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BIOACCESSIBILITY TESTS ACCURATELY ESTIMATE BIOAVAILABILITY OF LEAD TO QUAIL

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Abstract: Hazards of soil-borne lead (Pb) to wild birds may be more accurately quantified if the bioavailability of that Pb is known. To better understand the bioavailability of Pb to birds, the authors measured blood Pb concentrations in Japanese quail (*Coturnix japonica*) fed diets containing Pb-contaminated soils. Relative bioavailabilities were expressed by comparison with blood Pb concentrations in quail fed a Pb acetate reference diet. Diets containing soil from 5 Pb-contaminated Superfund sites had relative bioavailabilities from 33% to 63%, with a mean of approximately 50%. Treatment of 2 of the soils with phosphorus (P) significantly reduced the bioavailability of Pb. Bioaccessibility of Pb in the test soils was then measured in 6 in vitro tests and regressed on bioavailability: the relative bioavailability leaching procedure at pH 1.5, the same test conducted at pH 2.5, the Ohio State University in vitro gastrointestinal method, the urban soil bioaccessible lead test, the modified physiologically based extraction test, and the waterfowl physiologically based extraction test. All regressions had positive slopes. Based on criteria of slope and coefficient of determination, the relative bioavailability leaching procedure at pH 2.5 and Ohio State University in vitro gastrointestinal tests performed very well. Speciation by X-ray absorption spectroscopy demonstrated that, on average, most of the Pb in the sampled soils was sorbed to minerals (30%), bound to organic matter (24%), or present as Pb sulfate (18%). Additional Pb was associated with P (chloropyromorphite, hydroxypyromorphite, and tertiary Pb phosphate) and with Pb carbonates, leadhillite (a lead sulfate carbonate hydroxide), and Pb sulfide. The formation of chloropyromorphite reduced the bioavailability of Pb, and the amendment of Pb-contaminated soils with P may be a thermodynamically favored means to sequester Pb. *Environ Toxicol Chem* 2016;35:2311–2319. Published 2016 Wiley Periodicals Inc. on behalf of SETAC. This article is a US Government work and, as such, is in the public domain in the United States of America.

Keywords: Metal bioavailability Ecological risk assessment Soil contamination Wildlife toxicology

INTRODUCTION

Concern for children ingesting lead (Pb)–contaminated soil has prompted numerous investigations on the bioavailability of Pb [1]. In the present study, we evaluated the bioavailability of soil-borne Pb to Japanese quail (*Coturnix japonica*), which are well suited to controlled laboratory conditions and have been used previously in studies supporting ecological risk assessments of birds. Because risk assessments generally rely on dosing studies in which animals are fed a relatively soluble form of Pb, adjusting for differences in bioavailability affords a more accurate assessment of risk at a site. "Absolute bioavailability" refers to that fraction of an ingested test dose of Pb that is absorbed from the gut and taken up into tissues. "Relative bioavailability" (RBA) is generally more useful in ecological risk assessment. It is defined as the amount of Pb absorbed from a soil divided by the amount of Pb absorbed from a maximally available form of Pb [2]. "Bioaccessibility," in contrast, is a chemical concept, referring to the fraction of Pb that is soluble in an aqueous solution that mimics the chemical conditions in the stomach or intestines. Bioaccessibility tests are conducted under controlled laboratory conditions and provide replicable estimates of bioavailability without the need to dose animals. Such tests are verified only when their results are well correlated with bioavailability from animal testing [3].

Bioaccessibility of Pb has been successfully calibrated against bioavailability over a wide range of Pb-contaminated soils fed to mammals [2,3]. Swine-based, rat-based, or rabbit-based Pb bioaccessibility tests might also be expected to predict bioavailability of soil-borne Pb to birds, although this hypothesis should be verified in birds and the relation must be quantified for tests to be useful. Because children may ingest soil-borne Pb on an empty stomach, when absorption would be expected to be greatest, studies with children in mind usually have dosed animals on an empty stomach. In contrast, we incorporated soil-borne Pb into avian diets in our experiment, assuming that soil, when ingested by wild birds and mammals, is generally ingested with their diet. The diet adds sorption sites for Pb and decreases the acidity of the digesta. We assumed that the relation of the blood Pb concentration to the concentration of ingested Pb was linear, based on studies on Japanese quail [4] and waterfowl (Figure 1) [5]. The relation in mammals, in contrast, is nonlinear [6], requiring a more involved experimental design.

In both birds and mammals, Pb bioavailability and bioaccessibility are governed by the sorbed fraction of Pb, which may become solubilized in acidic gastric fluids [7]. As digesta pass into the small intestine, where Pb is absorbed, the pH rises to 5 to 7, precipitating and sorbing some of the solubilized Pb [7]. A bioaccessibility test should simulate the overall or most controlling process that affects bioavailability,

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often found to be gastric solubilization. The bioavailability of Pb to birds has previously been evaluated in Pb-contaminated floodplain soil along the Coeur d'Alene River basin in Idaho (USA). From a study in which soil was incorporated into the diet of mallard ducklings, the RBA of Pb was estimated to be 44% [8]. Working on another sample of soil from the same basin, Furman et al. [9] correlated blood Pb concentrations in mallards to log-soluble Pb concentrations measured in a physiologically based extraction method and showed how the use of phosphorus (P) to remediate contaminated soil reduced both the bioaccessibility and the bioavailability of Pb. Our aims were to estimate the RBA of Pb in several Superfund soils to birds, to evaluate the accuracy of 6 previously developed bioaccessibility methods in predicting the measured bioavailability, and, where possible, to relate observed differences in the bioavailability of Pb to particular soil variables or to soil mineral assemblages present.

METHODS

Soils, diets, and quail

The present study was designed to be applicable to wild birds inhabiting large mining and smelting sites that have been identified in the US Environmental Protection Agency's (USEPA's) Superfund program. We collected soils from 5 sites that we named for their 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 in Missouri. Soil from the Coeur d'Alene River basin was contaminated mainly with Pb in sediments from mine waste deposited on a floodplain of the lower Coeur d'Alene River near Dudley, Idaho, downstream from mining and smelting. Soils from the Big River site in southeastern Missouri reflect a similar pattern of contamination from riverine transport of mining-related wastes. Soils from the 3 other sites were contaminated mainly by smelter emissions. In addition, we obtained 3 treated soils: 1 from the Big River treated with P (0.75% triple superphosphate) and 2 from Joplin, treated with either 1% P (as phosphoric acid, 1% P [P-H_3PO_4], KCl, and lime) or with ComPro composted biosolids from Montgomery County, Maryland (USA) [1,10]. The treatments of the Joplin soil were applied in 1997, and the Big River soil was treated in 2011. Soils from Joplin [11] and the Big River [12] had been used in previous studies. Like Hoffman et al. [8], we used soil from the Coeur d'Alene River basin but from a different site within the basin. Uncontaminated reference soil (Beltsville loam) was collected from the Patuxent Wildlife Research Refuge in Laurel, Maryland. Portions of this Beltsville soil were treated with either 1000 mg Pb/kg as the acetate (lead[II] acetate trihydrate, 99.99+%; Sigma-Aldrich; dissolved in water) or a fine crystalline powder (<1 μm) of PbS (lead[II] sulfide, 99.9%; Aldrich), added at the rate of 2000 mg/kg. Lead acetate is a highly water-soluble and readily bioavailable form of Pb commonly used in toxicity studies and is suitable as a reference for use in estimating RBA [13]. We selected PbS because it is less soluble in water than Pb acetate and has been studied previously. All soils had been sieved (2 mm).

The basal diet for the quail contained 63% chow (dry wt, Purina® game bird maintenance chow), 27% ground corn (dry wt), and 10% water. The chow contained a minimum of 12.5% protein, a minimum of 0.5% P, and between 0.5% and 1.0% calcium (Ca). Because in preliminary work we found that quail differentially selected particles from mash while feeding, we pelletized the diets through a 6-mm die, thus ensuring that the quail ingested the soil at the nominal concentration in the diet.

Thirteen diets were prepared, 11 of which contained 4% soil (dry wt) and 2 of which contained no soil. Four reference diets were included: the basal diet, the basal diet with Pb acetate added at a rate of 40 mg Pb/kg (equivalent to adding 4% soil containing 1000 mg Pb/kg diet), the basal diet with 4% uncontaminated Beltsville soil, and the basal diet with 4% Beltsville soil to which Pb acetate (1000 mg Pb/kg of soil or 40 mg Pb/kg of diet) was added. Eight soils contaminated with Pb from the mining and smelting sites and the PbS-amended soil were mixed into 9 additional diets.

Healthy, male, 4-wk-old quail, obtained from a breeding colony at the Patuxent Wildlife Research Center, were acclimated to living in separate stainless steel cages and exposed to 16 h of light. On 29 August 2010, 8 quail were randomly assigned to the basal diet group exposed to neither soil nor Pb, and groups of 5 quail were randomly assigned to each of the 12 other treatments. Quail were provided with diets and water ad libitum, and they appeared healthy throughout the 15-d trial as they were observed and cared for daily. Because blood Pb concentrations are known to increase soon after exposure [14] and to remain relatively constant when birds are fed a constant dose [15,16], we sampled blood from quail only at the end of the trial. We weighed the quail and drew approximately 1.0 mL of blood from the jugular vein into a tuberculin syringe using a 25-gauge needle containing heparin in the needle hub (1000 USP units/mL; Sargent Pharmaceuticals). Whole blood was frozen at –20 °C, and the quail were euthanized. An additional 4 untreated 4-mo-old male quail from the Patuxent colony were euthanized. They were dissected, and the pHs of the contents in the gizzards were measured in place with an Orion Star A214 pH meter (Thermo Fisher Scientific) equipped with a micro–glass electrode. All procedures involving quail in the present study were approved by the Patuxent Animal Care and Use Committee.

Studies on mammals generally express concentrations of Pb in blood as micrograms of Pb per deciliter and express dietary dose as milligrams of Pb per kilogram of body weight per day. To facilitate comparison, we noted that the mean moisture content of the quail blood in the present study was 80% and we approximated 1 mL of blood to weigh 1.05 g. The average weight of the quail during the study was 95 g (standard deviation = 6.1g), and quail of similar age from the colony ingested an average of 15 g of diet per day. To convert from a concentration in the diet (milligrams per kilogram) to a dose, the concentration may be multiplied by 0.158 to estimate the dose in milligrams of Pb per kilogram of body weight per day.

Analytical methods

Blood samples from the quail and portions of the soils and diets were sent to the Columbia Environmental Research Center in Missouri to be analyzed for metals. Samples were analyzed concurrently with samples from a previously published study [4], which includes details of the methods. Briefly, freeze-dried blood samples were digested with HNO_3 followed by H_2O_2 in capped borosilicate test tubes that were heated to 110°C [17]. Feed samples were digested with HNO_3, and soil samples were digested in a mixture of HNO_3 and HCl (similar to USEPA Method 3051), each using a microwave digestion system. Lead concentrations in blood and feed and Pb, zinc (Zn), cadmium (Cd), and copper (Cu) concentrations in soil were then quantified by inductively coupled plasma mass spectrometry. Recoveries of Pb from standard reference materials were 100% in blood (Seronorm Trace Elements 201705 Whole Blood 3, Clinchek 8841 Whole Blood Control Level II) and from 76% to 100% in soil or sediment (NRCC MESS-3 Marine Sediment, NRCC PACS-1 Marine Sediment, NIST 2709 San Joaquin Soil). Recoveries of Pb from predigestion spikes ranged from 96% to 102% in blood, 92% to 102% in feed, and 92% to 110% in soil. The means of the percent relative standard deviations of replicate analyses were 14% (blood), 10% (feed), and 8.5% (soil). Metal concentrations were reported as milligrams per kilogram dry weight. The detection limits of Pb were 0.012 mg/kg in whole blood, 0.006 mg/kg in feed, and 0.004 mg/kg in soil.

Subsamples of the soil were sent to the Agricultural Analytical Services Laboratory of The Pennsylvania State University (University Park, PA, USA), where the texture was determined and soil pH, cation exchange capacity (CEC), organic matter content, and Ca, P, and Pb concentrations (extracted with Mehlich-3 solution, quantified by inductively coupled plasma-atomic emission spectroscopy) were measured.

Statistics

Blood Pb concentrations below the detection limit (7 of 13 birds not fed Pb) were approximated as one-half the detection limit in statistical calculations. Statistical tests were run with SigmaPlot® 12 software (Systat Software). Differences in weight changes of quail among treatment groups were evaluated with an analysis of variance. The degree of association between 5 soil variables and the relative bioavailabilities of Pb in the 8 contaminated field samples were estimated from Spearman rank correlation coefficients. The RBA (quail) of Pb in 10 contaminated soils was linearly regressed on the relative bioaccessibility of Pb for each of 6 methods. These regressions are referred to as in vitro–in vivo correlation regressions.

Determination of bioaccessible Pb

Bioaccessible soil Pb was determined by 6 in vitro gastro(intestinal) methods: 1) the relative bioavailability leaching procedure (RBALP) [2], which is also known as USEPA method 1340; 2) a modified RBALP method where an extraction solution of pH 2.5 was used instead of pH 1.5; 3) the waterfowl physiologically based extraction test (W-PBET) [9]; 4) the Ohio State University in vitro gastrointestinal method (pH 1.8; OSU IVG) [18]; 5) the urban soil bioaccessible Pb test (pH 2.5; USBLT) [19]; and 6) the physiologically based extraction test (pH 2.5) of Ruby et al. [20] with modifications [21]. The USBLT was designed to be a simplified test especially applicable to urban gardening. The W-PBET was designed to estimate bioavailability of Pb to birds. Although the authors transformed their bioaccessibility values to logarithms in their model, we used only untransformed data to facilitate comparison of the W-PBET with other methods, which may have reduced the predictive value of this method. A short description of each method follows.

RBALP (USEPA Method 1340 [2]). Soil (1.0 g, <250 μm) was placed in 100 mL of gastric solution (0.40 M glycine) preheated to 37 °C in a 125-mL high-density polyethylene bottle (HDPE) and placed into a rotator shaker located in a 37 °C incubator. Soil samples were rotated at 30 ± 2 rpm for 1 h. Solution pH was frequently checked and adjusted to 1.5 ± 0.05 using dropwise addition of 50% NaOH and/or 6 M trace metal HCl solution. After 1 h, an aliquot of suspension was collected with a syringe and filtered (0.45 μm) for Pb analysis by inductively coupled plasma optical emission spectroscopy (USEPA Method 6010C [22]).

RBALP (USEPA Method 1340) with pH modification. This modified procedure was as the RBALP method described in the previous paragraph, except the solution pH was adjusted to 2.5 ± 0.05 instead of 1.5 using dropwise addition of 50% NaOH and/or 6M trace metal HCl solution.

OSU IVG [18]. Soil (1.0 g, <250 μm) was placed in 150 mL of gastric solution (0.10 M NaCl and 1% [w/w] porcine pepsin) and heated in an open extraction vessel in a 37 °C water bath. The extraction solution was continuously mixed using a paddle stirrer to maintain a homogenous suspension, and the pH was continuously monitored and adjusted to 1.8 ± 0.1 using 6 M trace metal grade HCl. After 1 h, 10 mL of gastric solution was immediately centrifuged (11 160 *g* for 15 min) and then filtered (0.45 μm) for analysis of supernatant.

W-PBET [9]. Soil (3.6 g, <250 μm) was placed in 30 mL of gastric solution (0.10 M NaCl and 1% [w/w] porcine pepsin) preheated to 37 °C in a 125 mL HDPE bottle and placed on a rotator shaker in a 42 °C incubator. Soil extraction samples were rotated at 30 ± 2 rpm for 1 h, and solution pH was frequently checked and adjusted to 2.6 ± 0.05 using dropwise addition of 50% NaOH and/or 6M trace metal HCl solution. After 1 h, an aliquot of suspension was collected with a syringe and filtered (0.2 μm) for analysis.

USBLT [19]. Soil (5.0 g, <2 mm) was placed in 50 mL of gastric solution (0.40 M glycine, pH 2.5 at 22 °C) in a 125-mL urinalysis cup. The solution was mixed on a horizontal shaker at 100 rpm for 2 h, and samples were then filtered. The pH of each filtrate was determined and recorded to the nearest 0.01. The filtrate was analyzed for Pb by inductively coupled plasma atomic emission spectrometry. The relationship between bioavailable and bioaccessible Pb was established with the control and phosphate-amended soils from Joplin fed to humans, swine, and rats [1].

PBET (modified) [21]. Soil (1 g, <250 μm) was weighed into a 250-mL low-density polyethylene bottle and mixed with a gastric solution at a 1:100 ratio. The gastric solution was prepared by adding 1.25 g of pepsin powder (41707-1000, Acros Organics; with an activity of 800–2500 units/mg), 500 mg of sodium-l-malate, 500 mg of citrate, 500 μL of acetic acid, and 420 μL of lactic acid to about 500 mL of deionized water in a 1 L volumetric flask. Following thorough mixing, the pH of the solution was adjusted to 2.5 using trace metal grade concentrated HCl, and the volume was adjusted to 1 L with deionized water. The soil/gastric solution mixture was shaken for 1 h at 37 °C at 100 rpm. After 1 h, the extract was filtered through 0.45-μm syringe filters, and the pH was tested to ensure that it had not strayed from 2.5 ± 0.02. Samples were then analyzed by inductively coupled plasma spectrometry and compared with the total Pb concentration of the <250-μm fraction determined previously.

Calculation of in vitro bioaccessible Pb

Total Pb was determined for all soils by USEPA Method 3051A [23], which uses reverse aqua regia solution (1:3 HNO_3:HCl) in a microwave-assisted sample digestion.

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

$$\%\text{IVBA Pb} = ([\text{IVBA extractable Pb [mg/kg]}])/(\text{total soil Pb [mg/kg]}) \times 100\%$$

Pb speciation by X-ray absorption spectroscopy

X-ray absorption spectroscopy experiments to determine Pb speciation were conducted at the Materials Research

Collaborative Access Team beamline 10-ID, Sector 10, at the Advanced Photon Source of the Argonne National Laboratory [24]. The storage ring operated at 7 GeV in top-up mode. A liquid N_2 cooled double crystal Si (111) monochromator was used to select the incident photon energies, and a platinum-coated mirror was used for harmonic rejection. Calibration was performed by assigning the first derivative inflection point of the absorption LIII-edge of Pb metal (13035 eV), and each sample scan was collected simultaneously with a Pb metal foil. The samples were ground and pressed into pellets, affixed to a 20-hole sample holder, and mounted for analysis without any further modifications. Data collection was conducted in fluorescence (Ge detector; Canberra) and transmission modes for the samples. For some samples, the transmission data were unusable for analysis. Various Pb standards were used as reference spectra, including mineral sorbed Pb (Pb-ferrihydrite, Pb-kaolinite, Pb-goethite, Pb-gibbsite, Pb-birnessite, and Pb-montmorillonite in which each mineral was equilibrated with $Pb[NO_3]_2$ at pH 6 for a target surface loading of 2500 mg kg^{-1} after dialysis), organic bound Pb (Pb-fulvic acid and Pb-humic acid as reagent grade organic acids equilibrated with $Pb[NO_3]_2$ at pH 6 for a target loading of 1500 mg kg^{-1} after dialysis, and reagent-grade Pb acetate, Pb cysteine, and Pb citrate), Pb carbonate (Smithsonian Natural History Minerals Collection specimens of cerussite, hydrocerussite, and plumbonacrite with X-ray diffraction verification), PbO (massicot and litharge), Pb phosphates (chloropyromorphite, hydroxypyromorphite, $Pb_3[PO4]_2$, $PbHPO_4$, and Pb sorbed to apatite at pH 6 and surface loading of 2000 mg kg^{-1}), and other Pb minerals (leadhillite, magnetoplumbite, plumboferrite, plumbogummite, plumbojarosite, anglesite, and galena from the Smithsonian Natural History Minerals Collection with X-ray diffraction verification). All reference spectra were collected in transmission mode with dilution calculations determined by the program XAFSMass [25], mixed in binder, and pressed into a pellet. These spectra were acquired on the same beamline with identical scan parameters simultaneously with a Pb metal foil for calibration but on separate occasions from the samples.

All sample and standard spectra were calibrated to a Pb foil on the same energy grid, averaged, and normalized; and the background was removed by spline fitting using IFEFFIT software [26]. Principal components analyses were performed in the graphical interface SIXpack [27] on the normalized scans, and target factor analyses of each Pb standard were performed to determine the most appropriate standards to be used for linear combination fits analyses. Standards with SPOIL values <3.0 were used in the linear combination fits analyses, which included mineral sorbed Pb (sum of Pb-ferrihydrite, Pb-goethite, and Pb-birnessite), organic bound Pb (sum of Pb-fulvic acid and Pb-humic acid), Pb carbonate (sum of cerussite and hydrocerussite), PbO (sum of massicot and litharge), Pb phosphates (chloropyromorphite, hydroxypyromorphite, $Pb_3[PO4]_2$, and Pb sorbed to apatite), and other Pb minerals (leadhillite, plumboferrite, plumbojarosite, anglesite, and galena). The k-space functions of the standards and samples were used for all linear combination fitting. The Levenberg-Marquardt least squares algorithm was applied to a fit range of 0.6 $\mathring{A}^{-1}$ to 9.0 $\mathring{A}^{-1}$. Best-fit scenarios, defined as having the smallest residual error, also had sums of all fractions close to 1. A minimum of 2 components was necessary to fully describe any particular sample within 1% reproducible error. Results have a ± 10% accuracy.

RESULTS AND DISCUSSION

Bioavailability of soil-borne Pb to quail

The Beltsville reference soil contained 22 mg Pb/kg, and soils from contaminated sites contained from approximately 2000 mg Pb/kg to approximately 4700 mg Pb/kg. Study soils were loams with widely varying soil properties, including soil pH (4.4–7.7), CEC (8.3–23.7 mEq/100g), soil organic matter (3.0–12%), and Mehlich-3 extractable P (6–3300 mg/kg; Table 1). With the exception of Beltsville soil, soils were contaminated also with Zn, Cd, and/or Cu. Adding P increased concentrations of Mehlich-3 P from 42 mg/kg to 3300 mg/kg in Big River soil and from 74 mg/kg to 2900 mg/kg in Joplin soil. The basal diet contained 0.05 mg Pb kg^{-1}, 95 mg Zn kg^{-1}, 0.075 mg Cdnkg^{-1}, and 13 mg Cu kg^{-1}. Diets amended with soil from contaminated sites contained from 86 mg Pb kg^{-1} to 223 mg Pb kg^{-1} (Table 2).

Quail appeared healthy throughout the trial, and all birds gained weight, starting at a mean weight of 87 g and increasing an average of 18%. Final mean body weights of the groups were not significantly different from each other (analysis of variance $p = 0.60$). The average pH measured in the stomachs of the 4 quail that had eaten recently was 3.7, which is consistent with values previously reported in quail of 3.4 and 3.8 in Bonos et al. [28] and 3.7 in Yamamoto et al. [29]. Note, however, that gastric pH varies within the class, being much lower, for example, in avian raptors than in seed-eating birds [30].

Mean concentrations of Pb (about 0.02 mg kg^{-1}) in quail blood from the 2 low-Pb reference groups were close to the detection limit, and mean concentrations from the 11 other groups were in the range of 0.76 mg kg^{-1} to 2.26 mg kg^{-1}

Table 1. Element concentrations and properties of soils fed to quail

Soil	Strong acid–extractable metals (mg/kg dry wt) Pb	Zn	Cd	Cu	Mehlich 3-extractable elements (mg/kg dry wt) Pb	Ca	P	pH	Organic matter (%)	Cation exchange capacity (meq/100 g)
Beltsville	22	42	0.058	7.5	5.6	116	6	4.5	3.4	11.5
Pb acetate, Beltsville	1040	42	0.037	7.7	870	166	14	4.6	3.4	8.8
Coeur d'Alene	3730	1530	13	92	960	398	6	5.4	4	10.9
Helena	3340	2690	135	709	900	3330	110	6.5	11	21.7
Viburnum Trend	3560	260	7.2	96	1600	1150	19	5.1	12	14.2
Big River control	2240	654	9.8	66	1100	3030	42	7.6	3.1	20.4
Big River 0.75% P	2070	664	14	69	570	8350	3300	7.2	3	21.2
Joplin control	4710	14 800	98	42	1100	3800	74	7.0	7.3	17.5
Joplin 1% P	3830	4050	26	32	130	7840	2900	6.3	8	23.7
Joplin 10% compost	3530	3660	22	62	690	13 600	320	7.7	8.6	17.8
Pb sulfide, Beltsville	1733	41	0.047	7.8	1100	91	8	4.5	3.4	8.3

Table 2. Relative bioavailability of Pb calculated from concentrations of Pb in diets and in blood of Japanese quail

Treatment[a]	Pb in feed[b] (mg/kg dry wt)	Blood Pb (mean ± SE) (μg/g dry wt)	Adjusted mean[c] blood Pb (mg/kg)	Bioavailability ([μg/g blood]/[g/kg diet])	Relative bioavailability (%)[d]
Basic	0.05	0.019 ± 0.01	0.000	—	—
Pb acetate	41	1.25 ± 0.07	1.23	30.1	100
Beltsville soil	1.2	0.018 ± 0.01	–0.001	–1.04	–3.4
Pb acetate, Beltsville soil	43	0.96 ± 0.05	0.94	22.0	73
Coeur d'Alene soil	148	1.51 ± 0.08	1.49	10.0	33
Helena soil	123	2.12 ± 0.14	2.10	17.1	57
Viburnum Trend soil	137	2.26 ± 0.14	2.24	16.3	54
Big River control soil	86	1.65 ± 0.11	1.63	19.1	63
Big River 0.75% P soil	88	1.27 ± 0.10	1.25	14.2	47
Joplin control soil	161	1.94 ± 0.09	1.92	11.9	40
Joplin 1% P soil	223	1.15 ± 0.08	1.13	5.06	17
Joplin 10% compost soil	121	1.52 ± 0.05	1.50	12.4	41
Pb sulfide, Beltsville soil	70	0.76 ± 0.09	0.74	10.5	35

[a] The first 2 diets contained no soil, and the other diets contained 4% soil.

[b] The Pb concentration of 0.05 mg/kg in the maintenance diet was subtracted from each other measured concentration.

[c] Values were adjusted by subtracting 0.019 mg/kg from each.

[d] Calculated as bioavailability divided by bioavailability of Pb acetate.

SE = standard error.

(Table 2). Columns in Table 2 show mean measured blood Pb concentrations, adjusted blood Pb concentrations (background subtracted), bioavailability (blood Pb concentration divided by dietary Pb concentrations), and RBA (bioavailability divided by bioavailability of Pb acetate), expressed as a percentage. Based on our definition, the RBA of Pb in the diet treated with Pb acetate was 100%. The RBA of Pb in the soil-borne Pb acetate group was 73%, demonstrating that some of the Pb in the gut sorbed to the Beltsville soil, reducing its solubility/bioavailability. When uncontaminated Beltsville soil was added to the quail diet, the mean blood Pb concentration decreased slightly, resulting in a negative estimate of bioavailability (–3.4%). This means that the increase in sorption associated with the addition of soil more than offsets the increase in the dietary Pb concentration from the control soil. Bioavailabilities of the 5 untreated contaminated soils ranged from 33% to 63%, all reasonably close to 50%. Adding US Department of Agriculture compost to Joplin soil had no effect on bioavailability, although adding P reduced bioavailability by 25% (Big River soil) and by 57% (Joplin soil). The RBA of powdered PbS in soil was 35%, about one-half that of Pb acetate in soil.

Comparison to related studies

The range of RBAs of 33% to 63% at our 5 contaminated sites is consistent with the USEPA's [31] default estimate of 60%. None of our RBAs was as low as the lowest RBAs reported by Drexler and Brattin [2] (1–105%), based on feeding Pb-contaminated soils to swine. The value of 1% RBA in the Drexler and Brattin study referred to galena in soil, which is an insoluble crystal mineral that may become encrusted with other minerals. Although galena consists of PbS, it is quite different from the more soluble, amorphous PbS that we added to soil, with an RBA of 35%. Most of the soils at our sites were contaminated mainly from smelting, which tends to increase Pb's bioavailability [32]. Our values were also higher than the RBA's from 9% to 41% in rats reported by Ruby et al. [11], studying soils contaminated mainly from acid mining waste. The low RBAs associated with particulate Pb sulfate in those soils [33] contrast with the 63% RBA at our Big River site, where the calcareous waste contained Pb carbonate and relatively soluble Pb sorbed to minerals [34].

Three of our soils had been tested in previous bioavailability studies. Soil from Joplin, Missouri, had an RBA of Pb of 59% to 67% in swine (D. Mosby, 2000, Master's thesis, University of Missouri, Columbia, MO, USA) compared with 40% in our quail. A composite of soil contaminated from tailing piles near the Big River, Missouri, had a mean bioavailability of Pb to swine of 39% [11], based on blood analyses, compared with our estimate of 63% in quail. Soil from the Coeur d'Alene River basin had an RBA of Pb of 44%, based on ducklings [8], compared with our estimate of 33%.

Interactions of Pb with dietary Ca and P

Bioavailability of Pb to children has been estimated from feeding studies in which swine have been dosed on an empty stomach with Pb delivered in a ball of dough [11]. Food provides binding sites for soluble Pb and dilutes gastric acidity, which makes our feeding trial different from the model for children. Dietary Ca and P, in particular, reduce the uptake of Pb [35–38]. Potential sorptive effects of ingested soil may be partially masked by these dietary components. Consider the concentrations of Ca in the stomachs of our quail, for example. The expected concentration of Ca in the gut from feed (~0.5%) is about 1000 times the Mehlich-3 extractable Ca concentration associated with Beltsville soil added at 4% of the diet. Similarly, the pH of ingested noncalcareous soil may have only a slight effect on the pH of digesta in a stomach. Thus, it is not surprising that the values of none of the 5 soil variables (pH, percent organic matter, CEC, and Mehlich-3 Ca and P) we considered were statistically correlated with Pb bioavailability ($p > 0.05$ by Spearman rho). However, these soil variables might have had a noticeable influence if the soil had been ingested on an empty stomach.

Bioavailability depends on Pb speciation

The Pb speciation of our samples by X-ray absorption spectroscopic analysis enables us to relate the RBA of the soils to the forms of Pb present. Total soil Pb concentration was inversely related to RBA of Pb ($r = –0.55$), which suggests that the form of Pb in the soil, and not the total amount of Pb, is controlling the Pb bioavailability. Lead phases vary with each soil but have an overall trend of mineral sorbed Pb > organic bound Pb > Pb sulfate (anglesite) as the major components, with some contribution from Pb phosphate phases (chloropyromorphite, hydroxypyromorphite, and tertiary Pb phosphate), Pb carbonates, leadhillite, and galena (Table 3). The Pb in most

Table 3. Mineralogy of soils fed to quail

Soil	Mineral sorbed Pb	Organic bound Pb	Pb carbonate	Pb sulfate	Pb sulfide	Chloropyromorphite	Hydroxypyromorphite	$Pb_3(PO4)_2$	Leadhillite	χ^2
Beltsville	31.3		2.6	17.4		40.8			7.9	0.062
Pb acetate, Beltsville	23.0	45.2		11.8		2.6	12.8		4.6	0.001
Coeur d'Alene	39.2	13.8	5.3	16.7				24.9		0.001
Helena	38.4	1.7	3.0	18.0	3.1	17.3		18.6		0.001
Viburnum Trend	28.3	45.2	5.8	9.4				11.2		0.001
Big River control	60.3	1.0	20.2	3.9	3.0			11.6		0.001
Big River 0.75% P	38.9	24.7		9.6		4.0		8.7	14.0	0.001
Joplin control	20.0	36.8	2.2	31.1	9.9					0.001
Joplin 1% P	16.5	23.9	9.0	12.8		37.7				0.001
Joplin 10% compost	20.8	24.7	6.8	21.2				26.5		0.001
Pb sulfide, Beltsville	10.1	41.0		43.4	5.5					0.001

of these soils affected by mining and smelting activities is derived from Pb ore materials in which galena is often the prevalent form of Pb. Oxidation of galena can lead to a variety of Pb phases in soils and is dependent on the prevailing chemical conditions of the soil, such as pH, redox potential, water content, mineralogy, organic matter content, and presence of other elements in the soil solution. Anglesite (Pb sulfate) is a secondary mineral precipitate formed by galena oxidation as the sulfide in the galena structure is oxidized to sulfate. Release of Pb ions during galena oxidation can result in adsorption complexes with natural organic matter components or mineral surfaces such as clays and metal oxides [39]. Smelting of Pb ores produces Pb oxides, which may form relatively soluble forms in soil such as Pb carbonates and Pb sorbed to iron and manganese oxides [32]. Lastly, if phosphate is present or amended into soils with available Pb, the formation of Pb phosphates can be thermodynamically favored as a method to sequester Pb [40]. The addition of large quantities of P reduced bioavailability of Pb by 57% in Joplin soil and by 25% in Big River soil (Table 2). The influence of soil amendments on mineralogy is illustrated in the Joplin (control, 1% P, and compost) and Big River (control and treated) soils. The Joplin control contained primarily sorption complexes with mineral surfaces and organic matter followed by Pb sulfate and a small amount of Pb sulfide. Upon addition of compost to the Joplin soil, sorption complexes and Pb sulfate were still dominant, but about one-quarter of the Pb became associated with tertiary Pb phosphate from the compost. The 1% P Joplin soil also demonstrates the importance of sorption complexes and Pb sulfate. The dominant Pb phase, however, was chloropyromorphite (37.7%; Table 3), which is the probable cause of the low 17% bioavailability of Pb in this soil [34]. Chloropyromorphite formation was the goal of the phosphate treatment because it is stable and sparingly soluble at low pH [34].

Table 4. Bioaccessible Pb determined by 6 in vitro methods for soils fed to Japanese quail and associated relative bioavailabilities predicted from regression equations[a]

Soil	RBALP pH 1.5 1:100	OSU-IVG pH 1.8 1:150	RBALP pH 2.5 1:100	PBET pH 2.5 1:100	USBLT pH 2.5 1:10	W-PBET pH 2.6 1:8.3
Measured bioaccessibility of Pb (%)						
Beltsville	52	54	28	17	20	28
Pb acetate, Beltsville soil	96	87	77	47	104	59
Coeur d'Alene	67	54	45	25	46	30
Helena	89	74	50	14	32	16
Viburnum Trend	86	74	58	26	27	8.5
Big River control	101	93	81	55	21	11
Big River 0.75% P	97	87	51	31	5	1.7
Joplin control	91	80	63	16	15	1.8
Joplin 1% P	63	32	17	6.5	2	0.027
Joplin 10% compost	81	69	53	25	7	0.12
Pb sulfide, Beltsville soil	66	72	65	37	28	45
Predicted relative bioavailability of Pb (%)						
Beltsville	15	32	26	38	43	49
Pb acetate, Beltsville soil	58	57	62	60	71	59
Coeur d'Alene	30	33	38	43	52	50
Helena	51	47	42	35	47	46
Viburnum Trend	49	47	48	44	46	43
Big River control	63	61	65	65	43	44
Big River 0.75% P	59	57	43	48	38	41
Joplin control	53	52	52	37	42	41
Joplin 1% P	26	16	18	30	37	41
Joplin 10% compost	43	44	44	44	39	41
Pb sulfide, Beltsville soil	29	46	53	52	46	54

[a] Ratios shown represent soil (g):solution (mL).

RBALP = relative bioavailability leaching procedure; OSU IVG = Ohio State University in vitro gastrointestinal; PBET = physiologically based extraction test; W-PBET = waterfowl physiologically based extraction test; USBLT = urban soil bioaccessible lead test.

Chloropyromorphite is also easily formed and very stable in the gastrointestinal tract [40]. In contrast, only about 4% of the Pb in the Big River soil amended with P was associated with chloropyromorphite, and the bioavailability of Pb was 47%. The difference in the rates of chloropyromorphite formation may have been caused by the lower pH of the treated Joplin soil (pH 6.3 vs 7.2), given that the rate of formation of chloropyromorphite increases with soil acidity [41]. In addition, the Joplin soil had been treated in 1997, whereas the Big River soil was treated more recently, in 2011.

Various species of Pb (PbS, Pb acetate) were present in the control and treated Beltsville soils. The control soil shows the presence of chloropyromorphite, mineral sorbed Pb, and Pb sulfate. However, the concentration of Pb in this sample was low, the quality of the X-ray absorption spectroscopy spectrum was poor, and we caution that the corresponding linear combination fits fitting error is substantially higher than those from other samples. When Pb acetate was added to Beltsville soil, most of the Pb was sorbed to minerals or bound to organic matter, but approximately 12% became Pb sulfate and approximately 13% hydroxypyromorphite. When Pb sulfide was added to Beltsville soil, most of the Pb was bound to organic matter or identified as Pb sulfate, which is likely the result of the oxidation of sulfide (Table 3).

In vitro results

The percent IVBA Pb measured varied with the method, depending on both the acidity of the solutions and the ratio of soil to solution employed (top half of Table 4). Bioaccessibility of Pb was greatest when measured in RBALP at pH 1.5, followed by OSU IVG at pH 1.8, and least in the 4 methods conducted at pH 2.5. In addition, the ratio of soil to solution was important in the soils containing high concentrations of Mehlich-3 P. The columns of the 4 methods run at pH 2.5 are arranged left to right in Table 4, from a wide (1:100 in RALAP 2.5) to a narrow (1:8.3 in WPET) soil to solution ratio. In 5 of 6 soils with a P concentration of at least 40 mg/kg (3 Joplin soils, 2 Big River soils), RBA Pb decreased with a narrowing of the solution ratios (RBALP 2.5 > PBET > USBLT > WPET, Table 1). Reductions were especially pronounced in the P-treated soils, in which RBA Pb decreased from 51 mg/kg to 1.7 mg/kg (Big River soils) and from 17 mg/kg to 0.027 mg/kg (Joplin soils). No such pattern is observed in the 5 soils with lower Mehlich-3 P concentrations. These data suggest that employing a more concentrated solution limits Pb solubility through an interaction with P. The low IVBA Pb concentrations measured at narrow ratios may also interfere with a method's ability to accurately estimate the effect of treating a contaminated soil with P.

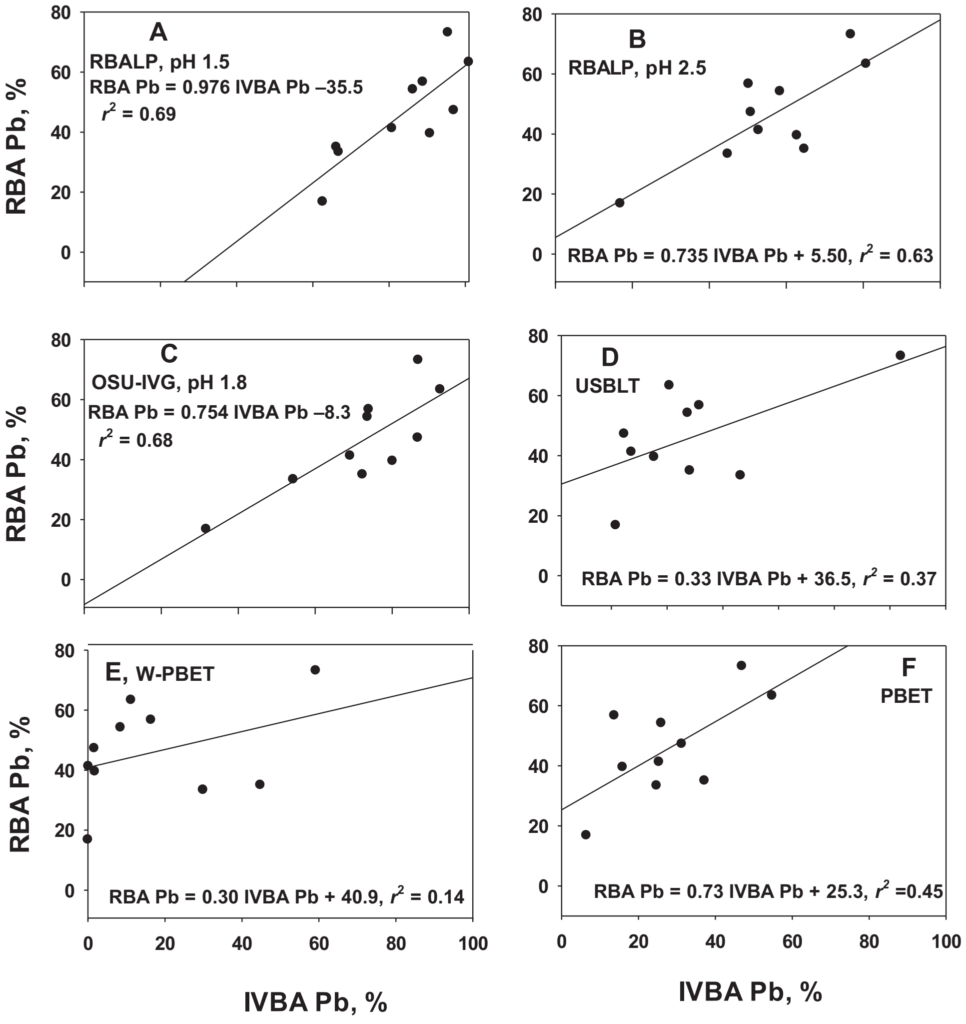

Figure 1. Percent relative bioavailable Pb (RBA Pb) versus percent bioaccessible Pb (IVBA Pb) measured by the respective in vitro gastro(intestinal) methods for 10 contaminated soils: (**A**) relative bioavailability leaching procedure (RBALP), (**B**) RBALP modified to pH 2.5, (**C**) Ohio State University in vitro gastrointestinal method (OSU-IVG), (**D**) urban soil bioaccessible Pb test (USBLT), (**E**) waterfowl physiologically based extraction test (W-PBET), and (**F**) modified physiologically based extraction test (PBET).

The bioaccessibility of Pb was lower in Beltsville soil than in untreated contaminated soils by the USO IVG and the 2 RBALP methods (Table 4). This finding is consistent with the reported low bioaccessibilities of Pb (median = 23%, by RBALP [pH 1.5]) measured in a survey of uncontaminated soils [42] and with the presence of insoluble forms (Table 3).

In vitro–In vivo correlation

The use of bioaccessibility tests is an efficient and practical alternative to measuring bioavailability directly in animals. It is essential, however, that the bioaccessibility tests be calibrated. The in vitro–in vivo correlation regressions in Figure 1 are the result of regressing the percent relative bioavailability of Pb (Table 2, 10 contaminated soils) on the percent bioaccessibility for each of the 6 methods (Table 4). All of the methods show a positive correlation between the 2 variables, with coefficients of determination from 0.14 to 0.69.

According to USEPA guidance [31] for human risk assessment, the 2 measured values need not be identical, but a regression on in vitro values should accurately predict in vivo values. The regression should have a high coefficient of determination, showing that the data fit the statistical model. In evaluating the suitability of bioaccessibility models, Wragg et al. [43] stated that a linear relationship between in vivo and in vitro data should have a correlation coefficient (r) > 0.8 and a slope > 0.8 and < 1.2. The criterion for the slope is based on matching 100% RBA to 100% bioaccessibility. In our case, however, the maximally available form of Pb in soil has an RBA of 0.73 rather than 1.0. This was a result of choosing a diet with Pb acetate but no soil as our divisor for RBA. Thus, the slope of the quail IVIVC regression should be within 20% of the ideal slope of 0.73. An IVIVC regression line should also pass close to the origin if the bioaccessibility test accurately mimics Pb absorption in the quail gut. The regression of the RBALP at pH 1.5, for example, has a negative intercept of –35.5%, meaning that the method extracts a predicted 35.5% of the Pb when the measured bioavailability is 0. This suggests that although the extraction solution might be appropriate for modeling Pb in an empty stomach, it is too strong to accurately model Pb solubility in a quail stomach containing food. The W-PBET solution is the weakest, extracting a predicted 0% Pb when the bioavailability is 41%.

A robust in vitro bioaccessibility method should accurately predict the effects of P and other amendments that could be used to reduce the bioavailability of Pb in contaminated soils. The addition of P to the Joplin soil caused a 57% reduction in Pb bioavailability to quail, similar to the 69% reduction in bioavailability to test humans [1]. This was close to predicted reductions of 52% by RBALP (at pH 1.5), 65% by RBALP (at pH 2.5), and 70% by OSU-IVG (from data in Table 4). The Big River soil had a higher pH and contained little chloropyromorphite compared with the Joplin soil. A 25% reduction in Pb bioavailability in that soil was close to predicted reductions of 34% by RBALP (at pH 2.5) and 26% by PBET. The RBALP at pH 2.5 was the only test that performed well on both soils. Further studies with more Pb-contaminated soils may improve the ability of these methods to predict reductions in Pb RBA in P-treated soils.

CONCLUSIONS

Japanese quail were well suited to the present 15-d dietary study estimating the bioavailability of Pb in soils contaminated from mining and smelting. Although Pb in some mineral forms, such as galena, is only slightly bioavailable [2,44], we found that that RBAs of Pb in soils contaminated from mining and smelting at 5 Superfund sites range from 33% to 63% of dietary Pb acetate. A reasonable default value for wild birds on these kinds of sites is 50%. The RBAs of Pb based on analyses of Pb in quail were correlated with estimates of bioaccessibility measured by 6 in vitro tests. Although 5 of the tests were developed for estimating the bioavailability of Pb to mammals, they predicted bioavailability to quail as well. Based on criteria for slope, coefficient of determination, and low intercepts, the RBALP (USEPA method 1340) [2] modified to pH 2.5 and the OSU-IVG methods performed the best of the 6 in vitro tests and are likely to be accurate over the range of soils tested.

The fraction of Pb solubilized was greatest in the tests conducted at the lowest pH. The pH of 1.5 in the original/official RBALP, selected to protect children by simulating the acidity of a child's empty stomach, seems to be too low to accurately predict bioavailability of Pb to wildlife ingesting soil with dietary items. The pH of the gastric solution in the quail stomachs was measured as 3.7, higher than that used in all of the methods. The addition of a large amount of P to Pb-contaminated soils decreased the bioavailability of Pb, consistent with a decrease in bioaccessibility; but other soil properties examined did not have a discernable effect on bioavailability.

Bioaccessibility estimates of Pb may be used in a risk assessment to account for the difference between the bioavailability of soil-borne Pb estimated at a contaminated site and the bioavailability of Pb associated with a toxicity reference value used in the assessment. Percent bioaccessibility measured should be adjusted with the calibration regressions in Figure 1 to yield a predicted estimate of RBA. Use of the bioaccessibility values without adjustment may be misleading, as demonstrated by comparing the measured bioaccessibility values to the predicted RBAs in Table 4. The bioaccessibility tests may also be used to predict the efficacy of remediating soils, such as with the addition of P.

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Data availability—Relative bioaccessibility data for all methods are shown in Table 4, and mineralogy data are shown in Table 3. Mean values of Pb concentrations in quail blood are shown in Table 2. Data on individual birds, as well as quality control details on metal analyses, are available from the corresponding author (nbeyer@usgs.gov).

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