played a role. The Ca content in corn may have been insufficient to precipitate phytate, contributing to higher IVBA Pb. Oats and layer feed contained similar phytate contents as corn but had much more calcium. Precipitation of phytate in these diets may have contributed to lower IVBA Pb compared to corn.

**Soil Comparison: Interaction of Diet and Soil Type**

Regressions of diet+soil IVBA Pb with the soil-alone IVBA Pb showed good relationships ($r^2=0.64-0.70$) (Figure 3). This suggests that the diet influences IVBA Pb similarly across the soils studied here. These findings agree with Schroder et al. (2004), who evaluated the IVBA Pb of 18 soils was evaluated with OSU IVG both with and without diet. The regression of IVBA Pb with and without diet showed a strong relationship ($r^2=0.81$, $p<0.0001$). Diet reduced IVBA Pb similarly in the studied soils, though these results should not be generalized to other soil types without additional research.
Source Comparison: Interaction of Diet and Pb Source

The regression of diet+soil IVBA Pb and diet+spike IVBA Pb had a strong relationship ($r^2=0.98$) (Figure 3), which suggests interactions of the six diets with soil Pb were similar to interactions with spike Pb. A regression slope greater than soil-alone IVBA Pb:total spike Pb would suggest diet reduces soil Pb more strongly than spike Pb. A lesser slope suggests diet reduces spike Pb more strongly. Diet interactions with Pb may differ between the various Pb minerals found in soil, and ions and minerals from diet could increase or decrease soil Pb dissolution. A slope similar to the soil-alone IVBA Pb, on the other hand, suggests that diet interacts with soil Pb and spike Pb similarly. The regression slope (0.58) was similar to the ratio of soil-alone IVBA Pb:total spike Pb (0.63) (Figure 4). As the slope of the regression was similar to the ratio of the soluble Pb fractions, this suggests diet may act on soluble Pb.

Conclusions

Accurately assessing exposure is critical to ERA to ensure cleanup efforts are focused on the most appropriate areas. In risk assessment, using RBA Pb rather than total Pb typically reduces calculated exposure, decreasing cleanup costs. Variations in diet+soil IVBA Pb suggest including effect of diet on soil RBA in exposure estimates will improve accuracy of ERA.

Stock diets tended to reduce IVBA Pb more than natural diets. A similar trend is
observed in animal feeding studies, where nutrient-dense diets tend to decrease RBA Pb relative to nutrient-poor diets (Aungst and Fung, 1985, O’Flaherty, Adams, et al., 1986, Wise and Gilburt, 1980). While this effect has been attributed to reduced nutrition, these results suggest that the stock diets themselves may reduce RBA Pb and are inappropriate for assessing soil IVBA Pb. Natural ingredient diets should be used rather than stock diets.

While diet P and mineral concentration influenced IVBA Pb here, other dietary components including fiber and protein shown to influence RBA Pb were not evaluated here (Barltrop and Khoo, 1975, Deluca, Hardy, et al., 1982, Flora, Kumar, et al., 1991, Fu and Cui, 2013, Wise and Gilburt, 1980). These should be examined in future in vitro bioaccessibility research.

This study suggests that diet impacts on soil IVBA Pb are independent of the diet+soil combination and that “diet-adjustment factors” could be used to adjust soil-alone IVBA Pb data in the soils examined here. It is important to note when deriving “diet-adjustment factors” or using in vitro bioaccessibility assays to predict in vivo bioavailability that IVBA Pb is not equal to RBA Pb. IVBA Pb data cannot be used directly to adjust exposure in ERA. Additionally, there are interactions between diet and Pb that are not simulated with in vitro bioaccessibility assays. Competitive absorption, changing permeability of the intestinal mucosa, and clearing Pb from tissue are mechanisms by which diet components including Ca, Fe, Mg, and organic acids influence Pb uptake in vivo. More research is needed to determine whether diet+soil in vitro bioaccessibility
assays are predictive of RBA Pb in ERA diets. Animal feeding studies with multiple diets are needed to develop predictive models for adjusting exposure in ecological risk assessment.
Figure 2.1. Schematic of Pb uptake. Soil is ingested and a portion of Pb solubilizes in the gastrointestinal (GI) solution. The soluble, or bioaccessible, Pb is able to be absorbed. A portion of bioaccessible Pb is absorbed into the bloodstream (bioavailable Pb). (Modified from White, 2005)
<table>
<thead>
<tr>
<th>Experiment</th>
<th>Design</th>
<th>Diet</th>
<th>Soil/Pb Source</th>
<th>Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Diet Comparison</td>
<td>7 diets x 3 <em>in vitro</em> bioaccessibility assays x 1 soil 7x3 factorial design</td>
<td>Purina ® Game Bird Maintenance Chow; Purina ® Game Bird Layer Chow; horsemeat; rodent chow; corn; oats; basal diet (70% Purina ® Game Bird Maintenance Chow, 30% corn)</td>
<td>CDA; Helena; Viburnum; Big River; Big River 0.75%P; Joplin; Joplin 1% P; Joplin 10% Compost</td>
<td>AOSU IVG; USEPA Method 1340 at pH 1.5; USEPA Method 1340 at pH 2.5</td>
</tr>
<tr>
<td>B. Soil Comparison</td>
<td>1 diet x 3 <em>in vitro</em> bioaccessibility assays x 8 soils 8x3 factorial design</td>
<td>Basal diet (70% Purina ® Game Bird Maintenance Chow, 30% corn)</td>
<td>CDA; Helena; Viburnum; Big River; Big River 0.75%P; Joplin; Joplin 1% P; Joplin 10% Compost</td>
<td>AOSU IVG; USEPA Method 1340 at pH 1.5; USEPA Method 1340 at pH 2.5</td>
</tr>
<tr>
<td>C. Source Comparison</td>
<td>6 diets x 1 <em>in vitro</em> bioaccessibility assays x 2 Pb sources 6x2 factorial design</td>
<td>Purina ® Game Bird Maintenance Chow; Purina ® Game Bird Layer Chow; horsemeat; rodent chow; corn; oats; basal diet (70% Purina ® Game Bird Maintenance Chow, 30% corn)</td>
<td>Joplin</td>
<td>AOSU IVG</td>
</tr>
</tbody>
</table>
Table 2.2. Total elemental, phytate, and moisture content of ecological risk assessment diets analyzed with *in vitro* bioaccessibility assays.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Phytate</th>
<th>Ca</th>
<th>K</th>
<th>Mg</th>
<th>Mn</th>
<th>Na</th>
<th>P</th>
<th>S</th>
<th>Moisture Content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Game Bird Maintenance</td>
<td>11600</td>
<td>10800</td>
<td>8010</td>
<td>2480</td>
<td>148</td>
<td>1350</td>
<td>7020</td>
<td>1840</td>
<td>8.77</td>
</tr>
<tr>
<td>Game Bird Layena ETTS</td>
<td>6180</td>
<td>29800</td>
<td>11200</td>
<td>2440</td>
<td>147</td>
<td>1030</td>
<td>7860</td>
<td>2580</td>
<td>8.86</td>
</tr>
<tr>
<td>Horsemeat</td>
<td>0</td>
<td>20</td>
<td>848</td>
<td>223</td>
<td>1</td>
<td>530</td>
<td>708</td>
<td>294</td>
<td>73.0</td>
</tr>
<tr>
<td>Rodent Chow</td>
<td>11100</td>
<td>8410</td>
<td>12800</td>
<td>2050</td>
<td>92</td>
<td>2850</td>
<td>6270</td>
<td>2740</td>
<td>9.89</td>
</tr>
<tr>
<td>Corn</td>
<td>5840</td>
<td>59</td>
<td>4430</td>
<td>1160</td>
<td>7</td>
<td>5</td>
<td>3060</td>
<td>886</td>
<td>7.99</td>
</tr>
<tr>
<td>Oats</td>
<td>6770</td>
<td>748</td>
<td>4970</td>
<td>1250</td>
<td>75</td>
<td>54</td>
<td>3310</td>
<td>1540</td>
<td>10.3</td>
</tr>
<tr>
<td>Basal Diet</td>
<td>10900</td>
<td>6660</td>
<td>5100</td>
<td>1310</td>
<td>82</td>
<td>948</td>
<td>4630</td>
<td>1550</td>
<td>8.45</td>
</tr>
</tbody>
</table>

*Average of n=3 subsamples. The relative standard deviation (RSD) of subsamples did not exceed 10%.
Table 2.3. Total elemental concentrations of study soils as determined with X-ray fluorescence.

<table>
<thead>
<tr>
<th>Soil</th>
<th>Ca</th>
<th>Cd</th>
<th>Fe</th>
<th>Pb</th>
<th>S</th>
<th>Zn</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDA&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2540 ±36</td>
<td>4 ±0.25</td>
<td>63600 ±398</td>
<td>3160 ±14</td>
<td>497 ±29</td>
<td>1340 ±12</td>
</tr>
<tr>
<td>Helena&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13200 ±151</td>
<td>99 ±3</td>
<td>28300 ±469</td>
<td>3810 ±199</td>
<td>1040 ±88</td>
<td>3090 ±184</td>
</tr>
<tr>
<td>Viburnum&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3230 ±195</td>
<td>&lt;8</td>
<td>6180 ±98</td>
<td>3200 ±54</td>
<td>629 ±56</td>
<td>231 ±16</td>
</tr>
<tr>
<td>Big River&lt;sup&gt;c&lt;/sup&gt;</td>
<td>32500 ±1680</td>
<td>3 ±2.02</td>
<td>19600 ±275</td>
<td>2150 ±13</td>
<td>441 ±39</td>
<td>597 ±4</td>
</tr>
<tr>
<td>Big River 0.75% P&lt;sup&gt;c&lt;/sup&gt;</td>
<td>50500 ±1260</td>
<td>8 ±0.96</td>
<td>18300 ±510</td>
<td>2000 ±76</td>
<td>520 ±24</td>
<td>632 ±28</td>
</tr>
<tr>
<td>Joplin&lt;sup&gt;c&lt;/sup&gt;</td>
<td>9380 ±885</td>
<td>8 ±1.62</td>
<td>15600 ±569</td>
<td>5120 ±84</td>
<td>1410 ±72</td>
<td>3980 ±162</td>
</tr>
<tr>
<td>Joplin 1% P&lt;sup&gt;c&lt;/sup&gt;</td>
<td>19400 ±983</td>
<td>9 ±0.46</td>
<td>15800 ±639</td>
<td>4270 ±56</td>
<td>1220 ±121</td>
<td>2980 ±157</td>
</tr>
<tr>
<td>Joplin 10% Compost&lt;sup&gt;c&lt;/sup&gt;</td>
<td>33000 ±1470</td>
<td>9 ±1.20</td>
<td>22890 ±799</td>
<td>3310 ±108</td>
<td>1010 ±66</td>
<td>2540 ±146</td>
</tr>
</tbody>
</table>

*Average of \(n=3\) subsamples
Background soil Pb (95<sup>th</sup> percentile) in a) Idaho=33 mg kg\(^{-1}\), b) Montana=40 mg kg\(^{-1}\) and c) Missouri=47 mg kg\(^{-1}\) (Smith, Cannon et al., 2013)
Table 2.4. Properties of study soils as reported in Beyer, Basta, et al. (2016).

<table>
<thead>
<tr>
<th>Soil</th>
<th>Pb</th>
<th>Ca</th>
<th>P</th>
<th>pH</th>
<th>Organic matter (%)</th>
<th>Cation exchange capacity (meq/100g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDA</td>
<td>960</td>
<td>398</td>
<td>6</td>
<td>5.4</td>
<td>4</td>
<td>10.9</td>
</tr>
<tr>
<td>Helena</td>
<td>900</td>
<td>3330</td>
<td>110</td>
<td>6.5</td>
<td>11</td>
<td>21.7</td>
</tr>
<tr>
<td>Viburnum</td>
<td>1600</td>
<td>1150</td>
<td>19</td>
<td>5.1</td>
<td>12</td>
<td>14.2</td>
</tr>
<tr>
<td>Big River</td>
<td>1100</td>
<td>3030</td>
<td>42</td>
<td>7.6</td>
<td>3.1</td>
<td>20.4</td>
</tr>
<tr>
<td>Big River 0.75% P</td>
<td>570</td>
<td>8350</td>
<td>3300</td>
<td>7.2</td>
<td>3</td>
<td>21.2</td>
</tr>
<tr>
<td>Joplin Control</td>
<td>1100</td>
<td>3800</td>
<td>74</td>
<td>7</td>
<td>7.3</td>
<td>17.5</td>
</tr>
<tr>
<td>Joplin 1% P</td>
<td>130</td>
<td>7840</td>
<td>2900</td>
<td>6.3</td>
<td>8</td>
<td>23.7</td>
</tr>
<tr>
<td>Joplin 10% Compost</td>
<td>6190</td>
<td>13600</td>
<td>320</td>
<td>7.7</td>
<td>8.6</td>
<td>17.8</td>
</tr>
</tbody>
</table>
Methods with different letters are significantly different according to a Tukey multiple comparison of means test.

*Average of n=2 subsamples. Relative percent difference (RPD) of subsamples averaged 8% and did not exceed 32%.

Table 2.5. *In vitro* bioaccessible (IVBA) Pb of Joplin soil with seven ecological risk assessment diets as determined with three *in vitro* bioaccessibility assays: Avian Ohio State University *in Vitro* Gastrointestinal evaluation (AOSU IVG), USEPA

<table>
<thead>
<tr>
<th>Diet</th>
<th>Bioaccessible Pb (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Diet</td>
<td>91</td>
</tr>
<tr>
<td>Purina® Game Bird Maintenance</td>
<td>63</td>
</tr>
<tr>
<td>Purina Game Bird Layena ETTS</td>
<td>60</td>
</tr>
<tr>
<td>Purina Game Bird Layena</td>
<td>57</td>
</tr>
<tr>
<td>Rodent Chow</td>
<td>24</td>
</tr>
<tr>
<td>Corn</td>
<td>24</td>
</tr>
<tr>
<td>Oats</td>
<td>22</td>
</tr>
<tr>
<td>Horsemeat</td>
<td>16</td>
</tr>
<tr>
<td>Feeding Study Diet</td>
<td>46</td>
</tr>
</tbody>
</table>

*Bioaccessible Pb (%)*

Methods with different letters are significantly different according to a Tukey multiple comparison of means test.

*Average of n=2 subsamples. Relative percent difference (RPD) of subsamples averaged 8% and did not exceed 32%.
Table 2.6. Results of a principal components analysis of ecological risk assessment diet mineral and phytate composition.

<table>
<thead>
<tr>
<th>Principal components</th>
<th>PC1</th>
<th>PC2</th>
<th>PC3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eigen value</td>
<td>5.73</td>
<td>1.03</td>
<td>0.93</td>
</tr>
<tr>
<td>Percent</td>
<td>71.6</td>
<td>12.9</td>
<td>11.7</td>
</tr>
<tr>
<td>Cumulative Percent</td>
<td>71.6</td>
<td>84.5</td>
<td>96.2</td>
</tr>
</tbody>
</table>

Eigen vectors\(^a\)

<table>
<thead>
<tr>
<th></th>
<th>PC1</th>
<th>PC2</th>
<th>PC3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phytate</td>
<td>-0.267</td>
<td>0.732</td>
<td>0.139</td>
</tr>
<tr>
<td>Ca.318.127</td>
<td>-0.329</td>
<td>-0.329</td>
<td>-0.397</td>
</tr>
<tr>
<td>K.766.491</td>
<td>-0.389</td>
<td>-0.389</td>
<td>-0.130</td>
</tr>
<tr>
<td>Mg.285.213</td>
<td>-0.388</td>
<td>-0.388</td>
<td>0.222</td>
</tr>
<tr>
<td>Mn.260.568</td>
<td>-0.375</td>
<td>-0.375</td>
<td>0.124</td>
</tr>
<tr>
<td>Na.589.592</td>
<td>-0.317</td>
<td>-0.317</td>
<td>0.000</td>
</tr>
<tr>
<td>P.213.618</td>
<td>-0.408</td>
<td>-0.408</td>
<td>0.030</td>
</tr>
<tr>
<td>S.180.669</td>
<td>-0.335</td>
<td>-0.474</td>
<td>0.313</td>
</tr>
</tbody>
</table>

Boldface factor loadings are considered highly weighted (Andrews, Karlen, et al., 2002)
Figure 2.2. Principal components analysis (PCA) plot of ecological risk assessment diets’ mineral and phytate compositions. Cluster 1 represents the stock diets (1. Purina® Game Bird Maintenance Chow; 2. Purina® Game Bird Layena ETTS, and 4. rodent chow). Cluster 2 represents the natural diets (3. horsemeat, 5. oats, and 6. corn). Basal diet (7), consisting of 70% Purina® Game Bird Maintenance Chow and 30% corn, was intermediate.
Figure 2.3. Diet+soil bioaccessible Pb (IVBA Pb) versus soil-alone IVBA Pb measured by the respective in vitro bioaccessibility assays for 8 contaminated soils: (A): Avian OSU IVG (AOSU IVG); (B) USEPA Method 1340 at pH 2.5; and (C) USEPA Method 1340 at pH 1.5.
Figure 2.4. Diet+soil bioaccessible Pb (IVBA Pb) regressed against diet+spike IVBA Pb as determined with Avian OSU IVG (AOSU IVG).
References


Urban agriculture consisting of backyard gardens, community gardening, and commercial agriculture is becoming an increasingly common solution to problems posed by post-industrial economic decline: vacant land, food insecurity, reduced economic opportunity, and community disintegration (Kaiser, Williams, et al., 2015, Kessler, 2013, Wortman and Lovell, 2013). Schilling and Logan (2008) reviewed benefits from urban greening and noted human health benefits such as improved mental health, access to healthy foods, and physical activity; economic benefits including increased consumer spending and property values; and environmental benefits such as mitigation of the urban heat island effect and storm water runoff management.

However, as communities redevelop vacant lots into gardens, parks, and play areas, the problems posed by soil lead (Pb) contamination are becoming increasingly apparent. Historical use of lead (Pb) in gasoline and paint, along with industrial activities, have left a legacy of Pb pollution in the soil (Minca and Basta, 2013). Exposure to lead is associated with numerous negative health effects. Toxic to all organ systems, Pb is particularly damaging to the kidneys, cardiovascular system, and nervous system (Schroder, Basta, et al., 2004). Childhood exposure to Pb can result in permanent neurological impairment. Soil Pb is a significant contributor to body Pb burden, especially in children as they have high rates of incidental soil ingestion (Mielke, Gonzales, et al., 1999). This can be seen in the “seasonality” of lead poisoning: blood
lead levels (BLLs) peak during the summer months (Laidlaw, Filippelli, et al., 2016). While a majority of urban soils do not have heavy metal concentrations that pose a significant risk to human health, it should not be assumed that a site is suitable for any green reuse (Minca and Basta, 2013). For example, in the City Park neighborhood of Appleton, Wisconsin, 40% of yard space had greater than 400 mg kg\(^{-1}\) Pb, the USEPA Residential soil screening limit (Clark and Knudsen, 2013; USEPA, 2018). A quarter of soils sampled from around Cleveland, Ohio’s Hough neighborhood exceeded 400 mg kg\(^{-1}\) Pb (Sharma, Basta, et al., 2015). In older cities with longer industrial histories, Pb pollution tends to be more widespread and severe (Duzgoren-Aydin, Wong, et al., 2006, Kelly, Thornton, et al., 1996).

Protecting people from soil Pb while gardening is particularly important in the context of urban agriculture as children in some cities are already more likely to have elevated BLLs (BLL >5ug/dL). While across the United States 2.5% of children had elevated BLLs between 2001-2010, 12% of tested children had elevated BLLs in Cleveland in 2016 (Cuyahoga County Board of Health, 2016). In the same year, Detroit, found that 9% of the 40% of children under 6 tested had elevated BLLs (Miller and Twichell, 2018). Lead exposure in this vulnerable population is therefore of great concern. Exposure from gardening could result from incidental soil ingestion of dust in the garden or tracked into the home, or of soil adhered to food crops; from inhalation of dust; or from ingestion of contaminated food crops. Research to quantify exposure from these pathways is limited and the findings are conflicting. Spliethoff, Mitchell, et al. (2016) found that incidental soil ingestion was the largest Pb contributor for children in gardens, while produce
consumption was the primary risk driver for adults. Hough, Breward, et al. (2004), on the other hand, concluded that Pb-contaminated produce was the most significant Pb source for gardeners of all ages.

It is unclear what health risks are posed by gardening on contaminated soils, and there is conflicting advice about how to lower risk when gardening. There is no USEPA Pb screening or cleanup limit for garden soils. State agency soil screening and cleanup levels vary from 80 to 2000 mg kg\(^{-1}\) Pb (USEPA 2014). Though very conservative screening limits are protective, they can exclude large tracts of land from being used for food production. As unused land can create its own problems for cities, it is important that soil screening and cleanup values protect the population while not needlessly restricting land use. Similarly, there is wide variation in best-practice recommendations (Surls et al., 2016; USEPA, 2014). These include soil barriers to reduce dust and splashing (mulch, plastic, pavement, plants); replacement of soil (raised beds, dilution, remove and replace); hygiene practices to reduce ingestion (handwashing, wearing gloves, removing shoes when home, monitoring hand-to-mouth behaviors); avoidance of high risk crops or plant parts (peeling root crops and removing outer leaves of leafy vegetables, or avoidance of these altogether); soil amendments to reduce soil Pb bioavailability to plants or humans (lime, organic matter, phosphorus). Few are validated with research and it is not clear which practices adequately reduce Pb exposure. Practices which are laborious, time-intensive, or expensive may not be followed, and the conflicting recommendations for gardening in contaminated soil may dissuade gardening altogether.
Discrepancies in soil and gardening recommendations from different agencies stems in part from disagreement over whether Pb consumption with food is considered a significant exposure pathway (Brown, Chaney, et al., 2016, Olowoyo and Lion, 2016, Spliethoff, Mitchell, et al., 2016). It is oft assumed that lead does not transfer readily from soil into plants (Amato-Lourenco, Moreira, et al., 2016, Chaney, Sterrett, et al., 1984). This is because Pb is held strongly in the soil matrix, so only a small fraction is available for plant uptake. Taking this view, incidental soil ingestion is the primary driver of exposure. In these cases, the USEPA residential SSL of 400 mg kg\(^{-1}\) Pb is often used as a no-concern gardening threshold since ingestion is the main risk driver in that calculation (Angima and Sullivan, 2008; USEPA, 2014).

Numerous reports suggest plants accumulate Pb when grown in contaminated soils (Brown, Chaney, et al., 2016, Finster, Gray, et al., 2004, Khan, Khan, et al., 2015, Preer, Akintoye, et al., 1984, Spittler and Feder, 1979). Plant Pb burden appears to be a combination of root uptake, soil and particulate matter (PM) adhesion, and foliar adsorption and absorption of soil and PM (Cary, Grunes, et al., 1997, McLaughlin, Smolders, et al., 2011, Olowoyo and Lion, 2016, Schreck, Foucault, et al., 2012). Several studies have found vegetables grown in contaminated soils to contain Pb levels in excess of the maximum limits set by the WHO/FAO for traded foods (0.1-0.3 mg Pb/kg fresh weight). Leafy and root vegetables in particular commonly surpass limits (Chaney, Sterrett, et al., 1984, McBride, Shayler, et al., 2014). While it is argued that soil Pb concentrations far exceed those in contaminated vegetables (40-15,000 mg kg\(^{-1}\) vs 0-80 mg kg\(^{-1}\)), vegetable consumption is much higher. Soil consumption from incidental
ingestion is around 50-200mg/day (Spliethoff, Mitchell, et al., 2016) USEPA, 2007). Average daily consumption of fresh vegetables in the United States was nearly 200g in 2015, or 1000 times greater (USDA ERS, 2017). This has called into question the assumption that incidental soil ingestion is the only exposure pathway of concern for gardening.

Agencies that include ingestion of vegetable Pb in their risk assessment and soil screening limit calculations produce limits that are much lower than those that only consider incidental soil ingestion (Angima and Sullivan, 2008; Surls et al., 2016; USEPA 2014). Additionally, risk assessments conducted with total vegetable Pb concentrations have suggested that this pathway poses a risk for gardeners (Hough, Breward, et al., 2004, Nabulo, Young, et al., 2010, Obiora, Chukwu, et al., 2016, Spliethoff, Mitchell, et al., 2016). Yet few studies have found increased blood Pb in individuals eating produce grown on contaminated soil (Gallacher, Elwood, et al., 1984). This discrepancy may be a result of using total Pb concentration in exposure calculations. Numerous studies have shown that food reduces the bioavailability of Pb, or the portion of Pb that is absorbed into the bloodstream (Barltrop and Khoo, 1975, Mylroie, Moore, et al., 1978, Wise and Gilburt, 1980). Maddaloni, Lolacono, et al. (1998) found that subjects absorbed 26% of the lead in soil while fasting, but this dropped to only 2.5% when dosed after breakfast, and James, Hilburn, et al. (1985) found similar results when Pb was administered following meals. Given that diet items can reduce soil Pb bioavailability, it may be the case that Pb consumed with vegetables is not very bioavailable. Food items differ significantly in their ability to reduce Pb uptake, however (James, Hilburn, et al., 1985,

While it is apparent that vegetables grown in contaminated soils have elevated levels of Pb, it is unclear whether these concentrations pose a health risk. Knowing the bioavailability of Pb consumed with vegetables could inform whether this pathway is worth considering when developing soil Pb thresholds for gardening. Here a worst-case scenario was examined: unwashed vegetables. Washing is a commonly recommended practice to reduce Pb exposure from vegetables, and washing removed a majority of Pb in studies by Nabulo, Young, et al. (2010), Kugonic and Kopusar (2000), and Zalud, Szakova, et al. (2012). However, gardeners may not wash their produce as thoroughly as researchers, if at all. Furthermore, the rough surface area of some vegetables are difficult to clean, and detergents that help remove adsorbed Pb are not commonly used (Defoe, Hettiarachchi, et al., 2014, Hinton, Kopp, et al., 1995). While other studies have examined the bioavailability of Pb taken up by vegetables, this is the first to look at vegetables’ impact on the availability of adhered soil Pb. This is important because soil Pb and endogenous Pb exist in different forms and media. Pb added to vegetables may have greater bioavailability than endogenous Pb. Crews, Burrell, et al. (1983) found endogenous Pb was much less bioavailable than Pb spikes in a variety of foods. The objective of this study, therefore, was to screen eight vegetables for their ability to reduce the *in vitro* bioaccessibility (IVBA) of soil Pb for two contaminated soils.
Materials and Methods

Diet Processing and Characterization

Approximately 500g of beefsteak tomatoes, Russet Burbank potatoes, snow peas, spinach, green leaf lettuce, zucchini, radish, and carrots were purchased from a local grocer. Vegetables were washed in tap water thoroughly so that all visible soil was removed, rinsed in deionized water (DI) (Barnstead Thermolyne Nanopure Ultrapure Water System D4751 6152s) and patted dry with paper towels (McBride, Shayler, et al., 2014). All vegetables were left raw, aside from potato, which was microwaved until thoroughly cooked. Vegetables were then ground with a blender until homogenized (Figure 1). Diets were stored at 4°C. Subsamples were dried at 60°C for 24 hours to obtain the moisture content. Total elemental content for each diet was determined through acid digestion. Dried diet (0.1g) was added to 0.6mL HCl and 2.0mL HNO₃, allowed to digest for 24 hours, and brought to 10mL with DI. Plant tissue digests were analyzed with inductively coupled plasma optical emission spectroscopy (ICP-OES) (USEPA Method 6010D, USEPA, 2000).

Soil Processing and Characterization

Two soils representing different contaminant sources were used in the analysis: a highly contaminated soil (5,120 mg kg⁻¹ Pb) sampled from Joplin, Missouri (from here on, Joplin) that was contaminated by Pb smelter emissions; and a moderately contaminated soil (702 mg kg⁻¹ Pb) sampled from a vacant lot in Cleveland, Ohio that was likely
contaminated with lead paint (from here on, Cleveland). Soil was air dried and sieved to ≤250 μm. This is the size fraction recommended by USEPA for use in \textit{in vitro} bioaccessibility assays as it is considered the fraction which sticks to hands (USEPA, 2007). This size fraction was used in all analyses. X-ray fluorescence (Thermo Scientific Niton FXL) was used to determine total Pb content of each soil.

\textit{Determination of In Vitro Bioaccessibility}

A new method was developed for evaluating soil Pb bioaccessibility with diet based on human diet and soil \textit{in vitro} bioaccessibility assays (Table 1). The method primarily drew from a soil Pb and As bioaccessibility analysis (OSU IVG as described by Schroder, Basta, et al. (2004) and the standardized static \textit{in vitro} bioaccessibility assay proposed by Minekus, Alminger, et al. (2014) for evaluating food. Only a gastric phase was used to evaluate ecological risk assessment diets in Chapters 1 and 2 as intestinal IVBA Pb was below detection for some diets. However, as Intawongse and Dean (2008) and Fu and Cui (2013) found that contaminated vegetables had higher intestinal IVBA Pb than gastric IVBA Pb both phases were included in this assay. Consideration was given to achieving a soil:solution ratio in line with other soil IVBA Pb methods while maintaining a soil loading rates that was realistic for unwashed vegetables. Soil was added to diet to achieve a target of 1% soil:fresh diet mass (0.5g:50g). The soil loading rate used here is in the upper range reported in the literature (Hinton et al., 1995; Cary and Kubota, 1990). A 50g:50mL diet:solution ratio was used. As the moisture content of most diets was near 100%, the resulting soil:solution ratio was approximately 1:200. The Pb concentration of Joplin-contaminated diets was 103 mg kg\(^{-1}\) and 14 mg kg\(^{-1}\) in the Cleveland-contaminated
diets. While the Joplin-contaminated vegetable Pb concentration exceeded most values reported in the literature, the Cleveland-contaminated vegetables were within the reported range (Finster, Gray, et al., 2004, Khan, Khan, et al., 2015). A high concentration ensured that IVBA Pb would be measurable.

The final method is detailed below, and references for each parameter are given in Table 8. Soil (0.5g, ≤250um) and 50g ground plant material (wet mass) or 50mL DI for soil-alone assays preheated to 37°C were stirred together in a 175mL HDPE bottle with 1.1g 1.7U mg⁻¹ amylase in 1mL DI. This gave a final amylase concentration of approximately 37.5U mL⁻¹ (Kejriwal, Bhandary, et al., 2014). After 2 minutes, 50 mL of preheated gastric solution (0.30M NaCl and 0.8% [w/w] 2000U mg⁻¹ porcine pepsin in DI adjusted with trace metal grade hydrochloric acid (TMG HCl) to pH 2.0) was added. This gave a final concentration of approximately 0.15M NaCl and 0.4% pepsin. The pH was immediately adjusted to 2.0 ±0.1 using dropwise addition of 2.3 M Na₂CO₃ and/or 6M TMG HCl solution. Bottles were placed into an end-over-end shaker inside a 37°C incubator. The pH was adjusted to 2.0 ±0.1 at one hour and after two hours 10mL of gastric solution was sampled and immediately centrifuged (9250 rpm; 14,500 X g for 10min). The supernatant was refrigerated at 4°C prior to analysis with ICP-OES. For the intestinal phase, the pH of the remaining suspension was adjusted to 6.5 using dropwise addition of 2.3 M Na₂CO₃. Subsequently, 5mL of intestinal solution (70g/L porcine bile, 70g/L porcine pancreatin, DI) was added to each bottle to obtain a final solution concentration of approximately 3.5% [w/w]. Bottles were rotated in an incubator for 2 hours, and midway the pH was adjusted to 6.5. Following the extraction, 10mL intestinal
solution was sampled and immediately centrifuged (9250 rpm, 14,500 X g for 10min). The supernatant refrigerated at 4°C prior to analysis with ICP-OES. Amylase, pepsin, bile, and pancreatin were sourced from Sigma-Aldrich (St. Louis, Missouri, USA) and the Na₂CO₃ and HCl from Thermo Fisher Scientific (Waltham, Massachusetts, USA). The impact of 8 vegetables on IVBA Pb of two soils was determined with the gastric and intestinal phases of this assay (8x2x2 factorial).

*Calculation of in Vitro Bioaccessible Pb.*

The *in vitro* bioaccessible Pb (IVBA Pb) was determined and expressed as a percentage of total Pb as follows:

\[
\%\text{IVBA Pb} = \frac{\text{IVBA extractable Pb [mg kg}^{-1}\text{]}}{\text{total soil Pb [mg kg}^{-1}\text{]}} \times 100\%
\]

*Geochemical Modeling*

Geochemical modeling of dissolved mineral (Ca²⁺, CI⁻, Fe³⁺, K¹⁺, Mg²⁺, Na⁺, PO₄³⁻, SO₄²⁻, Pb²⁺, Zn²⁺) and organic acid (malate²⁻, oxalate²⁻, and citrate³⁻) estimates from Suárez, Rodríguez, et al. (2008) and Bushway, Bureau, et al. (1984)) concentrations of tomato and potato *in vitro* extracts of both soils was performed with Visual Minteq (Version 3.1, 2018). Both gastric and intestinal phases were modeled to determine Pb saturation and possible precipitates.
Statistics and Quality Assurance

Statistical analysis was conducted with SigmaPlot 10 software (Systat Software) and R (R Core Team, 2018). Diet mineral concentrations were correlated with IVBA Pb for each method using Pearson’s correlations. Significance was assessed at $\alpha=0.05$.

The influence of diet type, soil, and gastric and intestinal phase on diet+soil IVBA Pb was explored using a linear mixed effects model. To explain the differences between diet+soil IVBA Pb and soil-alone IVBA Pb, the ratio of diet+soil:soil-alone was calculated to represent the change in IVBA Pb with the addition of diet (% of soil-alone IVBA Pb). This was modeled with diet type, soil, and phase with a linear mixed effects model. R (package lmne, function “lme”) was used to compare either IVBA Pb or % of soil-alone IVBA Pb as a function of diet, phase, soil and their interaction. The fixed effects were diet (factor with eight levels: all vegetables); soil (factor with two levels: Joplin and Cleveland); and phase (factor with two levels: gastric and intestinal).

Subsamples were fitted as a random effect (3 levels: $n=3$). Normality and homogeneity of variance were confirmed with a Normal Q-Q and residual plots, respectively.

Plant tissue digests and XRF analyses were conducted in duplicate. A blank and reference material were included with each batch for quality assurance and control. Blanks did not exceed 10% of the reporting limit for Pb (0.05mg/L) for both the in vitro assays and plant tissue digests. Pb recovery for a SiO$_2$ blank obtained with XRF was below the instrument detection limit (<3 mg kg$^{-1}$). A reference soil (National Institute of Standards and Technology (NIST) SRM 2711a Montana II Soil) was used to track interbatch variability.
The relative standard deviations of in vitro assays averaged 9% and did not exceed 25%. Variability between batches was low (4% RSD). The percent relative percent difference of in plant tissue digests did not exceed 15%. Accuracy of the plant tissue digest was verified using NIST 1573a Standard Reference Material (Tomato Leaves). Total elemental recoveries in % in parentheses were: Ca(81%); K(82%); Mg(77%); Mn(81%); Na(80%) P(90%); and S(111%). Accuracy of the soil total Pb analysis with XRF was verified with a RCRA multielement standard and NIST SRM 2709a (San Joaquin Soil). Total Pb recoveries were between 80-101%.

Results and Discussion

Diet and Soil Characterization.

The total elemental content of diets is given in Table 9. Total elemental content was not correlated with IVBA Pb (Table 10). Many of these elements have been found to reduce absorption of Pb in both in vivo and in vitro studies (Barltrop and Khoo, 1975, Fu and Cui, 2013, Gulson, Mizon, et al., 2006, Mylroie, Moore, et al., 1978). Relatively lower amounts of minerals, including Ca and P in vegetables compared to foods previously analyzed for their impact on IVBA Pb and RBA Pb may explain why such a relationship was not seen here (Marles, 2017).

Gastric In Vitro Bioaccessible Lead

Joplin soil-alone IVBA Pb was 65% Cleveland soil-alone IVBA Pb was 45% (Figure 3). With diet, however, IVBA Pb of the Joplin soil ranged from 2%-28% and for the
Cleveland soil 2%-19%. These results agree with studies of gastric IVBA Pb of vegetables contaminated by Pb uptake. Fu and Cui (2013) measured the IVBA Pb of pak choi (*Brassica rapa* L., *Chinensis Group*) and Malabar spinach (*Basella rubra* L.). IVBA Pb in these vegetables as determined by a physiologically-based extraction test (PBET, Ruby, Davis, et al., 1993) was between 11-16%. These also agree with Intawongse and Dean (2008), where IVBA Pb in uptake-contaminated spinach, carrot, radish and lettuce varied from 7-27%. Hu, Wu, et al. (2013), however, found higher IVBA Pb (16-42%) using a method similar to the one used here (2.0g/L pepsin, 0.15MNaCl, pH 1.8), possibly due to lower vegetable Pb concentrations. IVBA Pb was significantly different between diets (p<0.05) and phase (p<0.05), but not between soils. Diets had different impacts on IVBA Pb, which has been noted both *in vitro* and *in vivo* (Crews, Burrell, et al., 1983, Heard, Chamberlain, et al., 1983).

The change in diet+soil IVBA Pb compared to soil-alone IVBA (% of soil-alone IVBA Pb) significantly different between the Joplin and Cleveland soils (p<0.001) To explore this further, geochemical modeling of the gastric extractions of each soil were compared. Geochemical modeling of the Joplin gastric extraction with Visual Minteq showed saturation and potential formation of chloropyromorphite [Pb$_5$(PO$_4$)$_3$Cl]. When chlorpyromorphite was allowed to precipitate in the model, dissolved Pb was lower with vegetables compared to soil-alone, a trend observed in our experimental results. Precipitation of Pb minerals are a possible mechanism for diet-related reductions in IVBA Pb in the Joplin soil. Pb was not saturated in the Cleveland soil, which had a lower Pb content than Joplin. Precipitation likely did not cause reductions in gastric IVBA Pb.
for this soil. Alternative mechanisms include adsorption or chelation of Pb by soil and dietary components (fiber, pectin, amino acids, etc.) (Balaria and Schiewer, 2008, Flora, Kumar, et al., 1991). Different mechanisms of IVBA Pb reduction in the two soils may explain why the % soil-alone IVBA Pb were significantly different between the Joplin and Cleveland soils.

*Intestinal In Vitro Bioaccessible Lead*

IVBA Pb between gastric and intestinal phases were significantly different (p<0.05, Table 5A). The % of soil-alone IVBA Pb differed between phases as well (p<0.001, Table 5B). While all diets had lower gastric IVBA Pb than soil-alone IVBA, IVBA Pb in the Joplin soil with diet was between 45-218% of soil-alone IVBA Pb and IVBA Pb of the Cleveland soil with diet was between 212-898% of soil-alone IVBA Pb (Figure 4). Intestinal IVBA Pb is typically lower than gastric IVBA Pb when soil is analyzed without diet (Oomen, Hack, et al., 2002, Schroder, Basta, et al., 2004). Most bioaccessibility methods use an intestinal pH of 5.5-7.5 (the range of the human small intestinal tract), and lead is sparingly soluble at pH > 5.5. Oomen, Tolls, et al. (2003) examined Pb speciation in simulated chyme and found less than 1% was present as free Pb^{2+}. However, diet appears to stabilize dissolved Pb in a simulated intestinal extract. These results were consistent with Fu and Cui (2013), who found that average IVBA Pb in pakchoi increased from 11% to 20% in the gastric versus intestinal phase and from 16% to 24% in Malabar spinach. Intawongse and Dean (2008) also found greater IVBA Pb in the intestinal phase for carrots, radish, spinach, and lettuce. On the other hand, Hu, Wu, et al.
(2013), noted decreases in IVBA Pb in the intestinal phase compared to the gastric phase in market vegetables.

Geochemical modeling with Minteq suggested Pb was saturated in the intestinal phase for both soils, with and without diet, and that chloropyromorphite could precipitate. When chloropyromorphite was allowed to form, >99% of Pb precipitated in all cases. The model did not predict the influence of diet on intestinal IVBA Pb. Geochemical modeling only examined precipitation, not adsorption or chelation. Additionally, compounds including bile and organic acids complex with Pb during intestinal \textit{in vitro} bioaccessibility assays but could not be modeled with Minteq (Oomen, Tolls, et al., 2003, Ruby, Davis, et al., 1993). Bile increases IVBA Pb, but this does not explain the differences in IVBA Pb across diets. Organic acids from food can chelate lead, holding it in solution. While these acids are protonated at the low pH in the gastric phase, they are able to chelate metals in the neutral intestinal phase. Chelates may contribute to Pb absorption indirectly if they are labile, releasing Pb into solution as free Pb is absorbed. Metal chelates can also be absorbed directly (Heath, Soole, et al., 2018, Oomen, Tolls, et al., 2003). However, there is conflicting evidence as to whether Pb chelates increase or decrease Pb absorption. Everted sac experiments of rat jejunums conducted by Coleman et al. (1980) showed that chelating agents including ascorbate, citrate, and folic acid increased absorption of Pb into the serosal compartment. Lead chelates fed to rats increased absorption of Pb compared to Pb acetate (Jugo et al., 1975). However, when James, Hilburn, et al. (1985) fed human volunteers Pb with EDTA, uptake decreased by
86%. It is not clear what portion of IVBA Pb measured here was chelated, and of this how much would be labile or directly available for absorption.

*Gastric or Intestinal Phase?*

The linear mixed effects model of IVBA Pb showed an interaction between diet and phase (Table 5A, p<0.05), indicating differences between gastric and intestinal IVBA Pb are not constant across diets. Gastric and intestinal phases will give different IVBA Pb results depending on the diet. The changes in soil-alone IVBA Pb by diet model showed an interaction of phase and soil (Table 5B, p<0.001), suggesting changes in IVBA Pb with the addition of diet is dependent on phase. These experiments suggest gastric and intestinal IVBA Pb data from this assay are not comparable for different soils and diets. It is not clear here which phase is more appropriate for simulating incidental soil ingestion with vegetables. Gastric phases of soil-alone *in vitro* bioaccessibility assays tend to correlate better with *in vivo* trials, despite Pb absorption occurring in the small intestine (Ruby, Davis, et al., 1996, Schroder, Basta, et al., 2004). Lead is absorbed soon after chyme is released into the small intestine, before pH and Pb species equilibrate (Chaney, Mielke, et al., 1989). Gastric-soluble Pb thus appears to drive uptake for soil consumed while fasting, though it is unclear whether this is true in fed conditions. More research is needed to determine which phase is more predictive of RBA Pb of soil consumed with vegetables.

*Limitations of In Vitro Bioaccessibility Assays for Evaluating Soil Lead Bioaccessibility with Diet*
There are numerous factors that influence Pb bioavailability that are not captured within an *in vitro* bioaccessibility assay. Competitive absorption of Pb has been observed with Fe, Zn, and Ca (Aungst and Fung, 1985, Flora, Kumar, et al., 1991, Ragan, 1983). While soil is a more concentrated source of these minerals than diet, diet is consumed in far greater quantities. Pb interactions with Ca are also thought to change the permeability of tight junctions, allowing greater absorption of free Pb (Mushak, 1991). Pb precipitates may dissociate as lumen Pb concentration changes. Chelating agents such as organic and amino acids increase or decrease IVBA Pb depending on their permeability and lability (Coleman et al., 1980; Vieira, 2008). The possible influence of diet components on Pb absorption make it especially important that diet+soil *in vitro* assays are corroborated with *in vivo* trials.

**Conclusions**

When calculating Pb exposure from ingestion of contaminated produce, total Pb concentration is typically used (Hough, Breward, et al., 2004). These results suggest that using total Pb content may not be appropriate as IVBA Pb differed significantly with diet. IVBA Pb was dependent on phase, however, suggesting gastric IVBA Pb would not be equivalent to intestinal IVBA Pb for all diets. It is unclear whether a gastric or intestinal phase is more appropriate for predicting RBA. Previous research has shown soil Pb uptake in humans is lower when volunteers are dosed with or shortly after a meal (Chaney, Mielke, et al., 1989, James, Hilburn, et al., 1985, Maddaloni, Lolacono, et al., 1998). This is consistent with the decreases in gastric phase IVBA Pb relative to soil-alone IVBA Pb. The increases in IVBA Pb with diet during the intestinal phase, on the
other hand, do not reflect *in vivo* results. Further research with *in vivo* animal models (i.e., mice, pigs) are needed to determine whether *in vitro* bioaccessibility assays can predict RBA. This could facilitate research on whether Pb consumption with vegetables is a significant exposure pathway to those gardening on contaminated soil and help establish soil limits and best-practices.
Figure 3.1. Schematic of Pb uptake. Soil is ingested and a portion of Pb solubilizes in the gastrointestinal (GI) solution. The soluble, or bioaccessible, Pb is able to be absorbed. A portion of bioaccessible Pb is absorbed into the bloodstream (bioavailable Pb). (Modified from White, 2005)
Figure 3.2. Processing vegetables for *in vitro* bioaccessibility analysis. Top left: Vegetables were rinsed with tap water to remove all visible soil and then rinsed with deionized water. Top right: Vegetables were homogenized in a blender. Bottom: Homogenized vegetables.
Table 3.1. Parameters of the *in vitro* bioaccessibility assay. References justifying each parameter are given.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Final Concentration*</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mouth Phase</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amylase</td>
<td>37.5U/mL*</td>
<td>Kejriwal, Bhandary, et al., 2014</td>
</tr>
<tr>
<td>Extraction time (min)</td>
<td>2 min</td>
<td>Minekus, Alminger, et al., 2014</td>
</tr>
<tr>
<td><strong>Gastrointestinal Phases</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soil mass (g)</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Diet mass (g)</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Solution volume (mL)</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Soil: solution (w/v)</td>
<td>1:200*</td>
<td>Malagelada, Longstreh, et al., 1976, Schroder, Basta, et al., 2004</td>
</tr>
<tr>
<td>Diet:solution (w/v)</td>
<td>1:1</td>
<td>Minekus, Alminger, et al., 2014</td>
</tr>
<tr>
<td>Temperature (ºC)</td>
<td>37</td>
<td>Schroder et al., 2004; Minekus et al., 2016</td>
</tr>
<tr>
<td><strong>Gastric Phase</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>2±0.1</td>
<td>Johnson, 2013, Schroder, Basta, et al., 2004</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.15M*</td>
<td>Minekus, Alminger, et al., 2014, Schroder, Basta, et al., 2004</td>
</tr>
<tr>
<td>Pepsin</td>
<td>0.4%*</td>
<td>Wang, Zhao, et al., 2011</td>
</tr>
<tr>
<td>Extraction time (min)</td>
<td>120</td>
<td>Minekus, Alminger, et al., 2014</td>
</tr>
<tr>
<td><strong>Intestinal Phase</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>6.5±0.1</td>
<td>Johnson, 2013</td>
</tr>
<tr>
<td>Pancreatin</td>
<td>0.35%*</td>
<td>Crews, Burrell, et al., 1983</td>
</tr>
<tr>
<td>Extraction time (min)</td>
<td>120</td>
<td>Schroder, Basta, et al., 2004</td>
</tr>
</tbody>
</table>

*Assuming 50g diet contributes 50mL water
Table 3.2. Total elemental concentrations of study soils as determined with X-ray fluorescence.

<table>
<thead>
<tr>
<th>Soil</th>
<th>Ca</th>
<th>Cd</th>
<th>Fe</th>
<th>Pb**</th>
<th>S</th>
<th>Zn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Joplin(^a)</td>
<td>9380±885</td>
<td>8±1.62</td>
<td>15600±569</td>
<td>5120±84</td>
<td>1410±72</td>
<td>3980±162</td>
</tr>
<tr>
<td>Cleveland(^b)</td>
<td>7600±469</td>
<td>&lt;8</td>
<td>46200±813</td>
<td>702±11</td>
<td>280±63</td>
<td>630±9</td>
</tr>
</tbody>
</table>

*Average of \(n=3\) subsamples. Relative standard deviation (RSD) averaged 9% and did not exceed 25%.

**Background soil Pb (95\(^{th}\) percentile) in a) Missouri=47 mg kg\(^{-1}\), b) Ohio=50 mg kg\(^{-1}\) (Smith, Cannon et al., 2013)
Table 3.3. Total elemental content and moisture content of vegetables analyzed with the in vitro bioaccessibility assay.

<table>
<thead>
<tr>
<th>Diet</th>
<th>% Moisture*</th>
<th>Ca</th>
<th>K</th>
<th>Mg</th>
<th>P</th>
<th>Pb</th>
<th>S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tomato</td>
<td>95.0</td>
<td>52.0</td>
<td>1530</td>
<td>58.5</td>
<td>189</td>
<td>0.02</td>
<td>84.9</td>
</tr>
<tr>
<td>Potato</td>
<td>73.4</td>
<td>161</td>
<td>3510</td>
<td>230</td>
<td>582</td>
<td>0.07</td>
<td>396</td>
</tr>
<tr>
<td>Peas</td>
<td>84.9</td>
<td>453</td>
<td>1280</td>
<td>219</td>
<td>469</td>
<td>0.02</td>
<td>257</td>
</tr>
<tr>
<td>Spinach</td>
<td>92.0</td>
<td>813</td>
<td>5580</td>
<td>898</td>
<td>424</td>
<td>0.02</td>
<td>389</td>
</tr>
<tr>
<td>Lettuce</td>
<td>93.8</td>
<td>463</td>
<td>1710</td>
<td>145</td>
<td>322</td>
<td>0.03</td>
<td>161</td>
</tr>
<tr>
<td>Zucchini</td>
<td>94.1</td>
<td>222</td>
<td>1660</td>
<td>126</td>
<td>265</td>
<td>0.01</td>
<td>110</td>
</tr>
<tr>
<td>Radish</td>
<td>95.4</td>
<td>195</td>
<td>1310</td>
<td>62.9</td>
<td>127</td>
<td>0.01</td>
<td>216</td>
</tr>
<tr>
<td>Carrot</td>
<td>86.5</td>
<td>342</td>
<td>2750</td>
<td>138</td>
<td>330</td>
<td>0.02</td>
<td>192</td>
</tr>
</tbody>
</table>

*Average of n=2 subsamples. Relative percent difference (RPD) did not exceed 15%.
Figure 3.3. *In vitro* bioaccessible Pb (IVBA Pb) for 2 soils and 8 diets. Error bars are 1 standard deviation of $n=3$ subsamples. Total Pb content of Joplin soil is 5,120 mg kg$^{-1}$ and Cleveland soil is 702 mg kg$^{-1}$. 

---

Table: Bioaccessible Pb (IVBA Pb) for 2 soils and 8 diets.

<table>
<thead>
<tr>
<th>Food</th>
<th>Joplin</th>
<th>Cleveland</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tomato</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Potato</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peas</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spinach</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lettuce</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zucchini</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Radish</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carrots</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---

Total Pb content: Joplin soil = 5,120 mg kg$^{-1}$; Cleveland soil = 702 mg kg$^{-1}$. 

---

**Note:** The table above shows the bioaccessible Pb (IVBA Pb) for 2 soils and 8 diets. The error bars represent the 1 standard deviation of $n=3$ subsamples. The total Pb content of Joplin soil is 5,120 mg kg$^{-1}$, and Cleveland soil is 702 mg kg$^{-1}$. 

---
Figure 3.4. % of soil-alone IVBA Pb (calculated as diet+soil IVBA Pb/soil-alone IVBA Pb) with 8 diets and 2 soils. Error bars are 1 standard deviation of \( n=3 \) subsamples. Total Pb content of Joplin soil is 5,120 mg kg\(^{-1}\) and Cleveland soil is 702 mg kg\(^{-1}\).
Table 3.4. Pearson correlation matrix for diet components and diet+soil IVBA Pb for two soils and eight vegetables in gastric and intestinal *in vitro* bioaccessibility assay.

<table>
<thead>
<tr>
<th>Component</th>
<th>Gastric</th>
<th>Intestinal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Joplin</td>
<td>Cleveland</td>
</tr>
<tr>
<td>Ca</td>
<td>-0.33</td>
<td>-0.18</td>
</tr>
<tr>
<td>K</td>
<td>0.29</td>
<td>-0.32</td>
</tr>
<tr>
<td>Mg</td>
<td>0.02</td>
<td>-0.38</td>
</tr>
<tr>
<td>P</td>
<td>0.52</td>
<td>0.00</td>
</tr>
<tr>
<td>S</td>
<td>0.41</td>
<td>-0.36</td>
</tr>
<tr>
<td>% Moisture</td>
<td>-0.64</td>
<td>0.04</td>
</tr>
</tbody>
</table>

*Correlation is significant at the 0.05 level (2-tailed).
Table 3.5. Results of linear mixed effects models for A. IVBA Pb and relative to B. % of soil-alone IVBA Pb (diet+soil IVBA Pb/soil-alone IVBA Pb)

<table>
<thead>
<tr>
<th>Factor</th>
<th>A. IVBA Pb (%)</th>
<th>B. % of soil-alone IVBA Pb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diet</td>
<td>0.0126*</td>
<td>0.1871</td>
</tr>
<tr>
<td>Phase</td>
<td>0.0024**</td>
<td>0.0001***</td>
</tr>
<tr>
<td>Soil</td>
<td>0.0767</td>
<td>0.0004***</td>
</tr>
<tr>
<td>Diet:Phase</td>
<td>0.02*</td>
<td>0.209</td>
</tr>
<tr>
<td>Phase:Soil</td>
<td>0.2308</td>
<td>0.0005***</td>
</tr>
<tr>
<td>Diet:Soil</td>
<td>0.3337</td>
<td>0.4667</td>
</tr>
</tbody>
</table>

Factors are significant at the *0.05, **0.01, and 0.001 levels.
References


Bibliography

Chapter 1 References


Chapter 2 References


Chapter 3 References


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