

## Effects of earthworms on leachability of lead compounds in soil



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## **Declaration**

I hereby declare that this thesis has not been previously submitted to any other institution or university for a higher degree. Except where otherwise acknowledged, this thesis is comprised entirely of my own work.

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## Abstract

Contamination of soils by metals and metalloids is an important environmental problem in many residential and industrial sites around the world. Lead is a common contaminant, which enters the soil through mining, industrial activities and waste disposal. A range of technologies can be used to remediate soil lead, however most remediation technologies adversely affect the environment and particularly soil biota. In this research, I assessed the effects of vermiremediation (the use of earthworms for remediation) as an environmentally friendly alternative for remediation of lead in soil. Earthworms were introduced to a sandy soil spiked with the common lead compounds  $\text{PbCl}_2$ ,  $\text{PbCO}_3$ ,  $\text{PbO}$  or  $\text{PbS}$  at the concentration of  $1,000 \text{ mg (Pb).kg}^{-1}$ . Concentrations of lead in soil washing from contaminated soil were significantly lower (ANOVA,  $F=23.56$ ,  $p<0.05$ ) in soil containing earthworms than in soil without. Earthworms increased slightly the concentration of lead in pore water between soils with and without worms but these differences were not statistically significant (ANOVA,  $F=0.14$ ,  $p>0.05$ ), and there were no significant differences (ANOVA,  $F=0.38$ ,  $p<0.05$ ) in pore water concentrations between compounds. Earthworms accumulated 241, 276, 235 and 40  $\text{mg.kg}^{-1}$  (dry weight of earthworms) of lead in their bodies, for  $\text{PbO}$ ,  $\text{PbCl}_2$ ,  $\text{PbCO}_3$  and  $\text{PbS}$  respectively. The concentrations of Pb accumulated by earthworms in  $\text{PbS}$  soil were significantly (ANOVA,  $F=8.38$ ,  $p<0.05$ ) less than those in worms in other soils. Measurement of earthworm weight before and after the experiment showed that earthworms lost weight in all contaminated soils, except for those contaminated with  $\text{PbS}$ . Reasons for these patterns, and their implications for the use of earthworms for remediation are discussed.





## **Chapter 1 – Introduction**

### **1. Introduction**

Environmental pollution is of great concern globally. Rapid growth of the global human population to the current 7.5 B, coupled with strong innovation in the industrial sector, has led to the creation of a diversity of chemicals. Inevitably, accidents in the mining of metals and metalloids, the manufacture and transport of materials and products, or disposal of wastes and end of life products, have contaminated the environment affecting soil, water and biological receptors such as plants, animals and people (Hong et al., 2002; Williams et al., 2000). The present study focuses on soil pollution as it can act as both a sink and subsequent source of contaminants, allowing later redistribution through other environmental pathways including groundwater, surface water and biological receptors (Hutchinson & Meema, 1987).

Lead is an important contaminant in many urban and industrial areas globally (Hong et al., 2002; Jaishankar et al., 2014) and there is an ongoing need to remediate lead contaminated soil at a wide range of locations. Contaminated soil can be managed and treated in various ways (summarised in section 2.3), however they have varying cost, effectiveness and harm to the soil and its biota. Of these methods, vermiremediation (the use of earthworms for remediation) has advantages including low cost, and in principle does not damage the soil, and as a consequence is potentially of interest for remediation of contaminated soil. As earthworms dig through the soil, they might bioaccumulate contaminants in their tissue, but potentially of greater significance is potential changes to the mineralogy of the metal-bearing compounds which might affect their leachability or bioavailability for plants. Similarly, the accumulation of lead in animals, including earthworms may be related to different mineralogies of the lead in the soil reflecting the contamination source.

The potential role of earthworms for the remediation of lead contaminated soil is not yet fully understood (see review in section 2.4). The present study investigates the effects of earthworms on different lead compounds in soil, and specifically how metal leachability changes after earthworm activity over several months.

### **1.1. Aims & hypotheses**

The overall aim of this study is to investigate the effects of earthworms on the leachability of different lead compounds in the soil in order to better define the role of mineralogy on the potential for vermioremediation. The underpinning hypothesis for this study is that earthworms will affect the leachability of lead compounds in the soil.

This aim is achieved by a series of experiments that will test the following hypotheses:

1. Earthworm activities will affect the concentrations of metals leaching from soils, compared with a “no worm” soil control.
2. Earthworm activities in soil will differentially affect the extractable lead in pore water of different lead compounds.
3. Lead compounds in soil will affect the size and mass of earthworms and these effects will differ between lead compounds.
4. There will be a difference in the concentration of lead accumulated in earthworms from different lead compounds.

### **1.2. Thesis structure**

This chapter introduces the importance of metal remediation in soil, the benefits of vermioremediation and the need for further research. Chapter two is a literature review that describes briefly some different technologies for remediation of contaminated soil and explores the mechanism of vermioremediation and previous research in this area. Chapter three explains the methods and instruments which have been used for the present study along with sample preparation procedures for analysis. Chapter four contains the results from this experiment along with data analysis. Chapter five compares the results from this experiment with published research and tries to explain the mechanisms creating the observed trends. The final chapter summarises the findings and suggests further research topics.

## **Chapter 2 – Literature review**

### **2.1. Introduction**

This chapter reviews and explains metal(loid) contamination in soil and the importance of soil remediation. Different remediation technologies are described briefly in this chapter along with possible mechanisms which allow earthworms to accumulate or affect metal(loid)s in the soil. Also the use of earthworms in previous research for metal(loid) contaminated soil is described.

### **2.2. Sources of metal(loid) contamination**

In general, metal(loid)s have negative effects on living organisms when they are present above certain concentrations (Jaishankar et al., 2014). Most of these elements are essential for plant cellular metabolism including iron, copper and zinc (Alloway, 2013; Jaishankar et al., 2014; Williams et al., 2000). However, these essential and other non-essential metal(loid)s such as arsenic, lead and mercury can increase to thresholds more than plants and animals need, impacting on their health and ultimately, ability to live (Hong et al., 2002; Williams et al., 2000).

Metal(loid)s can also be dangerous for human health (Martin & Griswold 2009). The United States Environmental Protection Agency (USEPA) specified chromium, nickel, copper, zinc, arsenic, cadmium, mercury and lead as priority contaminants for remediation (Chen et al., 2015). For example, USEPA categorised lead as carcinogenic for humans and being in direct contact with lead for long periods also causes high blood pressure, and damage to the nervous system, kidney and brain (Martin & Griswold, 2009). Human health could also be affected by these contaminants through the food chain. For example, long-time consumption of food containing arsenic leads to skin lesions or cancer in different body organs (Alloway, 2013; Zhang et al., 2015). Furthermore, exposure to the lead decrease academic achievement and IQ and will cause socio-behavioural problems including attention deficit hyperactivity disorder and also young children exposure to low level of lead will cause severe neurological problems (Harvey et al., 2016; Taylor et al., 2011).

Metal(loid)s can enter the soil through different human activities. The use of different pesticides and fertilizers, industrial activities and wastewater usage for agriculture may introduce contaminants to the soil (Chen et al., 2016a). Industrial activity can release metals to the air as fine particles or volatile compounds, which settle on surface soils far from the generating activity (Chen et al., 2016b). Mining and ore processing can contaminate the environment with mine wastes, which can spread by soil erosion, wind-blown dust or surface waters (Gore et al., 2007; Hutchinson & Meema, 1987).

In Australia, mining and metal processing industries are considered dominant sources of soil pollution for over 80,000 contaminated sites in urban and industrial areas (Hutchinson & Meema, 1987; Naidu et al., 2003). Table 2.1 shows concentrations of contaminants in four former mining sites in New South Wales (NSW) and Queensland (Qld) (Chaudhry et al., 1998; Gore et al., 2007; Hayes et al., 2003; Munksgaard et al., 2012). Australian health investigation levels for residential, park lands and industrial places are in Table 2.2 (NSW EPA, 2011). Copper, zinc and lead were present in all sampling sites in these mines but arsenic and lead concentrations are higher than Australian Health Investigation Levels for industrial sites in most of the mining areas. The data suggest that lead could be an important environmental problem as it presents in all these mines and its concentration is higher than the permitted level (NSW EPA, 2011). Above all the lead concentration in Sydney residential garden soil was investigated recently in VegeSafe program which indicated that lead concentration in gardens at 40 % of the Sydney homes was exceeded the lead guideline for residential garden (Rouillon et al., 2017).

**Table 2.1 Contamination at selected mining areas in Australia.**

Place  Element	Broken Hill <sup>1</sup> (NSW)		Mount Isa <sup>1</sup> (Qld)	Conrad <sup>2</sup> (NSW)				Sunny Corner <sup>3</sup> (NSW)			Sunny Corner <sup>4</sup> (NSW)	
	Site 1	Site 2	Site 1	Site 2	Site 3	Site 4	Site 5	Site 1	Site 2	Daylight Creek	Site 1	Creek
Fe (mg.kg <sup>-1</sup> )	57.1	50.6	42.4	-	-	-	-	-	-	-	-	-
As (mg.kg <sup>-1</sup> )	90.6	42.4	64.7	9,610	6,040	7,110	2,130	-	-	-	-	-
Cd (mg.kg <sup>-1</sup> )	22.5	10.3	19.4	-	-	-	-	11.7	5.7	1.6	12	2
Cu (mg.kg <sup>-1</sup> )	155	97.5	2,920	1,025	880	702	1,220	3,290	6,150	425	3,290	425
Pb (wt%)	1.25	0.552	0.235	0.915	0.780	0.693	0.214	1.960	3.10	0.419	1.960	0.419
Mn (wt%)	6.39	4.43	0.055	-	-	-	-	-	-	-	-	-
Sb (mg.kg <sup>-1</sup> )	93.7	48.2	9.44	-	-	-	-	-	-	-	-	-
Zn (mg.kg <sup>-1</sup> )	7,060	4,670	1,070	14,800	2,720	5,940	494	1,780	1,220	315	1,790	315
pH	7.3	7.8	5.9	4.2	3.1	3.2	4.8	3.5	3.5	2.9	-	-

1. (Munksgaard et al., 2012)

2. (Gore et al., 2007)

3. (Hayes et al., 2003)

4. (Chaudhry et al., 1998)

**Table 2.2 Australian Health Investigation Levels for common metals and metalloids in soils in areas of different land use.**

Element	Australian Health Investigation Levels <sup>1</sup>			
	Residential A <sup>2</sup>	Residential B <sup>3</sup>	Residential C <sup>4</sup>	Commercial Industrial land
Arsenic (mg.kg <sup>-1</sup> )	100	500	300	3,000
Cadmium (mg.kg <sup>-1</sup> )	20	140	100	800
Copper (mg.kg <sup>-1</sup> )	7,000	30,000	20,000	25,000
Lead (mg.kg <sup>-1</sup> )	300	600	300	1,500
Manganese (mg.kg <sup>-1</sup> )	3,000	8,000	9,000	40,000
Nickel (mg.kg <sup>-1</sup> )	400	900	800	4,000
Zinc (mg.kg <sup>-1</sup> )	8,000	60,000	30000	400,000

1. (NSW EPA, 2011)

2. Standard residential with garden and access to soil

3. Residential with minimum access to the soil

4. Developed open space such as parks, playgrounds

### 2.3. Soil remediation technologies

Metal pollution in soils has been remediated using different technologies. The best remediation technologies depend on the soil properties, the contaminant types and concentrations and the proposed final use of the polluted area (Mulligan et al., 2001). Common remediation technologies are described briefly below.

#### 2.3.1. Isolation and containment

The goal of isolation and containment is to reduce the amount of water passing through the contaminated soil via the use of grout or low permeability materials such as bentonite clay for capping, or horizontal and vertical barriers. This approach reduces the permeability of the containment material to  $<1 \times 10^{-7} \text{ m.s}^{-1}$ , effectively preventing the transmission of water-borne contaminants from soil to other areas including groundwaters (Hamby, 1996; Jankaite & Vasarevičius, 2005).

### **2.3.2. Solidification and stabilization**

Stabilization methods change pollutants chemically to more stable and less soluble forms (Hamby, 1996; Wuana & Okieimen, 2011). Solidification occurs via addition of reagents, such as cement to the soil to make the contaminants more physically stable. In this approach binders such as cement is mixed or injected to the contaminated soil in order to prevent migration of the contaminants through the soil (Wuana & Okieimen, 2011). Vitrification is also a solidification and stabilisation process. In this technique, electrodes are placed in soil and an electric current raises the soil temperature to create a vitreous material (Hamby, 1996; Jankaite & Vasarevičius, 2005; Wuana & Okieimen, 2011).

### **2.3.3. Soil washing and flushing**

Soil washing and flushing works by passing water, reagents or gas through the contaminated soil to remove pollutants. The washing solution type and concentration is chosen based on the pollutant type and site characterization (Jankaite & Vasarevičius, 2005; Yao et al., 2012). Soil washing is an ex situ process during which soil is washed with different reagents at different pH in reactors or heap leaching systems (Jankaite & Vasarevičius, 2005). In contrast, soil flushing is an in situ process in which aqueous solutions are introduced to the soil from the top and react with the contaminants in soil. These aqueous solutions are then collected from wells using pumps (Jankaite & Vasarevičius, 2005).

### **2.3.4. Electrokinetics**

In electrokinetics remediation, electrodes are inserted into the soil to create a low intensity electric current in the contaminated media (Hamby, 1996; Mulligan et al., 2001). The electric gradient causes anions to accumulate near the positive electrode and cations near the negative electrode in wet soil (Mulligan et al., 2001). Contaminants may precipitate or adsorb onto electrodes or are extracted from soil by pumping out the water near the electrodes (Hamby, 1996).

### **2.3.5. Phytoremediation**

Some plants have the ability to remediate the soil by accumulating contaminants in their tissue. Furthermore, some organic contaminants can be transferred to the plant root zone where they are degraded by microbes. This technique is considered more environmentally friendly than other destructive chemical remediation methods (Peng et al., 2009), however it needs a longer remediation time, is constrained to shallow depths above the water table (except in the case of aquatic plants), and depends on climate, depth and bioavailability of the contaminants (Mulligan et al., 2001; Peng et al., 2009).

### **2.3.6. Vermiremediation**

Earthworms play an important role in soil fertilization in agriculture. They dig through the soil and improve soil structure, pH, aeration and increase available carbon, nitrogen and other nutrients for plants (Ruiz et al., 2009). Not only do earthworms improve soil quality, but they can also remediate contaminated soil (Nannoni et al., 2011). Earthworms can accumulate contaminants in their bodies or change the bioavailability of the contaminants (Nannoni et al., 2011; Pattnaik & Reddy, 2011). Vermiremediation can be carried out in situ accompanied by other remediation technologies such as phytoremediation or soil flushing and it is very cost effective (Sinha et al., 2010). Earthworms tolerate temperatures from 5 °C to 29 °C and the optimal relative humidity for them is 60-75 % (Sinha et al., 2008). Earthworm populations can grow rapidly, doubling every 60-70 days (Sinha et al., 2008).

### **2.4. Mechanisms of vermiremediation**

Earthworms remediate metals by different mechanisms. As soil is ingested and passes through earthworms, the pH of the soil is adjusted to ~pH 7, and coupled with changes in dissolved organic carbon, the mobility of metals can change (Sherameti & Varma, 2015). Despite this, some contaminants simply pass through the earthworm bodies without mineralogical change (Sherameti & Varma, 2015; Sinha et al., 2008). Earthworms adjust soil pH to ~7 via their alkaline urine and production of calcium carbonate compounds from their calciferous glands (Nardi 2009; Wen et al., 2004). The calciferous glands in earthworms excrete calcium compounds to remove the calcium from their bodies and also as an aid for digestion process (Darwin 1892; Wen et al., 2004). This pH change can lead to an increase in microbial activity (Nardi 2009; Wen et al., 2004). Furthermore, the soil organic matter content and aeration are improved with the presence of the earthworms which also increase microbes activity (Sizmur & Hodson, 2009).

Earthworm bodies consist of 65 % protein (on dry weight basis) (Sinha et al., 2008) which helps to accumulate metals in their tissue. Metals react with proteins called metallothioneins, which are present in different animals and can create organometallic compounds (Alloway, 2013; Sahariah et al., 2015). Metallothioneins are excreted to bind with metals, and the bind between metallothioneins and metals helps to reduce metal toxicity to the cells (Alloway, 2013). Another protein, called “Heat Shock Protein”, created by the earthworms can also bind with metals (Alloway, 2013).

Another mechanism for vermiremediation is associated with humic acids in the soil. As earthworms dig through the soil, the concentration of humic acids in the soil increases due to the humifying role of earthworms in the soil (Sizmur & Hodson, 2009). Humic acids bind with metals, creating organo-metal complexes which could be uptaken by plants. The role of earthworms in the production of

organo-metal compounds could be the key mechanism for increasing the rate of metal uptake by plants (Sizmur & Hodson, 2009).

Earthworms can be categorised into three groups, epigeic, anecic and endogeic, based on their life strategies. Epigeic worms live in the litter layer and feed on the organic matter in this layer (Sizmur et al., 2011a). Anecic earthworms dig permanent vertical burrows and transfer the organic matter from the surface to their burrows and use them as food (Sizmur et al., 2011a). Endogeic earthworms dig non-permanent horizontal burrows and feed on the organic matter in the soil. They don't come to surface (Sizmur et al., 2011a).

*Eisenia fetida* (epigeic) earthworms and maize (*Zea mays*) plants were used to investigate the effect of earthworms on cadmium concentrations in spiked soil (Du et al., 2014). Cadmium chloride ( $\text{CdCl}_2$ ) was added to the soil to create concentrations from 0 to  $1,000 \text{ mg.kg}^{-1}$  of dry soil. Fifteen *E. fetida* and ten maize seeds were introduced to the soil and 5.0 g of cow manure was used as a feed for the earthworms initially. During the experiment, 2.5 g of cow manure was added weekly to the soil. All of the earthworms resisted different cadmium concentrations and no mortality was observed except for the highest concentration of cadmium in the soil which had 15 % mortality. Earthworms and plants were cultured in that soil for 28 days and during the experiment, room humidity and temperature were 60 % and  $22\text{--}28^\circ\text{C}$  respectively. This experiment indicated that earthworms were able to accumulate cadmium up to  $\sim 500 \text{ mg.kg}^{-1}$  in the presence of maize and  $600 \text{ mg.kg}^{-1}$  without maize. At the end of the project, extraction of cadmium using water and 0.01 M calcium chloride ( $\text{CaCl}_2$ ) increased after introducing earthworms to the soil (Du et al., 2014).

Jusselme et al. (2012) investigated the effect of earthworms on lead uptake by plants. Soil in this experiment was spiked at 500 and  $1,000 \text{ mg(Pb).kg}^{-1}$  and was kept for one week prior to start of the experiment. Earthworms used in this experiment, *Pontoscolex corethrurus* (endogeic), were introduced to the soil at a density of  $100 \text{ g.m}^{-2}$  and lantana (*Lantana camara*) was the plant used for phytoremediation. Their results showed that lead uptake by plants with earthworms was two to three times greater than from soil without earthworms and also the plant growth doubled in mass.

*Eisenia fetida* was also used to understand the effect of this earthworm species on phytoremediation by barley (*Hordeum vulgare*) and maize (*Z. mays*) (Ruiz et al., 2009). The contaminated soil in this experiment was from a lead and zinc mine with a pH of 7.3 and the initial concentrations of contaminants were 896 and  $1,452 \text{ mg.kg}^{-1}$  for zinc and lead, respectively. Lower concentrations of copper and cadmium ( $34$  and  $6 \text{ mg.kg}^{-1}$  respectively) were also present in the soil. The mine soil was a sandy clay and 12 *E. fetida* were used in 1 kg of the soil for eight weeks. Temperature varied during the experiment ( $28^\circ\text{C}$  in the morning and  $10^\circ\text{C}$  at night). The concentration of metals in the worm



bodies in the absence of plants was  $\sim 250 \text{ mg.kg}^{-1}$  (of dry earthworms) for zinc and  $176 \text{ mg.kg}^{-1}$  (of dry earthworms) for lead. In the presence of plants, the accumulation of metals in earthworms decreased, probably due to uptake of available metals by plants. The mobility of these heavy metals was determined by washing the soil with water and  $0.01 \text{ M CaCl}_2$ . The concentrations of water extractable copper and cadmium decreased after introduction of the earthworms while water extractable lead and zinc increased. The  $0.01 \text{ M CaCl}_2$  extractable fraction for cadmium, copper, zinc and lead also increased (Ruiz et al., 2009).

Sahariah et al. (2015) evaluated the effect of *E. fetida* and *Metaphire posthuma* (endogeic) on remediation of municipal waste which contained manganese, iron, copper, zinc and lead at concentrations of 43, 42, 7, 25 and  $27 \text{ mg.kg}^{-1}$  respectively. Earthworms were cultured in a clean soil environment with a 50:50 w/w ratio of soil and cow dung. Different mixtures of municipal waste and cow dung were colonised with these two species of earthworms. Containers 60 cm in diameter and 45 cm high were each filled with approximately 1 kg of mixture and had 12 worms placed inside each container. After 60 days, both earthworms decreased the bioavailability of the metals. For example, lead bioavailability in municipal waste (average of four replicates) was  $27 \text{ mg.kg}^{-1}$  in the first day but decreased to  $17 \text{ mg.kg}^{-1}$  after introducing *E. fetida*. In the same way, *M. posthuma* decreased bioavailability of the lead (average of four replicates) from  $26 \text{ mg.kg}^{-1}$  to  $8 \text{ mg.kg}^{-1}$ . They also reported that as a result of earthworm activity, total organic carbon, cation exchange capacity and pH declined at the end of the experiment.

Remediation of metals from paper and pulp industry sludge was investigated using *E. fetida* (Suthar et al., 2014). Before starting remediation, earthworms were kept in clean soil media. In order to make sure that no pollution existed in earthworm's bodies, a second generation of earthworms, cultured in clean soil was used in the experiment. The sludge containing chromium, copper, cadmium and lead ( $187, 144, 9$  and  $63 \text{ mg.kg}^{-1}$  respectively), was mixed with different proportions of cow dung and placed in 1.5 L containers with 15 earthworms in each container. During the experiment, soil humidity was kept at 65-70 % and the ambient temperature was  $28^\circ\text{C}$ . After 60 days, lead in soil showed the greatest reduction (95.3-97.5 %). Removal of chromium, copper, and cadmium were 47, 69, and 32 % respectively. Metal reduction was related to absorption in earthworms, the soluble fraction extracted by leachate during the experiment and adsorption on the surface of the waste fraction. Final concentrations of chromium, copper, cadmium and lead in earthworm tissues were 36, 12, 3 and  $10 \text{ mg.kg}^{-1}$  dry earthworms respectively (Suthar et al., 2014).

In another experiment, bioaccumulation of metal(loid)s was investigated in two types of earthworm. Earthworms were collected from an old smelter area in northern Kosovo, Serbia (Nannoni et al., 2011). The samples were taken from 18 different sites with average concentrations of 43, 231, 33, 1, 6 and 426 mg.kg<sup>-1</sup> for copper, zinc, arsenic, cadmium, antimony and lead respectively. Analysis of collected earthworms indicated that the two species (*Allolobophora rosea* (endogeic) and *Nicodrilus caliginosus* (endogeic)) had about the same total accumulation of 51 and 45 mg.kg<sup>-1</sup> (of dry earthworms) respectively. The bioaccumulation factor for different metal(loid)s from this smelter site was in this order cadmium >> zinc >> copper > arsenic = lead = antimony. They also found that mobility of the metal(loid)s in the smelter site was cadmium >> zinc >> lead > copper = arsenic = antimony (Nannoni et al., 2011). A more recent study of soil from an Australian copper mine showed that cadmium had the highest bioaccumulation in *E. fetida* at 250 % (Nirola et al., 2016). Bioaccumulation of zinc and copper were 37.3 % and 15.4 % in that order and lead had the lowest bioaccumulation rate (1.7 %) after 28 days. During this period decaying lawn grass was used as source of food for earthworms (Nirola et al., 2016).

The effects of compost and earthworms (*Lumbricus terrestris* (anecic)) on remediation of contaminants in soil from two mining areas were investigated by Sizmur et al. (2011b,c). Lead/zinc (soil A) and copper/arsenic (soil B) mining area soils were combined with 0, 5, 10, 15 and 20 wt% concentrations of compost. The effect of earthworm number on remediation was investigated by adding *L. terrestris* to one earthworm to 200 g soil and two earthworms in 400 g soil. Soil A had initial concentration of 4,550 mg.kg<sup>-1</sup> for lead and 908 mg.kg<sup>-1</sup> for zinc, whereas soil B had 1,150 mg.kg<sup>-1</sup> arsenic and 362 mg.kg<sup>-1</sup> copper. Soils were poured into plastic bags and placed in cups with a surface area of 0.005 m<sup>2</sup> in order to have earthworm density of 500 m<sup>-2</sup>. The experiment was in process for 60 days at an ambient temperature of 16 °C. Soluble arsenic in soil B increased as more compost was added to the soil while for copper the trend was opposite. The addition of more earthworms to the soil decreased water soluble copper and arsenic. Above all, in soil A the amount of extractable lead increased with compost amendment but for zinc the effect was opposite. The addition of earthworms reduced lead solubility but increased the availability of zinc (Sizmur et al., 2011b).

Sizmur et al. (2011c) used the native earthworm *L. terrestris* to assess remediation of three contaminated soils from different sites in the United Kingdom. Concentrations of copper, zinc and lead were 38, 908 and 4550 mg.kg<sup>-1</sup> respectively for the first site and 120, 241 and 988 mg.kg<sup>-1</sup> respectively for the second site. The third site had 362, 89, 1,150 and 109 mg.kg<sup>-1</sup> of copper, zinc, arsenic and lead. They placed one earthworm in a plastic bag containing 300 g of soil. The bags were

inserted in cups with a surface area of 0.005 m<sup>2</sup> to create earthworm density of 500 m<sup>-2</sup>. Earthworms burrowed through the soil at an ambient temperature of 20 °C for 28 days. Their results clarified that water extractable lead was less in bulk soil compared to the control soil without earthworms while the earthworm casts appeared to make lead more available. Their results also indicated that water extractable metal(loid)s increased in the earthworm casts due to the activity of the earthworms but in the bulk soil metal(loid) availability decreased or did not change (Sizmur et al., 2011c).

Zhang et al. (2016) collected soil which had been in contact with mine effluent and contained 394, 405, 0.639 and 439 mg.kg<sup>-1</sup> of copper, zinc, cadmium and lead respectively. *Eisenia fetida* and *Amyntas morrisi* (endogeic) were introduced to the soil for remediation. Earthworms were kept in the soil for ~60 days. In this experiment, 15.0 g of earthworms was placed in 1.0 kg of soil in a 1.5 L container at an ambient temperature of 25 °C. Diethylene triamine penta acetic acid (DTPA) was used as an extractant to evaluate metal leachability. Activities of the earthworms decreased availability of the lead by 16.4 % and increased cadmium and zinc availability by 31 % and 78 % respectively (Zhang et al., 2016).

## 2.5. Summary

Use of earthworms for remediation of metal(loid)s from soil can be an effective and environmentally friendly solution which helps to manage environmental contamination. The effects of earthworms on metal(loid) availability and bioaccumulation reported in the scientific literature is partly contradictory. For example, in some research earthworms increased extractable lead and accumulated high concentrations of lead (Ruiz et al., 2009), while other research showed lead leachability to decrease and earthworms accumulated low amounts of lead (Nannoni et al., 2011; Sizmur et al., 2011b; Sizmur et al., 2011c). These contradictions created both the opportunity and need for the research which follows in this thesis.

Considering that different soil had been used in these experiments, the metal(loid) compounds that existed in those soils could be different. In this experiment, the effects of earthworms on sandy soil contaminated with four common lead compounds (PbCl<sub>2</sub>, PbCO<sub>3</sub>, PbO, PbS) were investigated in order to understand how earthworms might change the metal leachability and accumulate these lead compounds after passing through their bodies.

## Chapter 3 – Methods

### 3.1. Overview

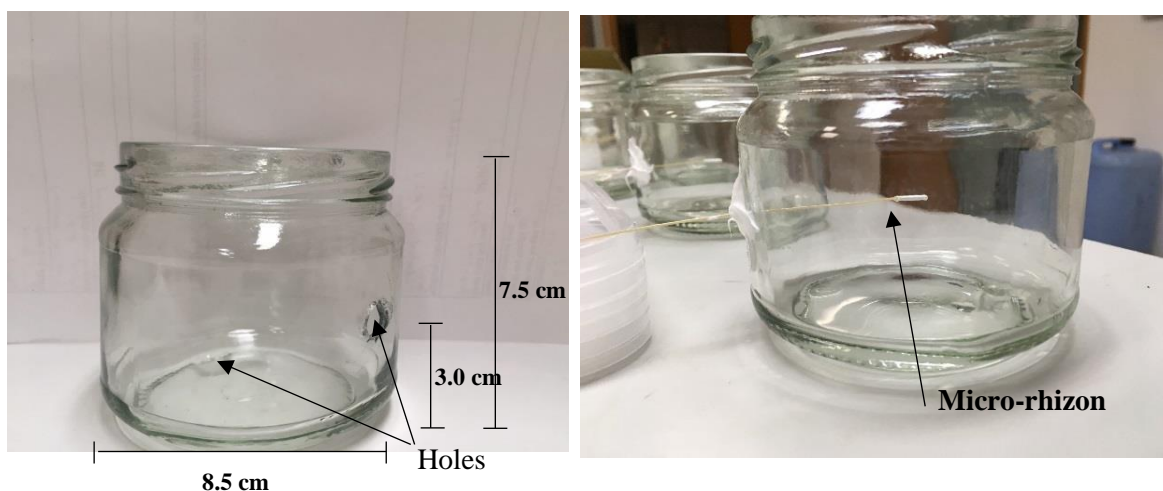
In this experiment, dry soil was contaminated with four lead minerals, lead sulfide (PbS), lead carbonate (PbCO<sub>3</sub>), lead oxide (PbO) and lead chloride (PbCl<sub>2</sub>), that are exemplar of inorganic lead compounds at mines and other contaminated sites. They were introduced at 1,000 mg.kg<sup>-1</sup> in 200 g of dry soil contained in 300 mL jars. Then 120 mL of water (Millipore Milli-Q grade) was added to the soil. There were six replicates of each contaminant treatment, including one control, leading to the experiment consisting of 60 containers (Table 3.1). Compost worms (*Eisenia fetida*) were used in this experiment. Ten mature earthworms (fully clitellate) were placed in each jar. This number and resulting density of earthworms was chosen to ensure thorough mixing and processing of the soil by the worms during the experimental period. The earthworms remained in the contaminated soil for 70 days to make sure that the earthworms dug through all of the soil. During the experiment, 2 g of oats and 2 mL of water (Millipore Milli-Q grade) were added to the soil every week and also micro-porous tubes (micro-rhizons) were used to extract pore water samples every two weeks. These water samples, combined with before-and-after deionized water leaching experiments using the soil, were used to determine changes to the leachability of lead in the pore water. Before and after the experiment, the weight and length of the earthworms were measured to assess any changes due to the lead compounds. Data analysis, consisting of one way and two way analysis of variance (ANOVA) with repeated measures and Tukey's pairwise significant difference of means, was performed using Minitab® v.17 for Windows.

**Table 3.1 Experimental design.**

<b><u>Factor</u></b> (in order of increasing solubility)	<b><u>kg soil</u></b> (no worms, no lead chemistry)	<b><u>kg soil with worms</u></b> (with worms, no lead chemistry)	<b><u>kg soil with lead</u></b> (no worms, with lead chemistry)	<b><u>kg soil with lead and worms</u></b> (with worms, with lead chemistry)	<b><u>kg of soil</u></b>
T0 – Control	6 * 0.2 = 1.2	6 * 0.2 = 1.2			2.4
T1 – PbS			6 * 0.2 = 1.2	6 * 0.2 = 1.2	2.4
T2 – PbCO <sub>3</sub>			6 * 0.2 = 1.2	6 * 0.2 = 1.2	2.4
T3 – PbO			6 * 0.2 = 1.2	6 * 0.2 = 1.2	2.4
T4 – PbCl <sub>2</sub>			6 * 0.2 = 1.2	6 * 0.2 = 1.2	2.4
				<b>Total kg of soil</b>	<b>12</b>
				<b># of jars</b>	<b>60</b>

### 3.2. Preparation of containers

Contaminated soil was placed in 300 mL jars which had 8.0 mm diameter holes drilled in the sides and bottoms (Figure 3.1). The bottom hole was used to drain the soil freely and the side hole was used for installing micro-porous sampling tubes (**micro-rhizons**; Rhizosphere Research Products B.V., The Netherlands) through which soil pore water was extracted at various times. Each jar had one micro-rhizon installed into the centre of the soil, and the hole through the jar was then sealed using silicone sealant.



**Figure 3.1** Position of micro-rhizon in the jar and the jar dimensions.

### 3.3. Soil preparation and characterisation

#### 3.3.1. Preparation of the soil

Soil, sourced from Australian Native Landscapes Pty Ltd (Terrey Hills, Sydney), was a mixture of 50:50 w/w potting mix:sandy soil. This soil was chosen as it provided a good source of organic matter for the earthworms. Prior to use, gravels and large organic particles were removed using a 2.0 mm mesh sieve (following Du et al. 2014, Nirola et al. 2016).

#### 3.3.2. Particle size analysis

Sieve analysis was used to help understand soil type and properties (Das 2002). Circa 500 g of air dry soil was passed through sieves with mesh sizes of 1,700, 850, 500, 355, 180, 106 and 75  $\mu\text{m}$ . Total mass loss on the sieves was kept to <2 wt%, following the method and calculations in Das (2002) (Equation 1);

$$\text{Mass loss during sieve analysis} = \frac{W - W_1}{W} * 100 \quad (\text{Equation 1})$$

Where:

w = Mass of the initial soil used for the analysis (g)

w<sub>1</sub> = Sum of the soil mass retained on the sieves or pan (g).

Particle size distribution curves were drawn and the arithmetic mean (D<sub>50</sub>) of the intermediate (b-axis) diameter calculated.

### 3.3.3. Soil Spiking

In order to investigate the effect of earthworms on lead compounds (PbS, PbCO<sub>3</sub>, PbO and PbCl<sub>2</sub>) and understand the changes in leachability of these compounds after earthworm burrowing and metabolic activities, lead compounds were added to the soil as reagent-grade powders to the soil. For each lead compound, the spiked soil was mixed for three hours on a rolling shaker (built in Macquarie University workshop) and operated at 60 rpm (Figure 3.2) and then three hours on a Ratek RM4 rotary end over end shaker operated at 60 rpm (Figure 3.3) to ensure that the soil was homogenized. Table 3.2 shows the mass of each compound that was used for spiking soil to achieve the target concentration of 1,000 mg.kg<sup>-1</sup> of lead. The amount of compound was calculated by Equations 2 and 3;

$$M_1 = \frac{\sum[M_a * M_c]}{M_L} \quad (\text{Equation 2})$$

Where:

M<sub>1</sub> = Mass of compound to spike 1.0 kg soil at 1,000 mg.kg<sup>-1</sup> (g)

M<sub>a</sub> = Relative atomic mass

M<sub>c</sub> = Molar mass constant 1 g.mol<sup>-1</sup>

M<sub>L</sub> = Molar mass of the lead (g.mol<sup>-1</sup>).

$$M_2 = M_1 * 0.2 \quad (\text{Equation 3})$$

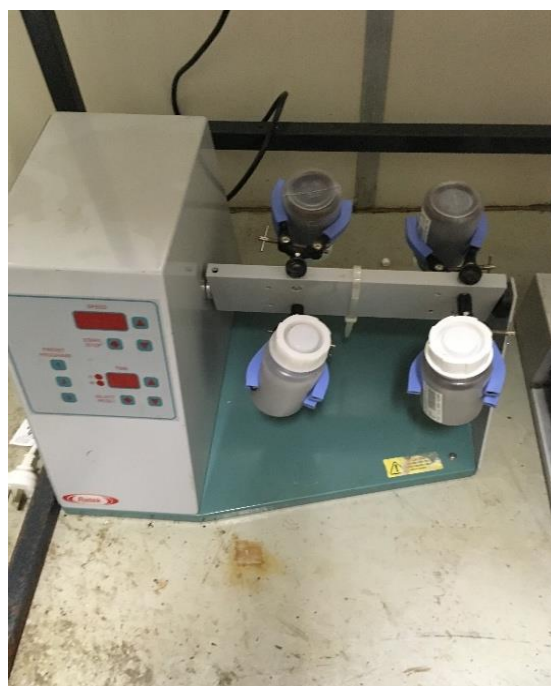
Where:

M<sub>2</sub> = Mass of compound to spike 0.2 kg dry soil at 1,000 mg.kg<sup>-1</sup> (g)

M<sub>1</sub> = Mass of compound to spike 1.0 kg dry soil at 1,000 mg.kg<sup>-1</sup> (g).

**Table 3.2 Mass of each lead compound added to spike soil.**

Compound	Formula	Mass (g) of compound per 1.0 kg soil (from Eqn 2)	Mass (g) of compound per 0.2 kg dry soil (from Eqn 3)
Lead sulfide	PbS	$[207.1+32.0]/207.1 = 1.15$	$1.15*0.2 = 0.230$
Lead carbonate	PbCO <sub>3</sub>	$[207.1+12.0+(3*16.0)]/207.1 = 1.29$	$1.29*0.2 = 0.258$
Lead oxide	PbO	$[207.1+16.0]/207.1 = 1.08$	$1.08*0.2 = 0.216$
Lead chloride	PbCl <sub>2</sub>	$[207.1+(2*35.4)]/207.1 = 1.34$	$1.34*0.2 = 0.268$

**Figure 3.2 Rolling shaker.****Figure 3.3 Rotary end over end shaker.**

### 3.3.4. Soil pH

Soil pH was measured based on the standard method USEPA 9045D, whereby 20 g of the soil was mixed with 20 mL of ASTM (ASTM D1193 – 06(2011)) Type I deionised water. The mixture was shaken by hand vigorously for 5 minutes, then left for 1 h to allow soil particles to settle, and then pH was measured using a pH meter. The pH meter was calibrated using three buffer solutions (4, 7 and 10) (USEPA, 2015).

### 3.3.5. Loss on Ignition

Loss on Ignition (LOI) allows estimation of total organic carbon in the soil (Schumacher, 2002). In this experiment, based on the European standard (BS EN 15169:2007) between 0.5 to 5.0 g of the dried soil was selected for determining LOI. Soil was first oven dried at 105 °C for at least 12 h to

make sure that the soil was dry. Soil (from 0.5 to 5.0 g) was then placed in nickel crucibles in a furnace that took 1 h to reach 550 °C and remained at 550 °C for 2 h, followed by a 2 h cooling period. The soil and crucible were then transferred to a desiccator and they were weighed to calculate the mass lost from the soil. The following equation was used for calculation of LOI (European Standard, 2009) (Equation 4);

$$W_{LOI} = \frac{W_d - W_c}{W_d - W_a} * 100 \quad (\text{Equation 4})$$

Where:

$W_a$  = is the mass of the empty crucible (g)

$W_c$  = is the mass of the crucible and soil after ignition (g)

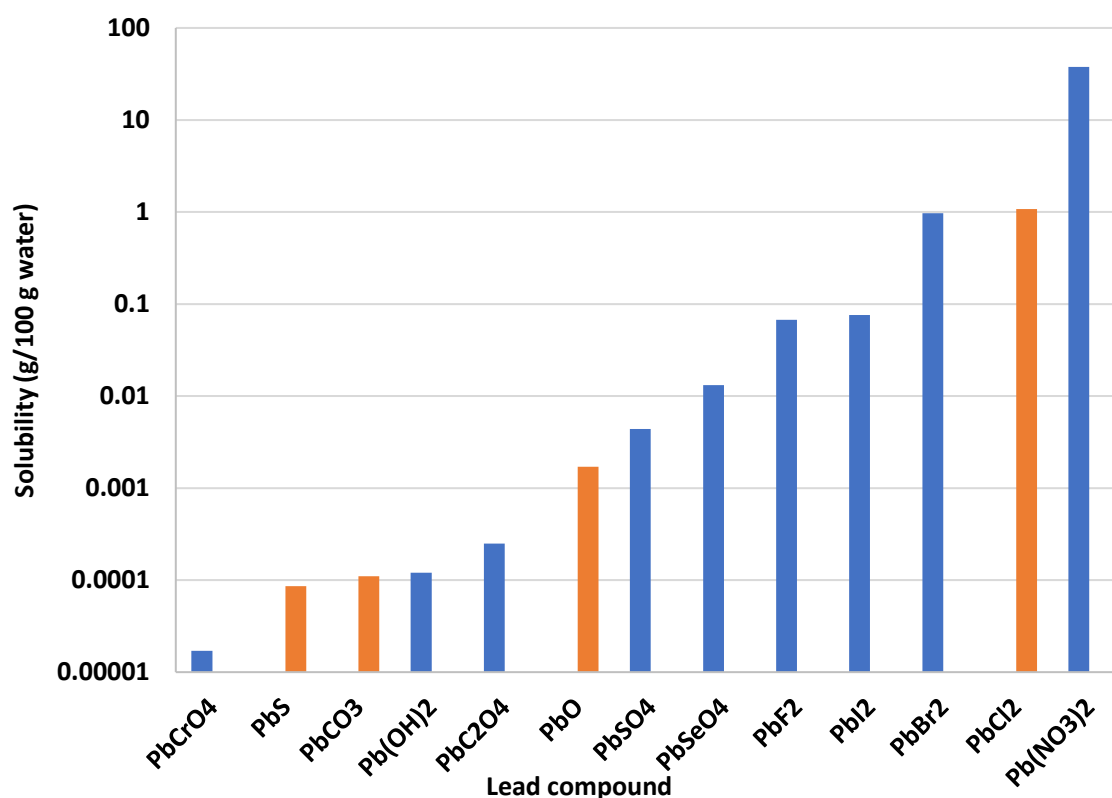
$W_d$  = is the mass of the crucible and dry soil before ignition (g)

$W_{LOI}$  = is the loss on ignition (%).

### 3.4. Lead compounds

The soil in this experiment was spiked with four lead compounds; lead chloride, lead carbonate, lead oxide and lead sulfide ( $PbCl_2$ ,  $PbCO_3$ ,  $PbO$ ,  $PbS$ ). The lead compounds chosen for this experiment cover a wide range of solubilities in water (Figure 3.4) (Abadin et al., 2007; Lide & Haynes, 2009). These compounds are also common minerals in different ore deposits. In Australia, 1,188 mines have galena ( $PbS$ ), 256 mines have cerussite ( $PbCO_3$ ) (Mindat, 2017a). Higher solubility lead compounds were represented by cotunnite ( $PbCl_2$ ), which is common in saline earth surface environments. The even more soluble lead nitrate ( $Pb(NO_3)_2$ ) (Figure 3.4) was not assessed here due to time and budgetary constraints.





**Figure 3.4 Solubility of common lead compounds. Compounds tested in the present study are coloured orange.**

### 3.5. Earthworms

Earthworms used in this experiment were *Eisenia fetida* (red worms, also commonly known as compost worms or tiger worms). This epigeic species was chosen for use here because it is widely used in laboratory studies (e.g. Sizmur & Hodson, 2009), its biology and ecology are well known, it is commercially available in sufficient quantities for testing and it is easy to keep in a laboratory environment. The worms were purchased from Wormlovers Pty Ltd (<http://www.wormlovers.com.au>), and prior to the experiment they were kept and cultured in a contaminant free environment. During the experiment a 2 g of oats was placed on top of each jar every week to ensure that the earthworms had enough nutrients to survive. Figure 3.5 shows an earthworm farm and Figure 3.6 an example of selected earthworms which had been used in the experiment.



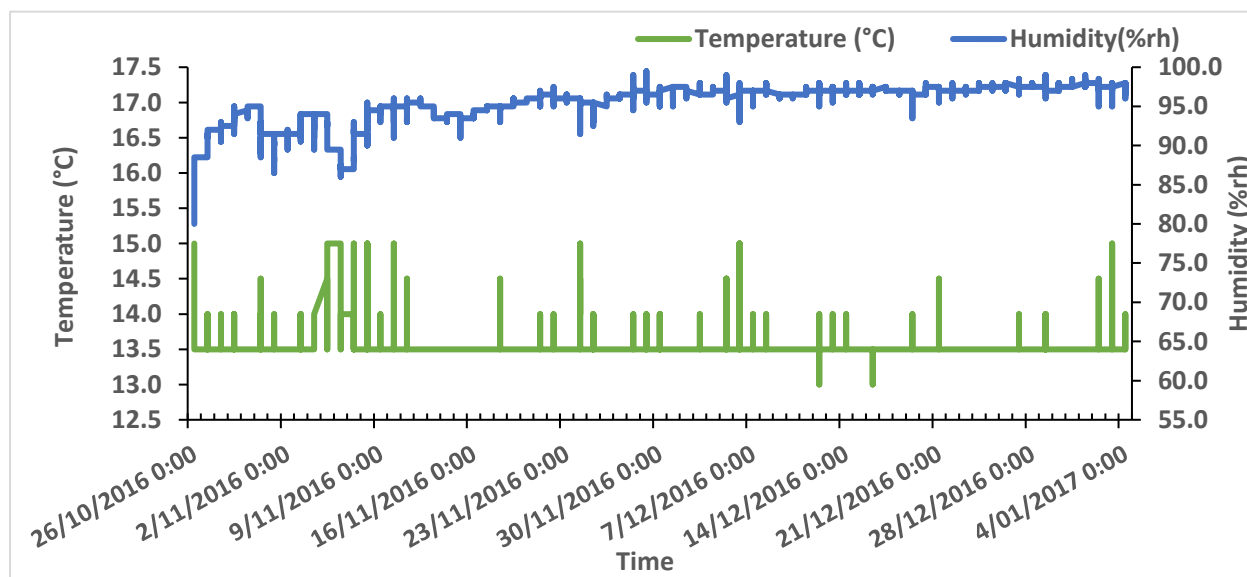
**Figure 3.5 Earthworms in clean soil before use in the experiment.**



**Figure 3.6 Selected earthworms used in the experiment.**

### 3.6. Room Temperature and humidity

The experiment was conducted in a controlled temperature room at  $14 \pm 1$  °C to be representative of sites in mid-latitudes typical of the eastern highlands in NSW. A data logger (EasyLog, EL-USB-2-LCD), set to record every 30 mins, was used to demonstrate that temperature and relative humidity were appropriate and stable in the room to make good conditions for earthworms (Figure 3.7). Short-lived periods of slightly higher temperature captured in Figure 3.7 reflect periodic opening of the controlled temperature room door.



**Figure 3.7 Data logger records of temperature and relative humidity recorded every 30 minutes during the experiment.**

### 3.7. Mass and length of earthworms

Earthworms were weighed and their lengths measured prior to placement in the soil. First, earthworms were washed quickly in water to remove soil particles attached to their bodies. They were then dried on tissue paper, weighed on a petri dish, photographed and their length measured using Snake Measure Tool software v. 2.2.1 (<https://sourceforge.net/projects/snakemt/>). At the end of the experiment, weight and length measurements were repeated to understand the effects of contaminants on earthworm weight and length.

### 3.8. Sampling and analysis

During the experiment, water samples were collected using micro-rhizons. Micro-rhizons are samplers consisting of an 8.0 mm long porous tip attached to a 1.0 mm outside diameter PEEK (polyether ether ketone) tube. Vacuum was applied, and sample collected, using a 5 mL syringe. Pore water samples were collected every two weeks, following watering of the soil. Figure 3.8 shows micro-rhizons attached to syringes installed in the jars. Earthworms were kept in the contaminated soil for 70 days. At the end of the experiment, earthworms were removed from the jars and their weight and length measured. Afterwards, earthworms were transferred to a container with dampened tissue paper and kept in a dark room for three days to remove the soil from their bodies. Earthworms were then killed by freezing at  $-30^{\circ}\text{C}$  and dried at  $65^{\circ}\text{C}$  for 2 days (Li et al., 2010). Dried earthworms were crushed using an agate mortar and pestle prior to analyses. Water (200 mL) was passed through the soil in the jars at the end of the experiment and the leachate collected to determine the leachability of the contaminants.



**Figure 3.8 Syringe and micro-rhizon installed in the jar.**

### 3.9. Sample analyses

#### 3.9.1. Leachate analyses

##### 3.9.1.1. Total Reflection X-ray Fluorescence (TXRF) Spectrometry

Total Reflection X-ray Fluorescence (TXRF) Spectrometry allows elemental determination of dissolved constituents in water to  $\mu\text{g.L}^{-1}$  concentrations. The method works by X-rays generated from the tube irradiating the sample, stimulating elements in the sample which produce “characteristic” fluorescent X-ray radiation with different energies. These X-rays allow identification of the elements, and their concentrations are determined from the area under their corresponding peaks in a spectrum. In TXRF Spectrometry, low background signals and low detection limits were achieved by directing a monochromatic X-ray beam on to the sample at a very low angle of around  $0.05^\circ$  (Brouwer 2006; Shaw et al., 2012; Towett et al., 2013). Most of the tube radiation shines past the sample, while X-rays generated from the sample are recorded using a detector closely coupled with the sample. The instrument used in this experiment was a Bruker S2 Picofox TXRF spectrometer with molybdenum anode X-ray tube.

Two millilitres of the water sample was filtered at  $0.45\ \mu\text{m}$  using a Sartorius MiniSart cellulose acetate syringe filter, and acidified using AnaLR grade concentrated nitric acid ( $\text{HNO}_3$ ) to  $< \text{pH } 2$ . Then,  $450\ \mu\text{L}$  of the acidified sample was mixed with  $50\ \mu\text{L}$  of  $20\ \text{mg.L}^{-1}$  of gallium which acted as an internal spike for quantification. Finally  $10\ \mu\text{L}$  of the prepared liquid was placed on a quartz disc sample carrier and left to dry on a hot plate at  $60\ ^\circ\text{C}$ . Prior to these steps, the sample carrier was cleaned by wiping with lint-free Kimwipes (Kimberly Clark Pty Ltd) soaked with acetone. The sample carrier was then soaked in tap water with washing detergent, rinsed with ASTM (ASTM D1193 – 06(2011)) Type I deionised water, then placed in a 10 % nitric acid:90 % Type I deionised water v/v acid bath overnight at room temperature. After the acid bath, carriers were double rinsed with ASTM (ASTM D1193 – 06(2011)) Type I deionised water and dried in an oven. Carriers were then wiped with acetone, and  $30\ \mu\text{L}$  of silicone in isopropanol (SERVA Electrophoresis GmbH) was applied to their surfaces and dried for 30 minutes at  $80\ ^\circ\text{C}$  (Riaño et al., 2016). The sample carrier was siliconized to become hydrophobic to prevent spreading of the liquid sample on the sample carrier. For quality assurance, some sample analyses were repeated six times in order to understand the quality of pipetting, drying and sample contamination during sample preparation. Measurement of replicate preparations had an average of 5.4 % relative standard deviation, showing that sample preparation was acceptable for this analysis (Appendix 1).

### 3.9.2. Solid phase analyses

#### 3.9.2.1. X-ray diffractometry (XRD)

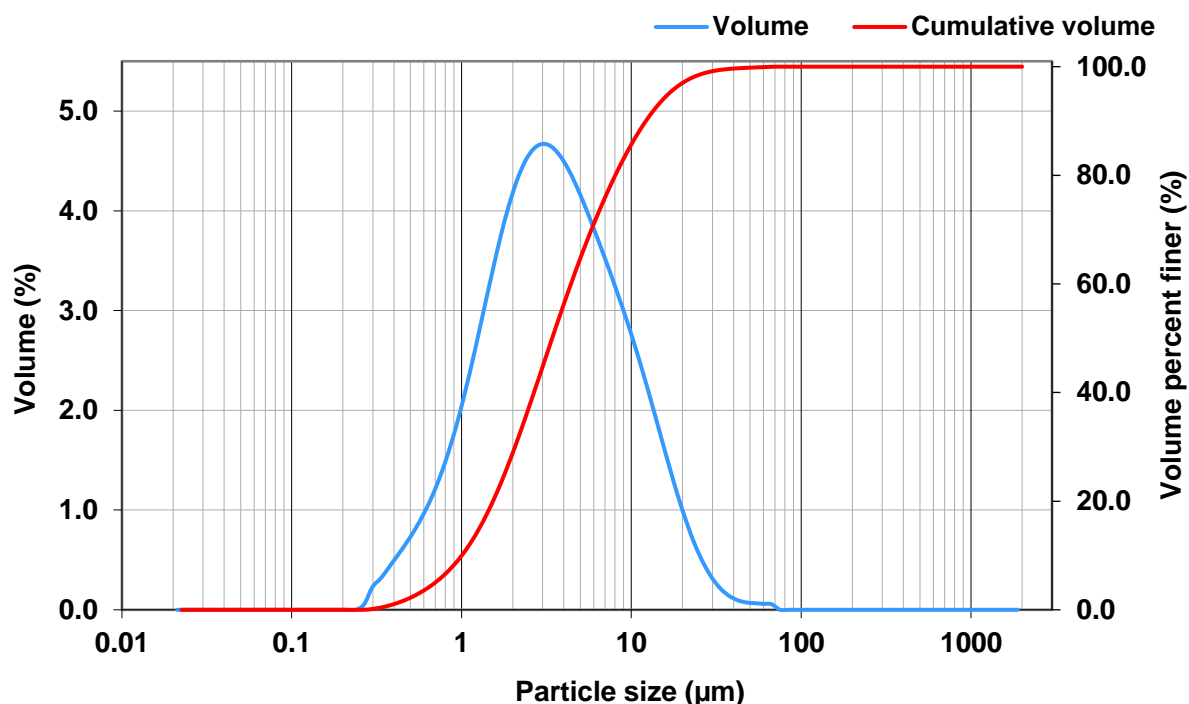
Lead compounds were introduced to the soil at concentrations of  $1,000 \text{ mg.kg}^{-1}$  (0.1 wt%). X-ray diffractometry (XRD), with a method detection limit of  $\sim 0.5 - 2 \text{ wt\%}$ , should not be able to identify these compounds in the soil. However, XRD analyses was conducted on selected samples in case of concentration of lead compounds by earthworm activity.

Mineralogy of the soil and earthworm's guts were measured using a PANalytical X'Pert Pro MPD X-ray diffractometer. In this method, X-rays generated by an X-ray tube irradiate the sample, which produces a unique diffraction pattern allowing identification of the mineral in the sample. Minerals are identified by comparing diffractograms with standard reference patterns (Trepczyńska-Lent & Szykowny, 2015) contained in the ICDD PDF2 inorganic database and PAN-ICSD structural database, and interrogated through the PANalytical software HighScore+ v2.2.1.

The ideal particle size for XRD is  $1.0$  to  $5.0 \text{ }\mu\text{m}$ . To achieve this particle size range, without deformation of the crystallites, a McCrone mill was used to mill the samples. Soil ( $3.5 \text{ g}$ ) and  $7 \text{ mL}$  of ethanol were placed in a grinding jar with 48 agate grinding parts (Figure 3.9). In order to find the appropriate duration of milling time to reach the ideal particle size, samples were milled for 5, 10 and 20 minutes. The best particle sizes were obtained after 20 minutes milling (Figure 3.10) which led to the final average particle size ( $D_{50}$ ) of  $3.4 \text{ }\mu\text{m}$  (Figure 3.11). The particle size distribution of the processed sample was determined by laser diffraction using a Malvern Mastersizer 2000 optical bench with Hydro 2000g accessory. A particle refractive index of 1.459 (quartz) and water refractive index of 1.333 were used (Connolly, 2013; Di Labio, 2015; Ermrich & Opper, 2013) (Figure 3.11).



**Figure 3.9 Grinding jar and agate grinding parts.** **Figure 3.10 Final milled sample.**



**Figure 3.11 Particle size distribution of a soil sample after milling for 20 minutes in the McCrone Mill.**

### 3.9.2.2. Total Reflection X-ray Fluorescence Spectrometry

In order to convert earthworm and extracted soil from their bodies into liquids, acid digestion of the samples were conducted following USEPA method 3051A (USEPA 2007). In this method, 10 mL concentrated nitric acid ( $\text{HNO}_3$ ) was added to earthworm bodies and gut contents in teflon-lined pressure vessels (Figure 3.12) and these were subjected to microwave digestion for 10 minutes at  $175 \pm 5^\circ\text{C}$ . The digested solution was concentrated by evaporation, then analysed using a Bruker S2 Picofox TXRF spectrometer. In order to check for contamination during sample preparation using microwave digestion, one vessel was filled with 10 mL nitric acid as a control and was prepared following the same procedure as the other samples in each run (Ruiz et al., 2009).



**Figure 3.12 Teflon-lined pressure vessels used for microwave digestion.**

### **3.9.2.3. X-ray Fluorescence (XRF) Spectrometry**

X-ray Fluorescence (XRF) Spectrometry characterizes elements by the energy or wavelength of the X-rays emitted from the sample. X-rays produced from an X-ray tube are used to excite the elements in the sample (Peinado et al., 2010), and the characteristic radiation produced by the sample. In this research an Olympus Delta Pro spectrometer with tantalum anode X-ray tube was used to analyse the lead in the soil. Prior to analysis, 5 g of the soils was milled using a Retsch MM301 mixer mill. Soil was placed in tungsten carbide grinding jars (Figure 3.13). In order to prevent cross contamination, the grinding jars were washed in hot water and then clean aliquots of acid washed quartz sand (UNILAB Ajax Finechem B/No. 1510204652) was milled twice between each sample. Milling was performed for 12 min at 25 Hz. Milled samples were lightly packed into polyethylene cups with a 3.6  $\mu\text{m}$  Mylar X-ray film base (Figure 3.14).





**Figure 3.13 Retsch MM301 Mixer Mill and tungsten carbide grinding jars.**



**Figure 3.14 Packed samples in the cups prior to analysis.**



## Chapter 4 – Results

### 4.1. Soil characteristics

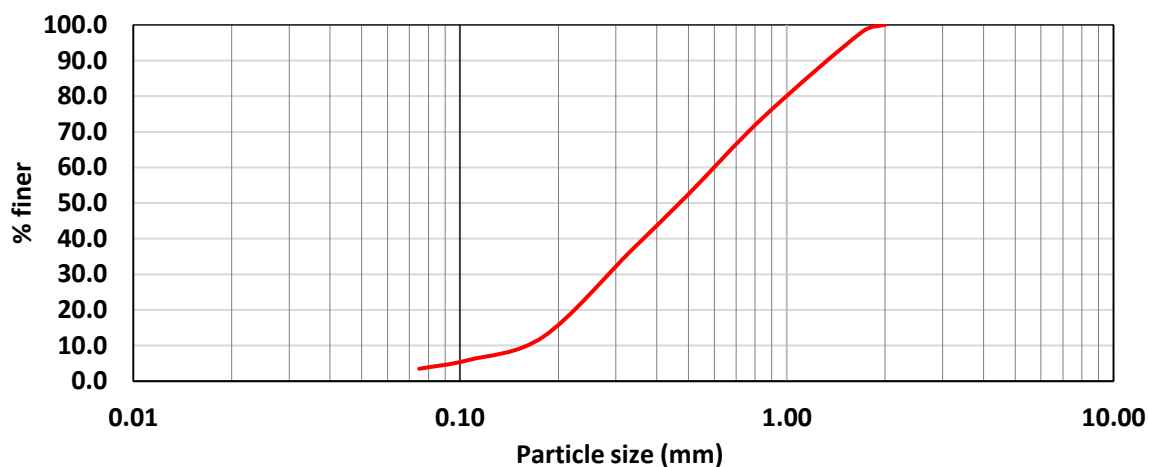
#### 4.1.1. Soil particle size

In order to characterise the soil type used in this experiment, sieve analysis was performed on circa 500 g of soil (Table 4.1). Soil particles with b-axis diameters of 2,000 to 50  $\mu\text{m}$  is considered by the U.S Department of Agriculture (USDA) to be sand (Das, 2010). The soil distribution curve shows that the soil was well graded (Figure 4.1). Particles with b-axis diameters  $> 75 \mu\text{m}$  comprised 97 % of the soil. Based on the USDA textural classification, the soil used in this experiment with a D50 of 480  $\mu\text{m}$ , and D90 of 1,430  $\mu\text{m}$ , is sand. According to the equation 1 the mass loss during this test was 0.03 % which was acceptable (Das, 2010).

$$\text{Mass loss during sieve analysis} = \frac{500.13 - 499.96}{500.13} * 100 = 0.034 \%$$

**Table 4.1 Sieve test data.**

Sieve No	Sieve Opening ( $\mu\text{m}$ )	Sieve weight (g)	Sieve weight + retained soil on each (g)	g of soil retained on each sieve	% retained	Cumulative % retained	Cumulative % finer than
10	2,000	320.00	320.00	0.00	0.00	0.00	100.00
12	1,700	318.05	327.88	9.83	1.97	1.97	98.03
20	850	349.12	468.33	119.21	23.84	25.80	74.20
35	500	265.77	374.38	108.61	21.72	47.52	52.48
45	340	246.44	324.93	78.49	15.69	63.21	36.79
80	180	258.38	379.77	121.39	24.27	87.48	12.52
140	110	207.49	240.37	32.88	6.57	94.06	5.94
200	75	206.37	218.56	12.19	2.44	96.49	3.51
under 200	$< 75$	196.00	213.36	17.36	3.47	99.97	
<b>Sum</b>				<b>499.96</b>			



**Figure 4.1 Cumulative particle size distribution of soil used for testing earthworm response to lead contamination.**

#### 4.1.2. Loss on Ignition

Loss on ignition, measured on ten soil samples (Table 4.2), showed that the soil used in this experiment had an average of 21.2 % organic matter.

**Table 4.2 Loss on Ignition measurement for soil organic matter.**

Sample	g dry soil	g soil after ignition	g lost	LOI (%)
1	1.0087	0.7152	0.2935	29.1
2	1.0365	0.8851	0.1514	14.6
3	1.0097	0.7766	0.2331	23.1
4	1.0190	0.8581	0.1609	15.8
5	1.0134	0.7755	0.2379	23.5
6	1.0558	0.8895	0.1663	15.8
7	1.0000	0.8574	0.1426	14.3
8	1.0537	0.7177	0.3360	31.9
9	1.0013	0.7190	0.2823	28.2
10	1.0034	0.8441	0.1593	15.9
Average				21.2

### 4.1.3. Soil pH

The soil used in this experiment had an initial pH of 4.76 and addition of the different lead compounds to the soil did not change soil pH markedly, with ranges from 4.60 to 4.84 (Table 4.3). Table 4.3 shows the pH values for soil with different lead compounds and leachate for treatments with and without earthworms.

**Table 4.3 Soil and water leachate pH.**

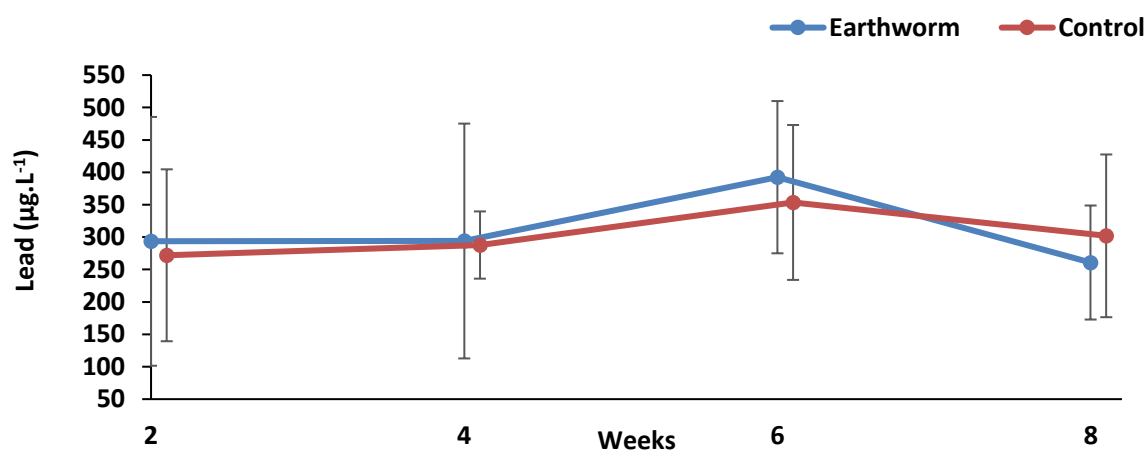
Compound	Formula	Soil pH	Water leachate (with earthworm) pH	Water leachate (without earthworm) pH
Lead sulfide	PbS	4.69	6.25	6.19
Lead carbonate	PbCO <sub>3</sub>	4.74	6.09	5.85
Lead oxide	PbO	4.84	6.44	6.30
Lead chloride	PbCl <sub>2</sub>	4.60	5.58	5.64
Control		4.76	5.82	5.93

### 4.2. Lead extracted in pore water

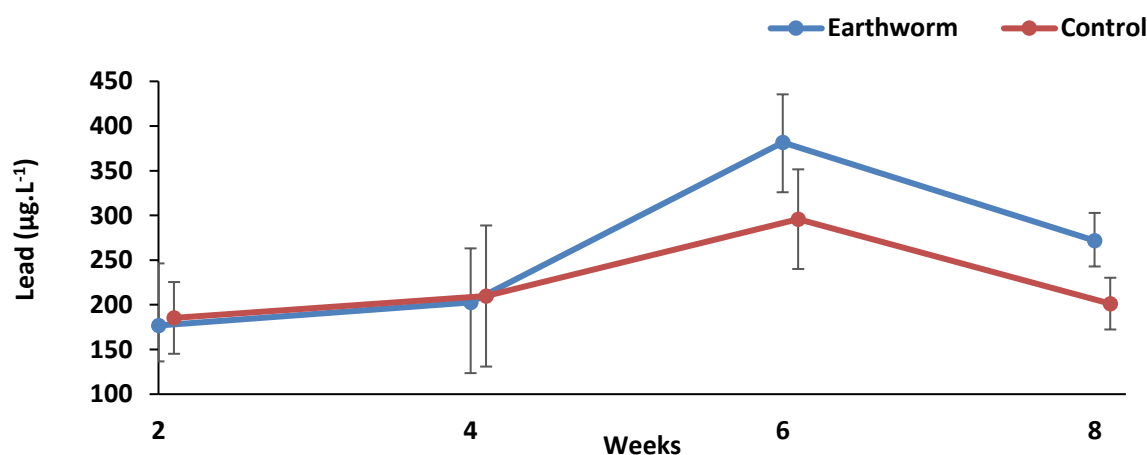
The average lead concentrations in pore water for each reagent applied are shown in Figures 4.2 to 4.5. Concentrations in these figures are averages of analyses from six replicates for each compound, sampled every two weeks, for eight weeks. The data show that the earthworms did not make any difference to concentrations of extracted lead from different compounds in the first two weeks. When earthworms had lived in the soil for four weeks the extracted lead increased slightly for PbCO<sub>3</sub>, PbO and PbCl<sub>2</sub>, consistent with Jusselme et al. (2012), Ma et al. (2002) and Ruiz et al. (2009) who found that worms increased the concentration of metals in plants and root zone. However, the pattern at four weeks was the opposite for PbS and the earthworms decreased the availability of lead in the pore water. After six weeks the lead in the pore water declined in all treatments and controls except for the PbS control which increased slightly. After six weeks, the PbCl<sub>2</sub> and PbCO<sub>3</sub> concentrations declined to equal or less than their control sets while PbO concentration remained higher than its control. A two-way analysis of variance (ANOVA) with repeated measures showed that there was no significant difference (ANOVA; d.f.=1, F=0.14, p=0.713) for the treatments with and without earthworms.

The Pb concentration from control sets (without any chemicals) both with and without earthworms were below detection limits of ~5 µg/L. This was the case for control samples in all of the Pb compound-spiked soils. Accordingly, further analyses focused only on the spiked soils. The results showed that there was a significant difference in Pb pore water concentration for different compounds

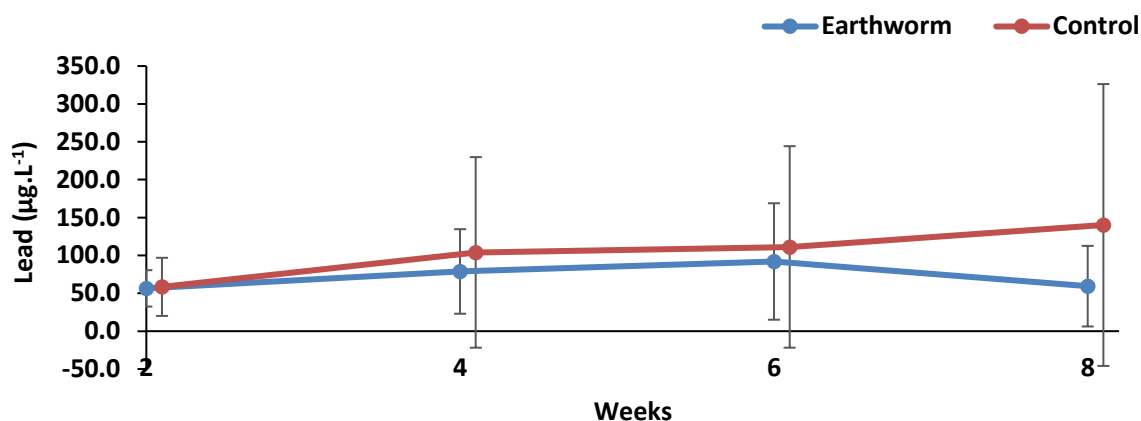
(ANOVA; d.f.=3,  $F=21.45$ ,  $p<0.001$ ) and across different sampling times (ANOVA; d.f.=3,  $F=17.34$ ,  $p<0.001$ ). Also, the interaction of compound type x time (ANOVA; d.f.=9,  $F=5.09$ ,  $p<0.001$ ) showed that different compounds behaved differently over time. Also, behaviour of earthworms over time (ANOVA; d.f.=3,  $F=1.83$ ,  $p=0.146$ ) and across different compounds (ANOVA; d.f.=3,  $F=0.38$ ,  $p=0.771$ ) were not significantly different.



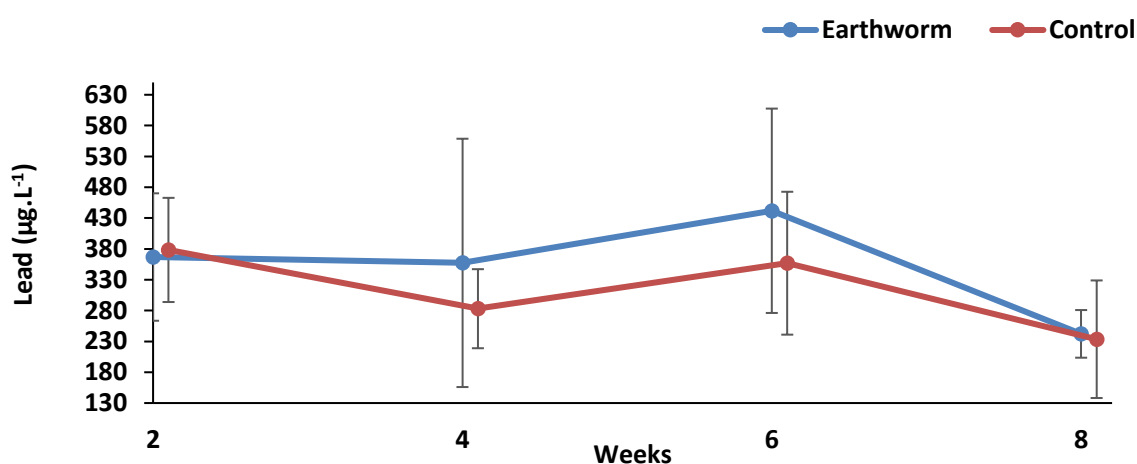
**Figure 4.2** Mean lead concentration ( $\pm 1$  standard deviation) in pore water extracted from lead carbonate contaminated soil containing worms or no worms (control) over time. Control data were offset slightly for clarity.  $n=6$ .



**Figure 4.3** Mean lead concentration ( $\pm 1$  standard deviation) in pore water extracted from lead oxide contaminated soil containing worms or no worms (control) over time. Control data were offset slightly for clarity.  $n=6$ .



**Figure 4.4** Mean lead concentration ( $\pm 1$  standard deviation) in pore water extracted from lead sulfide contaminated soil containing worms or no worms (control) over time. Control data were offset slightly for clarity.  $n=6$ .

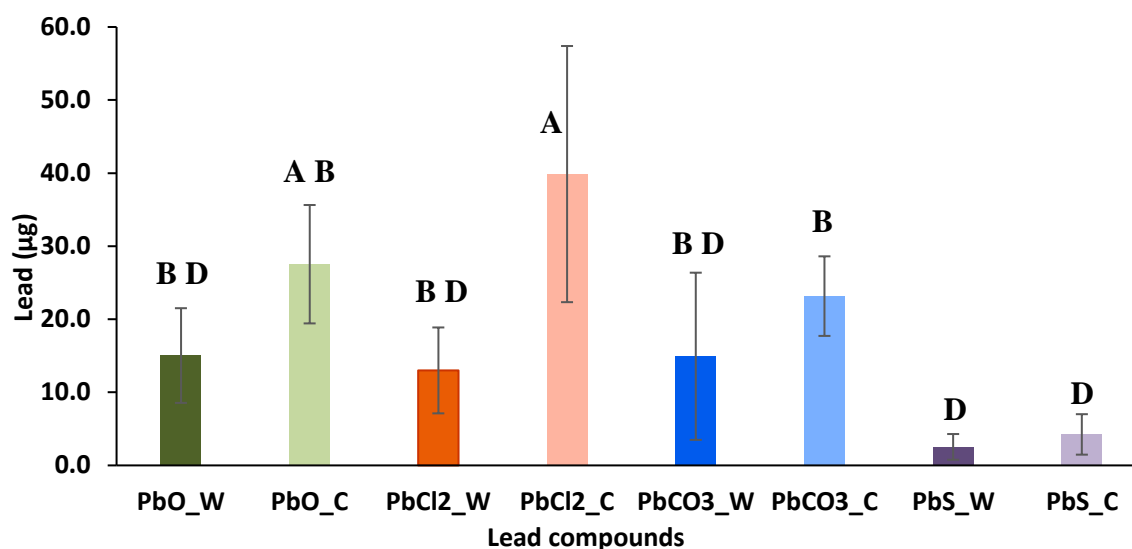


**Figure 4.5** Lead concentration ( $\pm 1$  standard deviation) in pore water extracted from lead chloride contaminated soil containing worms or no worms (control) over time. Control data were offset slightly for clarity.  $n=6$ .

#### 4.3. Lead extracted at the end of experiment by soil washing

Leachability of the lead decreased due to the activity of the earthworms (Figure 4.6) but the effect of the worms differed between compounds. There was a significant difference for treatments with and without earthworms (ANOVA; d.f.=1,  $F=23.56$ ,  $p<0.001$ ) and also between compounds (ANOVA; d.f.=3,  $F=15.28$ ,  $p<0.001$ ). Also, significant interaction of earthworm presence and compound type (ANOVA; d.f.=3,  $F=4.41$ ,  $p=0.009$ ) means that there was a significant difference between earthworms and no earthworms for some but not all of compound. Tukey's post-hoc test of significance showed that there was a significant difference in leachate concentrations for  $PbCl_2$

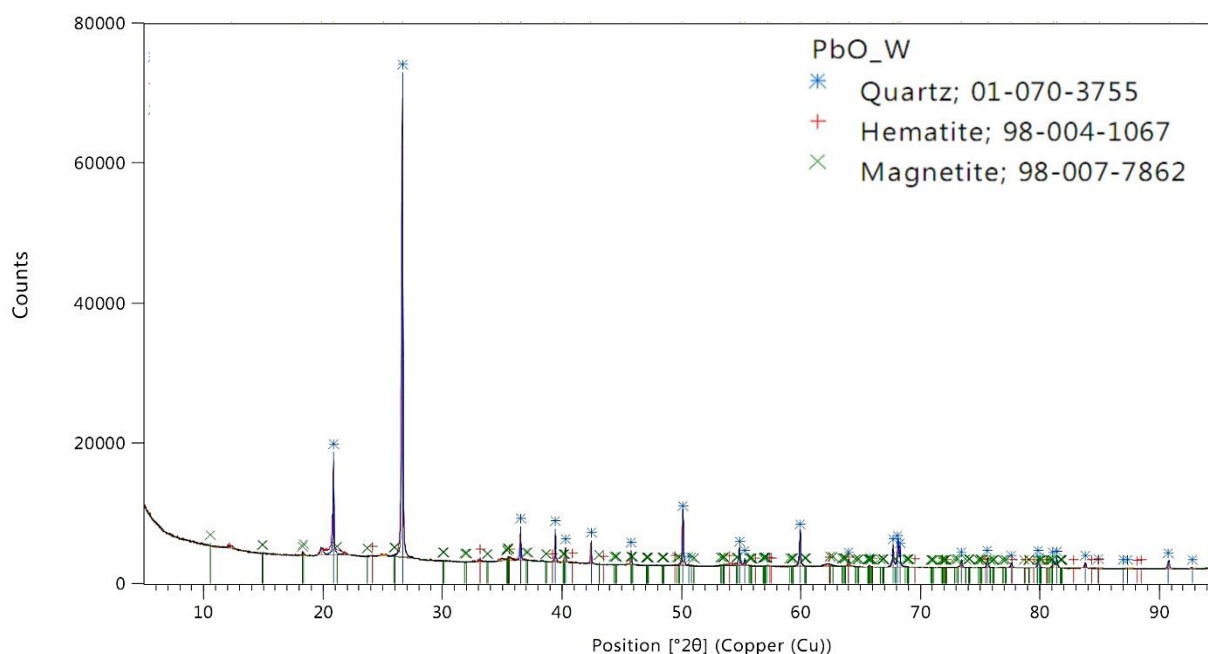
between jars with and without worms (Figure 4.6), but there was no significant difference for other compounds. Also,  $\text{PbCl}_2$  and  $\text{PbS}$  had the largest and smallest (respectively) leachability among the compounds in the absence of *E. fetida*. Although  $\text{PbCl}_2$  had the highest solubility ( $10.8 \text{ g.L}^{-1}$  at  $20^\circ\text{C}$  water) compared to other compounds, the *E. fetida* reduced leachability of this compound to less than  $\text{PbO}$  and  $\text{PbCO}_3$ . After the experiment, extracted lead from  $\text{PbS}$  contaminated soil decreased slightly and remained the lowest extractable compound after the activities of the earthworms.



**Figure 4.6 Lead extracted ( $\pm 1$  standard deviation) from soil by washing with water (columns sharing the same letter are not significantly different; W represents treatments with earthworms and C represents contaminated control soil without earthworms).  $n=6$ .**

#### 4.4. Mineralogy of soil after experiment

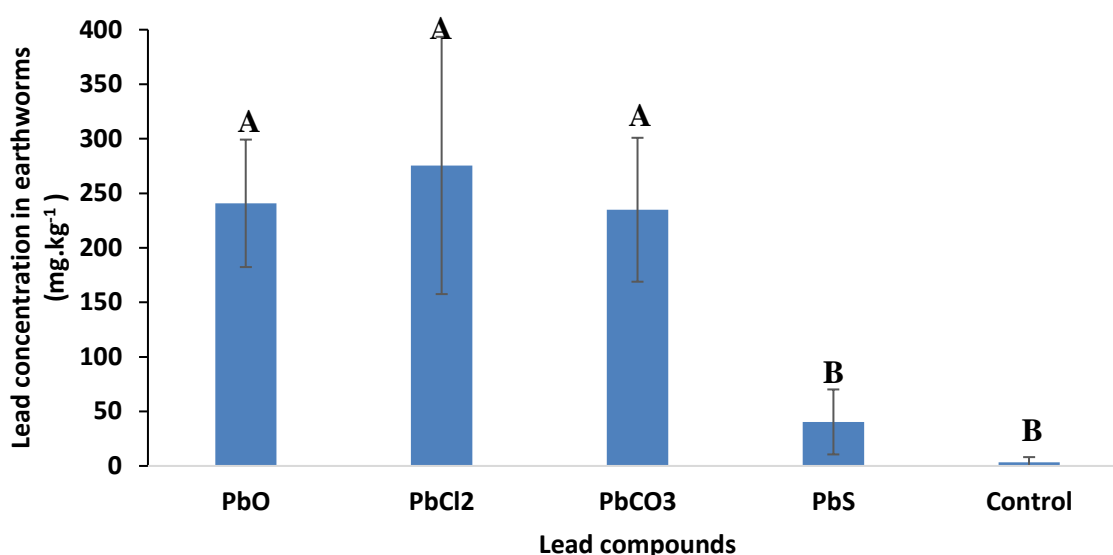
X-ray diffractometry for lead oxide contaminated soil in presence of the earthworms showed that, as expected, no lead minerals were detected. The soil used in this experiment contained quartz, hematite and magnetite minerals (Figure 4.7; Appendix 2).



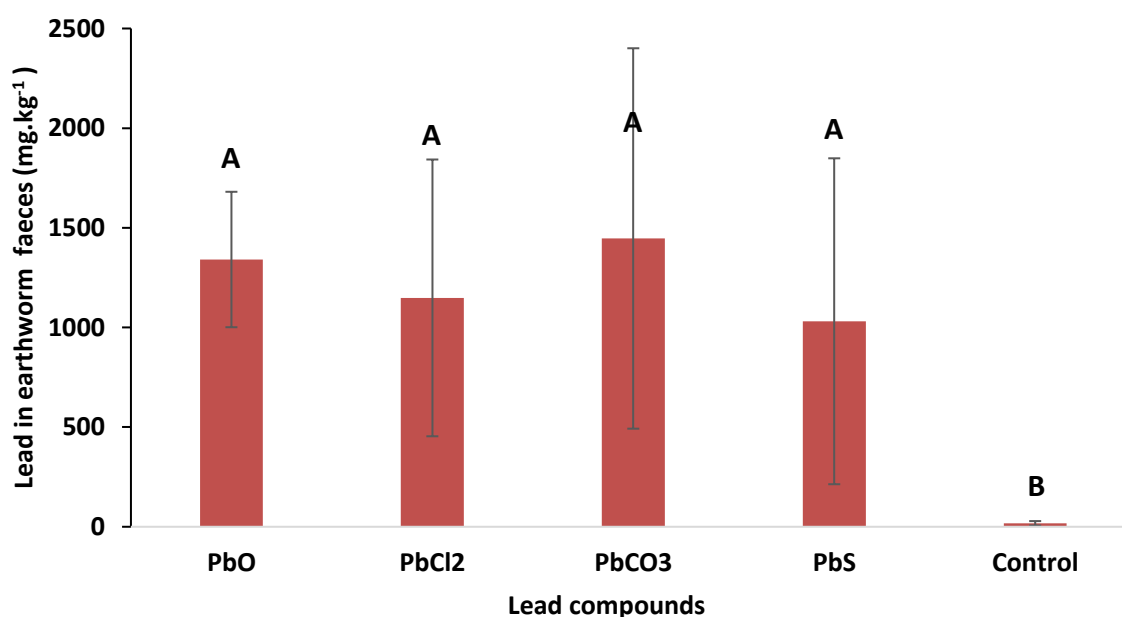
**Figure 4.7 X-ray powder diffractograms of lead oxide contaminated soil showing that no lead oxide mineral polymorph (litharge or massicot) is detectable at 0.1 wt(Pb)%.**

#### 4.5. Accumulated lead in earthworm bodies and soil inside earthworms

The concentrations of lead accumulated in *E. fetida* differed between the treated and the control soils. There was a significant difference in adsorbed lead between control and contaminated soil (ANOVA; d.f.=1,  $F=104.97$ ,  $p<0.001$ ) and also there was a significant difference between the compound types (ANOVA; d.f.=3,  $F=8.38$ ,  $p<0.001$ ). Also, the interaction of treatments and compound types was significant (ANOVA; d.f.=3,  $F=8.00$ ,  $p<0.001$ ). A Tukey's posthoc test of significance showed that the accumulated Pb for PbS was different from other compounds. There was no significant difference for accumulated lead in *E. fetida* for PbO, PbCl<sub>2</sub> and PbCO<sub>3</sub>. PbCl<sub>2</sub> had the highest accumulation rate (276 mg.kg<sup>-1</sup> of dry earthworm) and PbS had the lowest accumulation rate (40 mg.kg<sup>-1</sup> of dry earthworm) (Figure 4.8). Accumulated lead in *E. fetida* for PbO and PbCO<sub>3</sub> was 241 and 235 mg.kg<sup>-1</sup> of dry earthworm. However, there was no significant difference (ANOVA; d.f.=3,  $F=0.41$ ,  $p=0.750$ ) between the concentrations of lead, in soil from inside of *E. fetida* bodies for different compounds (Figure 4.9). Soil inside the earthworms had concentration of 1,340, 1,150, 1,450 and 1,030 mg.kg<sup>-1</sup> of dry soil for PbO, PbCl<sub>2</sub>, PbCO<sub>3</sub> and PbS respectively.



**Figure 4.8** Concentration of lead ( $\pm 1$  standard deviation) accumulated in the earthworm bodies (columns sharing the same letter are not significantly different). n=6.



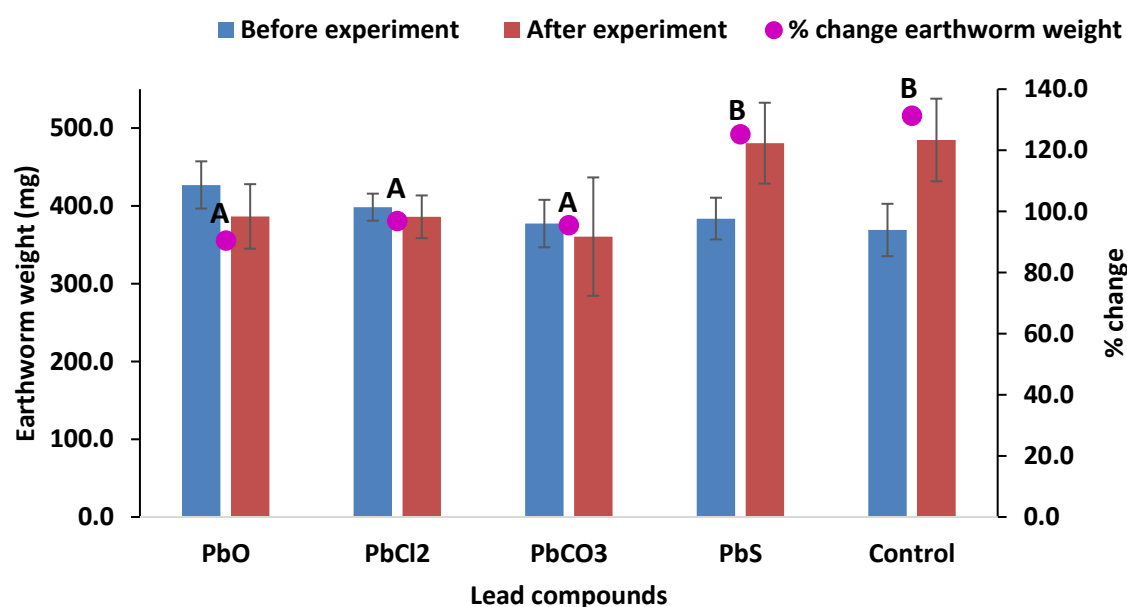
**Figure 4.9** Concentration of lead ( $\pm 1$  standard deviation) in soil removed from the earthworm bodies (columns sharing the same letter are not significantly different). n=6.

#### 4.6. Earthworm mass and length before and after the experiment

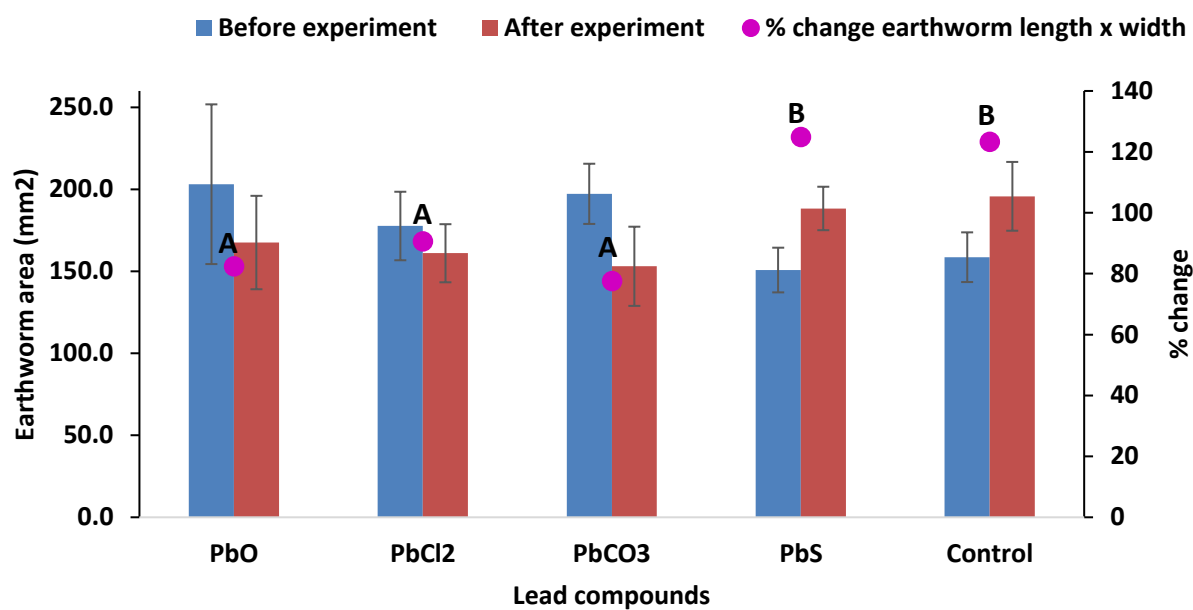
The presence of lead influenced *E. fetida* mass and length. The *E. fetida* in soil with PbO, PbCl<sub>2</sub> and PbCO<sub>3</sub> lost weight after the experiment while those in PbS and control soil gained weight (Figure 4.10). There was a significant difference (ANOVA; d.f.=1, F=45.93, p<0.001) for percent change of earthworm weight across control and treatments. Also, the percent change between different



compound type was significant (ANOVA; d.f.=3,  $F=3.85$ ,  $p=0.016$ ). The interaction of compound type and control/treatment (ANOVA; d.f.=3,  $F=3.82$ ,  $p=0.017$ ) means that there was a significant difference between percent weight change for some compounds but not all of them. The Tukey's post-hoc test of significance showed that the difference between weight changes between PbO, PbCl<sub>2</sub> and PbCO<sub>3</sub> was not significant, but between PbS and other compounds the difference was significant. As the length of the earthworms change when they stretch, their length and width were measured in order to estimate accurately changes in their body size. As a result, the data in Figure 4.11 is related to the average length x width of earthworms for each compound. The results showed the same trend as the weight of the earthworms. Earthworms in the control and PbS spiked samples increased in their length x width while for other compounds the presence of the lead compound decreased the length x width of the *E. fetida*. There was a significant difference in percent change of earthworm length x width across treatment and controls (ANOVA; d.f.=1,  $F=12.26$ ,  $p=0.001$ ) and between different compound type (ANOVA; d.f.=3,  $F=6.59$ ,  $p=0.001$ ). The interaction of treatment and compounds (ANOVA; d.f.=3,  $F=1.38$ ,  $p=0.263$ ) means that there was a significant difference between percent change of length x width for some compounds but not all of them.



**Figure 4.10** Average weights of ten unique earthworms ( $\pm 1$  standard deviation) weighed before and after the experiment along with the percent change in earthworm weight (dots sharing the same letter are not significantly different).  $n=6$ .



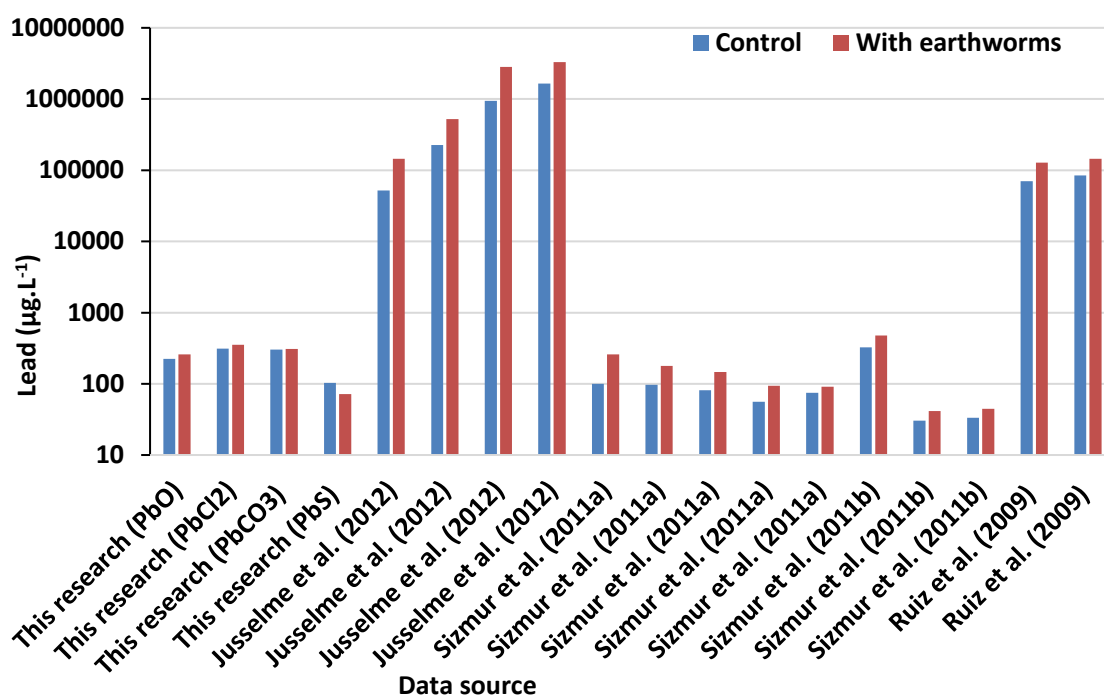
**Figure 4.11** Earthworm length x width ( $\pm 1$  standard deviation) before and after the experiment along with percent change in earthworm length x width (dots sharing the same letter are not significantly different).  $n=6$ .

## Chapter 5 – Discussion

### 5.1. Lead in pore water

Introduction of *E. fetida* to lead contaminated soil increased lead concentration in pore water for PbO, PbCl<sub>2</sub> and PbCO<sub>3</sub>, but for PbS, the earthworms decreased the concentration of lead in the pore water (Figure 5.1). Such increases in concentration of Pb in pore water is consistent with published studies: lead uptake by plants was increased two to three times in the presence of the earthworm *P. corethrurus* (Jusselme et al., 2012), probably due to more extractable Pb in earthworm casts (Jusselme et al., 2012). Furthermore, Ruiz et al (2009) showed that copper, zinc and lead accumulated in two plant species (*Hordeum vulgare* L. (barley) and *Z. mays* (maize)) was significantly greater as a result of earthworm (*E. fetida*) activities in the soil (Ruiz et al., 2009). Activities of *L. terrestris* increased the lead in pore water in soil from a lead/zinc mine and lead nitrate contaminated soil (Sizmur et al., 2011c). Also, *L. terrestris* was introduced to a mixture of soil from a lead/zinc mining area (containing cerussite (PbCO<sub>3</sub>) and galena (PbS), (Mindat, 2017b)) with different compost concentrations (0, 5, 10, 15 and 20 wt%) increased the lead concentration in pore water (Sizmur et al., 2011b).

The Pb in pore water and Pb taken into plant tissue show that the presence of earthworms increases Pb pore water concentrations (Figure 5.1). A possible reason for this increase is physical mixing of the soil by earthworms. In the control soil, contaminants are more likely to accumulate in soil in the lower part of the container because the flux of solutes and fine soil particles are downward, in the direction of water flow. Although water and some solutes move upwards with capillary action, due to the low solubility of PbS the bulk of the lead will move in particulate form which only migrates downwards with the incoming water and will not rise with capillary action. In contrast, earthworms mix the soil thoroughly and as a result, more contaminant particles are moved to the micro-rhizon intake area or, in the field, closer to plant roots. This mechanism of physical mixing could also be the reason for the Pb increase in pore water and plants observed in much of the literature. Different mineralogies and contaminant solubilities, as well as different interactions with soil biota such as earthworms, might result in different uptake of metals into plants or concentrations in pore water. However, in present study, the contrasting behaviour of PbS spiked soil versus the other three lead compounds, is probably mainly due to the very low solubility of this compound in water and thus less uptake into the earthworms. The pore water concentration in Jusselme et al. (2012) and Ruiz et al. (2009) study were the pore water uptaken by plants and this is the reason for the difference of their results with results from the present study and Sizmur et al. (2011b,c).



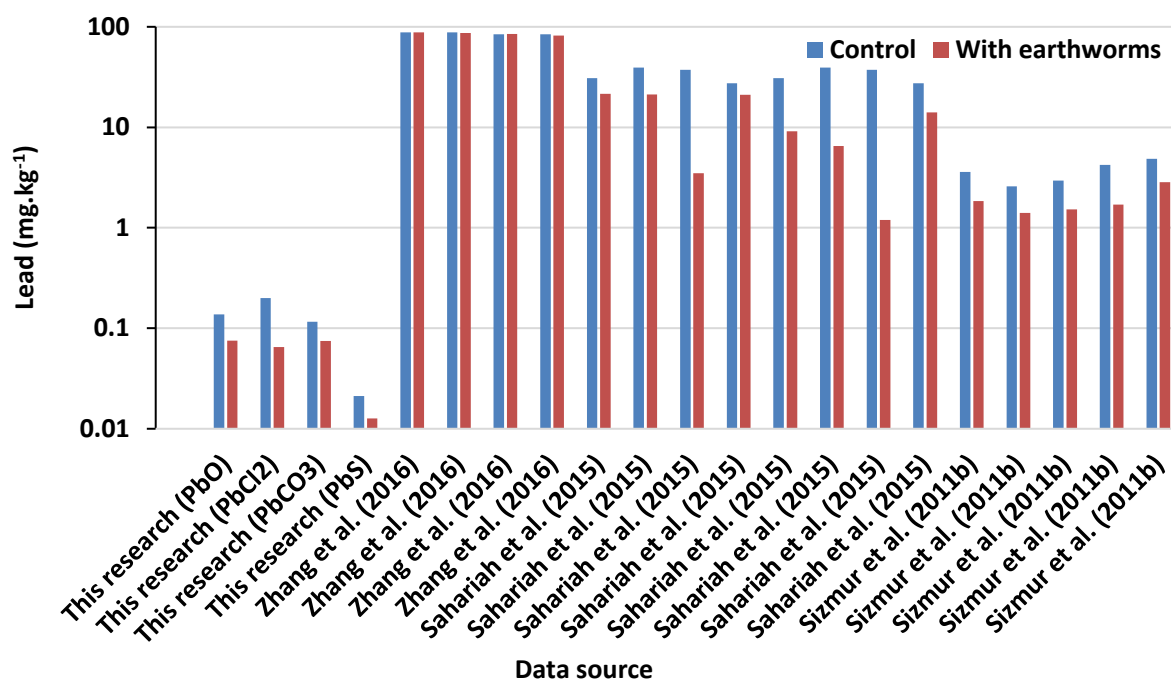
**Figure 5.1 Lead concentrations in pore water and plants in control and treatments with earthworms. (Data are presented in order of increasing research age. Pore water in this research represents an average concentration from four samplings over eight weeks).**

## 5.2. Lead Leachability

*Eisenia fetida* decreased the leachability of lead in this experiment for all lead compounds tested. The PbCl<sub>2</sub> spiked soil had the greatest lead leachability in contaminated soil without earthworms but earthworm activities in PbCl<sub>2</sub> soil decreased lead leachability to less than that of PbO, and PbCO<sub>3</sub>. Pb availability always decreased in the presence of earthworms in the four studies in Figure 5.2. *Eisenia fetida* and *A. morrisi* were used in a laboratory experiment to determine their effects on metal leachability. Soil contaminated with metals from deposition of sewage carried by a river from Dabaoshan (Guangdong Province, China) open cast mining area, was collected and subjected to the activity of the earthworms (Zhang et al., 2016). The minerology of the lead in that mining area is galena (PbS) (Mindat, 2017c). The chelating acid diethylene triamine penta acetic acid (DTPA) was used as the extractant to evaluate metal leachability (Zhang et al., 2016). Zhang et al. (2016) showed that there were 16.4 and 11.6 % decreases in leachability of lead in the presence of *E. fetida* and *A. morrisi* respectively. As earthworms dig through the soil, they decompose organic material and create low and high molecular weight organic acids (Zhang et al., 2016). As a result, Pb binds with organic acids producing organic matter complexes that reduce Pb leachability (Zhang et al., 2016). Earthworms (*E. fetida* and *M. posthuma*) were placed in a mixture of biodegradable municipal waste with cow dung in a laboratory experiment (Sahariah et al., 2015). *Eisenia fetida* decreased Pb

availability from 17 to 17 mg.kg<sup>-1</sup> and for *M. posthuma* from 26 to 8 mg.kg<sup>-1</sup>, probably due to the formation of bonds between Pb and metal binding proteins (metallothioneins), creating organo-metallic complexes which reduced the mobility of the lead (Sahariah et al., 2015). Soil from the Rookhope (England, UK) mining area (initial Pb 4,550 mg.kg<sup>-1</sup>) were subjected to the activity of earthworms (*L. terrestris*) along with addition of compost amendments (Sizmur et al., 2011c). Rookhope mining area contained cerussite (PbCO<sub>3</sub>) and galena (PbS) (Mindat, 2017b). In the absence of earthworms, the water extractable Pb increased along with increasing compost content in the soil but after addition of the earthworms, water extractable Pb decreased. The suggested mechanism was that Pb was extracted by the soluble portion of organic carbon. The decrease in water soluble organic carbon due to the activities of earthworms was believed to be the reason for the decrease in Pb extraction (Sizmur et al., 2011c). In the present study, PbCl<sub>2</sub> had the highest solubility of the compounds tested, and as it passes through the earthworms it is possible that Pb<sup>2+</sup> and the carbonate ion (produced from the calciferous glands in earthworms) will form cerussite (PbCO<sub>3</sub>). This could be the reason for the decrease in its leachability. This process is similar to the interaction of calcium carbonate or carbonate ions with lead in aqueous solutions which led to nucleation of the lead carbonate mineral (cerussite) (Godelitsas et al., 2003).

Earthworms may also decrease available Pb by two physical changes in the soil. First, earthworms accelerate soil water permeability via a network of interconnected burrows lined with biopolymers derived from their bodies (Ruiz et al., 2015). These channels cause uneven water distribution through soil, but generally as a result of the increased effective porosity, there will be fewer interactions of water with soil particles as water will drain faster. Secondly, earthworm mucus is hydrophobic (Ruiz et al., 2015) which could create a layer of hydrophobic material around the surface soil particles along with Pb. This hydrophobic layer might reduce interactions between water and Pb which could decrease Pb availability. Sizmur et al. (2010) also noted that earthworm mucus contains amino acids. They suggested that positive and negative sites of amino acids might bind with soil particles and metals and reduce the metal concentration in solution (Sizmur et al., 2010). In the present study, the soil containing earthworms darkened in colour. Changes in soil properties such as organic matter content, due to the activities of earthworms could be a reason for the change in leachability, and this possible relationship requires further research. The relatively low concentration of Pb in leachate in this experiment, in comparison with other research, could be due to the different spiking procedure used here in which compounds were added to the soil as reagent-grade powders. Alternatively, a gentle, single-pass soil washing procedure used in this research might have extracted less lead than those used in other studies, such as sequential extraction and DTPA extraction (Sahariah et al., 2015; Sizmur et al., 2011b,c; Zhang et al., 2015).



**Figure 5.2 Extractable lead before and after activity of earthworms. (Data are presented in order of increasing research age).**

### 5.3. Lead accumulated in earthworm tissue

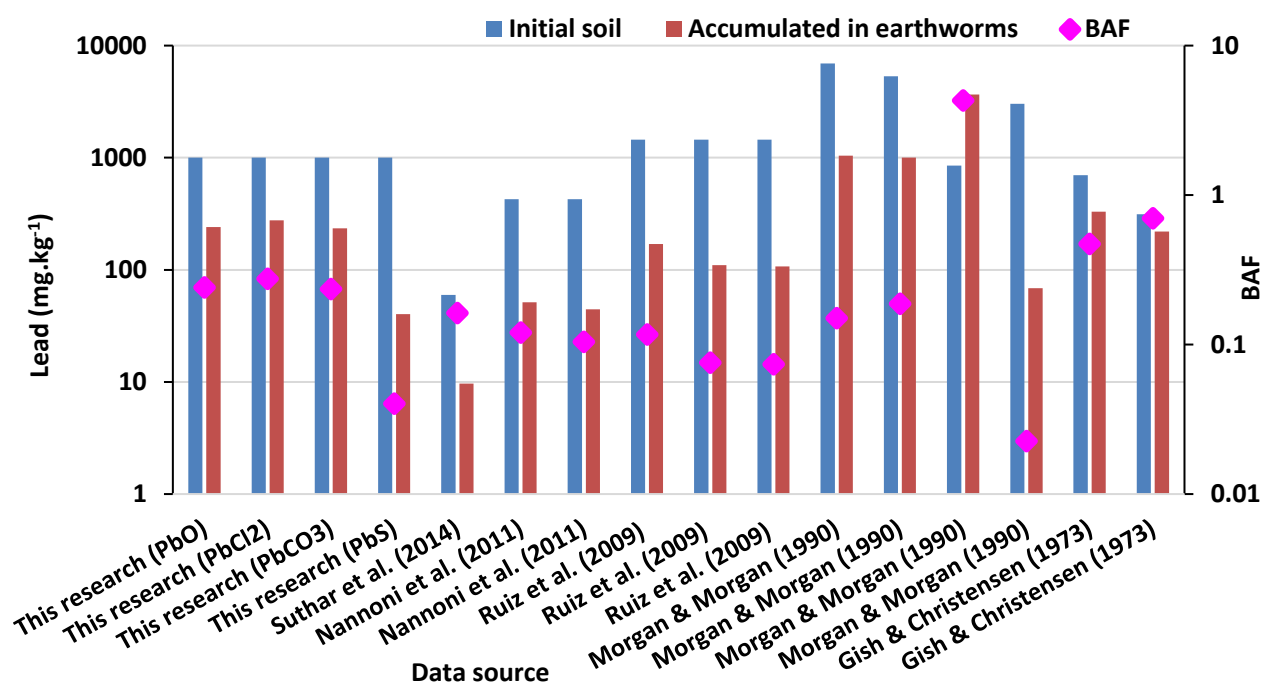
Measurement of lead accumulated in *E. fetida* tissue shows that worms in the PbS treatment had uptaken the least Pb (40 mg.kg<sup>-1</sup> of dry earthworms) and this result is significantly different from other compounds (ANOVA; d.f.=3, F=8.38, p<0.001). The concentration of Pb in *E. fetida* tissue for PbO, PbCl<sub>2</sub>, PbCO<sub>3</sub> and was 241, 276 and 235 mg.kg<sup>-1</sup> (of dry earthworms) respectively. The Tukey's post-hoc test of significance shows that these three compounds were not significantly different in the amount of lead the earthworms take from them. Large concentrations of Pb were present in soil inside the earthworm guts (PbS =1,030, PbO =1,340, PbCl<sub>2</sub>=1,150 and PbCO<sub>3</sub>=1,450 mg.kg<sup>-1</sup>) and there was no significant difference in Pb across these four compounds (ANOVA; d.f.=3, F=0.41, p=0.75). This shows that high concentration of Pb was present in soil inside the earthworms for different treatments.

The Bioaccumulation Factor (BAF) is a metric that reflects how much of an analyte, in this case Pb, is taken into an organism's tissue. It is calculated by dividing the metal accumulated in the earthworm tissue (in mg.kg<sup>-1</sup> dry earthworm) by the total soil metal content (in mg.kg<sup>-1</sup> dry weight). The BAF for these animals for these compounds were, in order, PbCl<sub>2</sub> (0.28) > PbO (0.24) > PbCO<sub>3</sub> (0.23) > PbS (0.04). This different accumulation is consistent with published studies: earthworms (*A. rosea*

and *N. caliginosus*) collected from an old lead smelter area in Kosovska Mitrovica, Kosovo (Pb concentration of 426 mg.kg<sup>-1</sup>) had average lead concentrations of 51.3 and 44.5 (BAF 0.12 and 0.10) mg.kg<sup>-1</sup> (of dry earthworms) respectively. The lead was present in that area (District of Kosovska Mitrovica, Kosovo) in a variety of mineralogies including anglesite (PbSO<sub>4</sub>), boulangerite (Pb<sub>5</sub>Sb<sub>4</sub>S<sub>11</sub>) and jamesonite (Pb<sub>4</sub>FeSb<sub>6</sub>S<sub>14</sub>) (Mindat, 2017d). Lead had the lowest BAF among other metals (Cd > Zn > Cu > As = Sb = Pb) (Nannoni et al., 2011). Although Cd is toxic, its mobility and availability along with its similarity to Zn make it available to accumulate in earthworm tissue. Zn is essential for respiratory control and tissue growth so earthworms accumulate a large amount in their tissue. Cu is essential for cells and tissue but due to its toxicity to earthworms, causing mortality, it accumulates only to low concentrations. The low accumulation of Pb is due to its very low mobility from binding with organic matter of the oxidisable fraction (Nannoni et al., 2011). *Eisenia fetida* was introduced to contaminated soil from a lead/zinc mining area (soil Pb = 1,451 mg.kg<sup>-1</sup>, dry earthworm Pb = 176 mg.kg<sup>-1</sup>, BAF = 0.12). The BAF for metals were, in order, Cd > Zn > Pb > Cu (Ruiz et al., 2009). Lead accumulated in *E. fetida* tissue from paper and pulp industry sludge (lead in soil 60 mg.kg<sup>-1</sup>) was 10 mg.kg<sup>-1</sup> (of dry earthworms) while for chromium, copper and cadmium (initial concentration of 124, 110 and 9 mg.kg<sup>-1</sup> respectively) the accumulated concentration were 36, 12 and 3 mg.kg<sup>-1</sup> (of dry earthworms). The BAF for these metals were Cd (0.30) > Cr (0.19) > Pb (0.15) > Cu (0.08) (Suthar et al., 2014). Lead accumulated in earthworms (*Lumbricus rubellus*) from different abandoned metalliferous mine areas indicates that earthworms from Ecton mining area (England, UK) with 3,022 mg.kg<sup>-1</sup> of lead in the soil accumulated 68 mg.kg<sup>-1</sup> (of dry earthworm tissue) (BAF Cd > Zn > Pb > Cu) while in soil from Cwmystwyth mining area (Wales, UK) with a concentration of 853 mg.kg<sup>-1</sup> of lead, accumulated lead in earthworm tissue to 3,667 mg.kg<sup>-1</sup> (of dry earthworms) (BAF Cd > Pb > Zn > Cu). The Ecton mining area (Pb BAF = 0.02) has galena (PbS) as the main lead-bearing mineral and cerussite (PbCO<sub>3</sub>) as the secondary lead mineral (Natural England 2017). Lead in Cwmystwyth mining area (Pb BAF = 4.3) has a variety of mineralogies including anglesite (PbSO<sub>4</sub>), linarite (PbCuSO<sub>4</sub>(OH)<sub>2</sub>), leadhillite (Pb(SO<sub>4</sub>)(CO<sub>3</sub>)(OH)<sub>2</sub>) and susannite (Pb(SO<sub>4</sub>)(CO<sub>3</sub>)<sub>2</sub>(OH)<sub>2</sub>) (Mindat, 2017e). Two other abandoned metalliferous mining area soils (Llantrisant and Draethen mines, Wales, UK) were used in the experiment with soil lead concentrations of 6,930 and 5,329 mg.kg<sup>-1</sup>. The accumulated lead in *L. rubellus* was 1,044 (BAF Cd > Zn > Cu > Pb) and 1,000 mg.kg<sup>-1</sup> (BAF Cd > Cu > Pb > Zn) (of dry earthworm) respectively (Morgan & Morgan, 1990). Lead in Llantrisant (Pb BAF = 0.15) and Draethen (Pb BAF = 0.19) mining areas (Wales, UK) is in anglesite (PbSO<sub>4</sub>), cerussite (PbCO<sub>3</sub>) and galena (PbS) (Mindat, 2017f,g). Earthworms collected from soil near two highways accumulated up to 670, 219, 38 and 15 mg.kg<sup>-1</sup> (of dry earthworm) of zinc, lead, nickel and cadmium respectively (Gish & Christensen, 1973). The initial concentration of these contaminants in the soil were 141, 313, 24 and 1 mg.kg<sup>-1</sup> in

that order. Although Pb had the lowest BAF among these metals ( $\text{Cd} (15) > \text{Zn} (4.7) > \text{Ni} (1.6) > \text{Pb} (0.7)$ ), earthworms accumulated high concentration of Pb in their tissue (Gish & Christensen, 1973). The majority of road side dust has lead in the form of anglesite ( $\text{PbSO}_4$ ), elemental lead, minium ( $\text{Pb}_3\text{O}_4$ ), lanarkite ( $\text{PbO} \cdot \text{PbSO}_4$ ), and white lead ( $2\text{PbCO}_3 \cdot \text{Pb}(\text{OH})_2$ ) (Abadin et al., 2007).

The results presented here show that earthworms accumulated different amounts of Pb and had different BAF in different studies (Figure 5.3). This experiment and other research on contaminated soil which had Pb in soluble forms such as cotunnite ( $\text{PbCl}_2$ ), anglesite ( $\text{PbSO}_4$ ) or had a variety of lead minerals, had higher BAF values. The presence of galena ( $\text{PbS}$ ) as the main lead-bearing mineral in soil leads to a low BAF value. The availability of Pb for interaction with metal binding proteins could affect the BAF value. The interaction of metallothioneins in earthworms with Pb creates lead-thionein which can accumulate in different organs including kidneys, the brain, lungs and the liver (Gonick, 2011). As  $\text{PbCl}_2$  is very soluble, the presence of  $\text{Pb}^{2+}$  and its interaction with protein chains could be the reason that it had highest accumulation in *E. fetida* while  $\text{PbS}$  is less soluble and as a result it had the lowest accumulation in the worms (Nahmani et al., 2007). Also, the BAF factor for  $\text{PbCO}_3$  and  $\text{PbO}$  was about the same and this could result from their similar solubilities. The contrasting results between the concentrations of lead accumulated in the earthworms is related to the solubility of the lead-bearing minerals in the soil and also the interaction of different lead minerals with different proteins in the earthworm bodies. This last aspect is little documented and could be a fruitful area for future research.

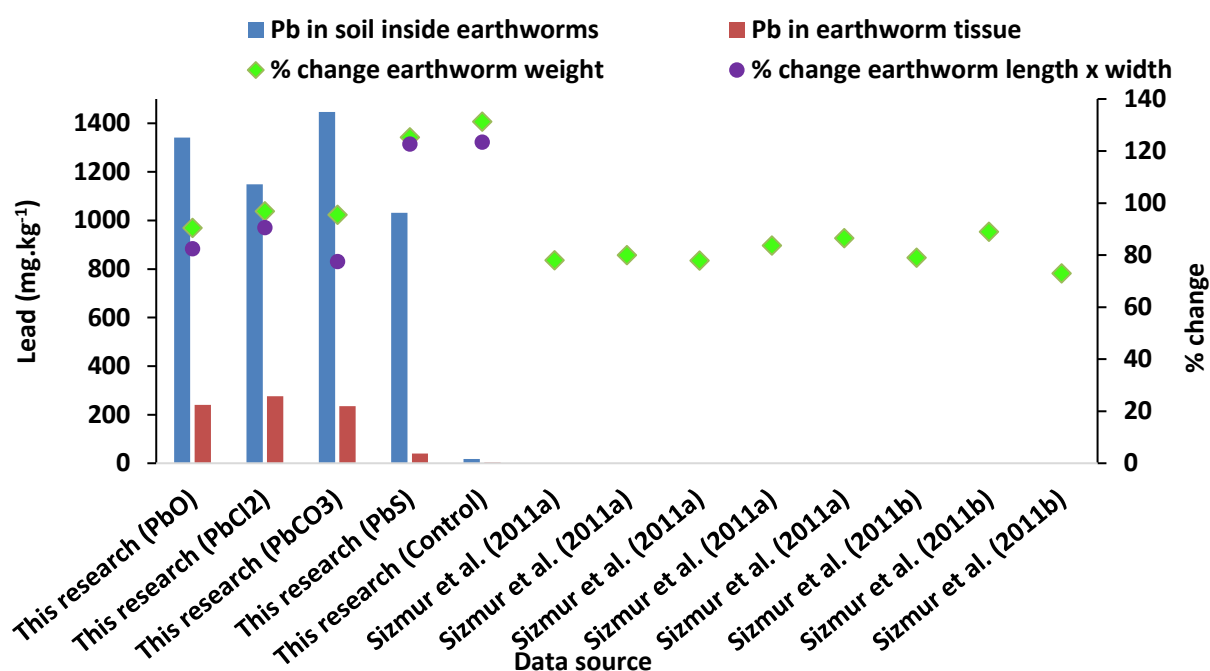


**Figure 5.3** Lead concentrations in initial soil and accumulated in earthworms. (Data are presented in order of increasing research age).



#### 5.4. Earthworm mass and length

During this experiment, the presence of lead compounds had negative effects on *E. fetida* except in PbS contaminated soil. Earthworms lost weight in PbO, PbCl<sub>2</sub> and PbCO<sub>3</sub> contaminated soil while the earthworms in PbS soil and control soil gained weight. The percent change in weight was significantly different across control and treatments (ANOVA; d.f.=1, F= 45.93, p<0.001), and between different contaminant types (ANOVA; d.f.=3, F= 3.85, p= 0.016). Also, earthworms in PbS soil had the lowest Pb accumulation, so the increase in mass of the earthworms could be from lower negative effects of lead on earthworm condition (Figure 5.4). Earthworms (*L. terrestris*) lost weight after activities in different contaminated soil from Pb/Zn, Cu/As mining areas and aged Pb(NO<sub>3</sub>)<sub>2</sub> contaminated soil (Sizmur et al., 2011b,c; Figure 5.4), probably (at least partly) since no food was added to the soil during that experiment. In the present study, oats were introduced to the surface of the soil to provide food for the earthworms so the increase in mass of earthworms in the control soil is related to presence of sufficient food for their growth. However, weight loss in earthworms when oats were presented to the soil is related to the presence of the lead compounds in the soil. Also, the length of the earthworms showed the same trend as the earthworm mass. The length x width metric decreased in the presence of the PbO, PbCl<sub>2</sub> and PbCO<sub>3</sub> while for the control and PbS it increased.



**Figure 5.4 Lead concentrations in soil inside earthworms and their tissue along with percent change in earthworm weight and length x width. (Data from other studies are only percent change of earthworm weight).**

## Chapter 6 – Conclusion

### 6.1. Major findings

The effect of earthworms on tissue accumulation and leachability of lead were different for some of the lead compounds. The presence of lead in the form of very insoluble minerals like galena (PbS) leads to very low accumulation in earthworm bodies. Also, the highest accumulation rates of those studied here, belonged to cotunnite (PbCl<sub>2</sub>) which had the greatest solubility among compounds used in this experiment. Cerussite (PbCO<sub>3</sub>) and massicot (PbO), which had similar solubilities also accumulated similar amounts in earthworm tissue. The apparently contradictory accumulation amounts of lead in different published research might be reconciled by reference to the source lead mineral solubilities. Unfortunately, this information is not always available in other studies, so this statement cannot be assessed further here.

Water extractable lead had decreased after introduction of earthworms to the contaminated soil. The greatest decline was observed in cotunnite (PbCl<sub>2</sub>) which showed that earthworm activity could reduce mobility of the lead in contaminated soil. This could result from more chemical interaction with soluble compounds in the earthworm bodies. Also, the extractable lead in pore water increased due to earthworm activity, except where the source of lead was PbS. The solubility of the compounds along with physical mixing of the soil could explain that increase while the very low solubility of PbS is the reason for its contradictory trend. Earthworm weight and length before and after the experiment for treatment and control soils showed that PbCl<sub>2</sub>, PbCO<sub>3</sub> and PbO had adverse effects on earthworms and decreased their weight and length after the experiment. Conversely, earthworms in control and PbS contaminated soil gained weight and grew longer.

### 6.2. Directions for future research

Future topics for research that comes from this study include;

1. Investigating effects of soil property changes on metal contamination in soil due to the activity of earthworms,
2. Determining interactions of lead compounds with different proteins in earthworm bodies, and
3. Investigating mineralogical changes in different compounds before and after earthworm activity.

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**Appendix 1: Quality assurance for sample preparation for TXRF analysis****Concentration of lead for repeated measurements of different samples.**

Sample Measurement	PbCl <sub>2</sub> _C	PbO_W	PbCl <sub>2</sub> _W	PbCl <sub>2</sub> _C	PbS_W
1 (mg.L <sup>-1</sup> )	0.474	0.053	0.098	0.106	0.065
2 (mg.L <sup>-1</sup> )	0.429	0.048	0.086	0.113	0.061
3 (mg.L <sup>-1</sup> )	0.429	0.055	0.085	0.108	0.068
4 (mg.L <sup>-1</sup> )	0.462	0.053	0.095	0.106	0.063
5 (mg.L <sup>-1</sup> )	0.429	0.049	0.091	0.106	0.072
6 (mg.L <sup>-1</sup> )	-	0.052	0.091	0.100	0.059
Average (mg.L <sup>-1</sup> )	<b>0.445</b>	<b>0.0517</b>	<b>0.091</b>	<b>0.107</b>	<b>0.0647</b>
Standard Deviation (mg.L <sup>-1</sup> )	<b>0.0218</b>	<b>0.00266</b>	<b>0.00502</b>	<b>0.00418</b>	<b>0.00476</b>
% Relative Standard Deviation (RSD)	<b>4.9</b>	<b>5.1</b>	<b>5.5</b>	<b>3.9</b>	<b>7.4</b>

**Appendix 2: X-ray powder diffractograms of contaminated soils. The sample is identified at the top left of each diffractogram.**

