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Abstract

This study compared lead (Pb) immobilization efficacies in mining/smelting impacted soil using phosphate and iron amendments via ingestion and inhalation pathways using *in vitro* and *in vivo* assays, in conjunction with investigating the dynamics of dust particles in the lungs and gastro-intestinal (GI) tract via X-Ray Fluorescence (XRF) microscopy. Phosphate amendments [phosphoric acid (PA), hydroxyapatite, mono-ammonium phosphate (MAP), triple super phosphate (TSP) and bone meal biochar] and hematite were applied at a molar ratio of Pb:Fe/P=1:5. Pb phosphates formation was investigated in the soil/post-IVBA residuals and in the mice lung via Extended X-Ray Absorption Fine Structure (EXAFS) and X-ray Absorption Near Edge Structures (XANES) spectroscopy respectively.EXAFS analysis revealed that anglesite was the dominant phase in the ingestible (<250 µm) and inhalable (<10 µm) particle fractions. Pb IVBA was significantly reduced (p<0.05) by phosphate amendments in the <250 µm fraction (solubility bioaccessibility research consortium assay) and by PA, MAP and TSP in the <10 µm fraction (inhalation-ingestion bioaccessibility assay). A 21.1% reduction in Pb RBA (<250 µm fraction) and 56.4% reduction in blood Pb concentration (<10 µm fraction) were observed via the ingestion and inhalation pathways respectively. XRF microscopy detected Pb in the stomach within 4 h, presumably via mucociliary clearance.

Keywords: Lead; Phosphate amendment; Relative bioavailability; Bioaccessibility; Immobilization; Dust

Highlights

- Pb exposure via inhalation and ingestion pathways was assessed

- *In-vivo*, *in-vitro*, XFM, EXAFS and XANES methods were used

- Oral Pb exposure was reduced by incorporating P amendments into contaminated soil

- Water soluble P amendments were more effective in reducing Pb inhalation exposure

- Pb bioavailability may be reduced through *in-vivo* formation of Pb-phosphates

Graphical Abstract
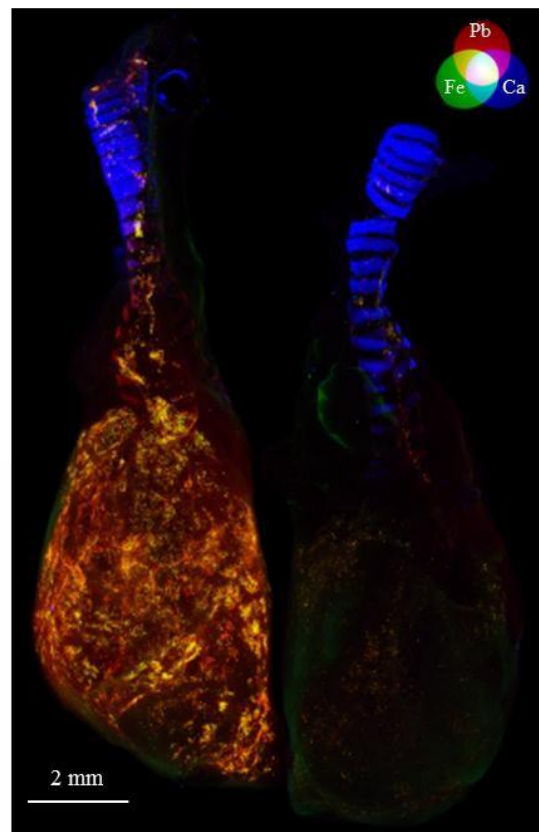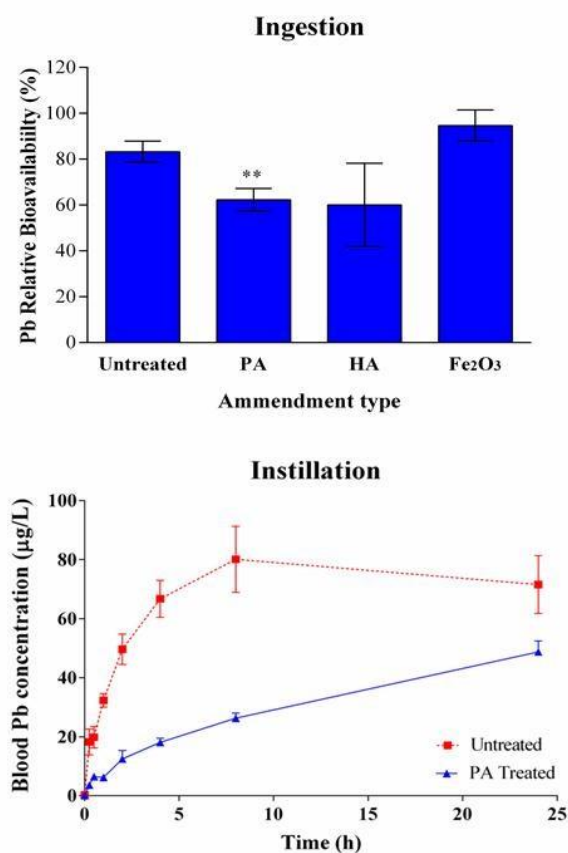
Introduction

Lead (Pb) exposure has been linked with approximately half a million deaths and nine million disability-adjusted life years worldwide by the Institute for Health Metrics and Evaluation[1]. Cognitive and neurological impairment due to childhood Pb exposure is well documented[2-4] and can occur at blood Pb concentration (PbB) as low as 3 µg/dL[5]. The two significant pathways for Pb exposure in humans are incidental ingestion of soil/soil borne dust and inhalation of re-suspended indoor surface/house dust[6]. Solubilization of Pb occurs in the stomach acid (pH 1.5-2.5)[7,8] following soil ingestion or in the lung surfactants and epithelial fluid (pH 7.0)[9] following dust inhalation. The proposed mechanisms for Pb absorption include passive and facilitated diffusion via the divalent metal transporter in the lungs, duodenum and ileum of the small intestine (pH 7.0)[10-12]. Children are the most at risk of Pb exposure from soil

ingestion or dust inhalation because of their higher occurrence of hand-to-mouth activities[7], as well as their increased respiratory frequency, leading to a higher deposition fraction of fine particulate matter (<4 µm) in the respiratory tract[13,14].

Reduction in Pb absorption into the systemic circulation using soil amendments (e.g. phosphates, metal oxides, clay minerals) may be a cost effective risk mitigation strategy compared to soil removal, replacement, washing and capping[7,15]. Particularly, phosphate amendments may reduce Pb bioavailability by promoting the formation of poorly soluble Pbphosphate species [e.g. pyromorphites ($Pb_5(PO_4)_3X$, where X=OH/Cl/F) and $Pb_3(PO_4)_2$][7,15,16]. Although Pb immobilization has been demonstrated in humans[17] and animal feeding

studies[15,17,18], its efficacy is governed by site-specific soil chemistry, Pb speciation and choice of amendment[7].

The predominant focus for Pb immobilization studies thus far has been demonstrating reduction of Pb bioavailability (absorption of Pb into systemic circulation) or bioaccessibility (dissolution of Pb in simulated biological solution) via the ingestion pathway. During the analysis of oral bioavailability/bioaccessibility, <250 µm soil particle fraction is generally used; however, Pb contaminated particulate matter, which may be <10 µm in aerodynamic diameter, may be inhaled in arid mining/smelting impacted regions due to resuspension of dust

inside or near households in the vicinity of the mining region[19-21]. The concentration of Pb (as well as other toxic elements present) may be several magnitudes higher in the <10 µm particle fraction compared to the < 250 µm counterpart[22]. Although the mass of inhaled dust may be less than the mass that is incidentally ingested (depending on the local concentration of particulate matter), Fent et al.[19] reported that bioavailability of Pb from contaminated soil via instillation is significantly higher than ingestion when identical doses for both pathways are used using blood, liver and kidney Pb concentration as bioavailability endpoints. When deposited in the respiratory system, rapid Pb absorption from the lungs of rats instilled with Pb-acetate or Pb contaminated soil may occur[19], presumably due to the high gas exchange

surface area (70 m^2), short distance between the air-blood barrier (0.5 µm) and high vascularization of the respiratory system[23]. Additionally, particles may reach gastro-intestinal (GI) tract via mucociliary escalator and further dissolve in the stomach, be absorbed in the intestine[24], translocate to extra-pulmonary organs (e.g. liver, heart, bone), be retained in the lung for weeks-years[25] and exert additional acute or systemic toxicity[12,26]. Therefore, incorporating Pb immobilization information for both ingestion and inhalation pathways may be considered a holistic approach when recommending an amendment strategy for risk minimization in arid regions.

Discovered in 1883, Broken Hill city contains the world's largest Pb, zinc (Zn) and silver (Ag) ore deposit known as the Line of Lode[27]. In addition to the continuous mining since discovery, a brief period of Pb and Zn smelting (1883-1898)[27] have resulted in widespread Pb contamination in the urban residential area of the Broken Hill city[28]. Dust suppression and Pb exposure mitigation strategies in the Broken Hill residential regions to date include the application of cracker dust in parts of the central southern urban area[29] and home remediation trial (encapsulation/removal of dust, sealing of cracks in the windows/walls)[20]. However, a high risk of chronic childhood Pb exposure was predicted in a recent study by Yang and

Cattle[28].

This study determined the efficacy of Pb immobilization via ingestion and inhalation pathways in Pb contaminated soil from Broken-Hill (Australia) using P and Fe amendments. In addition to assessing Pb exposure reduction using bioaccessibility and bioavailability, changes in Pb speciation in the pre- and post-bioaccessibility residuals and in the lungs were determined using Extended X-ray Absorption Fine Structure (EXAFS) and X-ray Absorption Near Edge Structures (XANES) spectroscopy, while Pb dynamics following *in vivo* inhalation exposure was assessed via X-Ray Fluorescence (XRF) microscopy.

Materials and methods

Soil collection and physicochemical characterization

Mining/smelting impacted topsoil (0-20 cm) was collected from Broken-Hill, Australia. After drying at 40°C, the soil was sieved to <2 mm and its water holding capacity (WHC), pH [soil(m):water(v)=1:5] and total organic carbon content (LECO TrueMac CNS) was measured (n=3). Subsamples were sieved to <250 µm (incidentally ingestible fraction) and <10 µm (inhalable or dust fraction) (Endecotts Octagon digital shaker). Each fraction (0.1 g, n=3) and a Standard Reference Material (SRM) from the National Institute of Standards and Technology (NIST) 2710a were pre-digested overnight in 5 mL aqua-regia, then digested in a MARS-6 microwave (CEM) using USEPA method 3051[30]. Digested samples were syringe filtered (0.45 µm, cellulose acetate), diluted with MilliQ water and the pseudo-total elemental concentrations analyzed using Inductively Coupled Plasma Mass Spectrometry (ICP-MS) (Agilent 8800) according to USEPA method 6020A[31]. The average recovery of Pb from SRM, check values, duplicates and spiked samples were within the limits specified in USPEA method 6020A[31]. See Supporting Information (SI) for further details.

Application of amendments

Soil amendment and ageing was conducted according to Juhasz et al.[32] using phosphoric acid (PA, Fisher Scientific), hydroxyapatite (HA, Sigma Aldrich), mono-ammonium phosphate (MAP, Sigma Aldrich), triple super phosphate (TSP, Richgro), bone meal biochar (Charcoal House LLC, Crawford, NE, USA) and synthetic hematite Fe_2O_3 (Sigma Aldrich). See SI for more details. After the application of amendments (Pb:P/Fe=1:5) at 80% WHC and ageing for two weeks (24±2°C)[32], soils were dried (40°C) and soil pH determined in a subsample. To remove excess P, soils were leached 5 times with natural rainwater collected from rooftop into rainwater tank (pH 5.8), followed by drying and sieving to recover the <250 µm and <10 µm particle size fractions. Pseudo-total elemental concentrations from each fraction were reanalyzed as described earlier.

Assessment of *in vitro* bioaccessibility (IVBA)

Oral Pb and Fe IVBA was assessed (n=3) in the <250 µm soil particle size fraction following the Solubility Bioaccessibility Research Consortium (SBRC) assay[33]. Inhalation Pb and Fe IVBA was assessed (n=3) in the <10 µm soil particle size fraction using the InhalationIngestion Bioaccessibility Assay (IIBA)[22] using Hatch's solution[34]. Percent IVBA was calculated using Eq. 1 given in Kastury et al.[22]. See SI for more details.

EXAFS assessment of Pb and Fe speciation

Lead speciation was assessed using EXAFS in pre- and post-amendment soil, as well as selected post-IVBA assay residuals (to provide speciation information on the non-bioaccessible fraction). Methodologies described in Kropf et al.[35] was used to conduct EXAFS analysisat the Materials Research Collaborative Access Team (MRCAT), Advanced Photon Source of the Argonne National Laboratory, U.S. (beamline 10-ID) . See SI for additional details.

Assessment of oral bioavailability via gavage

In vivo Pb relative bioavailability (RBA) studies were conducted using female Balb/C mice (46 weeks old), according to the Guidelines for the Care and Use of Laboratory Animals[36], with approval from the South Australian Health and Medical Research Institute Animal Ethics Committees (application number SAM268). Based on the Pb IVBA results using SBRC assay, unamended, PA, HA or Fe_2O_3 amended <250 µm soil particle fraction (20-108 µg of Pb suspended in 180 µL of Pb free water) was administered via gavage to fasted mice (20-25 g)[37]. Blood (~0.5 mL) was collected via cardiac puncture 8 h post exposure, stored at 4°C, and analyzed by ICP-MS after diluting ten-fold in blood diluent solution[38]. Equation 1 according to Juhasz et al.[32] was used to calculate Pb RBA(See SI for further details).

Assessment of Pb bioavailability via instillation

Based on the Pb IVBA results of IIBA, PA amended and unamended inhalable dust (< 10 µm fraction) was intra-tracheally instilled using female C57Bl/6 mice (8-10 weeks old) with

approval from the Animal Ethics Committee of the Women's and Children's Health Network, Adelaide, South Australia (WCHN AEC project No. AE1044/7/2019). Mice (n=6) were anaesthetized and intubated[39] and a 20 µL bolus dose (0.02 mg of dust/mL suspended in 0.9% NaCl) was administered directly into the trachea over ten seconds via the endotracheal tube. Mice were exposed for 0.25, 0.5, 1, 2, 4, 8 or 24 h, after which they were humanely killed by CO_2 asphyxiation. Blood was collected via cardiac puncture, with Pb concentration analysis undertaken as described above. Lungs and the entire GI tract were removed and immediately frozen at -86°C to prevent change in Pb speciation and stored at -80°C to -20°C until analysis.

Two sets of lungs and GI tracts for each time-point/treatment was reserved for further analysis using XRF described below, while the remaining tissues (n=4) were used to determine Pb concentration. The GI tracts were sectioned into stomachs and intestines. Stomachs were refrozen and freeze dried (Modulyod Freeze Dryer). Lungs, GI tracts and NIST SRM 2976 were digested using nitric acid (70%) in a block digester (A.I. Scientific AIM500) ramping up to a maximum temperature of 180°C[40]. Following digestion and evaporation of acid to ~1-2 mL, samples were diluted with Milli-Q water, syringe filtered (0.45 µm, cellulose acetate) and stored at 4°C until analyzed by ICP-MS. See SI for more details.

XRF and XANES analysis of lungs and GI tracts

In order to retain the morphology of organs during sample preparation for XRF, the mouse organs were freeze dried (Modulyod Freeze Dryer). The GI tracts were thawed, unraveled and positioned onto Kapton tape, while the lungs were dissected and the left lobes were suspended on Lecter coat hangers and frozen prior to freeze drying. The dehydrated organs were sandwiched between ultralene and x-ray fluorescence microscopy (XFM) performed (18.5 keV incident energy, velocity of 2 mm/sec and a pixel size of 20 µm) at the XFM beamline at the Australian Synchrotron. Regions of interest (0.2 x 0.2 mm) from the 24 h unamended and amended lungs were selected for XANES mapping (90 energy steps, mapped at a velocity of 0.5 mm/sec and a pixel size of 2 µm)[41]. See SI for additional details.

2.8 Statistical analysis

To determine the efficiency of each amendment in reducing ingestion and inhalation exposure, treatment effect ratio (TER) was calculated using Eq. 1

$$\text{TER} = \frac{Pb\ IVBA\ or\ RBA\ (\%)\ in\ amended\ sample}{Pb\ IVBA\ or\ RBA\ (\%)\ in\ unamended\ sample} \quad \ldots\ldots\ldots. \text{Eq. 1}$$

Significant difference in Pb concentrations in soils post-amendment was determined using One-Way ANOVA ($\alpha=0.05$). Statistical significances of IVBA and RBA outcomes between unamended and amended soils were assessed using t-test ($\alpha=0.05$).

Results and Discussion

Elemental concentration and Pb speciation in unamended soil

The pH, TOC and concentrations of elements in the bulk soil (<2 mm), incidentally ingestible (<250 µm) and inhalable (<10 µm) soil particle fractions, are listed in Table 1. The pH of the bulk soil was slightly acidic (5.2±0.08) with low total organic carbon (0.5±0.04%). The concentration of Pb in the <2 mm, <250 µm and <10 µm particle size fractions were 11,656±754 mg/kg, 14,874±157 mg/kg and 62,069±739 mg/kg, exhibiting Pb enrichments of 1.3 and 5.3 fold in the <250 and <10 µm fractions respectively. Among the other notable trace elements of interest, manganese (Mn) and zinc (Zn) were present at an elevated level (3334±754 and 5432±271 mg/kg respectively), while aluminum (Al) and iron (Fe) were the highest among the major elements (19035±960 and 25746±2140 mg/kg). Although metal cations, particularly Mn, Zn, calcium (Ca) are associated with lower PbB level[42,43], combined Pb and Mn exposure has been reported to exacerbate cognitive performance[44]. Similarly, when inhaled, Zn (18437±174 mg/kg in the <10 µm particle fraction) may potentially cause lung inflammation and fibrosis[45].

The predominant Pb species in the unamended <250 µm soil particle fraction was anglesite (PbSO$_4$) (66%) and organic matter bound Pb (31%), with the remainder of the Pb was mineral sorbed (e.g. adsorbed onto clays and oxides) (Table 2). Although the proportion of anglesite

did not change in the <10 μm soil particle fraction (67%), the majority of the remaining Pb was distributed among clays and oxides (20%), organic matter bound Pb (8%) and plumbojarosites ($PbFe^{3+}_6(SO_4)_4(OH)_{12}$) (7%).

Effect of soil amendments on pH and Pb/Fe speciation

Phosphoric acid and TSP amendments lowered soil pH from 5.2 to 3.7 and 4.8 respectively, while HA, MAP and Fe_2O_3 amendments had minimal effect on pH (Table S1). In contrast, biochar amendment resulted in an increase in soil pH to 6.5, presumably as a result of hydroxide ion release from biochar[46]. Speciation analysis of Pb (EXAFS) in the phosphate amended <250 μm soil particle fractions suggested small increases in anglesites phases with corresponding decreases in organic bound Pb (Table 2). The exception was biochar, where a greater proportion of Pb was organic bound (42%), presumably due to the interaction between Pb and organic ligands in the biochar. In the <10 μm dust fraction, minor redistribution of Pb phases between Pb adsorbed to clays and oxides and organic matter bound Pb were observed after phosphate amendment. Similar to the <250 μm soil particle fraction, biochar amended dust was the exception, where anglesite (45%) and organic bound Pb species (42%) were the dominant Pb phases. Fe speciation in the dust fraction (Table S2) revealed that Fe was present predominantly as ferrihydrite (24-42%) and associated with clay (26-40%), with the remainder distributed as goethite (8-15%), magnetite (5-9%), hematite (5-15%) and maghemite (2-10%).

The formation of Pb-phosphate species (pyromorphite and $Pb_3(PO_4)_2$) is thermodynamically favored in the presence of $H_3PO_4^0$ (at pH of <2.12) and $H_2PO_4^-$ (at pH between 2.12 to 7.21)[47]. Therefore, formation of Pb-phosphate species was expected following PA and TSP amendments (pH 3.7-4.8) (Table S1). However, EXAFS analysis did not detect Pb-phosphates in any phosphate amended soils for either the <250 or <10 μm soil particle size fractions, presumably because the rate of dissolution of anglesite (the dominant phase) is pH independent[48]. In the study of anglesite immobilization using of $Ca(H_2PO_4)_2.H_2O$ and HA, Cao et al.[49] and Zhang and Ryan[48] reported a rapid dissolution of anglesite in aqueous systems, with

the detection of chloropyromorphite. However, more complex interactions may occur in natural soil system, limiting Pb immobilization *in situ* (e.g. transient increases in soluble Pb, incomplete formation or dissolution of Pb-phosphates due to inadequate Pb dissolution, competition from high cation concentrations with phosphate for sorption sites)[50-52]. Higher phosphate application rates may be necessary in future studies in Broken Hill soil to compensate for competition posed by cations (e.g. Al, Ca, Fe, Mn, Zn).

Treatment efficacy - ingestion pathway

Pb IVBA and residual Pb speciation following gastric phase (SBRC-G) assessment

Pb IVBA in unamended soil was $88.6\pm0.8\%$ when assessed in the SBRC-G phase (Figure 1A). This value was at the high end of the range (23.7-89.3%) previously reported in Yang and Cattle[28] for Broken-Hill topsoil, who reported an average gastric phase Pb IVBA value of $61.2\pm14\%$. The lower average value in Yang and Cattle[28] most likely reflected the assessment of soils from a large geographic area with diverse Pb speciation influencing IVBA outcomes. In contrast, soil was collected near a former Pb ore crushing area in this study and contained anglesite ($PbSO_4$) as the dominant phase, which is a common occurrence in mining impacted soils with acidic pH and results from the oxidation of galena[7]. Anglesite's solubility over a wide range of pH (2-7)[48] may have resulted in higher Pb IVBA results.

No significant reduction (P>0.05; Figure 1A) in Pb IVBA was observed in P and Fe amended soil (TER=1.02-1.08; Table S3) using SBRC-G phase, which is similar to the results reported for amended mining soil when assessed at pH 1.5[16,53,54]. Pb speciation in SBRC-G residual soil identified that the majority of anglesite was solubilized (Figure 1B), which was expected due to anglesite's propensity for solubilization under low pH conditions[48].

Pb IVBA and residual Pb speciation following intestinal phase (SBRC-I) assessment

When IVBA assays were modified to reflect intestinal phase conditions (i.e. addition of bile and pancreatin, pH adjustment to 7.0), Pb IVBA in the unamended soil was reduced to

16.6±1.3%. The decrease in Pb IVBA corresponded to a 88% decrease in Fe IVBA (Figure S2A), resulting from the precipitation of amorphous Fe species following oversaturation of Fe during the pH change[55]. During the transition from the gastric to intestinal phases, the increase in pH may have caused Pb to precipitate and become re-adsorbed to the Fe oxides and/or soil matrix[37]. A corresponding increase in mineral sorbed Pb species in post-extraction intestinal phase residuals (Figure 1B) supports the hypothesis that Pb co-precipitates with Fe, as suggested by Smith et al.[37]. A significant reduction (p<0.001) in Pb IVBA was observed for all P amended soils (TER=0.02-0.1). However, Pb IVBA in Fe_2O_3 amended soil was similar to the unamended soil (18.0±0.7%), suggesting that Pb immobilization did not occur *in vitro*.

Among the phosphate amended soils, the lowest TER following SBRC-I assessment was obtained for HA and biochar amended soil (0.02 and 0.04 respectively), followed by PA, MAP and TSP (TERs of 0.1). As HA and biochar exhibit low solubility in water, higher Pb:P ratios (1:5.3 and 1:4.1 respectively) were observed in the <250 µm amended soil factions following rainwater leaching used to remove the excess P after the two-week incubation period (Table S1). Consequently, due to the dissolution of P during SBRC-G assessment, higher concentrations of P were available to react with Pb in the SBRC-I phase, resulting in lower TER. This is also supported in Figure 1B, which shows a 2.2-2.3 fold increase in Pb-phosphate species in the post-IVBA residuals of SBRC-I in all phosphate treated soils but not in the soil amended with Fe_2O_3. Although only PA amended post-IVBA residual was tested to represent water soluble amendments, presumably Pb-P would form in the MAP and TSP amended residuals as well.

Pb RBA

Although assessment of Pb IVBA using SBRC-I determined that all phosphate amendments were effective in reducing Pb IVBA due to the presence of Pb-phosphates in the nonbioaccessible residuals, Scheckel et al.[7] cautioned the use of IVBA outcomes (extracted at pH > 1.5) in interpreting phosphate amendment efficacies due to the potential formation of

pyromorphite *in vitro* as a result of the pH change. Therefore, in addition to IVBA, the efficacy of Pb immobilization was also assessed using Pb RBA in unamended and selected amended soils (PA, HA, Fe_2O_3). In the unamended soil, Pb RBA was $47.9 \pm 2.6\%$, which was higher compared to similar studies utilizing mining/smelting impacted Australian soils[32,37]. Low Pb RBA may occur when sparingly soluble Pb species are present, such as galena or Pb-phosphates[56,57]. However, the dominant Pb phase, anglesite, may have contributed to the high Pb RBA observed in this study because of its solubility in a wide pH range $(2-7)$[48].

When soil was amended with PA and HA, Pb RBA was reduced to $21.1 \pm 6.2\%$ (TER=0.79) and $35.8 \pm 10.7\%$ (TER=0.74) respectively, which supports the hypothesis of Juhasz et al.[32] that Pb immobilization may occur *in vivo*, even when *in situ* Pb-phosphate formation did not occur. Bradman et al.[18] also reported that when Pb speciation in ingested soil was compared to feces following administration in mice, Pb speciation changed during the transit through the GI tract. Despite both phosphate amendments reducing Pb RBA, a significant reduction was only observed in the PA amended soil ($p<0.01$). High variability in Pb RBA was observed in the HA amended soil, which may be attributed to the slow dissolution kinetics of HA in the stomach, resulting in the formation of a layer of Pb phosphate species on the outside of the HA particles, reducing the surface area available for reactions with Pb[48]. In contrast to the P amended sample, there was no reduction in Pb RBA in the Fe_2O_3 amended sample (TER=1.2).

Treatment efficacy - inhalation pathway

Pb IVBA and residual Pb speciation following IIBA lung phase (IIBA-L) assessment

In the unamended dust fraction, Pb IVBA was $61.7 \pm 0.5\%$ during the IIBA-L phase (Figure 2A). High Pb solubility in Hatch's solution was expected, given that anglesites was the dominant Pb phase with solubility in a wide pH range[48] and the high concentration of ligands and surfactants present in the extracting solution. The major speciation change in the IIBA-L phase residual was the reduction in anglesite concentration, indication mobilization of

anglesites in Hatch's solution (Figure 2B). Lung phase Pb IVBA in unamended dust was lower compared to the results obtained for the SBRC-G assessment (<250 µm soil particle fraction) reported in the preceding section, which may be attributed to the lower pH of the latter. However, despite IIBA-L and SBRC-I phases were conducted at neutral pH, IIBA-L Pb IVBA was 3.7 fold higher in the former. Lung surfactants and chelators in Hatch's solution may have rapidly transported the solubilized ions away from the surface of the dust particles, preventing their precipitation during IIBA-L assessment[22]. Additionally, the use of longer extraction period in IIBA-L (24 h) compared to SBRC-I (4 h) allowed a greater dissolution of Pb from the matrix, which was also observed in Kastury et al.[22] when assessing other mining/smelting impacted dusts.

In PA, MAP and TSP amended dust, Pb IVBA was significantly reduced from $61.7 \pm 0.5\%$ in the unamended dust to $20.0\pm0.9\%$ (PA) - $29.5\pm1.1\%$ (TSP) following assessment using IIBAL (p<0.01, TER=0.33-0.48). In contrast, no significant reduction in Pb IVBA was observed in HA, bone meal biochar and Fe_2O_3 amended dusts (TER=0.9-1.1), presumably due to the reduced anglesite dissolution observed in IIBA-L residuals (Figure 2B). The presence of Pbphosphates in the MAP and TSP amended IIBA-L residuals suggested the possibility that an outer layer of Pb-phosphates may have formed surrounding Pb particles during the assay, reducing further Pb dissolution[58].

Pb IVBA and residual Pb speciation following IIBA lung + intestinal phase (IIBA-I) assessment

Following a 24 h extraction in lung fluid, dust particles were transitioned to gastric (pH 1.5, 1 h) and intestinal solutions (pH 7.0, 4 h) to simulate the clearance of particles from the lungs and their passage through the GI tract. In the unamended dust, Pb IVBA in IIBA-I was $74.5\pm0.8\%$ (Figure 2A). Solubilization of Pb in the acidic pH of the preceding gastric phase may be attributed to the higher Pb IVBA following IIBA-I extraction compared to IIBA-L, which was also evident in the reduction in residual anglesite and an increase in residual

plumbojarosites (Figure 2B). Similar to the SBRC-I phase, co-precipitation with Fe during the transition from IIBA-G to IIBA-I may be attributed to the increase in residual plumbojarosites[37].

An important observation during the IIBA-L phase was that Pb IVBA was reduced from 74.5±0.8% in the unamended dust to 44.9±2.1% - 59.3±1.8% in PA, MAP and TSP amended dust (p<0.01, TER: 0.67-0.80), while no reduction was observed in HA, biochar and Fe_2O_3 amended dust (TER 0.97-1.13). EXAFS analysis identified Pb-phosphate species in the IIBAI residuals of PA, MAP and TSP amended dust, which explains the reduction in Pb IVBA in the IIBA-I phase. The combined results of Pb IVBA reduction in phosphate amended dust using IIBA-L and IIBA-I phases indicated that water soluble phosphate amendments may be the most suitable for amending Pb contaminated soil. This result is particularly germane in arid mining/smelting impacted regions of Australia, where exposure via aeolian transport of dust may be considered a significant contributor to childhood PbB[19-21].

Pb bioavailability following dust instillation into mouse lungs

Based on the Pb IVBA results, unamended and PA amended dust was instilled into mice lungs to ascertain whether a reduction in Pb absorption occurs *in vivo*. Reduction of inhalation bioavailability using phosphate amendment was deemed an important part of overall Pb exposure reduction in Broken Hill because human studies have demonstrated that up to 95% of the Pb in fine particles (<0.1 µm) may be absorbed upon inhalation[59,60]. A study conducted by Borelnad et al.[21] reported an influx of 166-1104 µg of P /m^2 during a 30 day period, which indicated that windblown dust in an arid environment such as Broken Hill may potentially contribute to childhood PbB via inhalation. The choice of instillation to assess Pb exposure as a surrogate to inhalation assays enabled a more precise delivery of small sample masses[19] compared to insufflation of dry dust, allowing direct comparison of Pb absorption in unamended and PA amended dust. Following exposure to the unamended dust, PbB increased rapidly from 0.29 µg/L (time=0) to 18.2±4.4 at 0.25 h and 49.7±5.1 µg/L after 2 h (Figure 2C).

Subsequently, a slower rate of Pb absorption between 2-8 h resulted in a peak PbB of 80.1±11.2 µg/L at 8 h. At the end of 24 h, a moderate decrease in PbB was observed (71.5±9.8 µg/L). The rapid absorption of Pb from Pb contaminated dust in this study agrees with the results reported in Fent et al.[19], who instilled dust from shooting range soil into rat lungs and reported a high Pb absorption, which peaked at 24 hours and slowly declined until 96 hours without PbB returning to baseline. Although the maximum exposure time-frame of 24 hours in this experiment was not sufficiently long for PbB to return to baseline, this information, together with the bio-distribution data obtained via the XRF images, yielded valuable insight into the bioavailability of Pb following exposure to Pb contaminated dust. Tricolor XRF images of lungs instilled with the unamended sample showed that Pb (red) became distributed throughout the lungs between 0.5 - 2 h (Figure 3A), presumably aided by gravity and lung surfactants[61,62]. While Fe (green) was mostly observed as associated with Pb (indicated by yellow), Ca (blue) was mostly observed in the upper respiratory tract, incorporated in the tracheal rings.Metal sulphates (e.g. anglesite) have been suggested to be water-soluble, therefore potentially bioavailable in the lungs[63,64]. As anglesite was the predominant Pb phase in the unamended dust, anglesite dissolution may have resulted in the majority of Pb absorption via the air-blood barrier.

Tricolor images of the GI tracts showed that Pb appeared in the stomach 8 h post instillation with the unamended dust (Figure 4A), presumably due to particle clearance from the lungs by the mucociliary escalator and the diffusion of Pb throughout the stomach. This was confirmed by Pb concentrations in the lungs (Pb_L) and stomach (Pb_S) in Figure 4C, where Pb_L decreased from 1.1±0.2 µg/organ at 0.25 h to 0.5±0.1 µg/organ at 8 h, while Pb_S increased from 0.06±0.1 µg/organ at 0.25 h to 1.5±0.3 µg/organ at 8 h.Low concentrations of Pb was detected in this compartment at 24 h (Figure 4A), presumably due to precipitation of non-absorbed Pb. Detection of Pb in the small intestinal tracts suggested that in addition to Pb absorption in the lungs, it is possible that a fraction of the PbB during 8-24 h resulted from the solubilization of Pb in the stomach, followed by absorption in the small intestine[10].

In contrast to the unamended dust, PbB in mice instilled with PA amended dust showed a slower rate of Pb absorption; PbB at 0.25 h being 3.7±0.5 µg/L and reaching a maximum of 48.7±3.7 µg/L at 24 h (Figure 2C). A comparison of the AUC of the unamended and PA amended dust suggested a 55.8±1.6% decrease in the total Pb absorbed into the systemic circulation as a consequence of PA amendment over 24 h. Although it was not clear from Figure 3C whether PbB had peaked during 8-24 h, or if it would continue to increase beyond

24 h, the reduction *in vivo* lies within the 32.4-67.5% reduction interval in the Pb IVBA using IIBA-L and IIBA-I phases. Additionally, because the reduced Pb absorption was more prominent during the first 8 h of the assay, it was theorized that similar to the Pb-phosphates formation in the small intestine during the gavage study described above, Pb-phosphates may have formed in the lung owing to its neutral pH. Further experiment with longer time frame may be conducted to estimate AUC once PbB has returned to baseline with dose normalization using Pb acetate as a reference dose.

XRF images of lungs and GI tracts depict Pb dynamics in the PA amended sample (Figures 3B and 4B). In contrast to the unamended dust, Pb particles from PA amended dust was dispersed more rapidly at 0.25 h. The discrepancy between the dispersion rates most likely resulted from inter-animal variation, which is inherent in instillation experiments[65]. The quicker dispersion of PA amended dust in the lungs may have contributed to its earlier detection in the stomach (4 h; Figure 4B). Pbs increased from 0.41±0.1 µg/organ at 0.25 h to 5.8±1.6 µg/organ at 4 h and peaked at 11.1±2.1 µg/organ after 8 h (Figure 4D), supporting the aforementioned suggestion that Pb from PA amended dust demonstrated reduced absorption in the lungs.

To further test this theory, a 0.2 x 0.2 mm region of the lung receiving the unamended and PA amended dust at 24 h was analyzed using XANES to investigate Pb speciation in the residual Pb particles. Within these regions, spectra corresponding to high and low Pb concentrations were obtained (unamended$_{high}$, unamended$_{low}$, PA amended$_{high}$ and PA amended$_{low}$, Figure S4). The spectra and their corresponding LCF fits are depicted in Figure 3C, while Pb speciation (weighted %) is provided in Figure 3D. Residual Pb speciation in the unamended$_{high}$ was

dominated by anglesites (61.3%) with the remainder consisting of Pb phosphates (38.8%), while that in the PA amended$_{high}$ was distributed as anglesite (48.5%) and Pb phosphates (51.5%), a 1.3 fold increase in Pb-phosphates in the PA amended dust residuals. . Similarly, when residual Pb speciation in the unamended$_{low}$ and PA amended$_{low}$ were assessed, a 1.3 fold increase in Pb-phosphate was detected in the lungs receiving the PA amended dust (39% in the unamended and 50.6% in the PA amended). The remainder of the unamended$_{low}$ Pb was comprised of 51% anglesite and 10% mineral sorbed, while that in PA amended$_{low}$ was 27.3% anglesite and 22.2% mineral sorbed. It is likely that similar to the results observed in the IIBAL residuals, the Pb ions on the surface of anglesites reacted to P and Cl in the lung lining fluid forming a coating of chloropyromorphite, preventing subsequent dissolution, which was also reported in Wragg and Klinck during an IVBA study using SLF[58]. It is noteworthy that although a small region of the lung was analyzed for residual Pb speciation, the formation of Pb-phosphates was 1.7 fold higher in the PA amended dust. Therefore, it is possible that even though Pb-phosphates did not form *in situ*, the presence of soluble P in the dust at the time of instillation increased the likelihood of Pb-phosphate formation thereby reducing Pb absorption in the lungs.

To the best of our knowledge, this is the first study to report a reduction of Pb exposure using phosphate amended dust via instillation, although a longer study may be required for PbB to return to (near) baseline. Nevertheless, this study highlighted that although P amendments with varying solubility reduced Pb exposure via the oral pathway, water soluble amendments were more effective in immobilizing Pb via the inhalation pathway, with important implications of improving Pb exposure mitigation strategies worldwide. As particle retention in the lung is longer than that in the GI tract, future inhalation bioavailability studies should focused on longer term bio-assays utilizing dust with different Pb phases to ascertain the influence of Pb speciation on Pb absorption in the lungs and GI tracts. Although the Pb concentration in urban soil typically <1000 mg/kg, urban soil Pb has been linked with children's PbB[66,67]. Additionally, remediation of homes to seal houses to prevent Pb entry via fugitive dust has

been shown to have limited impact on lowering children's PbB when initial Pb levels were not high[20]. Therefore, in urban soils and in arid regions, Pb immobilization using water-soluble phosphate sources may be an effective remediation strategy to mitigate Pb exposure.

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Table 1: Physico-chemical characterisation of the soil particle sizes fractions and amendments. N.D. = not determined

	pH	Total organic carbon (%)	Minor element concentration (mean ± SEM) (mg/kg)			Major element concentration (mean ± SEM) (mg/kg)					
			Pb	Mn	Zn	Al	Ca	Fe	K	Mg	P
Total soil < 2 mm	5.5 ± 0.1	0.05 ± 0.04	11656 ± 754	3337 ± 305	5432 ± 271	19035 ± 960	6202 ± 367	25746 ± 2140	5015 ± 379	2760 ± 182	712 ± 55.5
Ingestible fraction < 250 µm	N.D,	N.D,	14874 ± 157	4223 ± 190	6901 ± 122	23412 ± 706	8387 ± 194	31807 ± 311	5998 ± 18.7	3273 ± 12.5	855 ± 39.9
Inhalable fraction < 10 µm	N.D,	N.D,	62039 ± 736	4171 ± 6.8	18437 ± 174	56359 ± 2818	4207 ± 57.9	73746 ± 1049	11667 ± 296	5036 ± 51	1406 ± 10.2

Table 2: Pb speciation (weighted %) in the <250 and <10 µm soil particle fraction. PA = phosphoric acid, HA = hydroxyapatite, MAP = monoammonium phosphate, TSP = triple super phosphate.

	Adsorbed into clays and oxides	Organic matter bound	Anglesite ($PbSO_4$)	Plumbojarosite ($PbFe_6(SO_4)_4(OH)_{12}$)	Pbphosphates (pyromorphite + $Pb_3(PO_4)_2$)	Galena (PbS)	R factor
< 250 µm soil particle fraction							

Unamended	2	31	67	-	-	-	0.001
PA	9	9	82	-	-	-	0.004
HA	0	16	78	-	-	6	0.004
MAP	0	28	72	-	-	-	0.001
TSP	0	15	85	-	-	-	0.003
Bonemeal biochar	0	42	45	-	-	13	0.006
Fe_2O_3	0	26	74	-	-	-	0.005
< 10 µm soil particle fraction							
Unamended	20	8	66	7	-	-	
PA	11	14	66	9	-	-	
HA	2	24	74	-	-	-	0.003
MAP	14	10	68	8	-	-	
TSP	15	8	68	8	-	-	
Bonemeal biochar	0	44	44	-	-	12	0.005
Fe_2O_3	0	0	87	-	9	4	0.004
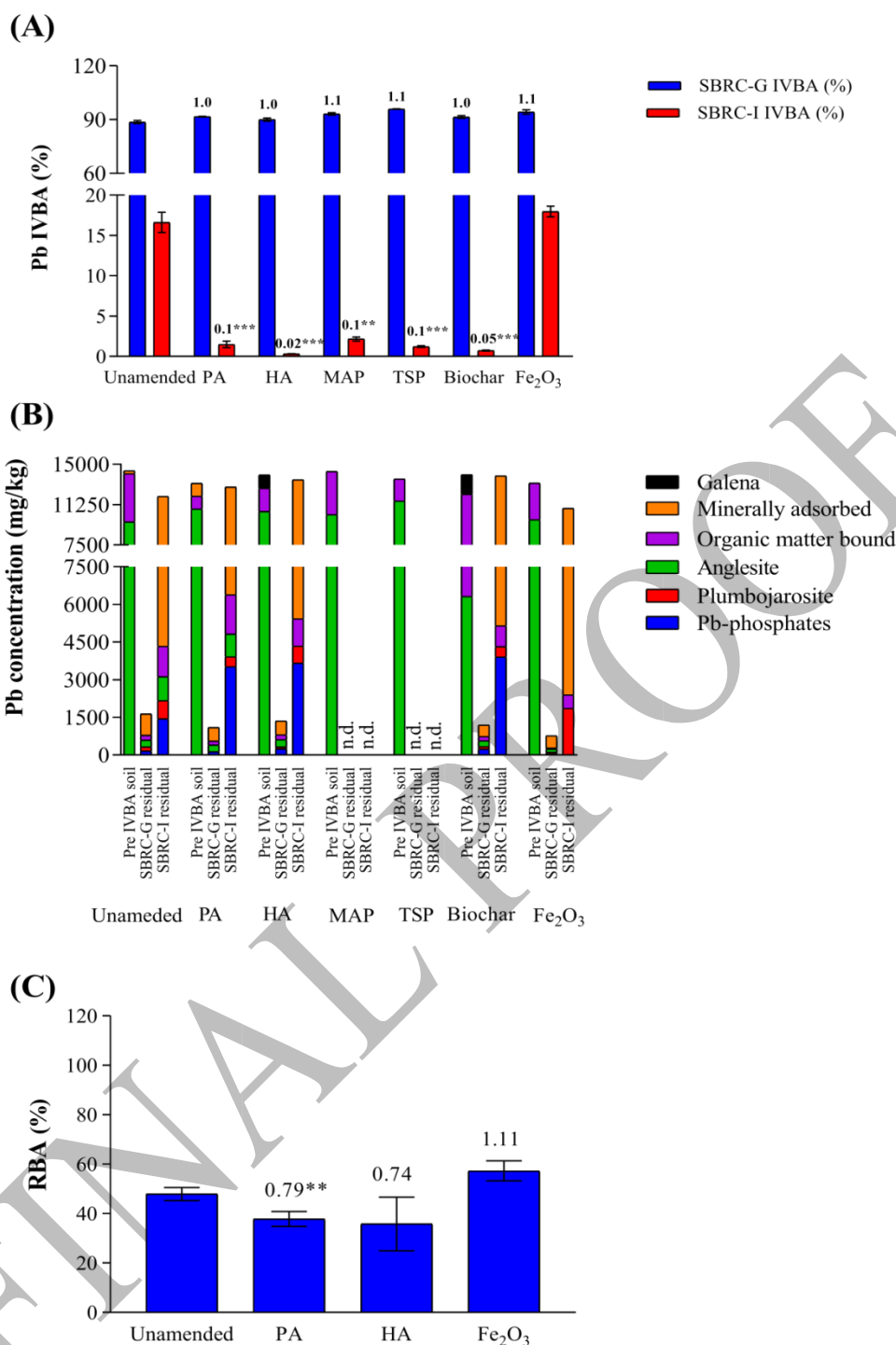
Figure 1: **(A)** Pb IVBA (%) using the Solubility Bioaccessibility Research Consortium (SBRC) assay where SBRC-G = extraction using the gastric phase and SBRC-I = extraction using the gastro-intestinal phase. (B) Pb speciation of pre- and post-SRBC assay residuals. (C) Pb relative bioavailability (RBA) in unamended and selected amended soil. PA = phosphoric acid, HA = hydroxyapatite, MAP = monoammonium phosphate, TSP = triple super phosphate, numbers on the top of bars of (A) and (C) represent treatment effect ratio (TER), asterix represents significance differences between unamended and amended soil ** = $p < 0.01$ and *** = $p < 0.001$ and n.d. = not determined.
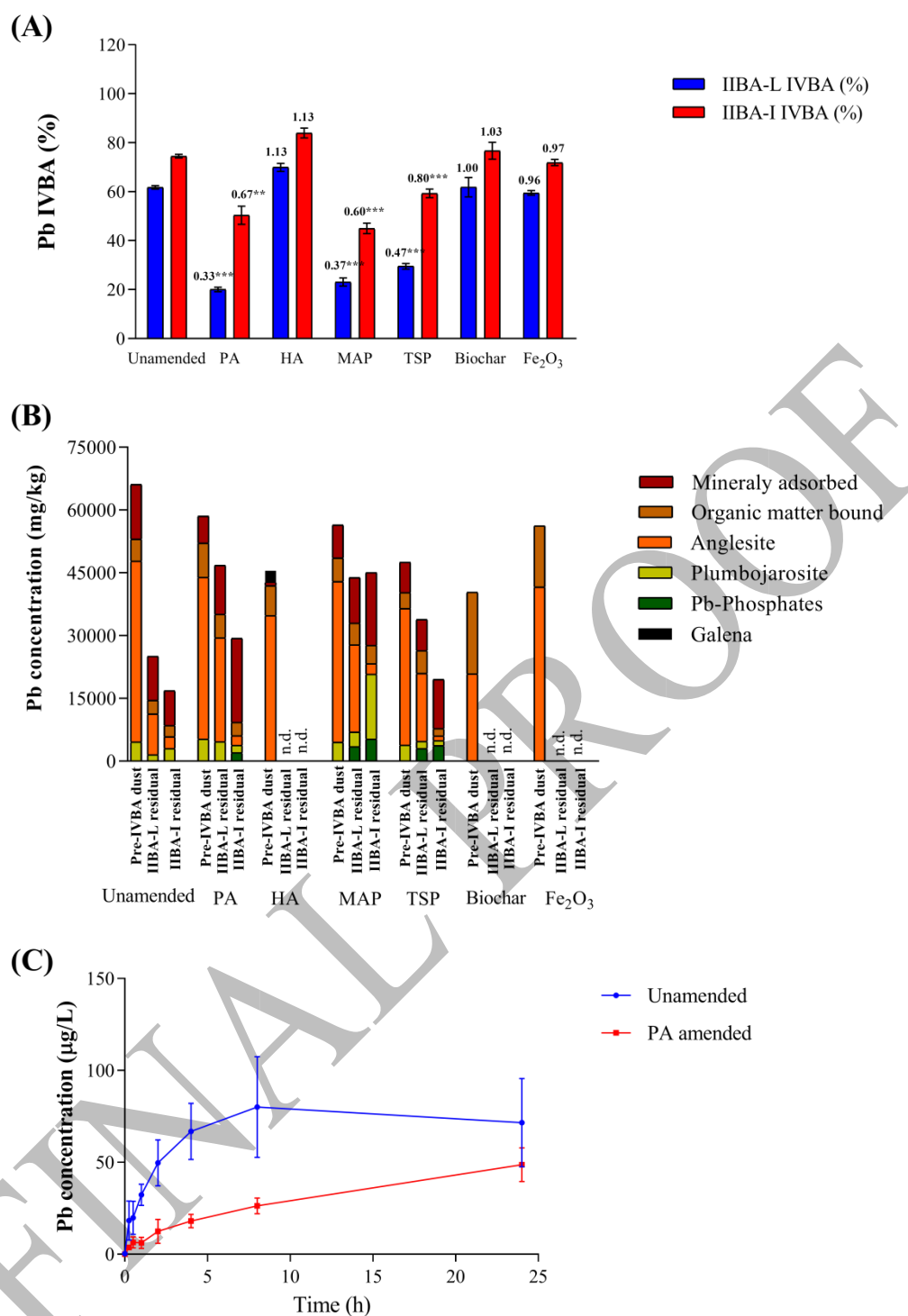
Figure 2: (A) Pb IVBA (%) using the Inhalation-Ingestion Bioaccessibility Assay (IIBA) where IIBA-L = extraction using the lung phase, IIBA-I = extraction using the lung+GI phase. (B) Pb speciation in pre- and post-IIBA assay residuals. (C) Blood Pb concentration following instillation of unamended and PA amended soil. PA = phosphoric acid, HA = hydroxyapatite, MAP = monoammonium phosphate, TSP = triple super phosphate, numbers on the top of bars of (A) represent treatment effect ratio (TER), asterix represents significance differences between unamended and amended soil $* = p < 0.05$, $** = p < 0.01$ and $*** = p < 0.001$ and n.d. = not determined.
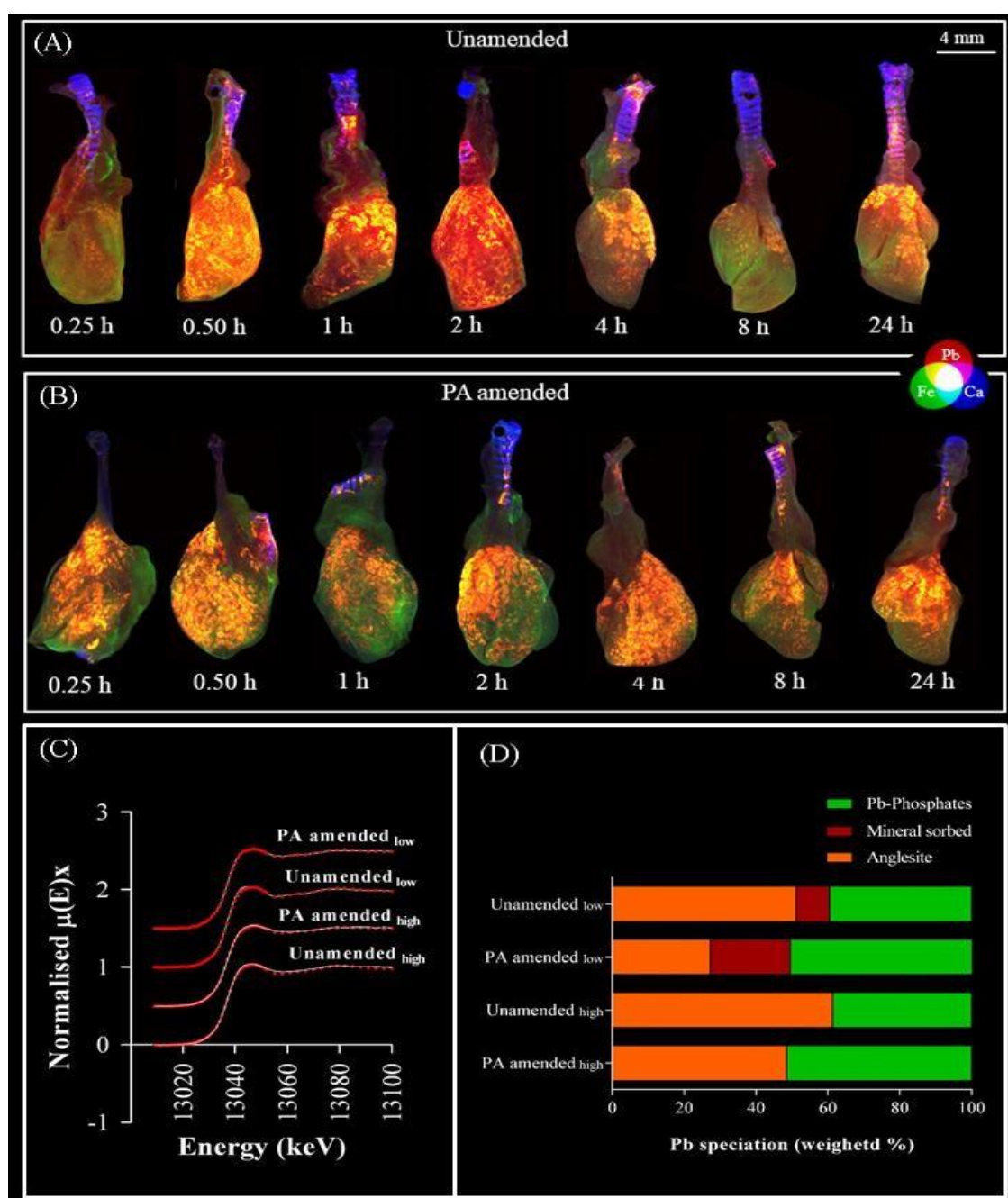
Figure 3: (A) and (B) 2D Elemental map (Pb = red, Fe = green and Ca = blue) using X-ray Fluorescence Microscopy (XFM) in mice lungs (including partial trachea) exposed to unamended and phosphoric acid (PA) amended dust via instillation over 24 h. (C) Pb speciation in regions of interest in the 24 h mouse lungs using X-ray Absorption Near Edge Structures (XANES). Linear combination fitting (LCF) is indicated using red dots and original spectra as white lines. (D) Final Pb speciation (weighted %) results obtained using XANES.
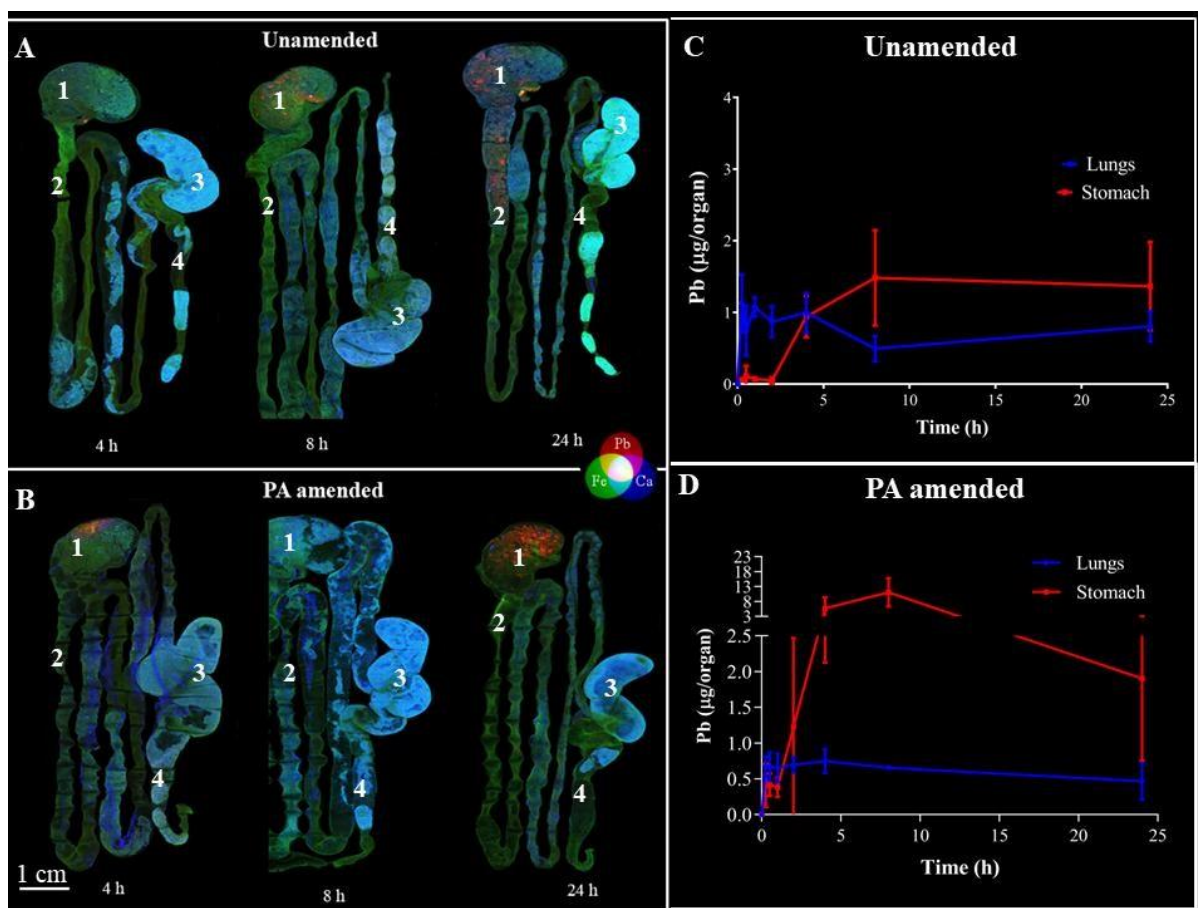
Figure 4: (A) and (B) 2D Elemental mapping (Pb = red, Fe = green and Ca = blue) in the gastro-intestinal (GI) tract of mice, 4-24 h post instillation with unamended and phosphoric acid (PA) amended dust (1: stomach, 2: small intestine, 3: cecum, 4: colon). (C) and (D) Pb concentration in lungs and stomach intestines between 0.25-24 h post instillation.

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Supporting Information: Details of methodologies (Quality assuarance and quality control of soil and tissue digestion, Application of amendments, Calculation of Pb *in vitro* bioaccessibility (IVBA), Calculation of Pb relative bioavailibility (RBA), Extended X-ray Absorption Fine Structure (EXAFS) spectroscopic assessment of Pb and Fe in phosphate amended Pb-contaminated soils and post IVBA assay residuals, X-ray fluorescence (XRF) microscopy images of lungs and gastrointestinal (GI) tracts and Pb speciation analysis in lungs using X-ray Absorption Near Edge Spectroscopy (XANES), Figure S1: Standard spectra used for LCF fitting using XANES, Figure S2: (A) Fe IVBA (%) using the Solubility Bioaccessibility Research Consortium (SBRC) assay where SBRC-G = extraction using the gastric phase and SBRC-I = extraction using the gastro-intestinal (GI) phase. (B) change in Fe speciation (mg/kg) in the pre- and post-SRBC assay residuals, Figure S3: (A) Fe IVBA (%) using the Inhalation-Ingestion Bioaccessibility Assay (IIBA) where IIBA-L = extraction using the lung phase, IIBA-I = extraction using the lung+GI phase. (B) Fe speciation in pre- and post-IIBA assay residuals, Figure S4: Regions of high and low Pb concentration used to obtain spectra for LCF fitting where (A) unamended and (B) amended samples, Table S1: Change in pH, Pb and P concentration before and after soil amendment, Table S2: Fe speciation (Weighted %) in the < 10 µm particle size fraction, **Table S3:** Summary of treatment effect ratios (TER) for Pb amended soils.