LEAD AND CADMIUM BIOAVAILABILITY: CYSTEINE- AND GLUTATHIONE-MEDIATED SOIL DESORPTION AND PLANT UPTAKE

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LEAD AND CADMIUM BIOAVAILABILITY: CYSTEINE- AND GLUTATHIONE-MEDIATED SOIL DESORPTION AND PLANT UPTAKE

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The use of plants in phytoremediation is limited by incomplete knowledge of transport mechanisms and low solubility of metals in soil. I have investigated the use of biogenic thiols cysteine and glutathione to chelate Pb and Cd in soils and to create a soluble metal-thiol species that may be actively transported into plants.

Short-term hydroponic experiments revealed that both cysteine and glutathione mediate uptake of Pb and Cd into roots of *Zea mays* and *Brassica napus*. Uptake rates were enhanced after pre-exposure to cysteine or glutathione and inhibited in the presence of vanadate, suggesting a biological mechanism of uptake. Increasing concentrations of glutathione resulted in decreasing Pb uptake rates, which indicates competition for transport between the free glutathione and Pb-glutathione species. Pb uptake in the presence of increasing cysteine concentrations resulted in decreased uptake initially but linearly increasing uptake at higher thiol concentrations. Uptake is possibly mediated by a peptide or amino acid transporter.

While cysteine and glutathione mediate uptake of Pb into roots, shoot translocation was not significant in wildtype *B. napus, Z. mays* or *Arabidopsis thaliana*. Experiments with *A. thaliana* tDNA insertion mutants deficient in OPT, PTR, or PDR transporters showed changes in root Pb accumulation as well as increased shoot translocation in the OPT5 knockout. Root vacuolar sequestration may

be occurring and will be verified in future experiments using TEM and x-ray spectroscopy.

Cysteine and glutathione are both effective at solubilizing Pb and Cd from several contaminated soils at pH 7 and 8. While glutathione is relatively stable in solution and maintains metal solubility, cysteine is rapidly oxidized resulting in short-lived solubility of metals. Sequential extractions showed that cysteine and glutathione removed from 20 to 100 % of the soil metal contaminant after 4 cycles depending on the soil.

In intact soil systems, Pb and Cd solubility ranged from 20 to 200 ppm due to thiol treatment, while Cd solubility was less than 10 ppm. Root and shoot uptake of Pb and Cd from soil into *Zea mays* did not result in significantly higher uptake than the control, but these initial experimental methods were inconclusive.

BIOGRAPHICAL SKETCH

Tim was a student of science all his life, persistently asking questions, sometimes to others' dismay. Math and science were always his best subjects and he pursued an undergraduate education at Rutgers University. Combining his concern for the environment and mathematical prowess, he pursued a dual degree program in Bioresource Engineering from Cook College and the College of Engineering. Tim also spent 4 years outside of the classroom working in greenhouses, first repotting orchids for a whole year and later building, installing and maintaining an open-roof greenhouse with Eugene Reiss and Arend-Jan Both.

Tim pursued graduate studies at Cornell University, majoring in environmental engineering and quickly found a place in the biogeochemistry and environmental biocomplexity group, thanks to both an IGERT fellowship and a great group of people. He was able to pursue research both in Dr. Ahner's laboratory and interdisciplinary research in carbon mitigation with the biogeochemistry group. In addition to his education in environmental sciences and engineering, he gained a strong background in interdisciplinary research, communication, and a broad biogeochemical perspective on environmental issues that will shape his research in the future.

Tim is planning on pursuing a career as an academic, looking forward to teaching the next generation of environmental scientists and engineers and never wanting to leave the independent, stimulating, and quirky world of academia.

ACKNOWLEDGMENTS

I have benefited greatly from the independence afforded me as well as the guidance provided by my advisor Beth Ahner. I appreciated the ability to pursue research both in and out of her laboratory as well as support to pursue other interests on campus. I have become both a better researcher and better writer under her direction. I would also like to thank my other advisors and mentors, Roger Spanswick, Murray McBride and Tim Fahey for their feedback, support, and suggestions regarding my research and education. I could also not have done my work without the help of David deVilliers in the greenhouse and Mike Rutzke for his donation of and my education in ICP technology.

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CHAPTER 1

A NEW TAKE ON CHELATE-ASSISTED PHYTOREMEDIATION

1.1 Introduction

In the 2005 CERCLA priority list of hazardous substances published by the EPA (http://www.atsdr.cdc.gov/cercla), the number 1, 2, 3, and 8 ranked priority pollutants are arsenic (As), lead (Pb), mercury (Hg), and cadmium (Cd), respectively, due to their potential harmful effects on human health and abundance at contaminated sites. These sites are primarily the result of historically poor mining or industrial disposal practices and are very costly to remediate. In addition, Pb is elevated in urban soils due to lead-based paints and historical leaded gasoline emissions. This study focuses on phytoremediation, a cost-effective plant-based means to remediate soil contamination of Pb, with some investigation of Cd, using biodegradable ligands that are relatively specific for these metals, thus addressing current criticisms of chelate-assisted phytoremediation (Nowack et al. 2006).

The successful application of phytoremediation relies on knowledge of metal availability in the soil solution and of plant metal uptake mechanisms. The soil is a complex matrix in which metals adsorb to soil particles, precipitate as minerals, or form soluble inorganic or organic species. Most well-characterized metal uptake mechanisms in plants involve the free metal ion, but there are some that mediate transport of a metal-ligand complex such as with Fe-phytosiderophores (Roberts et al. 2004). The uptake of toxic metals, more specifically non-essential elements such as Pb, is thought to occur through essential element transporters (see reviews by Clemens et al. 2002, Colangelo and Guerinot 2006). However, this is limited by the presence of competing elements, and uptake via these mechanisms is low in most plants. The so-called hyperaccumulator plants have greater uptake potential, but are limited in

biomass production, limiting total metal removal from soil. Ultimately, optimization of controlled uptake and/or sequestration mechanisms in high biomass crops will lead to more effective implementation of phytoremediation. The goal of this dissertation is to characterize the mechanisms of ligand-assisted Pb and Cd solubility and uptake and determine the extent to which they can be harnessed to augment phytoremediation of metal-contaminated soils.

1.2 Objectives

The objective is to describe and characterize novel transport mechanisms for Pb and Cd into plant root cells. Thiol-containing ligands, such as the amino acid cysteine and the tripeptide glutathione (GSH), are relatively specific for these metals and are also important biochemicals in plants. This study examines whether transporters specific for these ligands will also transport the metal-ligand complex. It also investigates the use of these same ligands to increase and maintain Pb and Cd solubility in soil. Increased solubility of the metal in a form that can be transported into the roots should increase the bioavailability of these metals and ultimately enhance remediation of contaminated soil.

1.3 Background

Phytoremediation has been used with moderate success to remediate a number of hazardous waste sites, ranging from sites contaminated with heavy metals to pesticides (Brown et al. 1994; Cunningham and Ow 1996; Salt et al. 1998). This technology relies on the ability of plants to transport heavy metals and other contaminants across the root membrane for the eventual sequestration and/or metabolism of these compounds. Genomic studies are currently revealing a large set of genes that have potential for manipulation with respect to trace metal transport and intracellular chelation (Clemens 2001).

Cd is a very labile metal in soil but uptake by plants is highly variable. Several species are known as hyperaccumulators of Cd but they typically have low biomass. The few crop plants that accumulate fairly high levels of Cd (e.g. 104-220 ppm per shoot dry weight in *Brassica juncea*, Kumar et al. 1995; Blaylock et al. 1997), have been well studied. Pb, on the other hand, is generally insoluble in soils and, like Cd, is not taken up directly by plants for any biological function. Because of the different availability of Cd and Pb in soil, the focus of scientific inquiry has been quite different. There has been more emphasis on transporters, intracellular speciation and sequestration, and genetic engineering in relation to phytoremediation for Cd. In the case of Pb, the focus has been on enhancing its solubility in the soil solution and uptake via passive mechanisms using synthetic ligands.

Factors affecting heavy metal uptake

The factors affecting metal uptake into plants include total soil metal concentration, metal speciation, metal solubility, soil pH, presence of competing ions, and transport mechanisms. Plants may alter metal uptake by reducing localized soil pH to promote proton exchange for required nutrient metals (Banuelos and Ajwa 1999), exuding metal chelating compounds such as phytosiderophores for Fe (Roberts et al. 2004), or forming symbiotic relationships with soil organisms such as mycorrhizal fungi (Vogel-Mikus et al. 2006). The generally accepted paradigm is that trace metals necessary for plant growth are taken up as free metal ions but, due to the complexity of the soil solution and metal-ligand relationships, it is possible that plants take up a variety of metal species that are typically present (Hassler et al. 2004; Parker et al. 2001).

Behavior of Pb and Cd in soils

The ability of plants to take up Pb and Cd are markedly different, and it is likely in part due to availability of each metal in the soil solution. The solubility and

activity of each metal is affected by many factors such as soil pH, organic matter content, cation exchange capacity, redox potential, and the presence of Mn and Fe oxides (Banuelos and Ajwa 1999; McBride 1994). While Pb is extremely insoluble in soils at pH levels relevant for plant growth and is usually present as stable phosphate or carbonate species (Blaylock et al. 1997; Chaney et al. 1988), Cd is more soluble at a pH lower than 6, but can also form precipitates such as $CdCO_3$ or complex with organic matter above pH 6 (McBride 1994).

Depending on the soil type and source of contamination, Pb or Cd will partition into different phases. Sequential extraction of soils for trace metal determination have been developed and divide the soil into five general fractions: (1) exchangeable, (2) bound to carbonates, (3) bound to iron and manganese oxides, (4) bound to organic matter, and (5) residual (Li et al. 1995; Tessier et al. 1979). The potential availability of metals from each of these fractions will differ; mining wastes may be in the form of more stable primary and secondary minerals (residual fraction) and more difficult to solubilize (Shen et al. 2002). The type and age of contamination will play a role in solubility of these metals.

Soil extractions with organic acids and synthetic ligands

It has been shown that the addition to soils of organic acids, such as citrate, increases the solubility of heavy metals (Blaylock et al. 1997; Wu et al. 2003) and the uptake of the metals to some extent (Chen et al. 2003; Pires et al. 2004). However, in one instance, a decrease in dissolved Pb was observed, potentially due to adsorption of the complex onto soil particles (Chen et al. 2003). Mineral phases, such as $PbHPO_4$, were shown to dissolve when exposed to cysteine or thiosulfate (Martinez et al. 2004). This has also been examined in model clays or peat by Fischer (2002), showing increased solubility of Pb and Cd with increasing concentration of cysteine or penicillamine.

Soil extractions with synthetic chelators

Proponents have argued that enhanced solubilization of Pb is critical for phytoremediation of Pb-contaminated soils (Blaylock et al. 1997; Epstein et al. 1999; Huang et al. 1997). In this strategy, plants are grown in soil contaminated with Pb and, once an optimal plant size is reached, the soil is treated with a high concentration of chelator to solubilize the Pb. Studies have shown that Pb accumulation in the tissues is proportional to the strength of the chelator and that the amount of Pb accumulated in plant tissues increases with increasing soluble Pb (Blaylock et al. 1997). This also applies to a lesser extent for Cd (Grĕman et al. 2003). One of the main drawbacks of this strategy is that there is the potential for leaching of long-lived metal-chelator complexes that are not taken up by the plant roots [complexes of EDTA can be found in pot experiments up to 1 year later (Kos and Lestan 2003; Tandy et al. 2004)]. This has led some researchers to examine chelators that might be rapidly degraded by microbes in the environment (Grĕman et al. 2003; Kos et al. 2003; Kos and Lestan 2003; Tandy et al. 2004), but they are not always as effective and still have the potential to leach metals.

The transport of metal-chelator complexes is a passive mechanism. It is thought that the entire metal-ligand complex is taken up by the plant and transpiration drives the movement of the metal into the shoot. The plant's own transport systems are bypassed as the membrane integrity is most likely compromised at the high chelator concentrations used in these studies (Epstein et al. 1999; Vassil et al. 1998); complexes are believed to "leak" through membranes. Uptake is limited to a short time following application of the chelator, but sequential applications of lower chelator concentrations have been shown to increase total extraction (Shen et al. 2002); however, the plant is quickly killed.

Uptake of Cd and Pb by roots

Early evidence for specific mechanisms of Cd transport into plant roots was obtained via competitive inhibition studies (Hart et al. 1998; Hart et al. 2002). More recently, direct evidence of Cd transport by several nutrient metal transporters has been obtained using molecular techniques (see recent review by Colangelo and Guerinot 2006). For example, a Zn transporter from a hyperaccumulator (ZNT1, Pence et al. 2000) and a putative Fe transporter from Arabidopsis (IRT1, Korshunova et al. 1999) have been shown to transport Cd. Other less specific transporters such as HMA2 (Eren and Argüello 2004) and the low affinity cation transporter LCT1 from wheat (Clemens et al. 1998) also transport Cd.

The mechanisms of Pb transport through the plasma membrane are largely unknown. Hydroponic studies of plants grown in the presence of Pb and excess Ca or Mg have shown less transport compared to control solutions (Kim et al. 2002), suggesting that Pb ions may pass through Ca or Mg channels (Kerper and Hinkle 1997). Arazi et al. (1999) found that a calmodulin-binding transporter in tobacco plants could mediate Pb transport as well. A human divalent metal transporter, DMT1, expressed in yeast has been suggested to transport Pb via a pH-dependent process (Bannon et al. 2002). These transporters rely on the presence of free Pb ion, which is extremely low in soils.

Amino acid, peptide, and glutathione transporters

Many recent studies have identified amino acid, peptide, and glutathione transporters in plants (see reviews by Fischer et al. 1998; Stacey et al. 2002). These transporters exist throughout the plant, including in leaf cells, stem cells, and root cells (Fischer et al. 1998). Genome sequencing and experimental results with *Arabidopsis thaliana* suggest the presence of at least 20 amino acid transporters, 50 sequences related to amino acid transporters, and 51 peptide transporters (Delrot et al. 2001).

Thus far, four broad classes of amino acid transporters have been identified: acidic amino acid symport, basic amino acid symport, and two neutral amino acid symporters (e.g. cotransport of one neutral amino acid with one proton, Fischer et al. 2002; Bush 1993). Several researchers have isolated and cloned various amino acid transporters (e.g. AAP1/NAT2, Boorer et al. 1996; Chang and Bush 1997; ANT1, Chen et al. 2001; and AAP1-6, Fischer et al. 2002) to characterize their structure and function.

Studies have also shown uptake of amino acids by plants from the environment (Dahlman et al. 2004; Henry and Jefferies 2002, 2003; Persson and Näsholm 2001, 2002). Persson and Nasholm (2002) also showed that amino acid transport decreased in the presence of elevated NH_4^+ and increased in N-limited plants. In addition, the transcription of amino acid transporter genes was increased by light in dark-adapted plants, possibly due to a response that decreases the C:N ratio via uptake of amino acids (Delrot et al. 2001).

Peptide transporters have been studied less extensively. Many members of this family, including the oligopeptide transporters (OPT), pleiotropic drug resistance transporters (PDR), or peptide transporters (PTR), can transport glutathione (GSH), the major form of reduced sulfur in plants. Transport of di- and tri-peptides has been characterized in cereal leaf tissues, such as barley (Delrot et al. 2001). GSH transport has been identified in leaf tissue and protoplasts (cells with the cell wall removed) of broad bean (Jamaï et al. 1996) as well as a rice GSH transporter cloned into yeast and characterized (Zhang et al. 2004). GSH transport has also been shown to be inhibited by oxidized glutathione (GSSG) and GSH conjugates, but several amino acids, including cysteine and other di-peptides, did not interfere with transport (Jamaï et al. 1996). Additionally, the use of carbonyl cyanide 3-chlorophenylhydrazone (CCCP), an inhibitor that dissipates the proton gradient, inhibited transport of GSH and GSSG in leaf protoplast cells, suggesting that transport is proton-dependent. In root plasma

membranes these transporters are hypothesized to play a role in the scavenging of recycled proteins and oxidized GSH from the apoplasm (Delrot et al. 2001, Zhang et al. 2004).

There is some evidence in the literature that metals can be transported across membranes as part of a complex with amino acids or GSH. Cannon et al. (2001) reported that Hg- cysteine complexes passed through amino acid transporters in a mammalian system. Several studies have found that metal transporters are more active in the presence of thiols (ZntA; Sharma et al. 2000) or even dependent on the presence of cysteine or GSH for metal transport activity (P_{1B}-type ATPases, Eren and Argüello 2004; ABC transporter, Lee et al. 2005; oligopeptide transporter, Cagnac et al. 2004). Transport of Cd into plant vacuoles is mediated by phytochelatins, small sulfhydryl-containing polypeptides produced by plants to detoxify metals, though a specific transporter has not been identified (Salt and Rauser, 1995). Given the abundance and non-specific nature of amino acid and peptide transporters found in plants, it is possible that a metal-ligand complex (e.g. Pb-Cys or Pb-GSH) can be actively transported across the plant root plasma membrane.

1.4 Hypotheses

It was the objective of this thesis to find a mechanistically-based strategy for chelate-assisted remediation of Pb-contaminated soil. Experiments utilized several plant species, including model crop species *Brassica napus* (canola) and *Zea mays* (corn) and a genetically tractable plant *Arabidopsis thaliana*. A mechanistic approach will, in the long term, allow for the manipulation of biochemical transport systems either through molecular biology or nutrient manipulation. Specifically, the following major hypotheses (**H**) examining both the uptake mechanisms and soil solubility of Pb and Cd were tested:

H1. Pb or Cd is accumulated into plant tissues from hydroponic medium as a result of transport of the metal-ligand complex through one or more biological transporters such as an amino acid, GSH, or peptide transporter.

H2. Sulfur limitation will enhance uptake of the reduced-sulfur containing biochemicals, cysteine and GSH, and therefore of the metal-thiol complex.

H3. Nitrogen limitation in plants will enhance uptake of amino acids or GSH rich in reduced N, and therefore of the metal-thiol complex.

H4. Transport of the metal-ligand complex is dependent on a proton gradient across the root plasma membranes.

H5. Knock-out mutants of specific transporters in *A. thaliana* will exhibit different patterns of Pb accumulation.

H6. Pb or Cd can be solubilized from the soil matrix by the sulfhydryl-containing peptide glutathione and the amino acid cysteine.

Physiological studies were performed hydroponically to examine **H1-H5** and are discussed in Chapter 2, 3, and 4. Chapter 2 presents data confirming **H1** and then examines the mechanisms and biological activity (**H4**) involved in transport of metal-thiol complexes. Appendix C further examines uptake under nitrogen or sulfur limited conditions (**H2-3**) as well as uptake of Pb in the presence of silver (Ag), a competing thiol binding metal. Chapter 3 then presents data on the long-term root and shoot uptake of Pb and Cd, providing more evidence for **H1**, and also examines uptake patterns of Pb in transporter knockouts of *Arabidopsis thaliana* (**H5**). The other major component of the remediation system, solubilization of Pb and Cd by cysteine and glutathione in soils is discussed in Chapter 4 (**H6**). Finally, an attempt at thiol-mediated Pb and Cd remediation was examined in pot studies and is presented in Chapter 5. Chapter 6 summarizes these results and discusses the broader impacts and the future potential for chelate-assisted phytoremediation.

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CHAPTER 2

CYSTEINE- AND GLUTATHIONE-MEDIATED UPTAKE OF LEAD AND CADMIUM INTO ZEA MAYS AND BRASSICA NAPUS

2.1 Introduction

Phytoremediation has been suggested as a cost-effective strategy for removal of metal or organic contaminants from soil. However, the remediation of metals is hindered by their low solubility in soil as well as a lack of biological transport mechanisms for toxic metals. Because of this, researchers have examined chelator-assisted phytoremediation which involves the addition of synthetic chelators to soil with mature plantings (Blaylock et al. 1997; Huang et al. 1997). However, the plant is quickly killed, limiting the time that metals can accumulate in the tissues and there is also the potential to leach metals into the groundwater (Nowack et al. 2006). Possible improvements to this strategy include using biodegradable ligands to limit leaching and those that form metal complexes that can be actively taken up by plants. The future success of phytoremediation relies on knowledge, optimization, and potential genetic modification of pertinent plant transport systems to increase metal transport and uptake.

The mechanisms of Pb transport into plant roots through the plasma membrane are largely unknown, whereas several are known for Cd. Studies have suggested that Pb may be transported through a Ca or Mg channel (Kerper and Hinkle 1997; Kim et al. 2002), or through a calmodulin-binding transporter in tobacco plants (Arazi et al. 1999). A human divalent metal transporter, DMT1, expressed in yeast has been shown to transport Pb via a pH-dependent process (Bannon et al. 2002). Nutrient metal transporters for Fe, Zn, and Ca have been shown to transport Cd^{2+} with reasonable efficiency (Clemens 2001). The active substrate for all of these transporters is typically a divalent cation, which for Pb is extremely low in soils.

Many recent studies have identified amino acid, glutathione (GSH; a tri-peptide and the major form of reduced sulfur in plants), and peptide transporters in plants (Fischer et al. 2002; Stacey et al. 2002). Genome sequencing of *Arabidopsis thaliana* suggests the presence of at least 20 amino acid transporters, 50 sequences related to amino acid transporters, and 51 peptide transporters (Delrot et al. 2001). Thus far, four broad classes of amino acid transporters have been identified: acidic amino acid symport, basic amino acid symport, and two neutral amino acid symporters (Bush 1993; Fischer et al. 2002). The few amino acid transporters that have been cloned have had fairly broad substrate specificity (Fischer et al. 1998). GSH transporters have been shown to transport oxidized glutathione and glutathione conjugates (Jamai et al. 1996) and are thought to be proton-dependent transporters (Bogs et al. 2003). Of the few general peptide transporters that have been examined, many also transport glutathione (Stacey et al. 2002).

There is some evidence in the literature that thiols are involved in the transport of metals, either directly as a thiol-metal complex or indirectly by activating a transport system. Examples of direct involvement include transport of Cd into plant vacuoles mediated by phytochelatins (Salt and Rauser 1995), transport of Ag-thiosulfate complexes into algae (Campbell et al. 2002), or increased sensitivity of yeast-mutants expressing an Arabidopsis peptide transporter to Cd and Pb in the presence of GSH (Cagnac et al. 2004). Also, an ABC-type (ATP-binding cassette) transporter has been shown to contribute to Pb resistence in Arabidopsis by mediating the export of Pb-GSH complexes (Lee et al. 2005). Several studies have found that transport of metals is increased in the presence of thiols, such as the Zn-transporter ZntA (Sharma et al. 2000), or even dependent on the presence of cysteine or GSH for activity such as HMA2, a P_{IB}-type ATPase (Eren and Argüello 2004).

No previous study has demonstrated direct uptake of metals into plant roots via amino acid or peptide transport, but given the abundance and non-specific nature of these transporters in plants, we hypothesized that the uptake of toxic metals could be enhanced via active or inadvertent transport of strong biogenic thiolate-metal complexes. Characterization of this uptake mechanism is the first step toward a potentially novel phytoremediation strategy that uses thiols in soil to solubilize metals, creating a bioavailable species that can be taken up by plants. The present study examines the mechanism of uptake of Pb and Cd into *Zea mays* (corn) and *Brassica napus* (canola) in the presence of the biogenic thiols cysteine and glutathione.

2.2 Materials and Methods

Plant growth

Z. mays (DeKalb 39-47) and *B. napus* (Quantum) were germinated on paper towels in the dark for approximately 3 and 6 d, respectively. Seedlings were then transferred to hydroponic solutions in the greenhouse, subject to a daily light integral of 17 moles/m^2 (an average of about 200 μmoles/m^2/s) and day/night temperatures of 24/18 °C for 12 and 18 d for *Z. mays* and *B. napus*, respectively. The *Z. mays* nutrient solution consisted of 4.4 mM KNO_3, 2.1 mM $Ca(NO_3)_2$, 0.18 mM NH_4NO_3, 1.0 mM KH_2PO_4, 0.5 mM $MgSO_4 \cdot 7H_2O$, 63 μM K_2SO_4, 15 μM H_3BO_3, 3.5 μM $ZnSO_4 \cdot 7H_2O$, 2.5 μM $MnSO_4 \cdot H_2O$, 0.37 μM $CuSO_4 \cdot 5H_2O$, 0.25 μM $Na_2MoO_4 \cdot 2H_2O$, 17 μM $Fe(NO_3)_3 \cdot 9H_2O$, and 17 μM $Na_2H_2EDTA \cdot 2H_2O$ (Ethylenediaminetetraacetic acid). The *B. napus* nutrient solution consisted of 1.2 mM KNO_3, 0.80 mM $Ca(NO_3)_2 \cdot 4H_2O$, 2.14 mM $NH_4H_2PO_4$, 2.4 mM $MgSO_4 \cdot 7H_2O$, 0.11 μM MoO_3, 10.7 μM KCl, 5.3 μM H_3BO_3, 0.43 μM $ZnSO_4 \cdot 7H_2O$, 0.43 μM $MnSO_4 \cdot H_2O$, and 0.11 μM $CuSO_4 \cdot 5H_2O$, 4.0 μM $Fe(NO_3)_3 \cdot 9H_2O$, and 4.0 μM Na_3HEDTA (N-(2-hydroxyethyl)ethylenediaminetetraacetic acid). Reagent grade chemicals were used. All Fe solutions were stored in the dark in amber bottles. While growing plants,

solutions were kept dark and continuously aerated, replenished to maintain an electrical conductivity of 1200-1400 μS/cm for *Z. mays* and 1000 μS/cm for *B. napus*, and pH adjusted daily with 1 M KOH or 1 M HNO_3 to within the range of 5.7-6.3. *Z. mays* seedlings were placed in the center of cut styrofoam test tube plugs (VWR) and inserted into holes cut in a styrofoam sheet, 5 cm between centers, and floated in the media. *B. napus* was grown in net-cups (available at nursery supply companies), 3 plants per cup, with black plastic beads as the support medium and similarly floated after placement in styrofoam sheets, 10 cm between centers.

Short-term metal hydroponic uptake experiments

Plants were exposed to various solution treatments containing Pb or Cd, ligands, competing compounds or other inhibitors to examine metal uptake by the roots. All experimental solutions contained a background ion concentration that was about the same ionic strength of the growth solution, comprised of 1 mM NH_4NO_3, 1 mM $Ca(NO_3)_2 \cdot 4H_2O$, 3 mM KCl, 2 mM KNO_3, 1 mM $MgCl_2 \cdot 6H_2O$, and 1 mM HEPES, typically buffered at pH 7 (adjusted with 10 mM NaOH). During metal uptake experiments, the solution also contained 1.0 μM $Pb(NO_3)_2$ or 1.0 μM $Cd(Cl)_2$ and varying ligand concentrations selected to fix the Me^{2+} concentration at 10^{-8} M at pH 7 as modeled in CHEAQS (Verweij 2005) using the constants shown in Table 2.1 (Martell and Smith 2004). The metal speciation in the various uptake solutions is shown in Table 2.2. In order to maintain relatively constant thiol-metal chelate concentrations during the short-term uptake experiments, replicate plants were placed in a single large-volume container to establish a high volume to root mass ratio, thereby minimizing sorption of metals to container walls. Both thiol and metal concentrations were monitored throughout the time course (data not shown) and there was typically a proportional decrease in metal and thiol concentration. Slight deviation from the original ratios due to metal sorption, uptake of metals or thiols, or

oxidation of thiols was not sufficient to alter the speciation significantly. In contrast, the majority of Pb was lost from the solution due to root sorption when uptake experiments were performed in the absence of chelators.

Table 2.1: Binding constants of relevant Pb- or Cd-thiol species obtained from Martell and Smith (2004). I = 0.

Species	Pb	Cd
	Log β	Log β
Me-cys	13.1	11.1
Me-H-cys	17.7	16.6
Me-cys_2	19.2	17.9
Me-H-cys_2	28.2	26.2
Me-H_2-cys_2	-	32.4
Me-cys_3	-	20.1
Me-H-cys_3	-	30.3
Me-penicillamine	14.0	12.4
Me-GSH	11.4	11.0
Me-H-GSH	18.5	18.0
Me-H_2-GSH	25.1	-
Me-GSH_2	15.4	15.8
Me-H-GSH_2	25.1	26.1
Me-H_2-GSH_2	34.4	34.4

Table 2.2: Initial solution speciation for experiments testing Pb or Cd uptake with cysteine, glutathione, penicillamine, or EDTA present in solution.

Metal	Ligand (L)	Total L (μM)	Me-L (% Me) *	Me-H-L (% Me)*	Me-H_2-L (% Me) *	Me-H-L_2 (% Me) *	Me-H_2-L_2 (% Me) *
1 μM Pb_T 1% Pb^{2+}	Cysteine	2.8	98	-	-	-	-
	Glutathione	72	37	37	16	-	-
	Penicillamine	1.3	99	-	-	-	-
	EDTA	1	99	-	-	-	-
1 μM Cd_T 1% Cd^{2+}	Cysteine	200	79	3	-	14	2
	Glutathione	215	24	21	-	4	50
	EDTA	1	99	-	-	-	-

* values below 1 % are omitted

Prior to the start of each uptake experiment, plant roots were rinsed in background ion solution for 20 min. Initial experiments to examine Pb uptake were conducted with 12 plants in 8 L of solution. Solutions were bubbled with N_2 gas during the course of the experiment to limit the oxidation of thiols. Plant roots were exposed to the Pb-containing solutions for 4 hours and 3 plants were removed at 30 min, 1 hr, 2 hr, and 4 hr. Plant roots were rinsed for 10 minutes each in 4 successive 1 mM EDTA solutions (adjusted to pH 7 with 10 mM NaOH) to remove the majority of reversibly-bound Pb (data not shown), and then cut at the base of the stem and dried at 80^{o}C to a constant weight. Later experiments utilized only three plants in 2 L of solution, all of which were sampled at the 4 hr time point.

In several experiments, plants were pre-exposed to thiols in order to examine activation and up-regulation of their respective transport systems. Plants were exposed to a 1 mM solution of cysteine or glutathione for up to 6 hours prior to running Pb-uptake experiments. Other experiments tested the pre-exposure to other amino acids (lysine, aspartate, methionine), competition of Pb uptake in the presence of amino acids (proline, lysine, aspartate, glycine) or the presence of the H^+-ATPase inhibitor vanadate. The experimental conditions are specified where data is presented.

Rates of uptake were calculated based on the slope of the line for experiments with four time points, and are presented as a rate ± SE calculated on the basis of the standard deviations of the individual data points, or in later experiments using only the four hour time points with no correction for the y-intercept. Replicate experiments were conducted for those uptake experiments shown in Figures 2 and 4 and calculated rates were within 5% of each other.

Metal analysis

Dry root tissue samples from each experiment were digested in 5 mL of concentrated HNO_3 (70%; EMD Chemicals, Inc.) and heated at 90 ºC for 15 minutes,

followed by the addition of 3 mL of H_2O_2 (30%; Mallinckrodt) and heating at 110 ºC for 10 minutes. Samples were diluted and then analyzed by ICP-OES (Thermo Jarrell Ash ICAP 61). Pb measurements were normalized to root dry weights. All data points are an average (± SD) of three plants.

2.3 Results

The uptake of Pb^{2+} from solution was non-linear for both plant species examined. In *Z. mays,* root-associated Pb remained below 75 μg Pb g^{-1} DW (Figure 2.1) and was variable, which we attribute to irreversible non-specific sorption. In *B. napus*, Pb concentrations saturated during the uptake experiment at a higher concentration (200 μg Pb g^{-1} DW; Figure 2.1) after two hours. The higher apparent uptake of Pb^{2+} by *B. napus* is likely in large part due to a finer root structure and therefore a greater root surface area resulting in more irreversible, non-specific sorption. It is also possible that there is some transport of Pb^{2+} through a non-specific divalent metal transporter though the environmental relevance of transport at this high a free Pb concentration is unclear given its extremely low concentrations in soil solutions.

In contrast to the non-linear uptake seen with Pb^{2+}, when *Z. mays* or *B. napus* roots were exposed to Pb in the presence of either cysteine or glutathione (when 99% of the Pb is bound as a thiol complex), linear rates of uptake were observed (Figure 2). For *Z. mays*, the rate of uptake in the presence of cysteine or glutathione was about 30 ± 5 and 25 ± 5 μg Pb g^{-1} DW hr^{-1}, respectively (Figure 2.2a). Similarly, for *B. napus*, uptake in the presence of cysteine or glutathione was about 32 ± 1 and 15 ± 3 μg Pb g^{-1} DW hr^{-1}, respectively (Figure 2.2b). No uptake of Pb was seen for either plant species in the presence of 1 μM EDTA (Figure 2.2a,b) and very little uptake in the presence of 1.3 μM penicillamine, a thiol ligand very similar in structure to cysteine

(Figure A.1), containing two methyl groups adjacent to the thiol group, at a rate of 2 ± 1 μg Pb g^{-1} DW hr^{-1} for both plant species (Figure 2.2a,b).

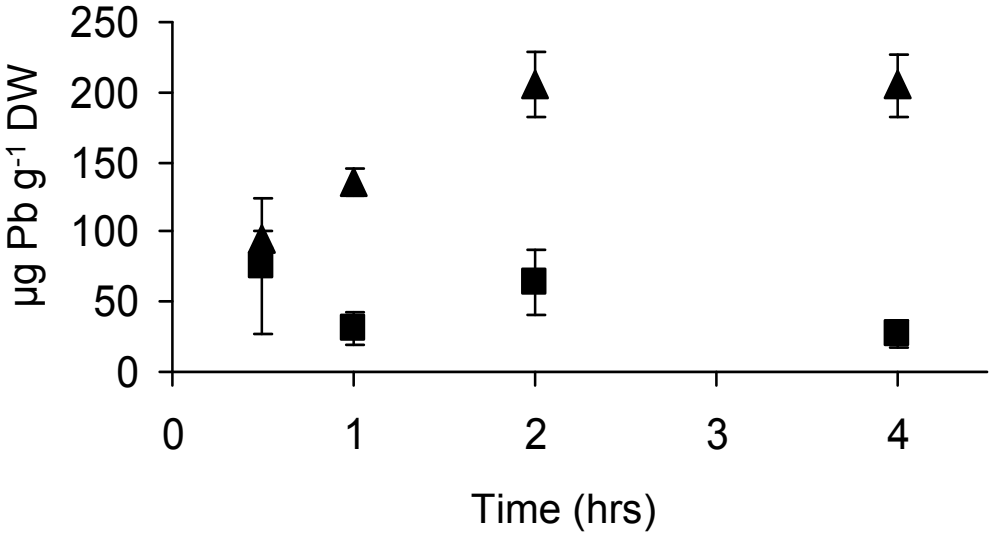

Figure 2.1: Pb concentrations in *B. napus* (▲) and *Z. mays* (■) roots after exposure to 1 μM Pb^{2+}. Values are ± SD (n=3).

In order to confirm biological activity, we included a metabolic inhibitor during the uptake experiments. *B. napus* was exposed to 0.5 mM sodium orthovanadate, an inhibitor of the plasma membrane H^+-ATPase, during uptake experiments containing cysteine or glutathione. Pb uptake was inhibited by about 50% in the presence of both ligands (Figure 2.3). This suggests the transport of Pb mediated by cysteine or glutathione is dependent on a proton gradient across the membrane.

In addition to Pb uptake, Cd uptake was also examined. When no ligands were added, linear rates of Cd uptake were measured to be 18 ± 1 μg Cd g^{-1} DW hr^{-1} in *Z. mays* and 12 ± 15 μg Cd g^{-1} DW hr^{-1} in *B. napus* (Figure 2.4). Similar rates were observed in the presence of GSH, whereas cysteine-mediated Cd uptake rates were significantly higher, by about 2-fold and 4-fold over Cd^{2+} uptake in *Z. mays* and *B. napus*, respectively (Figure 2.4). When EDTA was present, no uptake was measured.

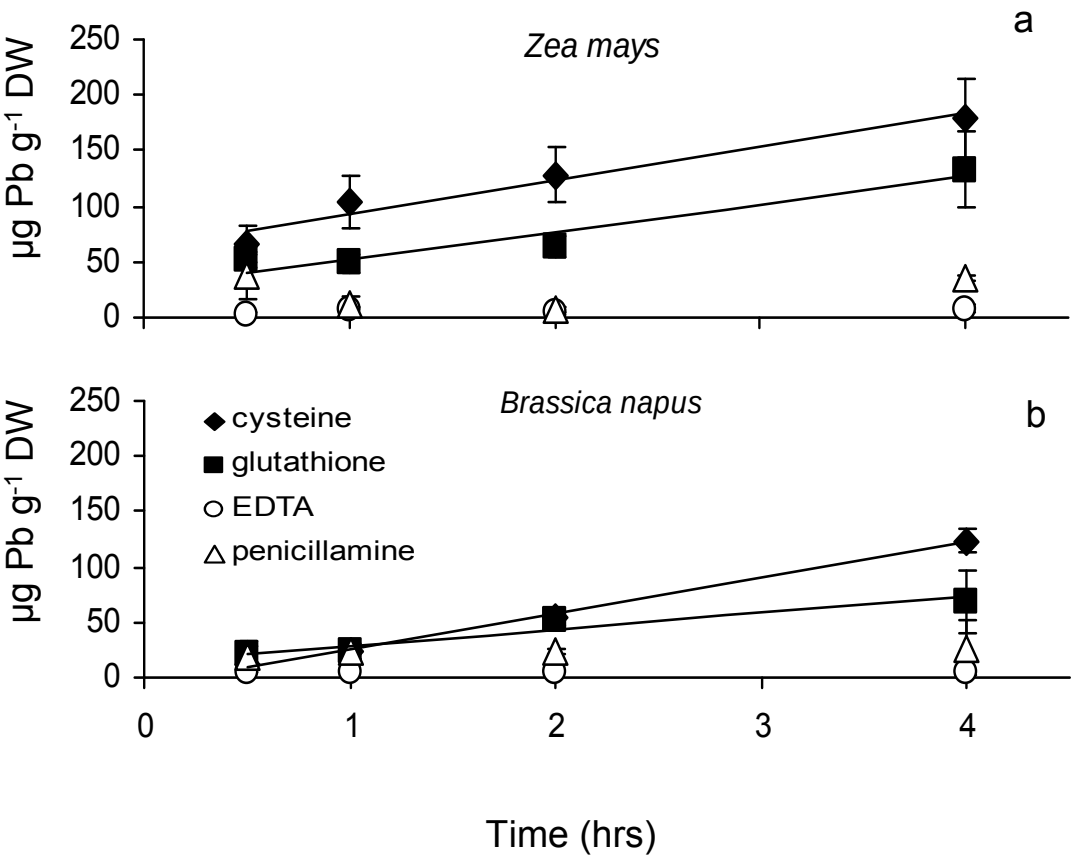

Figure 2.2: Uptake of Pb into plant roots from hydroponic solution while exposed to 1 µM Pb and 2.8 µM cysteine, 72 µM GSH, 1 µM EDTA, or 1.3 µM penicillamine. Values are ± SD (n=3).

In order to examine potential up-regulation of transport systems, *B. napus* plants were pre-exposed to 1 mM thiols prior to Pb uptake experiments for variable amounts of time. As we increased the pre-exposure time from 5 min up to 6 hr, we saw an increasing rate of Pb uptake that began to level off after 2 hours of pre-exposure at about 50 to 70 µg Pb g^{-1} DW hr^{-1} for cysteine- and glutathione-treated plants (Figure 2.5). The time-scale of increasing uptake rates is more consistent with biological activation or up-regulation of specific amino acid or glutathione transporters as opposed to a chemically induced change in the number of binding sites. Typically, we saw a 2 to 3-fold increase in rate for both *Z. mays* (sometimes up to 9-

fold, data not shown) and *B. napus* (e.g. Figure 2.5) following 6 hours of pre-exposure.

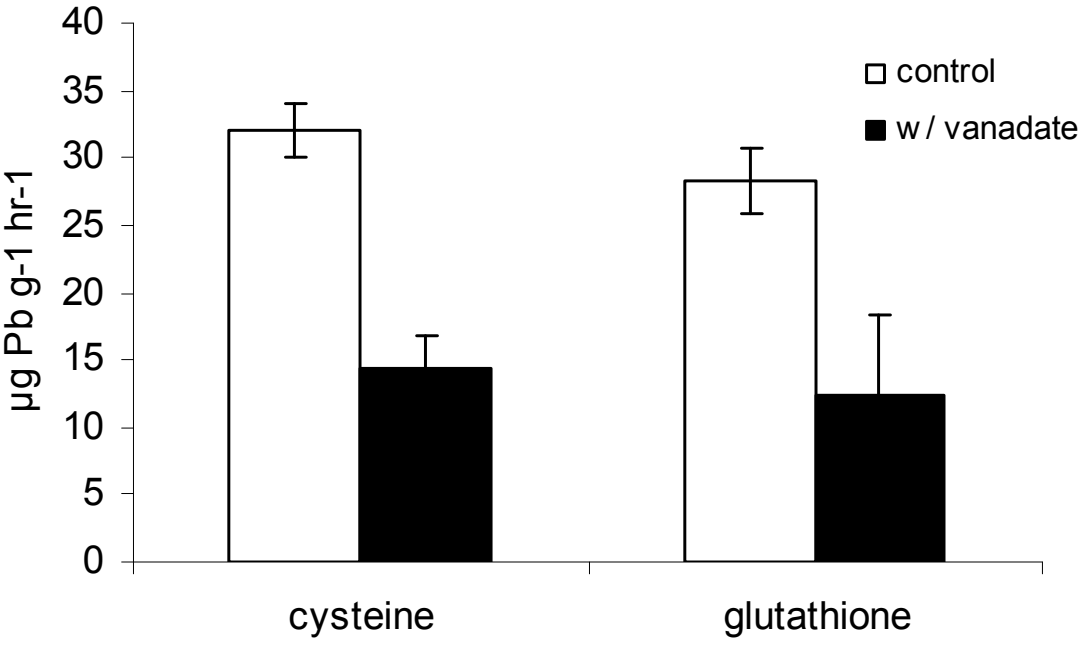

Figure 2.3: Inhibition of Pb uptake when exposed to 0.5 mM sodium orthovanadate for 1 hour prior to and during the 4 hour uptake experiment. Values are ± SD (n=3).

We also examined uptake of Pb in *B. napus* in the presence of increasing ligand concentrations. As the ratio of Pb:GSH increased from 1:1 to 1:400, the uptake of Pb decreased by a factor of 2.5 (Figure 2.6a). Increasing cysteine concentrations, initially from a Pb:cys ratio of 1:1 up to 1:20, resulted in slightly decreasing Pb uptake rates (see inset Figure 2.6b) but then rates began to increase linearly up to a ratio of 1:1000, with no apparent saturation (Figure 2.6b).

Adjusting the pH of the uptake solution or varying the total Pb concentration had significant effects on uptake rates (Table 2.3). In the GSH experiments, the reduction in pH from 7 to 6 resulted in a 176% increase in the rate of accumulation of Pb in the roots, while no significant difference was observed at pH 8. The observed

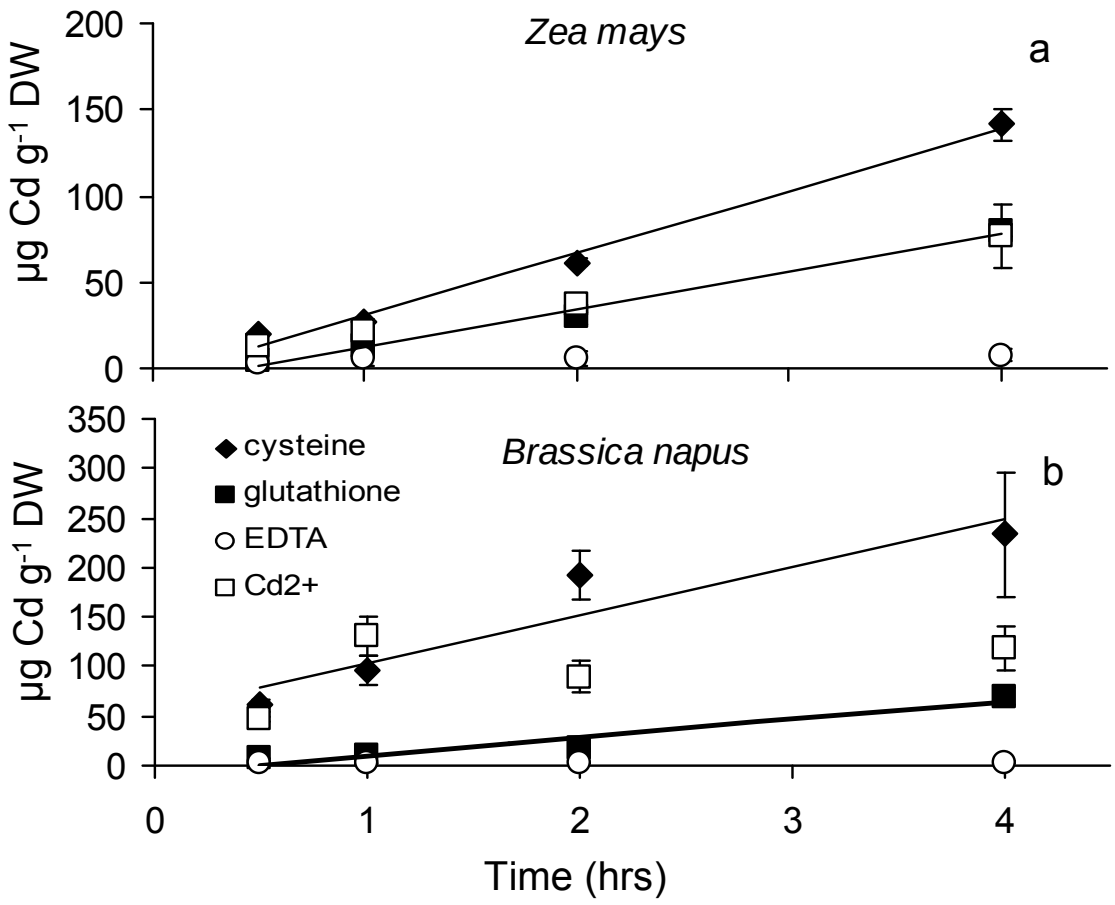

Figure 2.4: Uptake of Cd into plant roots from hydroponic solution while exposed to only 1 µM Cd or 1 µM Cd and 200 µM cysteine, 215 µM glutathione or 1 µM EDTA. Values are ± SD (n=3).

increase could be the result of a shift in the dominant form of GSH from Pb-H-GSH at pH 7 (Table 2.2) to Pb-H_2-GSH^+ at pH 6 (75% of the Pb species). This charged species may be the compound actively taken up by the transporter.

In the cysteine experiments, changing the pH up or down slightly lowered the rate of uptake, however only at pH 6 does the solution speciation change significantly (more free Pb as compared to mostly Pb-cys at the higher pHs), and perhaps the lower proton gradients resulted in less uptake at pH 8. As expected, linear changes in

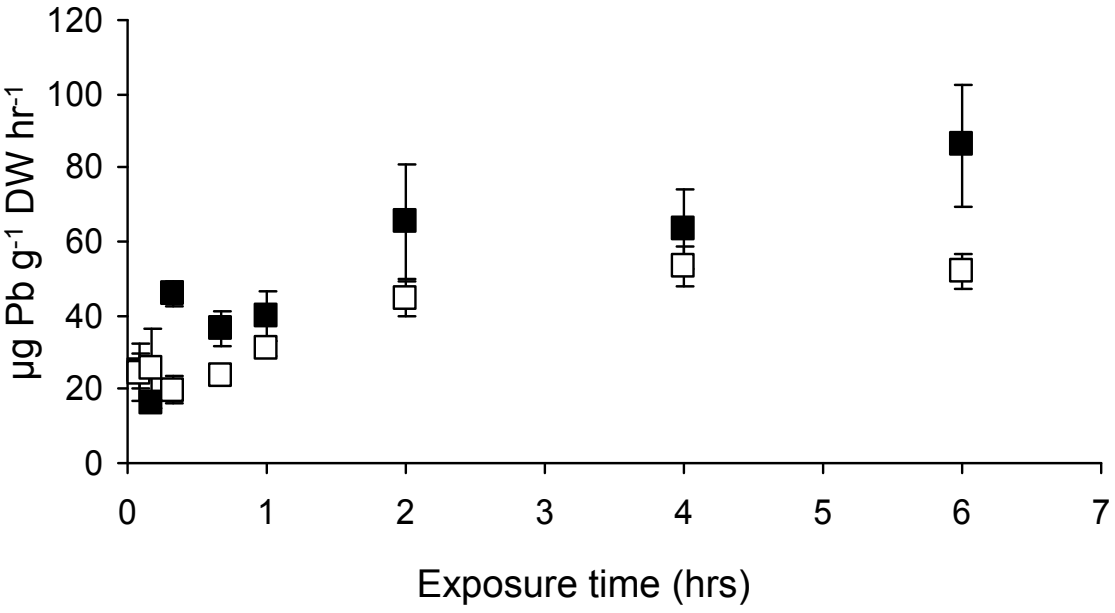

Figure 2.5: Effect of pre-exposure time to 1 mM thiols on Pb uptake rate in *B. napus*. Uptake experiments were conducted for 4 hours with 2.8 µM cysteine (■) or 72 µM GSH (□). Rates were calculated from the average of 3 plants (± SD) at the 4 hour time point only.

glutathione-mediated Pb uptake rates were observed as the Pb concentration was changed from 0.5 to 2.0 µM (Table 2.3). Likewise, cysteine-mediated Pb uptake increased from 0.5 to 1.0 µM, but remained relatively constant from 1.0 to 2.0 µM.

Experiments were also conducted in the presence of other amino acids (negative, neutral, or positive species) to examine the specificity of the transporter. In most cases, cysteine-Pb uptake in the presence of proline, lysine, aspartate, or glycine (100 or 500 µM) resulted in no significant difference in Pb uptake compared to a control (Table A.1). We also examined pre-exposure to 1 mM lysine, asparatate, or methionine and also saw no apparent up-regulation of active Pb transport. One experiment in which plants were pre-exposed to 1 mM cysteine and then exposed to increasing concentrations of methionine (100-800 µM) resulted in large increases in uptake rate (up to 500%) compared to the control.

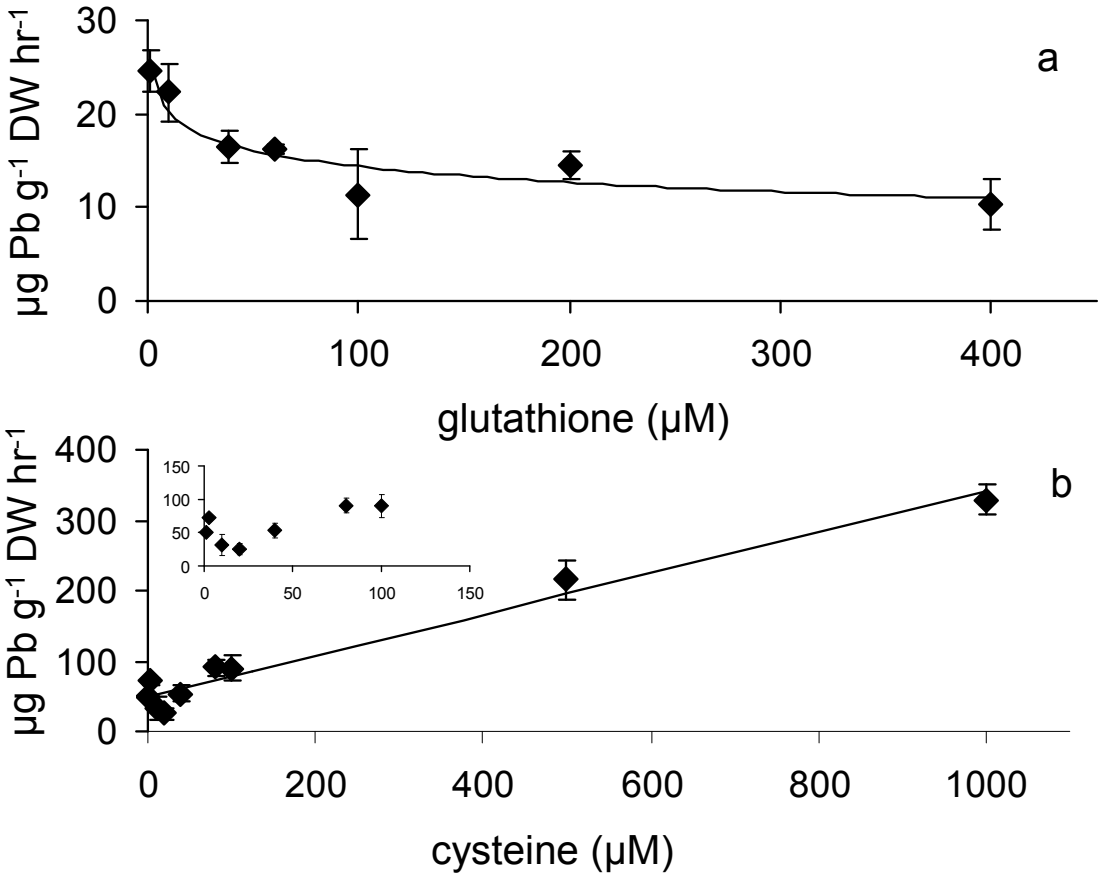

Figure 2.6: Pb uptake rates into *B. napus* roots over varying solution concentrations of thiol ligands glutathione (a) or cysteine (b) at a constant 1 µM Pb concentration. Values are ± SD (n=3)

Table 2.3: Percent change in Pb uptake in *B. napus* due to a change in solution pH to 6 or 8 compared to pH 7 and in the presence of 0.5 or 2.0 µM total Pb compared to 1.0 µM. Values are compared to a base rate of uptake of 32±1 µg/g/hr for cysteine and 15 ±3 µg/g/hr for glutathione.

Uptake solution conditions*	2.8 µM Cys	72 µM GSH
1 µM Pb, pH 6	-30%	+176%
1 µM Pb, pH 8	-42%	+5%
0.5 µM Pb, pH 7	-42%	-50%
2 µM Pb, pH 7	+16%	+217%

* other conditions unchanged

2.4 Discussion

This study examined the uptake of Pb and Cd into *B. napus* and *Z. mays* in the presence of the biological ligands cysteine and glutathione. The observed uptake appears to be specific for these two biological ligands as no uptake was seen in the presence of the synthetic chelator EDTA or the thiolate-containing amino acid penicillamine. Our results suggest that the uptake is mediated by transporters since pre-exposure to cysteine or glutathione resulted in elevated uptake rates and uptake was inhibited in the presence of vanadate. Inhibition of Pb uptake rates by increasing GSH concentrations and enhanced rates with increasing cysteine concentrations may reveal mechanistic clues about the specific transporters involved.

The GSH-mediated uptake of Pb and Cd observed here is possibly facilitated by one or more peptide transporters present in the plasma membrane of the plant roots, possibly from the PTR family. These transporters are believed to be responsible for redox control and possibly retrieval of GSSG, GS conjugates and nitrogen-containing peptides from the cell wall (Delrot et al. 2001; Zhang et al. 2004). Several members of this transporter family have been shown to transport GSH-conjugates (Zhang et al. 2004) or GSH-metal complexes (Cagnac et al. 2004) and at least one member is transcriptionally regulated by Cd (Bogs et al. 2003). Here, the observed inhibition of Pb uptake in the presence of increasing GSH concentration (Figure 2.6) and the apparent up-regulation of Pb uptake following pre-exposure to GSH (Figure 2.5) are consistent with transport via a peptide transporter that does not differentiate between GSH and the Pb complex. In addition, lowering the pH of the hydroponic medium from 7 to 6 also increased uptake; both Bogs et al. (2003) and Zhang et al. (2004) found a pH optimum of 5 for a peptide transporter from *B. juncea* and rice, respectively. It is not known whether this is due to a change in the protonation of the substrate as previously mentioned for GSH. Peptide transport is thought to occur via a

proton co-transport system (it is sensitive to an uncoupler, CCCP) and thus would be sensitive to pH. Also consistent with our conclusion that Pb transport is mediated by peptide transporters is the nearly linear variation of Pb uptake with Pb concentration. Linear changes in uptake rate are expected at concentrations below the K_m, and reported K_ms for so-called high affinity glutathione transport systems range from 7 – 55 μM (Foyer et al. 2001).

An amino acid transporter may be responsible for the observed transport in the presence of cysteine, but it is also possible that uptake is mediated by a peptide transporter that recognizes the Pb- or Cd-cysteine complex. Several transporters in the PTR family have been shown to transport amino acids, di- or tri-peptides, and nitrate (Zhou et al. 1998) and some are known to be expressed in the root epidermis (Stacey et al. 2002; Hirner et al. 2006). In our study, when other amino acids were added to solution to compete for uptake or potentially up-regulate transport via a general amino acid transporter, we saw no significant change in Pb uptake. However, we did see a large increase in uptake from cysteine pre-treated plants when methionine was present, which suggests that a methionine transporter may actively take up a Pb-cysteine complex as the structures of these two substrates are very similar, or that methionine may activate a cysteine or peptide transporter. Though no methionine transporters have been identified in plants, there is evidence for them in *E. coli* (Kadner and Watson 1974).

Inhibition of Pb uptake by cysteine was only evident at low concentrations and then increasing uptake rates were observed with increasing cysteine concentration (Figure 2.6b). This is perhaps indicative of a switch from one type of transport to another. In this experiment, the dominant speciation of the Pb changes from the mono to the bis complex as we increased cysteine concentration. The bis complex may be

more similar in size and structure to the di-peptides transported by the PTR (peptide transporter) family and therefore may be more efficiently taken up.

Whole-plant experiments of this nature cannot distinguish between multiple transport systems and therefore our results may be confounded by several processes occurring at the same time. For example, there is indirect evidence that GSH mediates efflux of metals across the plasma membrane via an ABC-type transporter (Kim et al. 2007) and specific ones increase resistance of plants to Pb (Lee et al. 2005). ABC-type transporters are fairly specific for GSH-conjugates and not inhibited by oxidized or free reduced GSH. Also, plant P-type ATPases with specificity for Cd have been shown to mediate efflux of the free metal (Eren and Argüello 2004) and similar transporters from *E. coli* export Pb across the plasma membrane (Sharma et al. 2000); several of these are dependent upon the presence of reduced thiols though the mechanism is not clear. In our plants, we don't know if efflux is significant or up-regulated in some experiments, but this may also influence the observed uptake rates. It is also difficult to distinguish the effects of a change in membrane permeability or membrane potential due to experimental conditions on the rates of uptake, but no literature is available on the effect of thiols on these parameters.

The identification of the biological mechanism explored in this chapter is just the first step toward an effective application in phytoremediation. In particular, we must demonstrate that there is subsequent movement of metals to the shoots so that metals can be harvested with the above ground biomass. In our short-term experiments, and also in longer-term experiments at higher Pb concentrations (Chapter 4), no Pb transport to the shoot was observed. Pb is likely sequestered in the vacuoles of the root cells via specific transporters (Martinoia et al. 1993; Gravot et al. 2004). If pertinent membrane transporters could be turned off, the plant would be forced to transport metals to its shoots for sequestration. Another possibility is that since our

plants were grown in high phosphate medium prior to uptake experiments, the Pb is trapped in the roots in Pb-phosphate precipitates. Electron microscope studies are planned to compare Pb compartmentalization in root cells following uptake of free Pb and Pb uptake in the presence of thiol ligands. An additional challenge to the ultimate application of this strategy in the field is economically feasible delivery of reduced thiols to the root zone or generation of thiols *in situ* in a manner that promotes Pb solubility. This may be accomplished in the future by novel time-release materials or potentially genetic modification of plants or associated microbes.

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CHAPTER 3

LEAD UPTAKE AND DISTRIBUTION IN *BRASSICA NAPUS* AND *ARABIDOPSIS THALIANA*

3.1 Introduction

The success of phytoextraction relies partly on a mechanism of transport of the contaminant metal across the root plasma membrane, as demonstrated in Chapter 2, and also on translocation in the plant from the roots to the shoots. The use of biogenic thiol ligands cysteine and glutathione as compared to previous research on chelate-assisted phytoremediation with synthetic ligands (Blaylock et al. 1997, Wu et al. 1999), likely leads to different mechanisms of uptake, transport within the plant and ultimately different compartmentalization of the metal within the plant. The uptake of Pb when bound to EDTA (ethylenediaminetetratacetic acid) is thought to be driven by transpiration via a non-specific or passive mechanism across the root plasma membrane, ultimately accumulating in the shoots as Pb-EDTA complexes as has been observed in shoot tissue of *Brassica juncea* (Vassil et al. 1998). This is mainly due to the strong binding constant of the chelator such that there is little exchange with ligands, enzymes or transporters that may bind other metals in the cytoplasm. In contrast, cysteine and glutathione are known components of a plant cell's biochemistry, present at mM concentrations in the cytoplasm. When cysteine or glutathione bind Pb, this likely leads to transport across the plasma membrane via an amino acid or peptide transporter (Chapter 2). There are several transporters that may play a role in both plasma membrane and vacuolar membrane transport of metal thiol complexes (Foyer et al. 2001; Fischer et al. 1998). However, it is unknown how plant biochemistry may interact with the Pb-thiol species once that compound enters the cytoplasm and whether shoot translocation or vacuolar sequestration may occur.

There are relatively few studies on transport systems that translocate glutathione-conjugates, such as oxidized glutathione or metal-glutathione complexes, and none that examine transport of amino acid-metal conjugates in plants. Several members of the oligopeptide transporter family (OPT) in *Arabidopsis thaliana* have been shown to transport glutathione and glutathione conjugates, possibly Cd-GSH (Cagnac et al. 2004). OPTs are typically expressed in the plasma membranes of the plant (Koh et al. 2002). There is also evidence that AtPDR8 and AtPDR12 (pleiotropic drug resistance family) are plasma membrane transporters that confer resistance to Pb and Cd via extrusion of a free metal (Kim et al. 2007) or metal-conjugate (Lee et al. 2005).

Once toxic metals reach the cytoplasm, particularly Cd, a detoxification mechanism involving phytochelatin is induced. Phytochelatins (PC), small sulfhydryl-containing chains of glutathione molecules, strongly bind metals such as Cd and Pb in the cytoplasm. The metal-glutathione molecule is likely the substrate for phytochelatin synthase (Vatamaniuk et al. 2000) and possibly the inducer of the mRNA as well. Once PC-metal complexes are formed, this complex may be transported across the vacuolar membrane (Salt and Rauser 1995; Kotrba et al. 1999) though no specific plant vacuolar transporters have been identified (Clemens 2006). Thus, if Pb- or Cd-glutathione molecules enter the root cells, they may trigger this detoxification mechanism in the root cells, leading to sequestration in the root vacuoles. An alternative means of vacuolar sequestration may be the translocation of free metal ions into the vacuole via a heavy metal transporter, such as HMA3, which is thought to be responsible for Cd influx into vacuoles (Gravot et al. 2004).

This study examines the long-term uptake of Pb and Cd into both roots and shoots of *Brassica napus* under cysteine and glutathione exposure. In addition, differences in uptake are examined in *Arabidopsis thaliana* tDNA insertion mutants

for several transporters, including some of the peptide and heavy metal transporters mentioned above. Amino acid transporter knockouts were not available and therefore could not be examined. Finally, transmission electron microscopy was used to observe differences in Pb uptake in *Brassica napus* roots between control and thiol treatments.

3.2 Materials and Methods

Plant growth

B. napus (Quantum) was germinated on paper towels in the dark for 6 d. *A. thaliana* (Columbia) seeds were first cold-treated for 3 days by placement on wet paper towel lined petri dishes in the refrigerator at 4 °C. *B. napus* seedlings were placed in net-cups (available from nursery supply companies), initially 3 plants per cup and later 1 plant per cup, with black plastic beads as the support medium. Several *A. thaliana* seeds (3-6 seeds) were placed in rockwool blocks that used 2 mL microcentrifuge tubes, with the bottoms cut off, as support. The net-cups or microcentrifuge tubes were then placed in styrofoam sheets and floated in the nutrient solution with *B. napus* placed at 10 cm between centers, and *A. thaliana* placed at 3 cm between centers. *B. napus* plants were subject to a daily light integral of 17 moles/m^2 (an average of about 200 μmoles/m^2/s) and day/night temperatures of 24/18 °C for 18 d. *A. thaliana* plants were placed in a growth chamber with average light levels of 340 μmol m^{-2} s^{-1} for 20 hours and at 25 °C for 28 d. The nutrient solution consisted of 1.2 mM KNO_3, 0.80 mM $Ca(NO_3)_2 \cdot 4H_2O$, 2.14 mM $NH_4H_2PO_4$, 2.4 mM $MgSO_4 \cdot 7H_2O$, 0.11 μM MoO_3, 10.7 μM KCl, 5.3 μM H_3BO_3, 0.43 μM $ZnSO_4 \cdot 7H_2O$, 0.43 μM $MnSO_4 \cdot H_2O$, and 0.11 μM $CuSO_4 \cdot 5H_2O$, 4.0 μM $Fe(NO_3)_3 \cdot 9H_2O$, and 4.0 μM Na_3HEDTA (N-(2-hydroxyethyl)ethylenediaminetetraacetic acid). Reagent grade chemicals were used. All Fe-chelate stocks were stored in the dark in amber bottles. While growing plants, solutions were kept dark and continuously aerated, replenished

to maintain an electrical conductivity of 1000 μS/cm, and pH adjusted daily with 1 M KOH or 1 M HNO_3 to within the range of 5.7-6.3.

Long-term hydroponic metal uptake experiments

In initial *B. napus* experiments, net-cups containing 3 plants were exposed for 5 days to various solutions containing 10 μM $Pb(NO_3)_2$ or $CdCl_2$ with or without ligand concentrations of 10 μM disodium ethylenediaminetetraacetic acid (Na_2EDTA), or a one time (first day) or daily addition of 28 μM cysteine or 380 μM glutathione. In later experiments, *B. napus* in individual net-cups, one age 18 d plant per net-cup, and *A. thaliana* in microcentrifuge tubes, 2-4 age 28 d plants per tube, were exposed to these same solutions exchanged daily for 5 days. For electron microscope samples, two sets of experiments were run using *B. napus*, one containing 10 μM $Pb(NO_3)_2$ and the ligands as above, and the other containing 50 μM $Pb(NO_3)_2$ with or without ligand concentrations of 50 μM Na_2EDTA, 140 μM cysteine, or 1900 μM glutathione. All experimental solutions contained a background ion concentration that was about the same ionic strength of the growth solution, comprised of 1 mM NH_4NO_3, 1 mM $Ca(NO_3)_2 \cdot 4H_2O$, 3 mM KCl, 2 mM KNO_3, 1 mM $MgCl_2 \cdot 6H_2O$, and 1 mM HEPES, buffered at pH 7 (adjusted with 10 mM NaOH or 10 mM HNO_3). Volumes of 2 L and 1 L were used for *B. napus* and *A. thaliana*, respectively. For *A. thaliana*, 6 microcentrifuge tubes with 2-4 plants each were exposed to the experimental solution and roots and shoot samples were pooled to obtain sufficient biomass. Roots were sampled after 5 days exposure and were rinsed for 10 minutes each in 4 successive 1 mM EDTA solutions (adjusted to pH 7 with 10 mM NaOH) to remove surface bound Pb. Root samples of both species and shoot samples from *A. thaliana* were placed in glass test tubes while shoot samples from *B. napus* were placed in paper bags prior to drying at 70 ^{o}C to a constant weight.

Electron microscope sample preparation and analysis

Root samples for the transmission electron microscope were taken from the 5-day *B. napus* Pb uptake experiment in the presence of both 10 and 50 μM Pb and ligands as described above as well as a control plant not exposed to Pb. Electron microscope slides were prepared by the Cornell Center for Materials Research Integrated Advanced Microscopy facility. Live samples, mostly root tips, were first fixed with glutaraldehyde to cross link proteins and then with osmium tetroxide to stabilize lipid molecules (Sabatini et al. 1963). Samples were then dehydrated in successively higher concentration ethanol solutions and infiltrated with a resin. Once the samples are fully cured and embedded in the epoxy resin, thin slices were taken and placed on a copper electron microscope grid for use. Samples were examined using a Tecnai T-12 transmission electron microscope. Representative samples from each experimental treatment are presented in the results.

Arabidopsis thaliana T-DNA insertion mutants

A. thaliana T-DNA insertion mutants based on the Columbia (Col) ecotype were obtained from SALK Institute lines distributed by the Arabidopsis Biological Resource Center (ABRC, Ohio State University, Columbus, OH, USA). The mutant identification numbers and gene information is described in Table 3.1.

Metal analysis

Dry *B. napus* shoot samples were ground with a mortar and pestle and about 0.1 g was weighed into glass test tubes for digestion. All *B. napus* samples and shoot samples of *A. thaliana* were digested first with 5 mL of concentrated HNO_3 (70%; EMD Chemicals, Inc.) and heated at 90 ºC for 15 minutes, followed by the addition of 3 mL of H_2O_2 (30%; Mallinckrodt) and heating at 110 ºC for 10 minutes. Due to the low biomass of *A. thaliana* roots, samples were digested with 2 mL concentrated HNO_3, followed by 1 mL 30% H_2O_2 using the same grade chemicals and heating scheme.

Table 3.1 *Arabidopsis thaliana* SALK Institute T-DNA insertion mutants obtained from the Arabidopsis Biological Resource Center.

Identification number	Knockout gene	Reference*
SALK_113350	OPT7	
SALK_038178	OPT5	
SALK_013945	PDR12	Lee et al. 2005
SALK_003119C	PTR3	
SALK_025186	PTR3	
SALK_000578C	PDR8	Kim et al. 2007
SALK_020948[a]	PDR8	Kim et al. 2007
SALK_073511	HMA3	Gravot et al. 2004
SALK_088015	HMA3	Gravot et al. 2004

*Knockouts without a reference have not been specifically studied and/or have not been studied in relation to metal transport.
[a] This mutant did not grow

Samples were diluted and then analyzed by ICP-OES (Thermo Jarrell Ash ICAP 61). Pb measurements were normalized to root dry weights.

Statistical analyses

A one-way analysis of variance (ANOVA) was performed on the Pb uptake data using Excel.

3.3 Results and Discussion

Long-term hydroponic metal uptake

As expected, the presence of EDTA limited uptake of Cd or Pb into roots (Figure 3.1, 3.2). However, uptake of free Cd after five days was higher than uptake under any of the thiol treatments (Figure 3.1), which is in contrast to uptake of free Pb (Figure 3.2). Neither the one time or daily additions of thiols in Cd or Pb treatments resulted in significantly different metal uptake from each other; this is likely due to the

resultant uptake all occurring over day one since greater than 90% of the metal in both cases was removed from solution after only one day (data not shown). Compared to the EDTA treatment in both the Cd and Pb experiments, the presence of cysteine and glutathione resulted in significantly increased uptake of the metal. The difference in uptake for Pb was not as high, with uptake in the presence of cysteine or glutathione at about 1 to 6 times higher than in the presence of EDTA, respectively (Figure 3.2).

Since all the metal was lost from solution within the first day, in part due to uptake and part due to root sorption, experiments were repeated with Pb treatment solutions exchanged daily. Uptake of Pb in the presence of glutathione was much higher than all the other treatments, at about 35000 ppm Pb in the roots (Table 3.2). The rate of uptake was about 295 μg/g/hr, which is 5-20 times higher than the rates calculated in short-term experiments (Chapter 2). The uptake of Pb in the presence of cysteine was lower than glutathione, but still significantly higher than both free Pb uptake and uptake in the presence of EDTA, at about 4000 ppm in the roots and at a rate not significantly different from the short-term uptake experiments (Chapter 2). Uptake into the shoots was not observed in most treatments, except for about 35 ± 15 ppm Pb in shoots in the EDTA treatment (Table 3.2).

Uptake of free Cd is known to occur in plants via a variety of transporters, including several Zn specific transporters or other divalent metal transporters (Clemens 2001). It seems that the presence of thiol ligands increased uptake of Cd bound to thiols as compared to the EDTA control, but does not mediate uptake to a greater extent than free Cd. However, some of this uptake may be due to oxidation of the thiols and therefore release and subsequent transport of free Cd. In contrast, the thiol-mediated Pb uptake is large and likely due to transport via an amino acid or peptide transporter as described in Chapter 2.

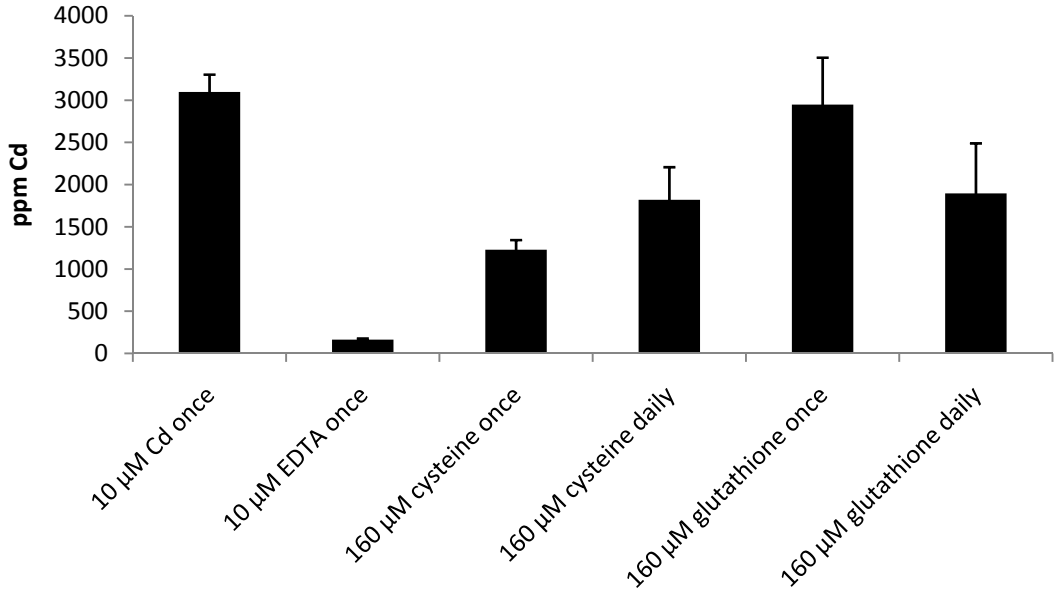

Figure 3.1 Root uptake of Cd in *Brassica napus* exposed to a one time or daily addition of ligand as shown. Values are ± SD (n=3).

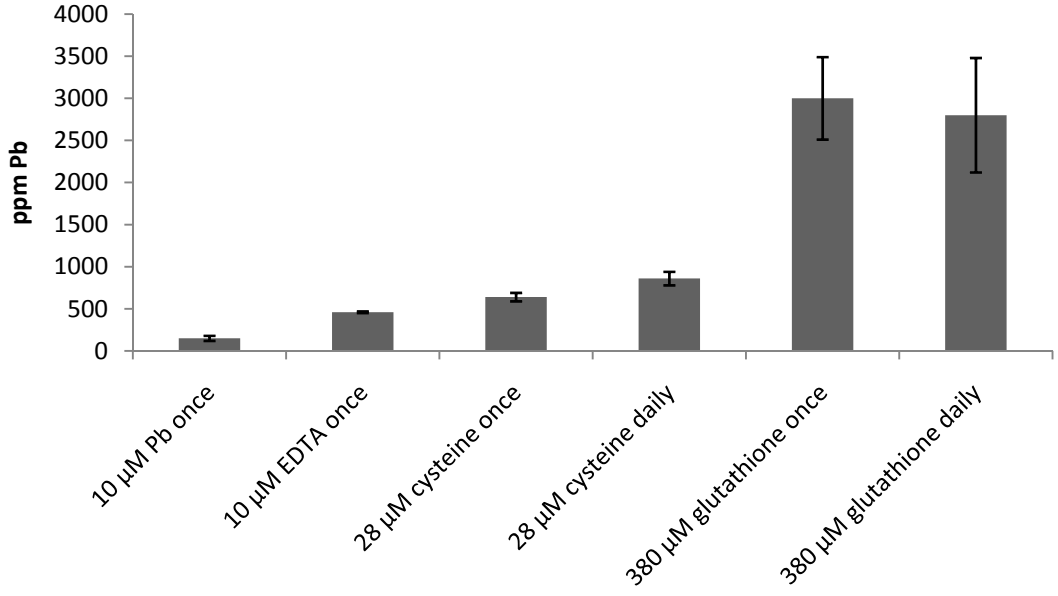

Figure 3.2 Root uptake of Pb in *Brassica napus* exposed to a one time (first day) or daily addition of ligand as shown over 5 days. Values are ± SD (n=3).

Table 3.2 Root uptake of Pb in *Brassica napus* exposed to solutions containing 10 μM Pb with or without a ligand, exchanged daily for 5 days. Values are ± SD (n=3).

Ligand treatment	Root Pb uptake (ppm)	Shoot Pb uptake (ppm)
No ligand	2800 ± 200	b.d.
10 μM EDTA	900 ± 130	35 ± 10
28 μM cysteine	4100 ± 600	b.d.
380 μM glutathione	35500 ± 7500	b.d.

b.d. = below detection (< 10 ppm)

In addition, no shoot translocation was observed, which may be in part due to the low concentrations used and exposure time of only 5 days, but may also suggest a strong sequestration mechanism in the roots. In particular, the Pb-glutathione taken up into the roots may be directly transported into the vacuole or may trigger a phytochelatin detoxification mechanism which ultimately sequesters metals in the vacuole (Kotrba et al. 1999).

Uptake experiments were repeated under varying thiol concentrations. Increasing the glutathione concentration increased uptake, but leveled off at about 32000 ppm Pb over the range of glutathione concentrations used here (Figure 3.3). The Pb-glutathione species may be the active substrate for the transporters involved and thus when Pb-glutathione species reach their maximum concentration at 380 μM glutathione and above, the uptake rate is at its maximum. Results from Chapter 2 suggested that free glutathione competes with Pb-glutathione uptake in the short-term. However, over a longer time period a homeostasis may have been reached between uptake, export and vacuolar sequestration of both Pb-glutathione and free glutathione species, resulting in similar total uptake even at higher free glutathione concentrations. In contrast, increasing cysteine concentrations showed about a doubling in uptake

from 1:1 to 2.8:1 cys:Pb ratios, then a drop in uptake at 10:1 cys:Pb ratios and a very large increase in Pb uptake at 100:1 cys:Pb ratios, up to about 15000 ppm Pb in the roots (Figure 3.4). The pattern in cysteine mediated Pb uptake may be in part due to a switch in speciation from 1:1 Pb:cys species to 1:2 Pb:cys species at higher concentrations, which may be a more active substrate for peptide transporters. Once again, shoot translocation did not occur (data not shown).

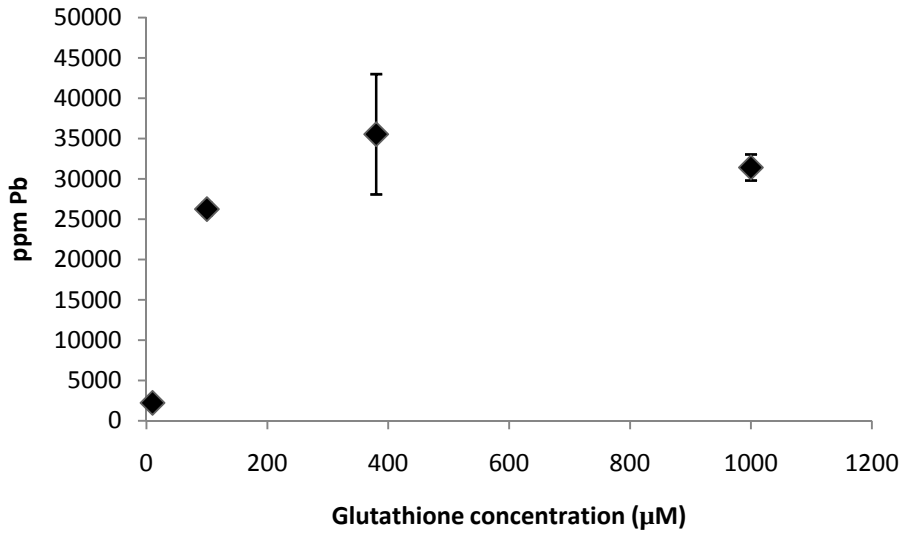

Figure 3.3 Uptake of Pb in *Brassica napus* roots over 5 days in solution containing 10 µM Pb and varying glutathione concentrations exchanged daily. Values are ± SD (n=3).

Arabidopsis thaliana knockouts

Six mutant lines of *Arabidopsis thaliana*, each containing a tDNA insertion disrupting the transcription of one transport gene linked to either glutathione or free metal transport were examined for the uptake of Pb into roots and shoots in the presence of glutathione. In four knockout lines, uptake of Pb was much greater than in the wildtype, including those lacking the AtOPT7, AtPDR12, AtPTR3, and AtHMA3 transporters (Figure 3.5a). AtOPT7, AtPDR12, and AtPTR3 are known plasma membrane transporters and although the substrates of these three transporters have not

been identified, related transporters are thought to export glutathione across the plasma membrane (AtPTR1; Dietrich et al. 2004; AtOPT6; Cagnac et al. 2004).

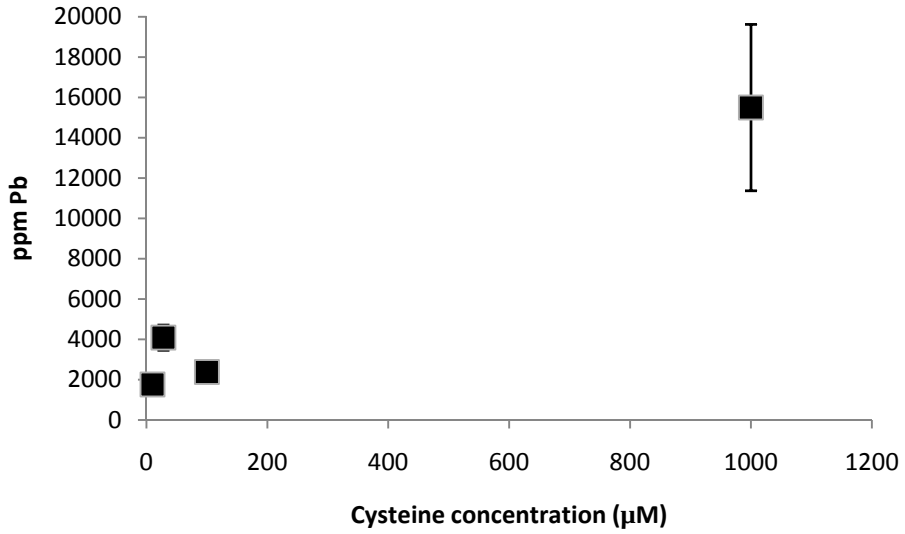

Figure 3.4 Uptake of Pb in *Brassica napus* roots over 5 days in solution containing 10 µM Pb and varying cysteine concentrations exchanged daily. Values are ± SD (n=3).

Increased accumulation of Pb in root tissue is consistent with a putative role in Pb export. Cagnac et al. (2004) also observed that AtOPT7 did not restore growth in yeast grown with glutathione as the sole sulfur source, possibly speaking to its role as an exporter and not an importer. It is unknown why uptake is higher with the AtHMA3 knockout since it is thought to play a role in root vacuolar sequestration of Pb and Cd (Gravot et al. 2004).

Uptake in the AtPDR8 knockout, a transporter thought responsible for Pb^{2+} export (Kim et al. 2007), was not significantly different from the wildtype, suggesting it plays no role in Pb homeostasis in this instance. Since any Pb transported into the cell is likely in the form of Pb-glutathione, the AtPDR8 protein may not be able to compete for binding of Pb.

Root uptake was significantly lower than the wildtype in only one knockout species, AtOPT5 (Figure 3.5a). The function and substrate of AtOPT5 has not been studied but it is a plasma membrane transporter and other members of the OPT family are known to transport glutathione (Cagnac et al. 2004). Similar to *B. napus*, shoot translocation was not observed in most knockouts, except in the case of the AtOPT5 knockout, resulting in shoot concentrations of about 150 ppm Pb (Figure 3.5b). The sum of the total mass of Pb in the roots and shoots of the OPT5 knockout accounted for about 90% of the Pb in the wildtype (normalized to total biomass). One possibility for the observed phenotypes is the upregulation of a less discriminating transporter in the absence of OPT5, possibly playing a role in loading the xylem with glutathione and allowing transport of Pb-glutathione into the xylem and ultimately the shoots.

Transmission electron microscopy

The transmission electron microscope was used to visualize *B. napus* root cells under various treatments Pb and ligand treatments. The one sample taken for total Pb analysis and root concentrations at 10 μM Pb was similar to previous experiments (Table 3.3). Higher solution Pb concentrations resulted in higher root concentrations for no ligand, cysteine, and glutathione treatments, but were about the same in the EDTA treatment.

Transmission electron micrographs of control plant roots (no Pb treatment) are shown in Figure 3.6 and 3.7. Several cell components are visible, including the cell wall, cytoplasm, various organelles including mitochondria, and vacuoles. In subsequent Pb treated samples, the dark spots (electron dense) present are likely Pb precipitates, possibly lead phosphate minerals in the plant tissue. However, there may also be dissolved Pb species throughout the cell which are fixed in place throughout the epoxy resin and not visible to the naked eye.

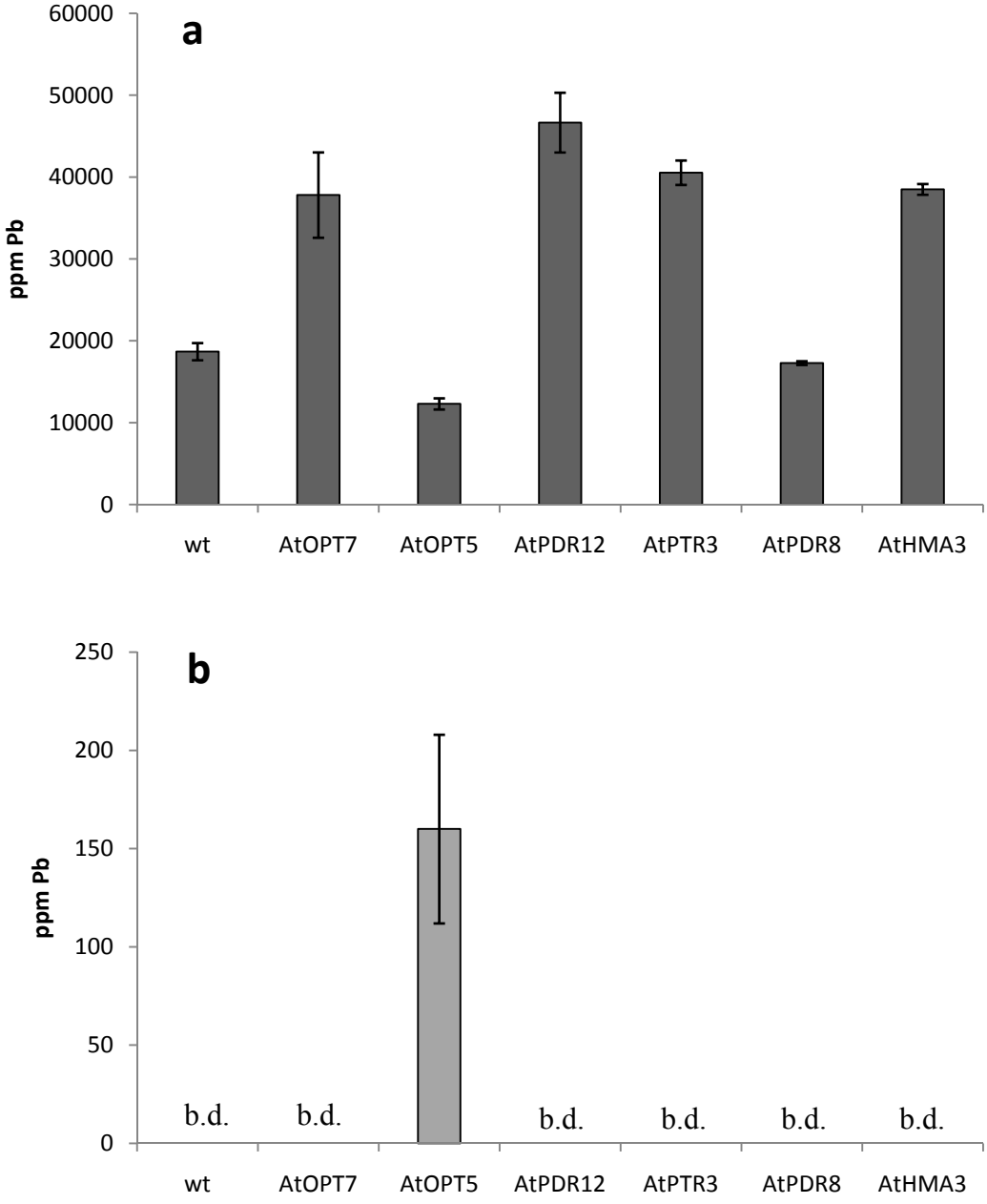

Figure 3.5 Root (a) and shoot (b) Pb content of *Arabidopsis thaliana* wildtype (wt) and knockout plants after 5 days exposure to 10 μM Pb and 380 μM glutathione solutions exchanged daily. AtHMA3 data shown from SALK_073511, and AtPTR3 data shown from SALK_003119C. Results from alternatives to those mutants were similar. Values are ± SD (n=3). b.d. = below detection (< 10 ppm)

Table 3.3 Root uptake of Pb in *Brassica napus* exposed to solutions containing 10 μM or 50 μM Pb with or without a ligand exchanged daily for 5 days. Values represent only one sample for each treatment.

Ligand treatment	Pb (μM)	Root Pb uptake (ppm)
No ligand	10	2800
No ligand	50	9400
10 μM EDTA	10	500
50 μM EDTA	50	600
28 μM cysteine	10	4100
140 μM cysteine	50	18000
380 μM glutathione	10	37000
1900 μM glutathione	50	57000

The transmission electron micrographs of Pb treated plants, both with cysteine or glutathione or without a ligand, showed some morphological differences compared to the control. Cells were typically less organized, with more disperse cytoplasm and multiple smaller, scattered vacuoles (Figures 3.6, 3.8, 3.9). Also, organelles were not as clearly visible in Pb treated cells, possibly due to toxicity effects on the root tip cells. Not many cells were present on the EDTA treatment slides and therefore it is difficult to generalize about the morphological differences.

In terms of potential Pb particulates within the root cells, very little difference was observed between the free Pb or EDTA treatments and the control (Figures 3.7, 3.10, 3.11), though there were several instances of electron dense areas present in the cell wall matrix under free Pb treatment compared to the control (Figures 3.12, 3.13).

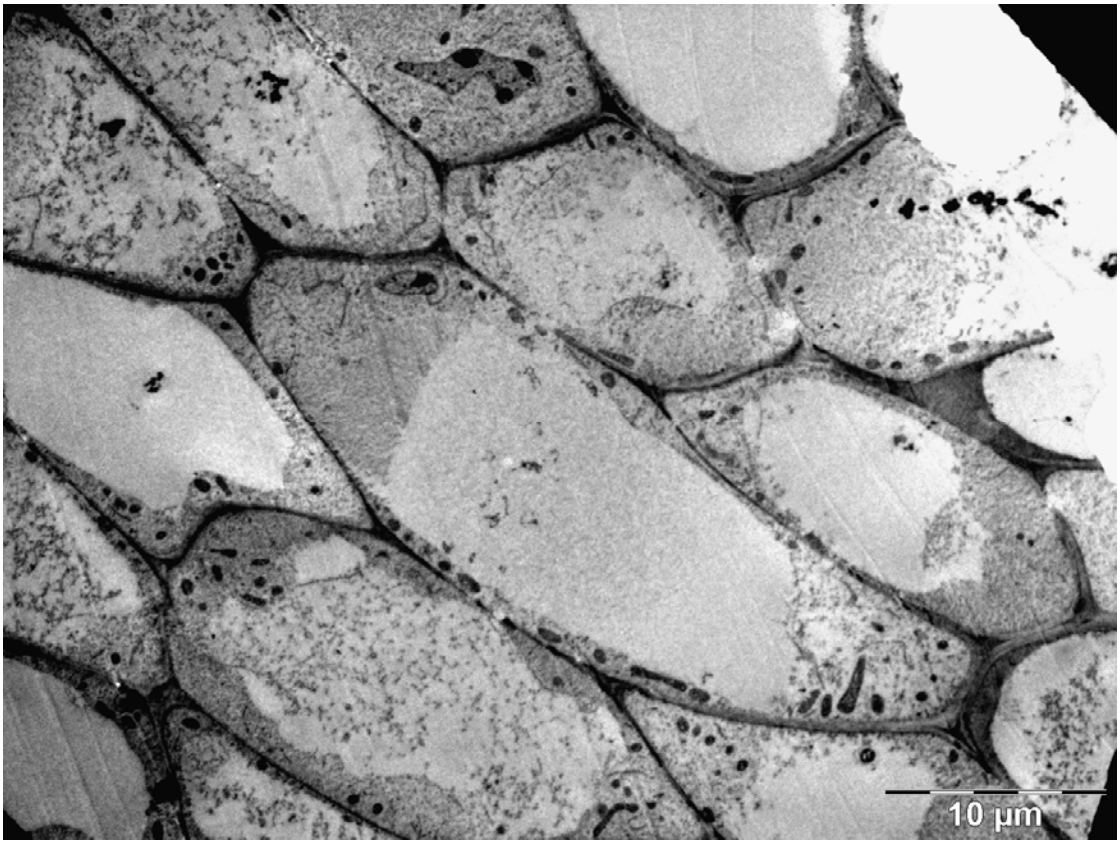

Figure 3.6 Transmission electron micrograph of Brassica napus root cells. Non Pb-exposed tissue (control).

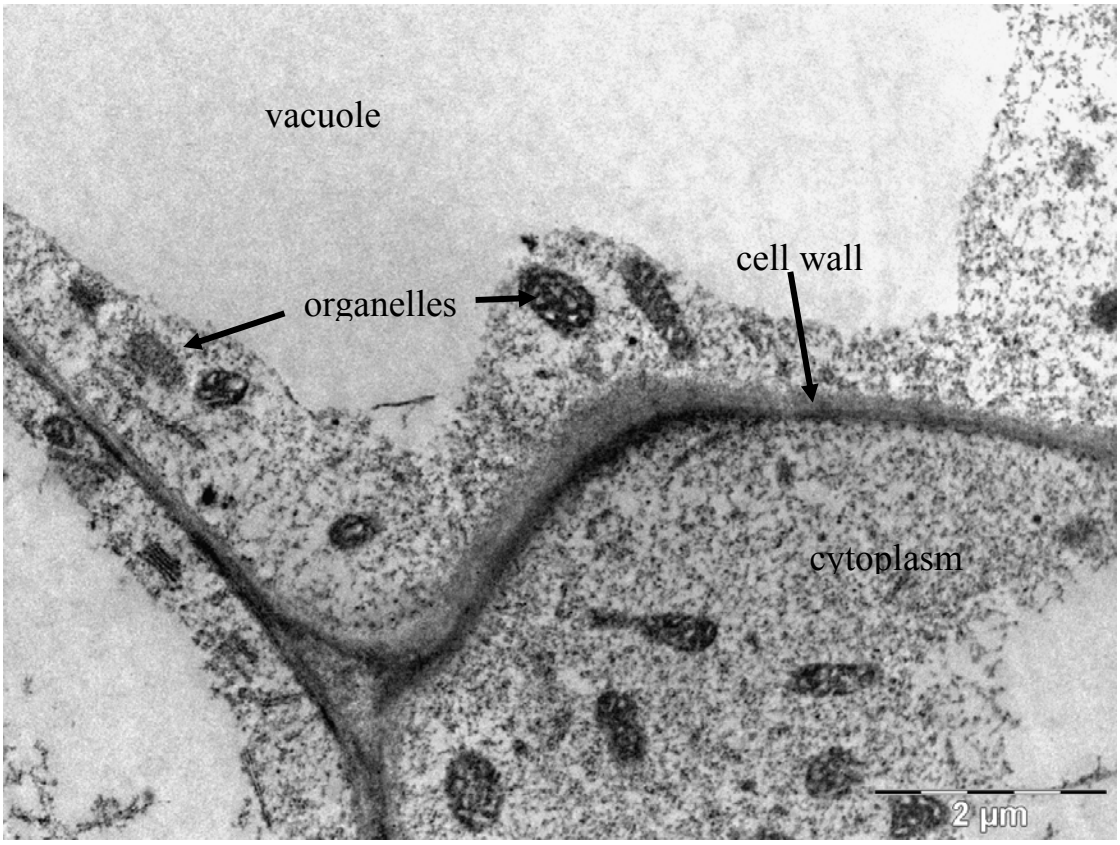

Figure 3.7 Transmission electron micrograph of Brassica napus root cells. Non Pb-exposed tissue (control).

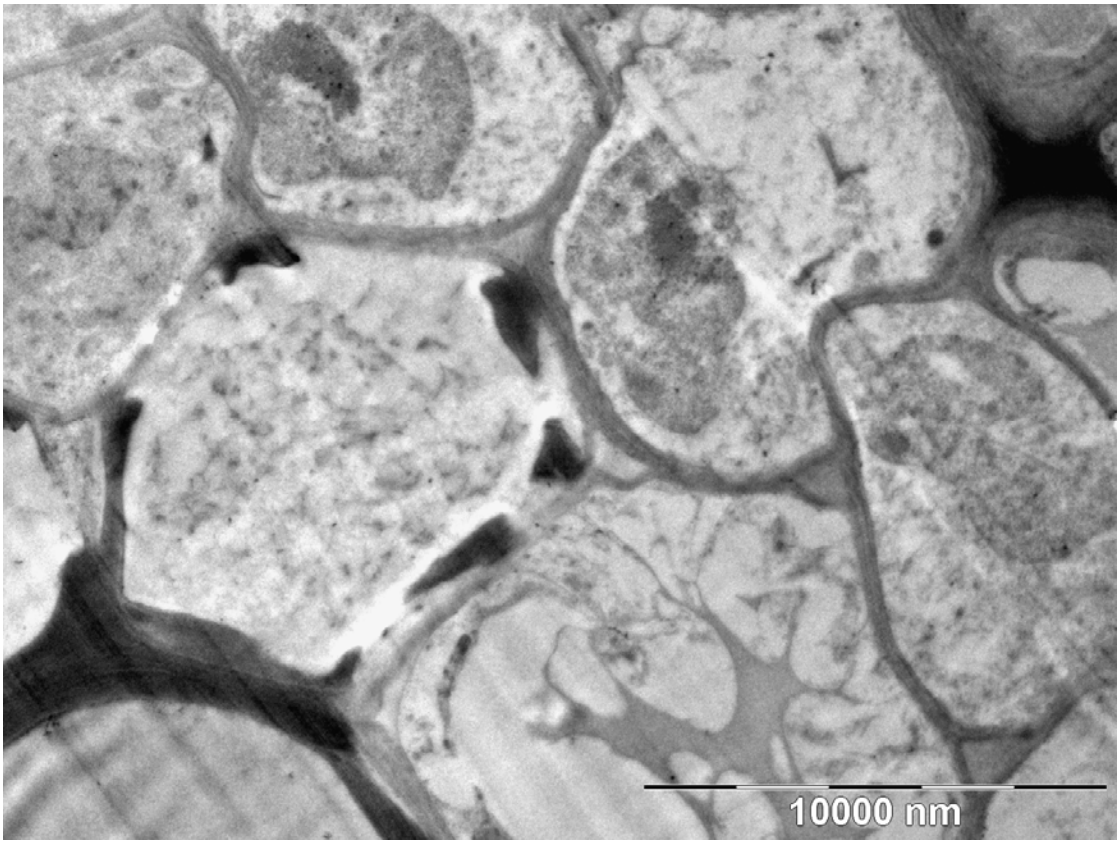

Figure 3.8 Transmission electron micrograph of Brassica napus root cells. Tissue exposed to 10 μM Pb and 28 μM cysteine, exchanged daily, for 5 days.

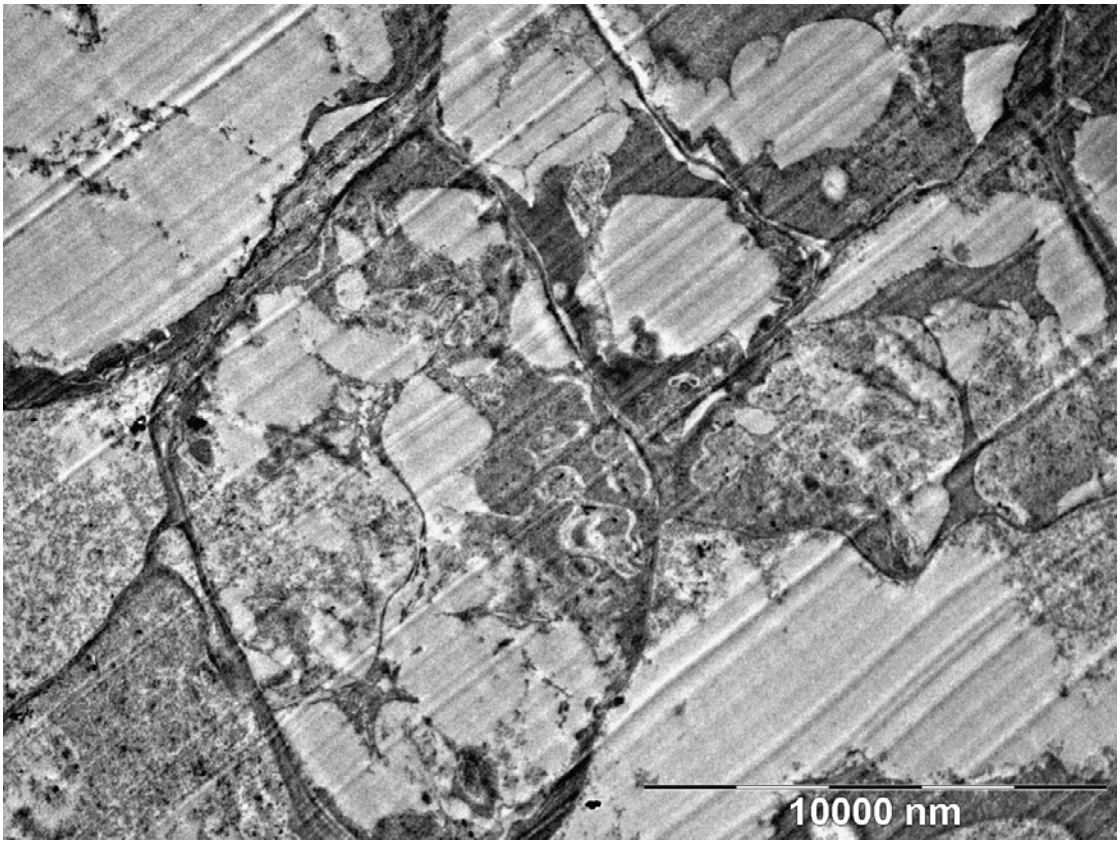

Figure 3.9 Transmission electron micrograph of Brassica napus root cells. Tissue exposed to 10 μM Pb and 380 μM glutathione, exchanged daily, for 5 days.

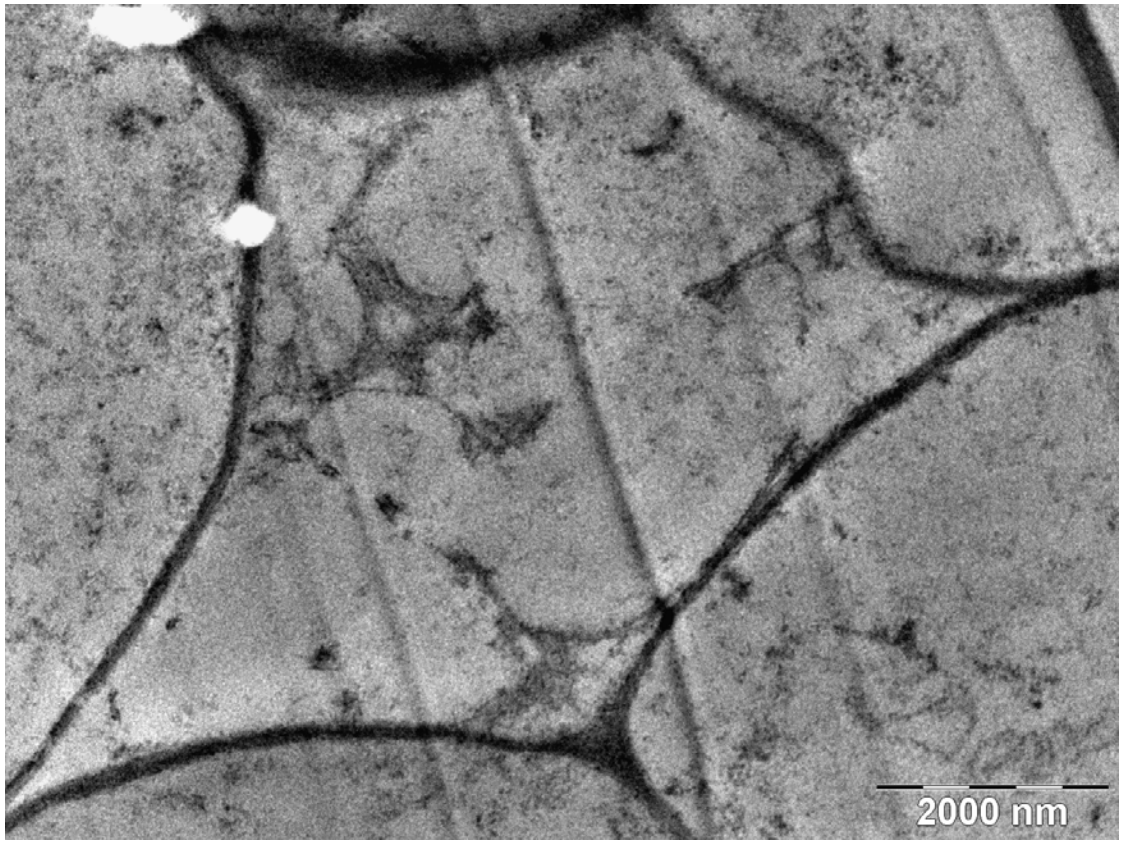

Figure 3.10 Transmission electron micrograph of Brassica napus root cells. Tissue exposed to 10 μM Pb, exchanged daily, for 5 days.

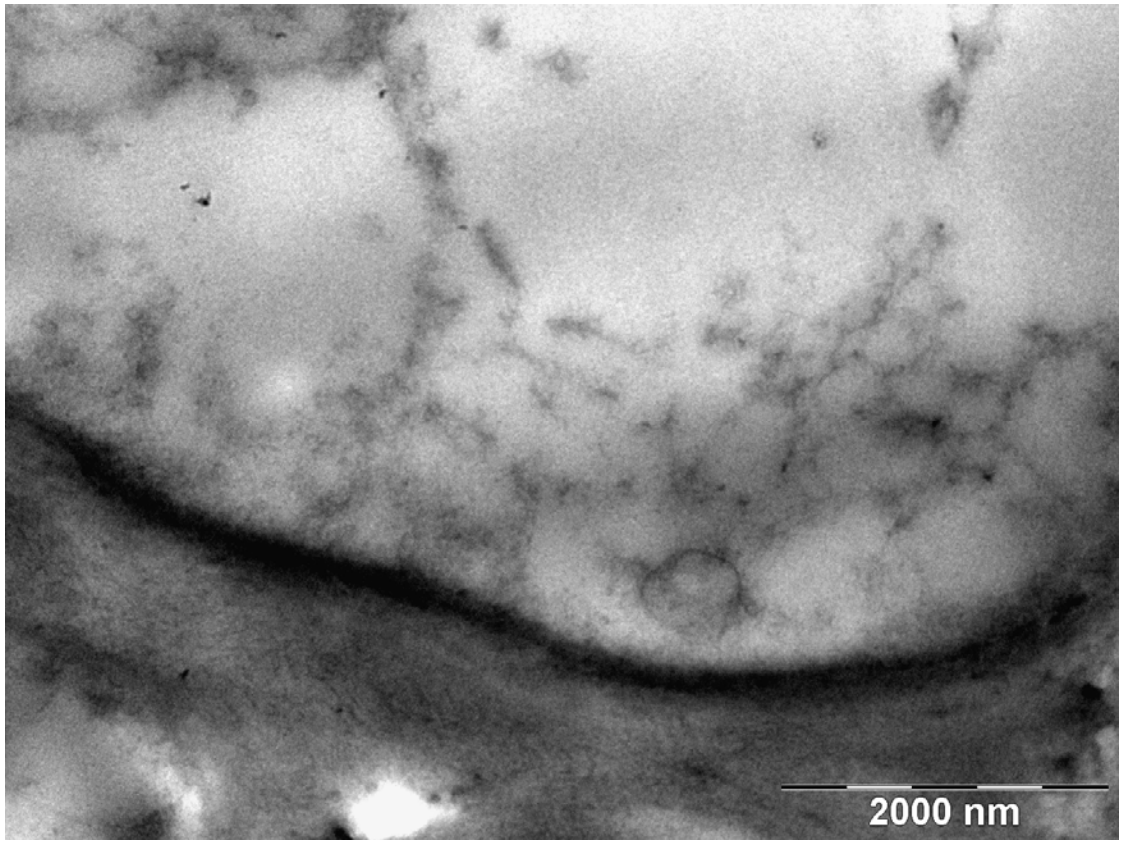

Figure 3.11 Transmission electron micrograph of *Brassica napus* root cells. Tissue exposed to 10 μM Pb and 10 μM EDTA, exchanged daily, for 5 days.

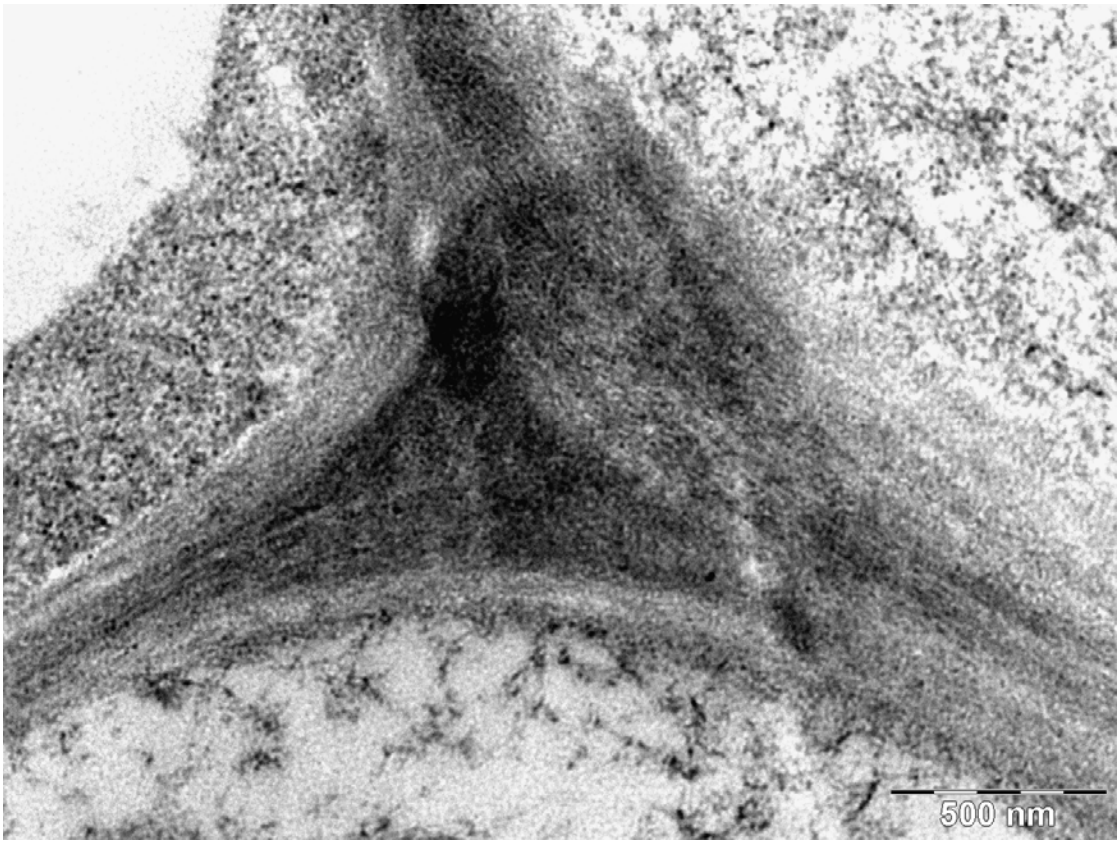

Figure 3.12 Transmission electron micrograph of Brassica napus root cell wall junction. Non Pb-exposed tissue (control).

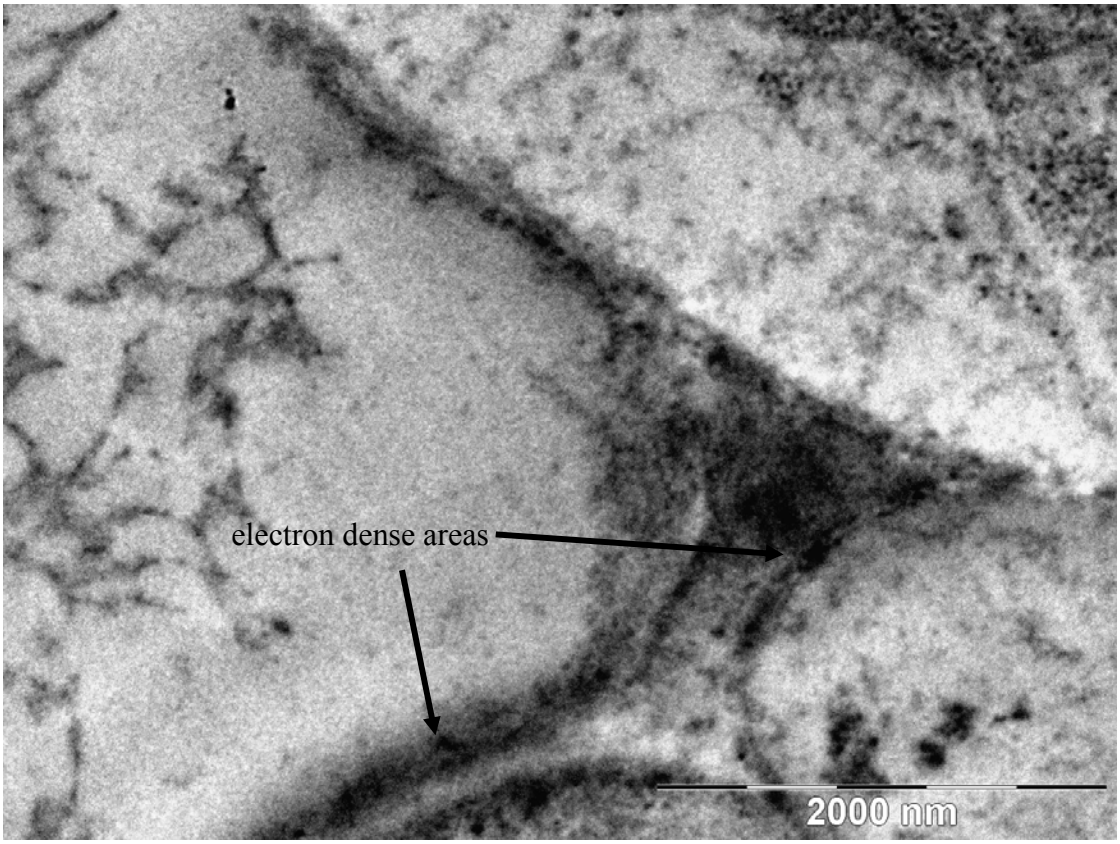

Figure 3.13 Transmission electron micrograph of *Brassica napus* showing electron dense areas in the root cell wall junction. Tissue exposed to 10 µM Pb, exchanged daily, for 5 days.

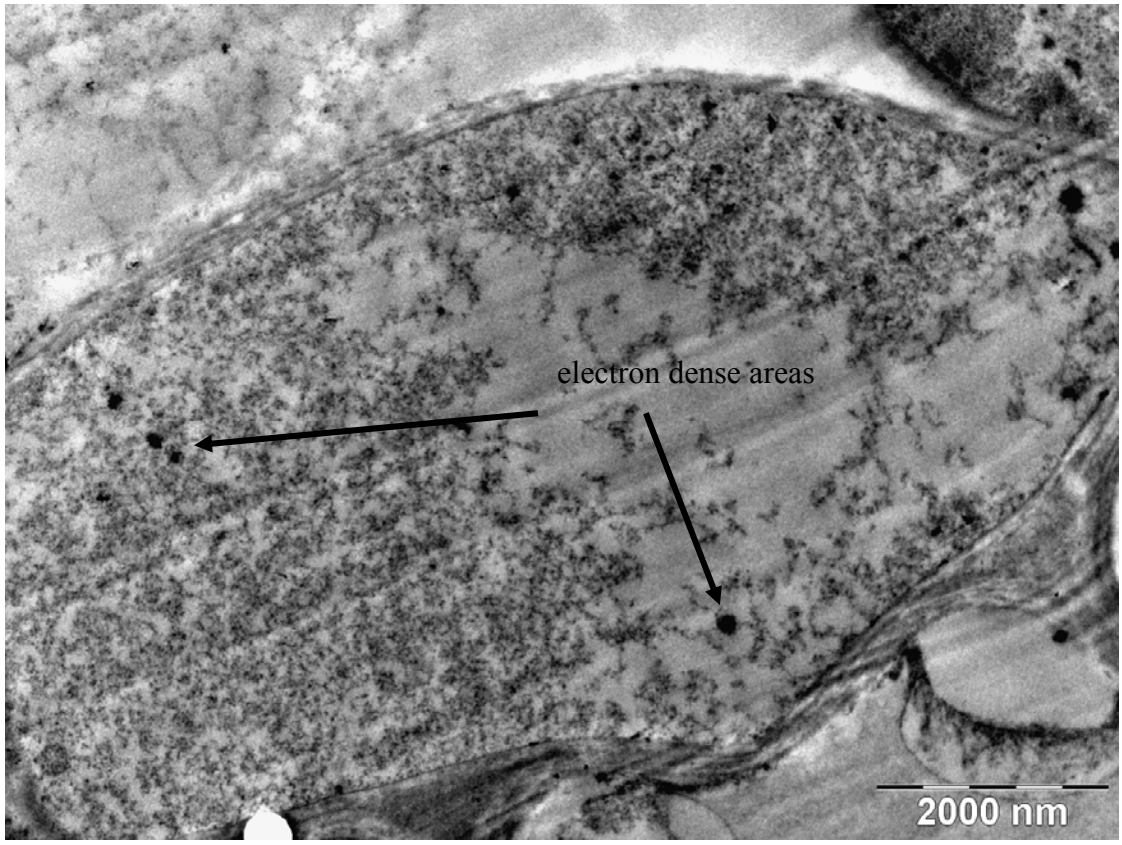

Figure 3.14 Transmission electron micrograph of Brassica napus root cells showing electron dense areas in the cytoplasm. Tissue exposed to 10 μM Pb and 28 μM cysteine, exchanged daily, for 5 days.

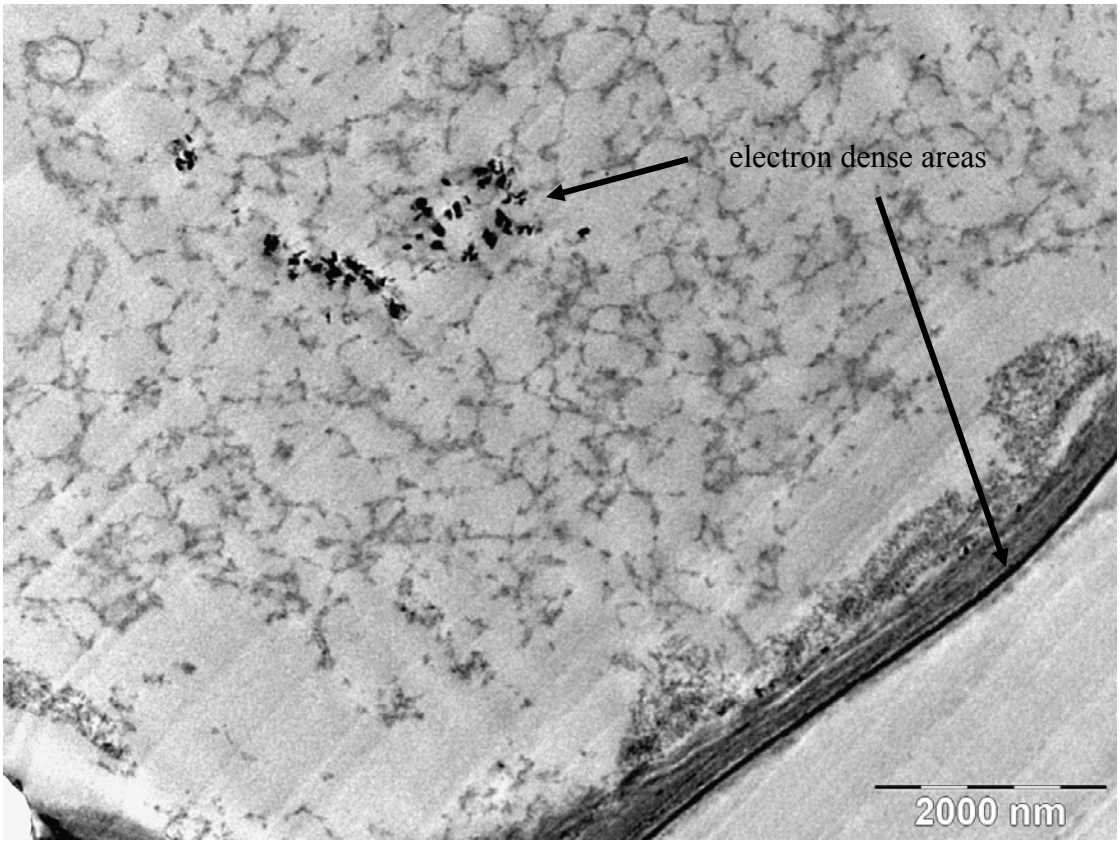

Figure 3.15 Transmission electron micrograph of Brassica napus root cells showing electron dense areas in the cytoplasm. Tissue exposed to 10 μM Pb and 380 μM glutathione, exchanged daily, for 5 days.

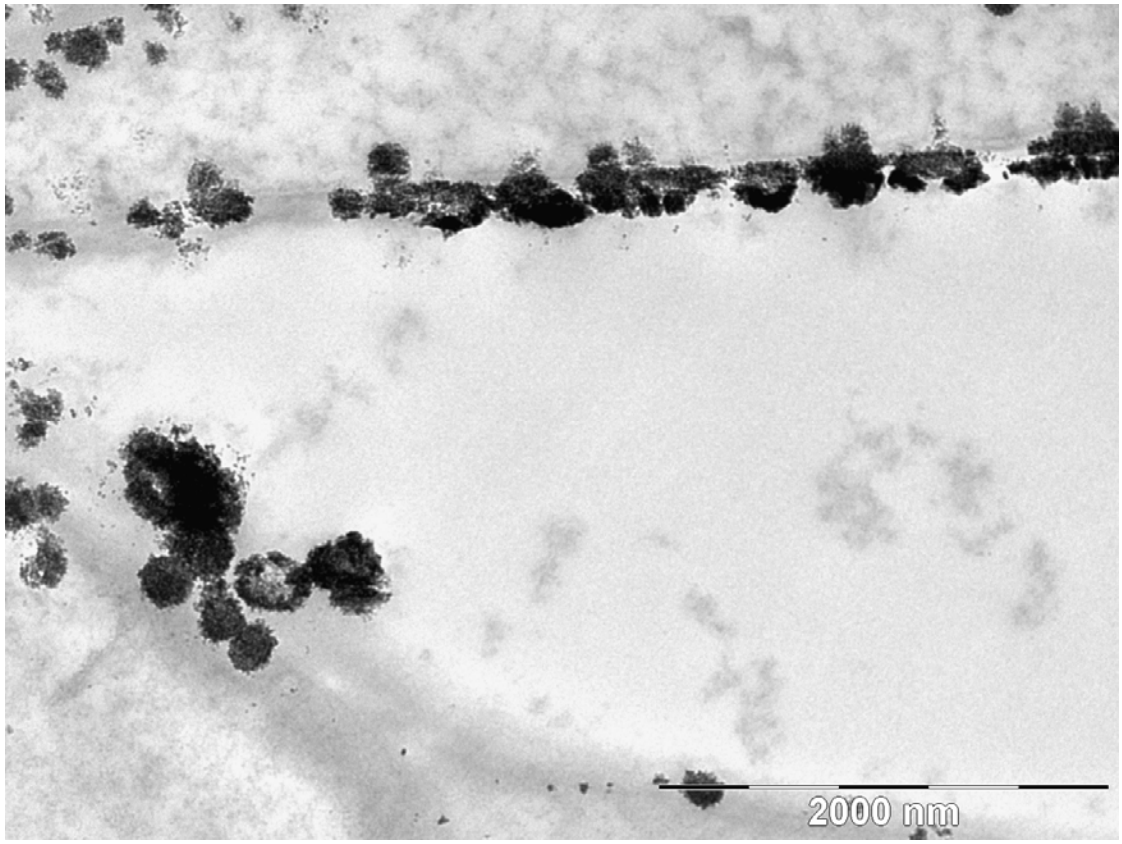

Figure 3.16 Transmission electron micrograph of Brassica napus root cells showing electron dense areas along the plasma membranes. Tissue exposed to 10 µM Pb and 380 µM glutathione, exchanged daily, for 5 days.

Under cysteine or glutathione treatment, more electron dense areas were observed within the cytoplasm (Figures 3.14, 3.15). Similar studies have been conducted with plants exposed to high free Pb concentrations and electron dense lead particles were only observed in the intercellular spaces (Jarvis et al. 2002). In some cases of the glutathione treatment, electron dense areas were observed along the plasma membrane (Figures 3.15, 3.16), though it is uncertain whether some are internal or external to the cell.

Although we measured much higher concentrations of Pb in plants exposed to glutathione versus cysteine, visually, the electron micrographs were similar. This is likely due to the presence of dissolved Pb species distributed throughout the plant roots. Research suggests that plants exposed to high concentrations of free Pb in solution store Pb in the form of Pb acetate, Pb sulfur (Sharma et al. 2004), or Pb carbonate bonds (Sarret et al. 2001). In the future, it would be valuable to measure the spatial distribution and speciation of Pb within these root cells, as others have done with trace metals using synchrotron x-ray fluorescence (Naftel et al. 2001; Twining et al. 2007) or energy dispersive x-ray spectroscopy with the transmission electron microscope (Van Belleghem et al. 2007). If high vacuolar concentrations of Pb are found in the glutathione treatment compared to the controls, it would suggest the role of a vacuolar transporter for Pb-glutathione leading to root concentration and no shoot translocation. Further research in identifying the responsible plasma and vacuolar membrane transporters will help understand the movement of Pb and Pb-thiols throughout the cell. This could lead to genetic modification of plants to maximize shoot translocation by limiting root vacuolar sequestration.

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CHAPTER 4

EXTRACTION OF LEAD AND CADMIUM FROM SOILS BY CYSTEINE AND GLUTATHIONE

4.1 Introduction

Lead (Pb) and cadmium (Cd) are recognized as priority pollutants by the United States Environmental Protection Agency due to their potential harmful effects to humans and abundance at contaminated sites. Cost-effective strategies to remediate and remove heavy metals from these sites are lacking and soil is typically either stabilized or excavated and disposed of as hazardous waste. These are not long-term solutions and a more ideal strategy would be extraction from the soil matrix, either chemically or through the use of plants.

Phytoextraction is the less energy intensive, less invasive, and less expensive of the two processes, but extraction efficiencies are not nearly as high as for chemical extraction. Phytoextraction is particularly limited by the solubility of metals in the soil matrix and the uptake mechanism of the plant. Enhanced phytoextraction has focused on the use of synthetic chelators to solubilize soil metals and increase uptake into the plant via a passive mechanism (Blaylock et al. 1997; Epstein et al. 1999; Huang et al. 1997), however the plant is quickly killed and leaching may occur (Nowack et al. 2006). Recent studies in our laboratory have examined thiol enhanced phytoextraction, using cysteine or glutathione as a ligand, resulting in greater uptake of Pb and Cd from hydroponic solutions, likely due to transport of the metal-thiol species (Chapter 2). This approach appears to take advantage of an active plant transport mechanism, allows a longer period of extraction, and may reduce leaching potential due to the high biodegradability of these particular ligands, thus addressing current concerns with chelate-assisted phytoextraction. However, in order for this to

be a viable strategy, we must also examine the ability of these ligands to enhance the solubility of Pb and Cd in soils.

The solubility of metals in the soil pore water is affected by many factors such as soil pH, organic matter content, cation exchange capacity, redox potential, and the presence of Mn and Fe oxides (Banuelos and Ajwa 1999; McBride 1994). Pb is extremely insoluble in soils at pH levels relevant for plant growth (5-8) and is usually present as stable phosphate, carbonate, or organic matter species (Blaylock et al. 1997; Chaney et al. 1988). Cd^{2+} is increasingly soluble as pH decreases, but can also form soluble complexes with organic matter above pH 6 or precipitates such as $CdCO_3$ (McBride 1994).

The synthetic chelators such as ethylenediaminetriacetic acid (EDTA) used in many chemical or phyto-extraction studies are strong ligands, particularly with iron (Fe), but with high stability constants for many metals (on the order of $10^{17} – 10^{27}$; Martell and Smith, 2004). Thiol ligands, such as cysteine and glutathione, have a sulfhydryl group that is particularly compatible with soft metals such as mercury (Hg), Cd, and Pb with stability constants on the order of $10^{11} – 10^{15}$ (Martell and Smith, 2004). While thiol ligands may not be as strong as synthetic chelators, they have been shown to be effective at solubilizing metals in several model systems. A Pb mineral phase, $PbHPO_4$, was partially dissolved when exposed to cysteine or thiosulfate (Martinez et al. 2004). Fischer (2002) also examined the extraction of heavy metals by cysteine and penicillamine from several soil components (peat, bentonite, and illite) and found effective removal (> 50%) of Pb and Cd from all soil components over the first 24 hours, but re-precipitation later was attributed to oxidation of the thiols. To our knowledge, there are no studies examining the extraction of metals from soils by glutathione.

The source, type, and age of contamination will likely play a role in determining extraction effectiveness. Depending on the soil fractions Pb or Cd are partitioned into, i.e. (1) exchangeable, (2) bound to carbonates, (3) bound to iron and manganese oxides, (4) bound to organic matter, and (5) residual (Li et al. 1995; Tessier et al. 1979), cysteine or glutathione may be more or less effective at solubilizing metals from those fractions.

The purpose of this study was to examine how effectively cysteine and glutathione solubilize Pb and Cd from contaminated soils under a variety of conditions. We hypothesized that cysteine and glutathione could be used to increase the solubility of Pb and Cd in soils and that extraction would be pH dependent and more effective at higher pH (6-8) due to the pKa's of the sulfhydryl groups on cysteine and glutathione. We also examined the effect of sorption and oxidation of the thiols on metal solubility.

4.2 Materials and Methods

Soil collection and preparation

Soils were collected from a fallow field in Ithaca, NY, smelting contaminated sites (labeled LP-44 Pb and LP-53 Pb) in Montreal, Canada, and the Cornell University apple orchard (labeled orchard Pb) in Ithaca, NY. The fallow field soil was artificially contaminated with Pb or Cd (labeled Hanshaw Pb and Hanshaw Cd) to target values of 2000 ppm for Pb and 400 ppm for Cd. Lead acetate or cadmium chloride was dissolved in water and sprayed onto soil over several layers and mixed, repeatedly until all the solution was used. All soil was air dried and sieved to 2 mm. Size distributed soil samples were sequentially sieved through several smaller pore size sieves (1.4 mm, 1 mm, 0.5 mm, 0.25 mm, and 0.125 mm).

Soil properties

Soil pH was measured by combining 10 mL air-dried and 2 mm sieved soil sample and 10 mL distilled water, stirred and allowed to equilibrate for 10 minutes before measuring pH. Soil organic matter was analyzed using the weight loss on ignition method (Nelson and Sommers, 1982).

Soil metal analysis

Initial measurements of soil metal content followed EPA Method 3050b (1995) for "environmentally available" metals (digestion with concentration HCl and HNO_3). Sequential extractions of soils generally followed the procedure outlined by X. Li et al. (1995). Five fractions were analyzed: 1) exchangeable, 2) bound to carbonate and specifically adsorbed, 3) bound to Fe-Mn oxides, 4) bound to organic matter and sulfide, and 5) residual phase. The digestion of the residual phase was different from X. Li's procedure (1995) and was completed using only 2.5 mL HNO_3 (70% w/w) and 2.5 mL $HClO_4$ (60% w/w) before heating to dryness. Solution samples were diluted as necessary, adjusted to contain 5% nitric acid and analyzed on a Thermo Jarrell Ash ICAP 61 Inductively coupled plasma optical emissions spectrometer (ICP-OES) for metal content.

Thiol extractions

Soil samples of 1 ± 0.02 g were weighed into 15 mL centrifuge tubes. Thiol solutions containing 10 mM cysteine, glutathione or thiopropionate, various buffers (0.01 M acetate for pH 4 and 5, 0.08 M MES for pH 6, 0.08 M HEPES for pH 7, and 0.1 M HEPES for pH 8), and 0.01 M $CaCl_2$ were prepared. At the start of each experiment, 10 mL of thiol solution was added to triplicate tubes of soil, the tube was sealed, vortexed to suspend soil, and placed in a covered rack lying sideways on a rotary shaker at 200 rpm. When sterile experiments were necessary, all glassware, utensils, and soil was autoclaved, and all procedures up to and including sealing the centrifuge tubes were completed in a laminar flow hood. To reduce oxidation of

thiols, several experiments were completed under reduced oxygen conditions. Sterilized water was purged with N_2 gas prior to and after mixing thiol solutions and the headspace in each centrifuge tube was flushed with N_2 gas prior to sealing. All thiol solutions were filtered through 0.2 μm glass fiber filters (Pall Supur®).

To separate the soil phase from solution for metal and reduced thiol analyses, a 1.5 mL sample was transferred to 2 mL microcentrifuge tubes and centrifuged at 9.6 g for 30 minutes. From that, 1 mL was taken for metals analysis (see below) and about 20 μL for reduced thiol analysis (see below).

Reduced thiol analysis

Reduced thiols were measured using Ellman's reagent, adapting the protocol of Riener et al. (2002). Samples were diluted with 10 mM $CaCl_2$ in microcentrifuge tubes to a total volume of 750 μL. To that solution was added 750 μL of PBS-EDTA buffer (137 mM NaCl, 10.1 mM Na_2HPO_4, 1.76 mM KH_2PO_4, 1 mM EDTA, and was adjusted to pH 7.4 with H_3PO_4) and 20 μL of Ellman's reagent solution (10 mM DTNB; 5,5'-Dithiobis(2-nitrobenzoic acid), 100 mM NaH_2PO_4, 0.2 mM EDTA, and adjusted to pH 7 with NaOH). Solutions were shaken, allowed to react for 5 minutes and then transferred to UV/vis spectrophotometer cells for analysis at 412 nm on a Perkin Elmer Lambda EZ-201 UV/vis spectrophotometer.

Statistical analyses

A one-way analysis of variance (ANOVA) was performed on the extracted metal data using Excel.

4.3 Results

Soil characterization

The soil pH of the Hanshaw soils after contamination with Pb and Cd were about 4.8 and 4.2, respectively, while the orchard soil was about 6.1, and LP-44 and LP-53 soils were higher at approximately 7.8 (Table 4.1). Organic matter was higher

in the Hanshaw soils, at about 70 g/kg, and lower in the orchard and LP soils, at 32 and 20 g/kg, respectively. The measured soil Pb or Cd content was about 2000 ppm in Hanshaw Pb and 430 ppm in Hanshaw Cd, close to their target values. The orchard soil had very low levels of Pb contamination, about 200 ppm, mostly from past use of lead arsenic pesticides. Although LP-44 and LP-53 Pb soils were from the same site, they had very different Pb contamination levels, at about 1400 and 500 ppm, respectively.

Table 4.1 Soil pH, % organic matter and total contaminant metal concentrations of artificially and field contaminated soils.

Soil	pH	organic matter (g/kg)	Total Pb (ppm)	Total Cd (ppm)
Hanshaw Pb	4.75 ± 0.11	70.3 ± 2.8	2002	b.d.
Hanshaw Cd	4.21 ± 0.01	69.4 ± 1.1	b.d.	427
Orchard Pb	6.08 ± 0.01	32.3 ± 16.1	227	b.d.
LP-44 Pb	7.77 ± 0.08	20.2 ± 7.4	1423	b.d.
LP-53 Pb	7.76 ± 0.03	20.2 ± 7.4	494	b.d.

b.d. = below detection (< 10 ppm)

Effect of thiols on metal extraction

Metal extraction from laboratory contaminated soils was dependent on buffered pH of the extraction solution. With control solutions, Pb extraction was similar at pH 4 and pH 5 (about 100 µg Pb extracted/g soil) and decreased to very low levels from pH 6 to pH 8 (Figure 4.1). Conversely, extraction of Pb from contaminated soil by solutions of 10 mM cysteine or glutathione generally increased as pH increased and were significantly different from controls at pH 5 and higher for cysteine and greater than pH 5 for glutathione. The most Pb was extracted by cysteine at pH 7 (about 800 µg Pb extracted/g soil or 40% of total Pb), whereas glutathione

extracted the most Pb at pH 8 (about 40 % of total Pb). Generally cysteine resulted in greater solubility except at pH 8 where the solubility of Pb was very low.

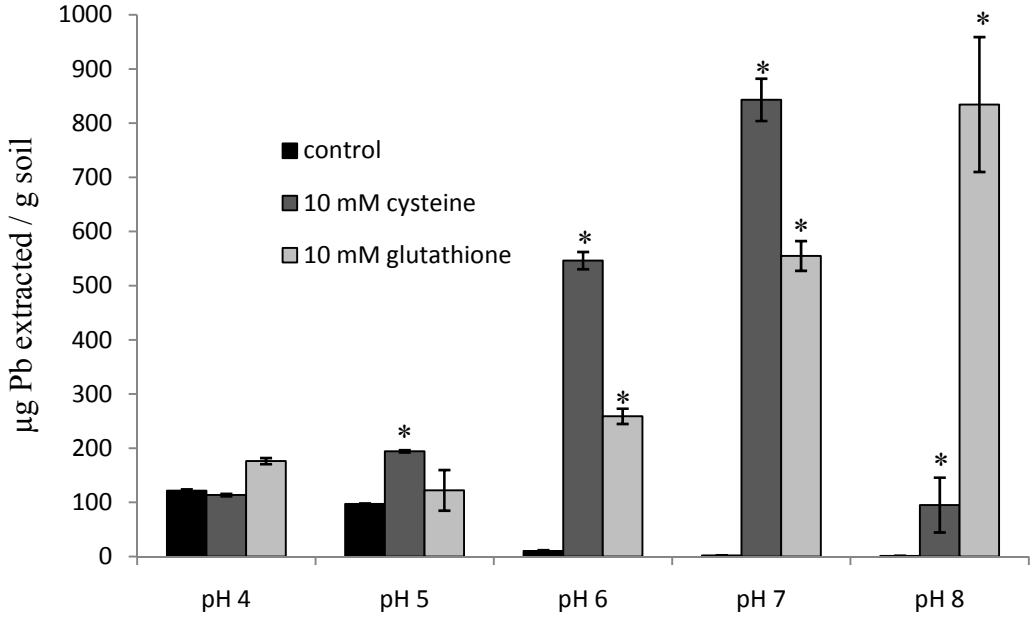

Figure 4.1 Total metal extracted (µg/g) from Hanshaw Pb soils after 1 hr exposure to the control or 10 mM solutions of cysteine or glutathione over a pH range of 4 to 8. Error bars represent one standard deviation (n = 3). * significantly different from control ($p < 0.05$)

In contrast to Pb, extraction of Cd from soils treated with 10 mM cysteine or glutathione solutions was generally lower than controls except at pH 7 and 8. The control solutions extracted high concentrations of soluble Cd at pH 4 and pH 5, about 80% of total Cd, with decreasing solubility up to pH 8, where extractable Cd was only about 15% of total Cd (Figure 4.2). For both cysteine and glutathione treatments at pH 4, 5 and 6, Cd solubility was significantly lower than the control, but was significantly higher than the control at pH 8 for cysteine and pH 7 and 8 for glutathione, reaching about 25% and 45% of total Cd, respectively at pH 8.

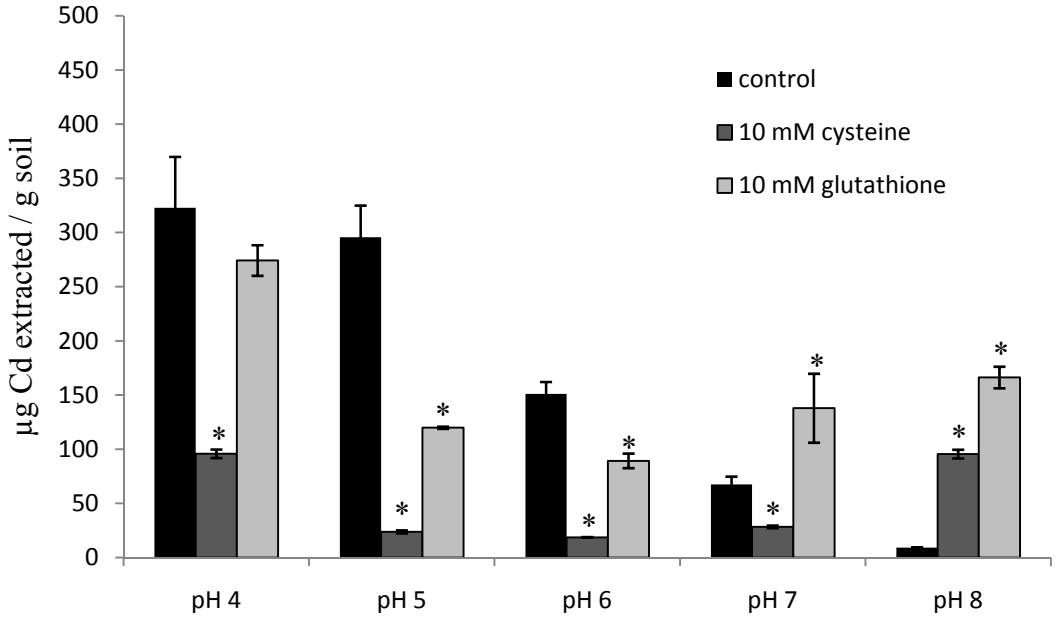

Figure 4.2 Total metal extracted (µg/g) from Hanshaw Cd soils after 1 hr exposure with a control or 10 mM solutions of cysteine or glutathione over a pH range of 4-8. Error bars represent one standard deviation (n = 3). * significantly different from control ($p < 0.05$)

For each metal extraction experiment (Figure 4.1 and 4.2), the reduced thiol concentration remaining in the initial 10 mM extraction solution following the one hour incubation was measured. The concentration of reduced glutathione remaining in Pb extraction solutions was independent of pH and nearly constant at about 6.5 mM, whereas cysteine concentrations dropped significantly from about 6 mM at pH 4 and 5 to less than 1 mM at pH 7 and 8 (Figure 4.3). Similar results and trends were observed in Hanshaw Cd soils. To evaluate whether the protonated amine played a role in the loss of reduced cysteine, an analogue to cysteine, thiopropionate, which has the same structure (Figure A.1) but lacks the amine group, was tested. Measurements of reduced thiopropionate following a one hour incubation were very similar to those of glutathione over the entire pH range (Figure 4.3), suggesting the amine group may play a role in the mechanism of loss of reduced cysteine from solution.

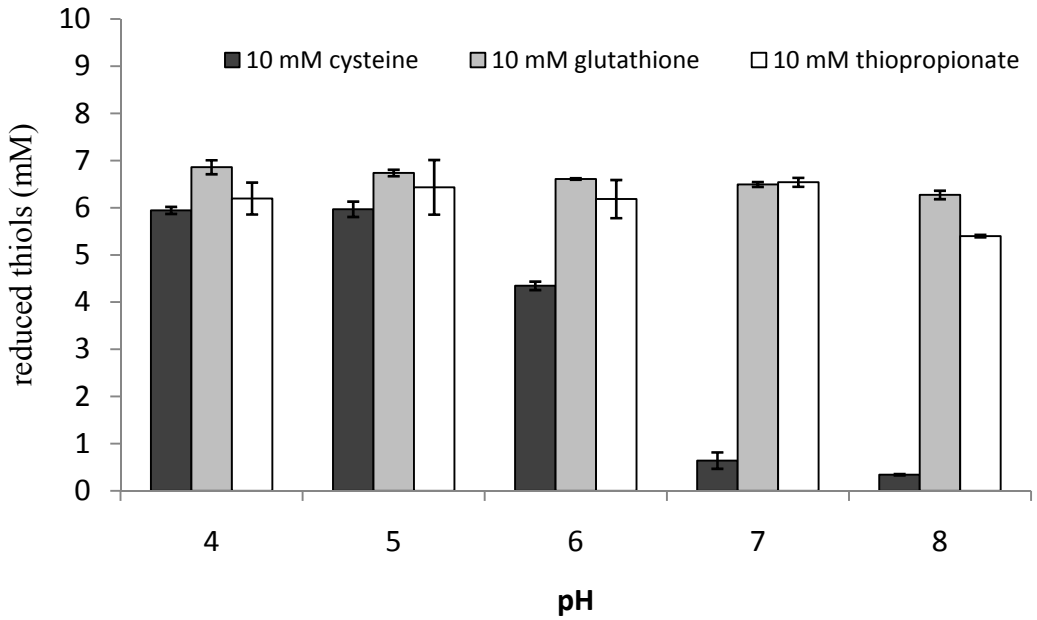

Figure 4.3 Reduced thiol concentrations remaining in Hanshaw Pb extraction solutions containing initially 10 mM cysteine, glutathione, or thiopropionate after 1 hr over a pH range of 4-8. Error bars represent one standard deviation (n = 3).

The pH dependent loss of cysteine from solution may be due to degradation, sorption, and/or oxidation of the thiol. Reduced cysteine concentrations in buffered extraction solutions containing no soil remained essentially unchanged at 10 mM over the first hour at both pH 5 and pH 7 (data not shown). In order to examine sorption of cysteine to soils, an experiment exposing different particle size distributions of Hanshaw Pb soil to 10 mM cysteine solutions at pH 5 and 7 for 10 minutes was conducted. At pH 5, the amount of cysteine lost from solution was about 45% at the largest particle size, whereas slightly less was lost from solution for the four smaller particle size distributions, about 30% (Figure 4.4). At pH 7, the loss of cysteine was about 60% of the total added and was not significantly different at any particle size distribution. While there is a nearly immediate loss of cysteine from solution in each case, the fact that it does not differ depending on surface area suggests sorption may not play a significant role.

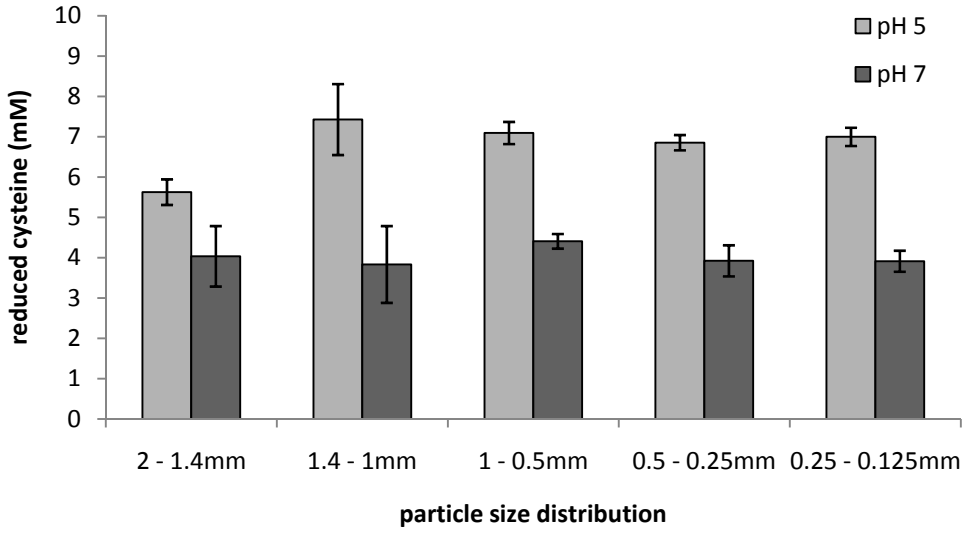

Figure 4.4 Reduced cysteine concentrations remaining in solution after exposure of Hanshaw Pb soils of different particle size distributions to 10 mM cysteine at pH 5 and 7 for 10 minutes. Error bars represent one standard deviation (n=3).

To test whether oxidation and/or degradation of cysteine or glutathione were occurring during the experiments, extractions were repeated under both sterile and reduced oxygen conditions (flushing with N_2). For Pb extractions, there were significant increases in Pb solubility at pH 6 and 8 with cysteine, whereas there were significant decreases at pH 7 and 8 with glutathione (Figure 4.5). Significant increases in Cd extraction were also observed at pH 6 and 7 with cysteine, and at pH 6 and 8 for glutathione (Figure 4.6). In general, the average metal extracted increased across the pH range for both Hanshaw Pb and Cd with cysteine or Hanshaw Cd with glutathione, but decreased with Hanshaw Pb with glutathione.

To examine extraction from field contaminated soils, extractions were repeated with control and 10 mM thiols under both sterile and N_2 flushed conditions for the orchard Pb, LP44 Pb and LP53 Pb soils. Control extractions resulted in undetectable Pb concentrations for each of these soils (data not shown). Measurable Pb was extracted from all three of these soils by both thiols at all pHs tested (Figure 4.7). In

general, extraction of Pb was more effective with cysteine than glutathione in each Pb soil type (Figure 4.7). As with artificially contaminated soils, metal extraction was dependent on pH, increasing as pH increased, but the % of the total metal extracted was much lower. For example, the total Pb extracted from LP44 soils at pH 8 with cysteine was about 28%, compared to about 50% in the cysteine treated Hanshaw Pb soil (Figure 4.5). This is likely due to the different source and type of Pb phase present in the soil. The lack of an increase in total Pb extraction at pH 8 in the presence of cysteine for both the orchard Pb and LP53 Pb soils may be due to some contamination with oxygen during the experiment.

Kinetics of cysteine and glutathione extractions

The kinetics of both metal solubility and reduced thiol concentrations remaining in extraction solutions were examined for artificially contaminated soils exposed to 10 mM cysteine at pH 7 or 10 mM glutathione at pH 8 under sterile conditions with or without flushing with N_2. In the presence of glutathione, Pb solubilized rapidly with levels reaching 400 µg/g in 5 minutes with levels up to over 700 µg/g after 15 minutes; the initial time course was independent of flushing with N_2 but during the later time points somewhat greater extraction was achieved with flushed solutions (at 6 and 12 hours; Figure 4.8). Similar to glutathione, initial extractions with cysteine (10 minutes or less) were independent of flushing, but at 15 minutes, Pb solubility without N_2 flushing decreased exponentially whereas the extraction flushed with N_2 led to a peak in Pb solubility at 6 hours and then gradually decreased throughout the experiment (Figure 4.8). Similar results were observed in Cd extraction experiments (Figure B.1).

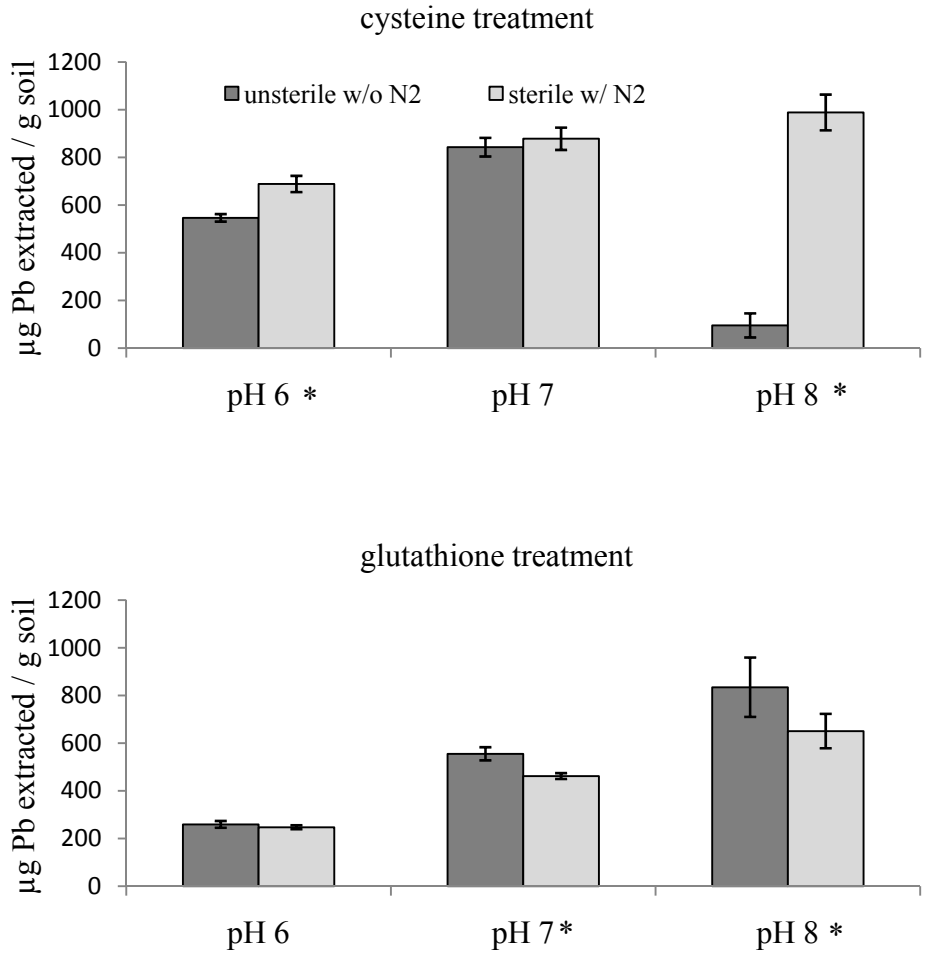

Figure 4.5 Total metal extracted from Hanshaw Pb soil after 1 hour exposure to 10 mM cysteine or 10 mM glutathione under unsterile w/o N_2 flushing or sterile w/ N_2 flushing conditions at pH 6, 7, and 8. Error bars represent one standard deviation (n=3). * significantly different from each other ($p < 0.05$)

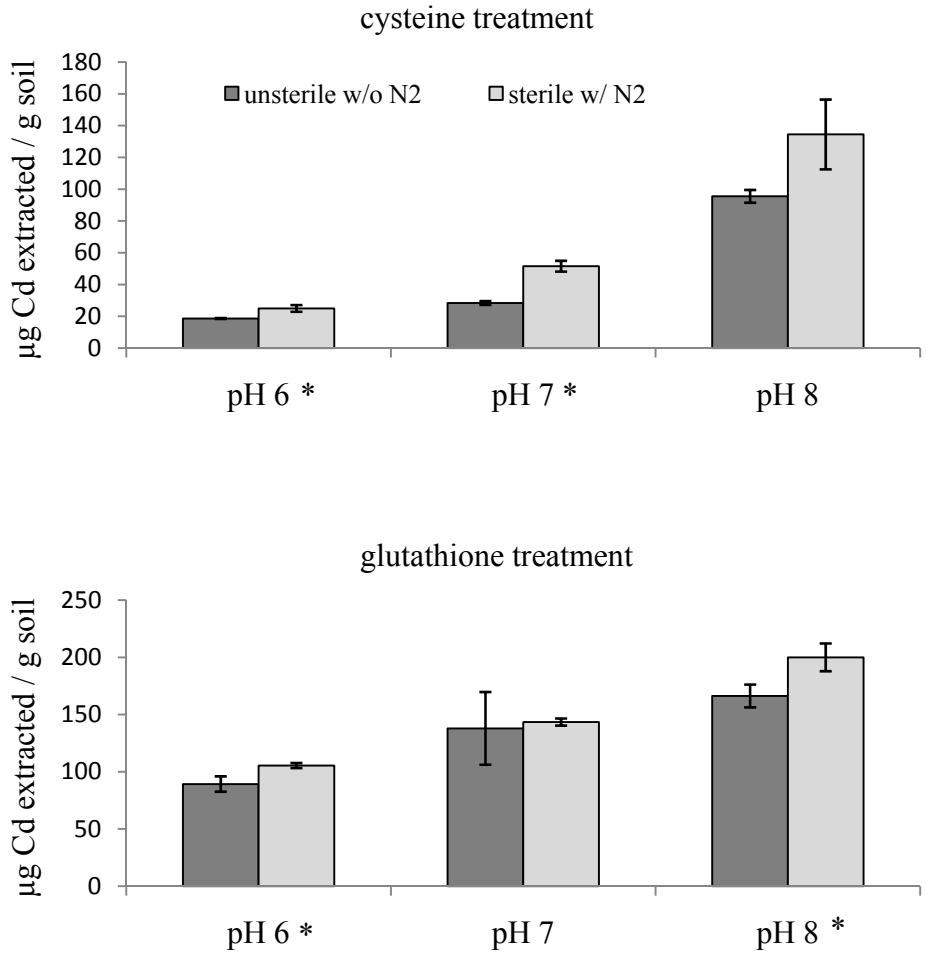

Figure 4.6 Total metal extracted from Hanshaw Cd soil after 1 hour exposure to 10 mM cysteine or 10 mM glutathione under unsterile w/o N_2 flushing or sterile w/ N_2 flushing conditions at pH 6, 7, and 8. Error bars represent one standard deviation (n=3). * significantly different from each other ($p < 0.05$)

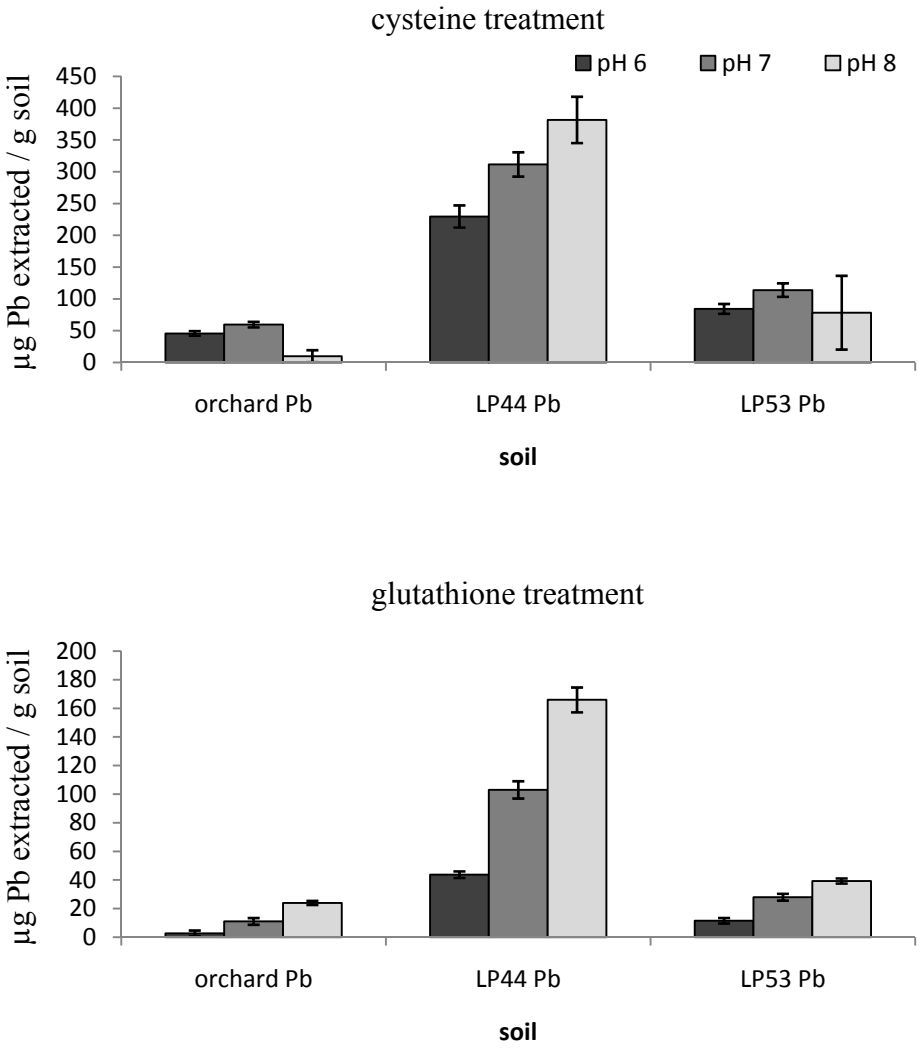

Figure 4.7 Total metal extracted from Orchard Pb, LP44 Pb and LP53 Pb soils after 1 hour exposure to 10 mM cysteine or 10 mM glutathione under sterile w/ N_2 flushing conditions at pH 6, 7, and 8. Control buffer extractions resulted in undetectable Pb concentrations (< 0.5 µg/g). Error bars represent one standard deviation (n=3).

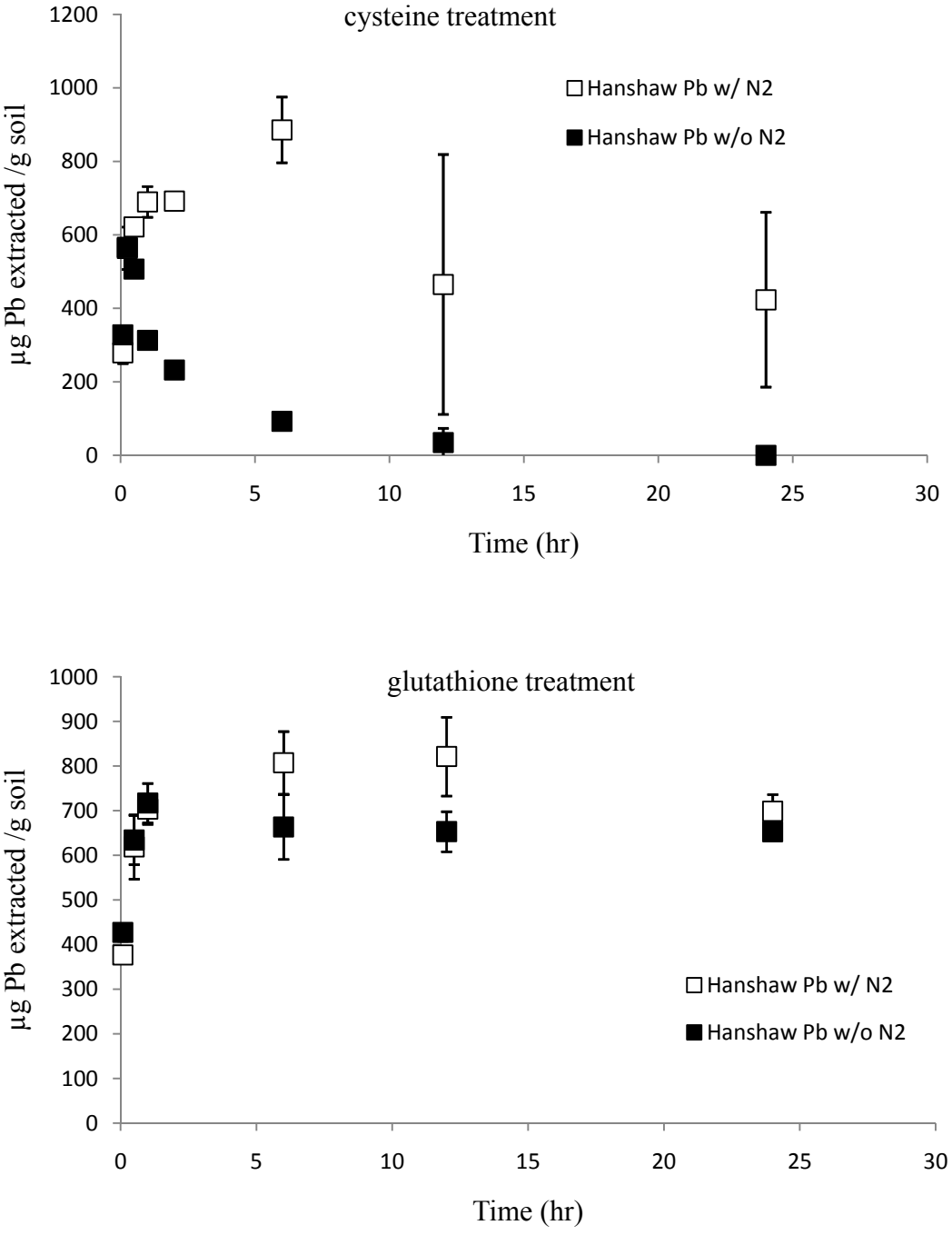

Figure 4.8 Total Pb extracted over time over the course of 10 mM cysteine at pH 7 and 10 mM glutathione at pH 8 sterile Hanshaw Pb soil extractions with and without flushing solutions and headspace with N_2 gas. Error bars represent one standard deviation (n=3).

During the course of the kinetic experiments described above, thiols not only resulted in increased Pb and Cd solubility, but increased solubility of other elements as well. While the solubility of potassium, phosphorus, manganese, and magnesium remained about the same whether flushed with N_2 or not, the iron concentrations differed greatly in the cysteine treatment. The dissolved Fe concentration in Hanshaw Pb samples treated with cysteine was high after only 1 minute in both flushed and unflushed soils, indicative of Fe (III) reduction by cysteine, and remained high and relatively constant under N_2 flushed conditions (Figure 4.9). Without N_2 flushing, dissolved Fe concentrations decreased exponentially throughout the time course. Fe concentrations were below detection (< 10 µg/g) in both N_2 flushed or unflushed glutathione treatments. Similar results were observed in Cd extraction experiments (Figure B.2).

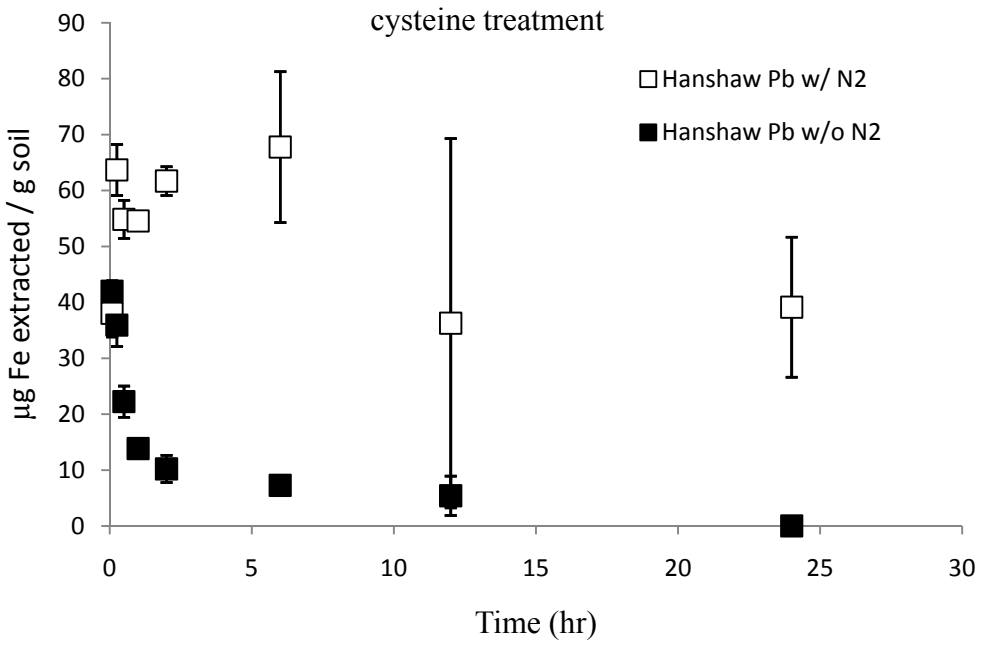

Figure 4.9 Total Fe extracted over time over the course of 10 mM cysteine at pH 7 sterile Hanshaw Pb soil extractions with and without flushing solutions and headspace with N_2 gas. Error bars represent one standard deviation (n=3).

Dissolved thiol concentrations in these experiments reveal very different results for cysteine and glutathione. While reduced cysteine concentrations in both N_2 flushed and unflushed experiments dropped to less than 1 mM after only 1 hour, reduced glutathione concentrations gradually decreased over 24 hours to about half the initial concentration (Figure 4.8). The half-life of cysteine loss in N_2 flushed Pb soil samples was about 0.65 hr (assuming first order kinetics and using the first hour of data points), while it was even shorter in unflushed samples, with a half-life of only 0.2 hrs. Glutathione loss rates were much lower, with half-lives of about 91 hr and 53 hr for N_2 flushed and not flushed, respectively. Similar rates were calculated for Cd soil experiments (Figure B.3).

Sequential thiol extractions of soils

Multiple extractions under nonsterile and oxygenated conditions were performed on the same soil to estimate total extraction potential of the thiol ligands. Solutions of 10 mM cysteine at pH 7 continued to remove metals from all soils even after 4 extractions, removing the most from Hanshaw Pb soil, reaching about 70 % of total Pb (Figure 4.11), and the least from orchard Pb soil, removing only about 23% of total Pb (Figure 4.13). Solutions of 10 mM glutathione at pH 8 typically removed a greater amount of total metal from the soils, reaching 100% removal from Hanshaw Cd soils (Figure 4.12), nearly 90 % removal from Hanshaw Pb (Figure 4.11), and were least effective for LP53 Pb soil, reaching only about 35% total Pb removal after 4 extractions (Figure 4.15). Each subsequent extraction typically removed less than the previous extraction, with only two exceptions, perhaps due to incomplete rinsing between samples. Typically, glutathione was more effective at removing metals, likely in part due to limited oxidation of the thiol, except in the LP53 Pb soil where cysteine and glutathione removed about the same amount of Pb, which could be due to

the relative amounts of Pb and their availability to cysteine or glutathione in the different soil fractions.

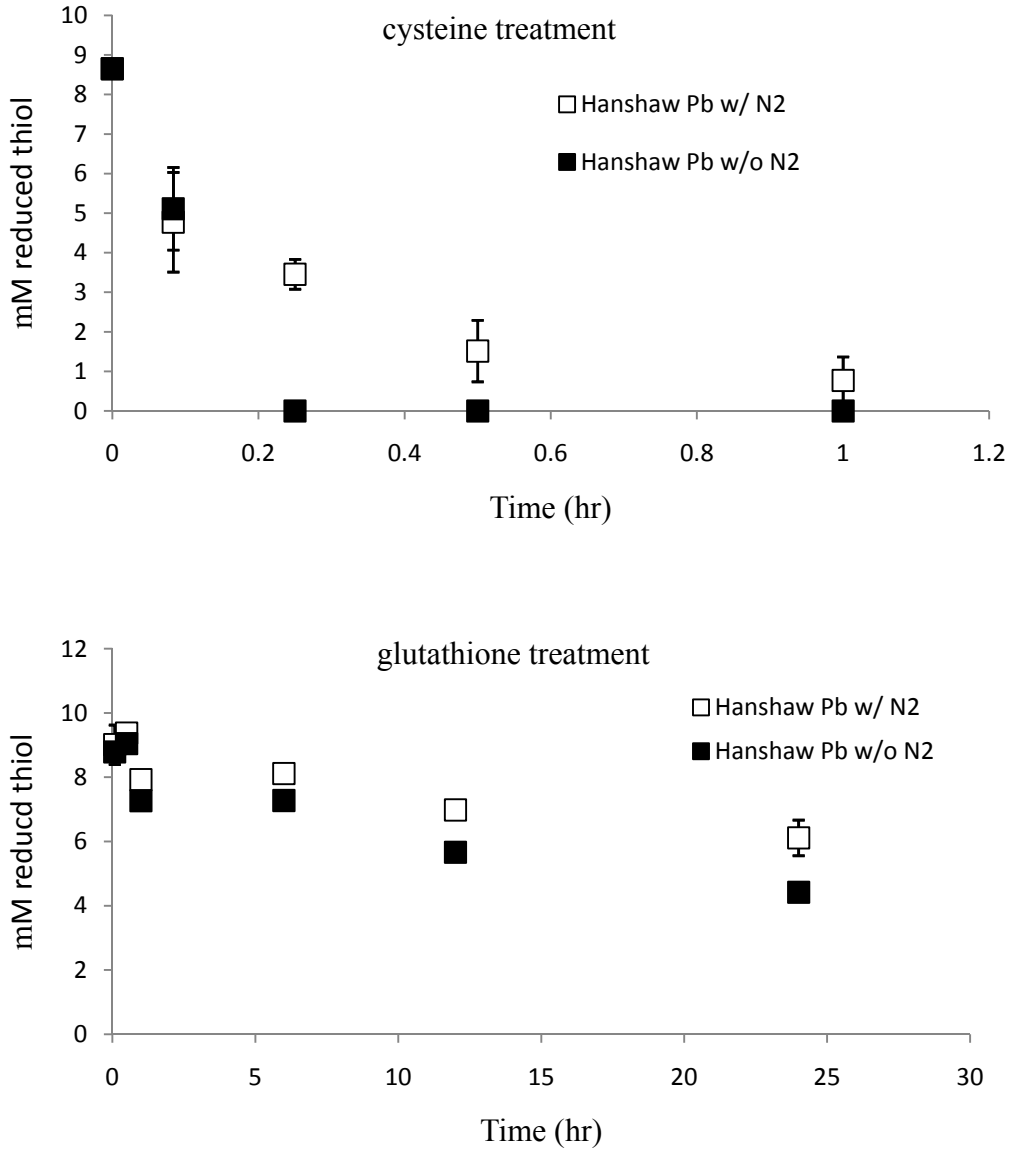

Figure 4.10 Reduced thiol concentrations remaining in extraction solution over time over the course of 10 mM cysteine at pH 7 (a) and 10 mM glutathione at pH 8 (b) sterile Hanshaw Pb soil extractions with and without flushing solutions and headspace with N_2 gas. Error bars represent one standard deviation (n=3).

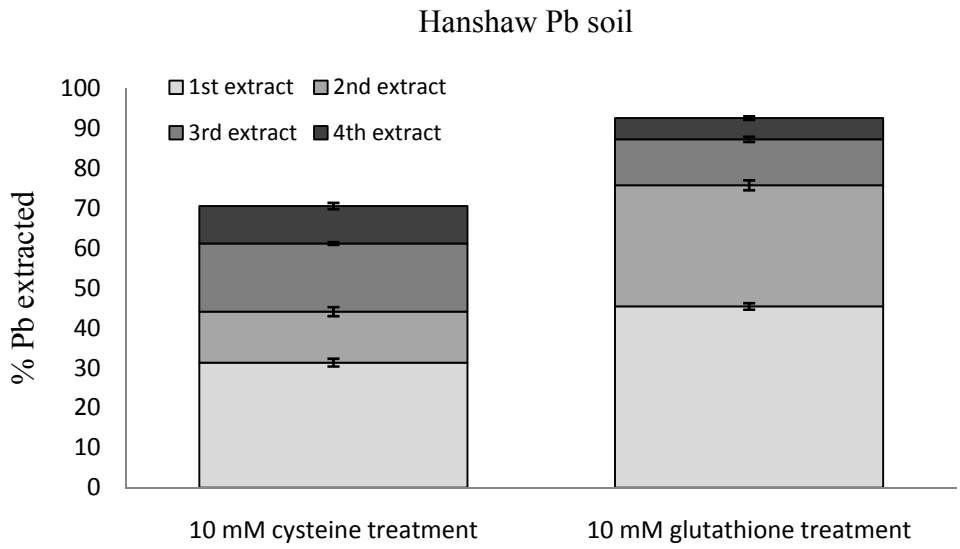

Figure 4.11 Sequential 1 hour extractions of Hanshaw Pb soils with 10 mM cysteine at pH 7 and 10 mM glutathione at pH 8. Error bars represent one standard deviation (n=3).

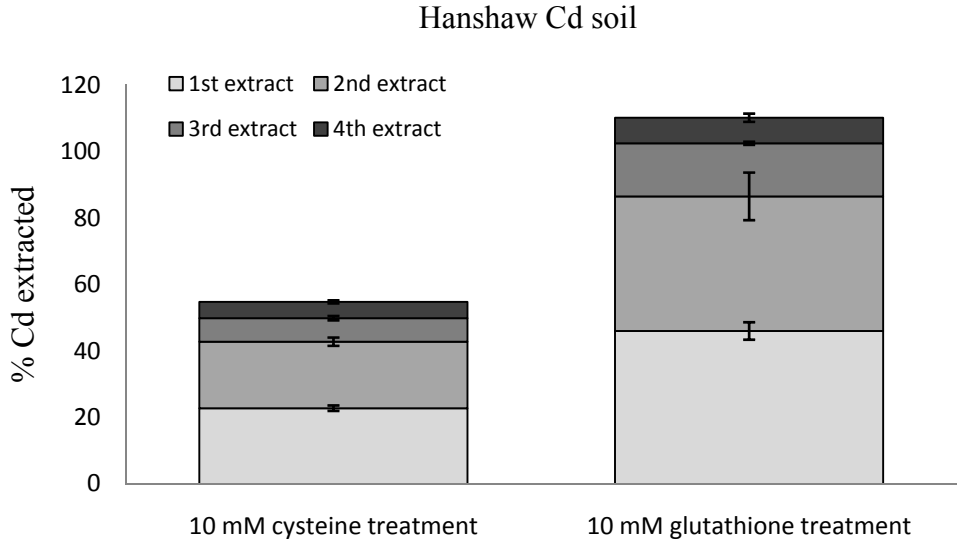

Figure 4.12 Sequential 1 hour extractions of Hanshaw Cd soils with 10 mM cysteine at pH 7 and 10 mM glutathione at pH 8. Error bars represent one standard deviation (n=3).

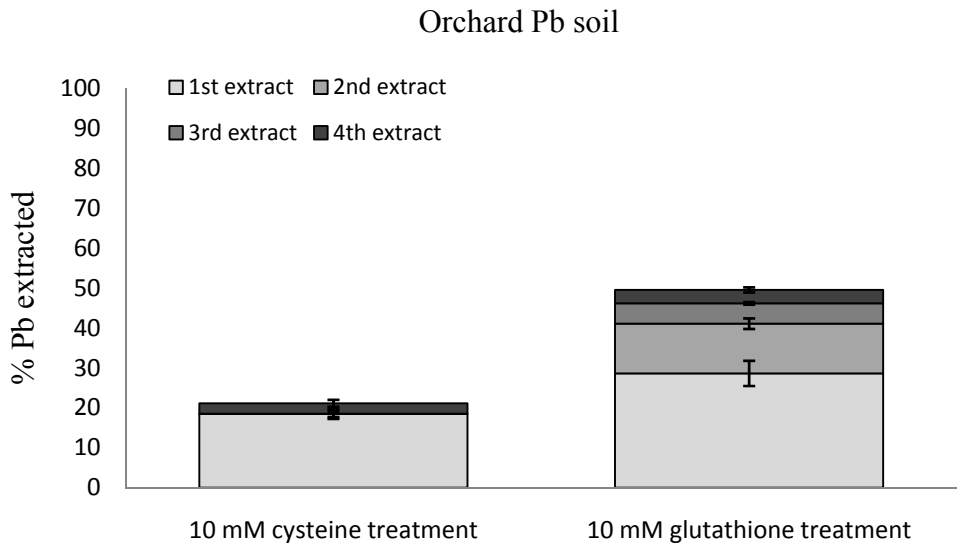

Figure 4.13 Sequential 1 hour extractions of Orchard Pb soils with 10 mM cysteine at pH 7 and 10 mM glutathione at pH 8. Error bars represent one standard deviation (n=3).

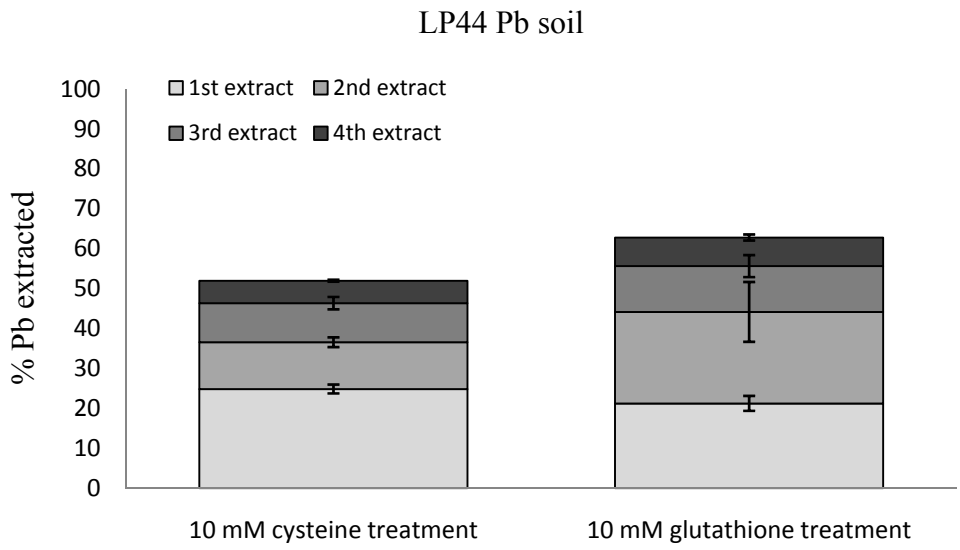

Figure 4.14 Sequential 1 hour extractions of LP44 Pb soils with 10 mM cysteine at pH 7 and 10 mM glutathione at pH 8. Error bars represent one standard deviation (n=3).

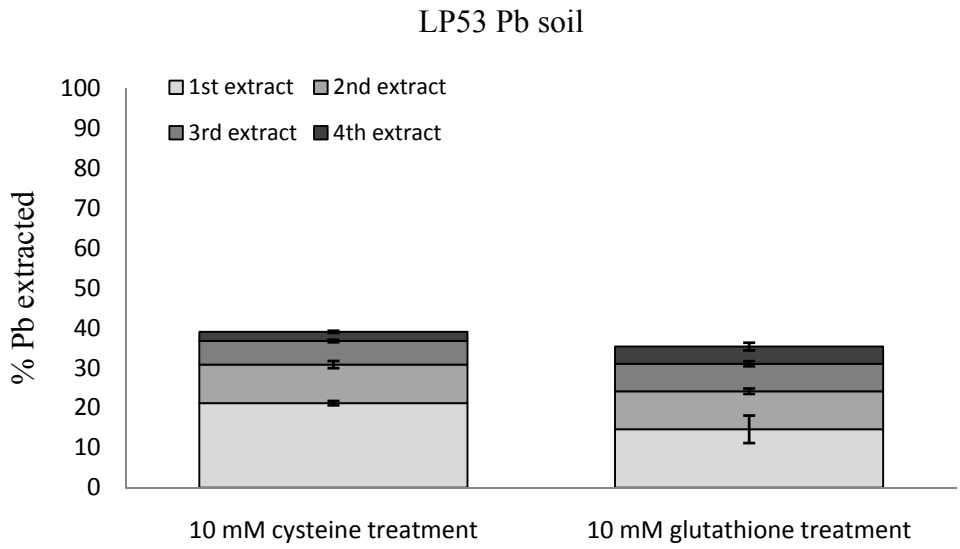

Figure 4.15 Sequential 1 hour extractions of LP53 Pb soils with 10 mM cysteine at pH 7 and 10 mM glutathione at pH 8. Error bars represent one standard deviation (n=3).

Sequential metal extraction from experimental soils

To determine what chemical fraction of Pb or Cd was solubilized by the thiols in the various contaminated soils, standard sequential extractions were performed on soils before and after 1 hour thiol extraction experiments. Prior to thiol extraction, the exchangeable fraction of metals in all soils was relatively low, less than 4% of total Pb or Cd content (Table 4.2). The $CaCO_3$ and Fe/Mn-oxide bound phases typically had the highest percentages of Pb or Cd, although the organic/sulfide bound fractions were relatively high in the orchard, LP44, and LP53 soils. In the Hanshaw Pb soils, both cysteine and glutathione were effective at removing Pb from the exchangeable and the Fe/Mn-oxide fraction, while only cysteine was effective at removing Pb from the organic/sulfide fraction. Glutathione treatment of Hanshaw Cd soils resulted in significant removal of Cd from all soil fractions, with the most from the Fe/Mn-oxide fraction at about 80% removal, but with cysteine treatment, significant removal only occurred in the exchangeable fraction. Glutathione was also effective at removing Pb

from the first two fractions of orchard soil, while cysteine removed Pb from the exchangeable and organic/sulfide fractions. In one instance, soil Pb increased in the Fe/Mn-oxide fraction of the glutathione treated soil, possibly due to an artifact of rinsing between the fractionation protocol. In both the LP44 and LP53 soils, cysteine treatment resulted in about 50 % removal from the organic/sulfide fraction, while glutathione treatment significantly removed Pb from the $CaCO_3$ fraction, removing 40 and 70 %, respectively. In all the Pb soils, cysteine treatment was effective at removing Pb from the organic/sulfide fraction. The amount of metal removed from the residual fraction was very low and not typically significant, due to the very stable nature of that fraction.

4.4 Discussion

This study examined extraction of Pb and Cd from contaminated soils via the thiol ligands cysteine and glutathione. Pb extraction under nonsterile, oxygenated conditions peaked at pH 7 for cysteine and pH 8 for glutathione, while Cd extraction was most effective at pH 8 for both thiols. The longevity of Pb or Cd solubility was influenced by the reduced thiol concentration remaining in solution, particularly for cysteine where the loss of thiol was substantial after only one hour. Overall total metal extraction reached at least 40% removal in most soils and up to 100% removal in Hanshaw Cd after four sequential extractions with 10 mM cysteine or glutathione at their optimum extraction pH.

The total Pb and Cd extracted after 4 sequential extractions were lower than studies with synthetic chelators, such as EDTA or DTPA (diethylenetriaminepentaacetic acid), which in some cases remove nearly 100% of the contaminant metals (Nowack et al. 2006). Other studies have examined the effectiveness of the amino acid histidine to remove Cd and Pb from contaminated soil (Chen et al. 2007). They reported about 30 % total removal at pH 6.5 with a

Table 4.2 Pb and Cd content in soil fractions of control or soils exposed to 1 hour treatments of 10 mM cysteine at pH 7 or 10 mM glutathione at pH 8.

	Soil	Exchangeable (ppm)	$CaCO_3$ (ppm)	Fe/Mn-oxide (ppm)	Organic/sulfide (ppm)	Residual (ppm)
Hanshaw Pb	Control	61 ± 1.0	500 ± 10	860 ± 200	310 ± 40	160 ± 20
	10 mM cys pH 7	3.0 ± 0.4*	470 ± 14	360 ± 120*	200 ± 40*	140 ± 10
	10 mM gsh pH 8	3.6 ± 0.2*	510 ± 13	500 ± 40*	230 ± 40	110 ± 20
Hanshaw Cd	Control	11 ± 0.0	85 ± 1	200 ± 30	53 ± 1	12 ± 2
	10 mM cys pH 7	6.4 ± 0.7*	77 ± 6	160 ± 20	57 ± 1	10 ± 1
	10 mM gsh pH 8	4.2 ± 2.5*	63 ± 5*	36 ± 1*	24 ± 1*	2 ± 1*
Orchard Pb	Control	6.6 ± 0.5	61 ± 1	54 ± 5	110 ± 8	53 ± 1
	10 mM cys pH 7	1.0 ± 0.3*	51 ± 4	60 ± 20	70 ± 5*	51 ± 2
	10 mM gsh pH 8	0.5 ± 0.2*	33 ± 3*	80 ± 4*	70 ±24	61 ± 6
LP44 Pb	Control	4.0 ± 2.2	480 ± 55	400 ± 70	360 ± 30	110 ± 50
	10 mM cys pH 7	1.6 ± 0.2	320 ± 7*	400 ± 50	130 ± 60*	190 ± 30
	10 mM gsh pH 8	1.6 ± 0.5	270 ± 130*	530 ± 90	320 ± 60	160 ± 10
LP53 Pb	Control	3.0 ± 1.3	120 ± 30	200 ± 20	150 ± 10	90 ± 10
	10 mM cys pH 7	1.3 ± 0.3	120 ± 24	230 ± 30	80 ± 10*	90 ± 6
	10 mM gsh pH 8	0.8 ± 0.1*	35 ± 2*	180 ± 30	130 ± 20	110 ± 10

*significantly different from the control ($p<0.05$)

10 mM histidine concentration (total Cd was only 25 ppm as opposed to about 400 ppm here), and found only about 1 % desorption from soil artificially contaminated with 1000 ppm Pb. They achieved from 25 to 45 % desorption with 10 mM oxalic acid or citric acid at pH 6.5.

The pH of the extraction solutions had a very strong effect on metal solubility. At a soil pH of 4 or 5, Pb solubility is extremely low, but Cd solubility is substantially higher due to its weak adsorption to soil phases (McBride, 1994); the solubility of both was likely augmented by the high concentration of acetic acid used here to buffer pH. However, when cysteine and glutathione were added at these pHs, Cd solubility decreased. This could be due to sorption of the Cd-thiol complex to the soil surfaces (Kozlowski et al., 1990). Once pH rose above 6, solubility of Pb greatly increased even though soil surface charges became more negative, likely due to the strong metal binding abilities of cysteine and glutathione at higher pH where competition of the sulfhydryl group with the H^+ ion has less influence.

The binding constants of cysteine and glutathione with Pb are $10^{13.1}$ and $10^{11.4}$, respectively, and with Cd are $10^{11.1}$ and 10^{11}, respectively (Martell and Smith, 2004), and the most stable complexes are typically formed at pH 7-9 where both hydrogen ions do not compete as effectively for the thiol group and hydroxide ions do not compete as effectively for the metals. The difference in binding constants with Pb likely plays a role in the higher Pb desorption in cysteine treatments. Since the binding constants of the 1:1 thiol and Cd complexes are nearly the same for cysteine and glutathione, the slightly higher desorption with glutathione as pH increases in Hanshaw Cd soils (Figure 4.2) is likely in part due to the stronger 2:1 thiol:Cd complexes potentially formed with glutathione at the higher pHs.

The sterilization and N_2 flushing of soils produced a large change in Pb extracted from Hanshaw Pb soil under cysteine treatment at pH 8, likely due to a

decrease in oxidation of cysteine as evidenced by the kinetic studies. However, under glutathione treatment, extraction of Pb from Hanshaw Pb soil across the pH range was significantly lower at pH 7 and 8, perhaps due to an alteration of the soil structure by autoclaving that affects glutathiones' ability to interact with Pb. Autoclaving soil alters the structure, releasing more elements from microbes and sequestered positions and destroying some of the larger organic matter complexes. Under these conditions, perhaps glutathione more selectively binds other elements or there is more chemisorption of glutathione to the soil, limiting its ability to solubilize Pb.

At the same time as Pb and Cd solubility was increasing with increased pH, reduced glutathione concentrations in the extraction solution were roughly constant whereas reduced cysteine in the extraction solution decreased significantly. In part, the almost immediate drop in concentration of both thiols may be due to sorption to the soil surface. Sorption of these organic acids may then increase the negative charge of the soil surfaces and create alternative binding sites for metals, thereby creating more re-sorption sites for the solubilized metals. However, the rapid drop in cysteine concentration in the kinetic experiments at pH 7 was likely due to metal catalyzed oxidation because sorption should have been similar whether N_2 flushed or not and cysteine was not oxidized in solution without soil after one hour.

Several factors may play a role in the rapid oxidation of cysteine. At pHs above neutral, cysteine oxidizes more rapidly than glutathione in solution although it is enhanced significantly by the presence of a metal catalyst (Dorćak et al. 2007). The presence of the protonated amine group at higher pH may enhance oxidation as it does with glutathione due to the presence of a salt bridge between the amine and the sulfhydryl (Krezel and Bal 2004). This would explain why there was a large loss of reduced cysteine from the extraction solution compared to essentially no loss of thiopropionate. In addition, metal catalyzed oxidation may occur through molecular

oxygen, as suggested by Bagiyan et al. (2003, 2004), where dissolved metals such as Cu, Mn, Fe, and Ni may catalyze oxidation at neutral to slightly alkaline pH. Oxidation may also occur via a surface catalyzed process by Fe- or Mn-oxides (Stone, 1987; Ulrich and Stone, 1989); cysteine has been shown to reductively dissolve Fe(III)oxides (Doong and Schink, 2002). The thiol group may exchange with the hydroxide group on a surface (eq 5.1), which then transfers an electron from the reduced thiol group to the Fe(III) (eq 5.2). Afterwards, both the reduced iron and oxidized thiol would be released from the soil surface and lead to the formation of cystine in solution and free Fe(II).

$$surface \equiv Fe(III) - OH + R - S - H \leftrightarrow surface \equiv Fe(III) - S - R + H_2O \quad \text{(eq 5.1)}$$

$$surface \equiv Fe(III) - S - R \leftrightarrow surface \equiv Fe(II),\cdot S - R \quad \text{(eq 5.2)}$$

$$surface \equiv Fe(II),\cdot S - R + H_2O \leftrightarrow surface \equiv Fe(II) - OH_2 + \cdot S - R \quad \text{(eq 5.3)}$$

$$surface \equiv Fe(II) - OH_2 \leftrightarrow Fe^{2+}(aq) + free\ site \quad \text{(eq 5.4)}$$

In these experiments, both aqueous and surface catalyzed oxidation may be occurring. The surface processes are fast and may have resulted in the rapid initial loss of reduced cysteine from solution as well as the higher concentrations of soluble Fe. The mechanism of soluble metal catalyzed oxidation is complex. Free Fe(II) is rapidly oxidized in the presence of molecular oxygen at high pH, which likely explains the difference between the soluble Fe concentrations in N_2 flushed and unflushed samples. Some of the Fe(III) formed may then bind to reduced cysteine and help photolytically catalyze the oxidation of cysteine to cystine in solution.

Compared to cysteine, glutathione is larger and therefore may have more difficulty interacting with the soil surface; glutathione also does not bind Fe(III) as effectively as cysteine. Together, this may explain why Fe does not catalyze glutathione oxidation as it does cysteine oxidation. In addition, the molar ratio of GSH to dissolved Pb was approximately 2:1 for the last 18 hours of the kinetic

experiment (Figures 4.8,4.10). The coordination of glutathione with Cd or Pb in solution, perhaps forming a 2:1 complex, may help protect the sulfhydryl group from oxidation, much like $Hg(GSH)_2$ was protected from oxidation by hydrogen peroxide and Cu(II) in another study (Hsu-Kim 2007).

In the environment, the oxidation of thiols and microbial activity would play a role in the loss of thiols over the long term. However, cysteine is still effective at solubilizing Pb and Cd in the short term and perhaps a balance could be met between continuous reduced cysteine inputs for the purpose of maintaining soluble Pb- or Cd-cysteine species for plant uptake. Glutathione is not as rapidly oxidized and is generally more effective at solubilizing metals and would likely require less input over the long term. In using these expensive molecules for solubilizing metals in a phytoremediation system, the lower the concentration required the better. In the future, systems could integrate either plant or microbe generation and exudation of cysteine or glutathione to solubilize contaminant metals in the rhizosphere.

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CHAPTER 5

UPTAKE OF PB AND CD BY *ZEA MAYS* IN SOIL SYSTEMS WITH THIOL TREATMENT

5.1 Introduction

Ultimately, for phytoremediation to be successful, it must be applicable in the field. Proponents for phytoremediation insist that shoot translocation must occur to a significant degree, with the contaminant representing at least a few percent of the shoot biomass for phytoremediation to be successful. For Pb, very few plants have been found that naturally accumulate the metal to that extent in the shoots (Vogel-Mikuŝ 2005), and none are high biomass species. Furthermore, synthetic chelator assisted phytoremediation will result in significant translocation, but the plant is quickly killed and leaching is an issue (Nowack et al. 2006).

The application of biogenic thiol mediated phytoremediation may not be ready for commercial use due to insignificant transport to the shoots (Chapter 3). However, the field application of the thiols cysteine and glutathione to solubilize metals from contaminated soil and mediate transport into roots in a soil system may still be tested. This chapter examines application of thiols to Hanshaw Pb and Hanshaw Cd soils at various pHs based on previous results (Chapter 4) and uptake into *Zea mays*, both roots and shoots, in pot systems in the greenhouse. The solubilization of Pb and Cd is also examined in the soil pore water.

5.2 Materials and Methods

Soil contamination and pH adjustment

Soil from a fallow agricultural field was used for these experiments. The soil was artificially contaminated with Pb or Cd (labeled Hanshaw Pb and Hanshaw Cd) to target values of 2000 ppm for Pb and 400 ppm for Cd. Lead acetate ($Pb(C_2H_3O_2)_2$; EMD Chemicals) or cadmium chloride ($CdCl_2$; Fluka) was dissolved in water and

sprayed onto soil over several layers and mixed, repeatedly until all the solution was used. All soil was air dried and sieved to 2 mm. The initial pH of Pb and Cd soils were 4.75 ± 0.11 and 4.21 ± 0.01, respectively. For these experiments, soils were limed by shaking in $CaCO_3$ fine powder, wetting soil and mixing, to achieve several target pH levels (Table 5.1), including Pb soils at pH 6, 6.5, and 8 and Cd soils at pH 8. The Mehlich buffer method (Mehlich 1976; Ssali and Nywamnya 1981) was used to estimate lime levels required to achieve up to pH 7, and trial and error was used to increase pH above 7. Soils will be identified by the contaminant metal and the actual pH of the soil, i.e. Pb 6.2 soil or Cd 7.9 soil.

Table 5.1 Target and actual soil pH of Hanshaw Pb and Hanshaw Cd contaminated soils after liming with CaCO3. Values are average ± SD (n=3).

Soil	Target pH	Actual pH
Hanshaw Pb	6	6.17±.08
Hanshaw Pb	6.5	6.84±.04
Hanshaw Pb	8	7.82±.02
Hanshaw Cd	8	7.85±.02

Plant growth

Four inch pots were filled with 200 ± 20 g of air-dried contaminated soil and watered to field capacity. One seed of *Zea mays* (DeKalb 39-47) was planted in the center of each pot, 1 inch below the surface. Plants were grown in the greenhouse subject to a daily light integral of 17 moles/m^2 (an average of about 200 μmoles/m^2/s) and day/night temperatures of 24/18 °C for 14 d.

Soil uptake experiments

After two weeks of growth, triplicate pots with plants were exposed to treatments of 10 mmole/kg, 1 mmole/kg, or 0.1 mmole/kg cysteine or glutathione solutions to examine Pb and Cd uptake from soils. Treatments were based on the

more effective extractions as identified in Chapter 4, including examining uptake of Pb in the presence of cysteine at soil pH 6.2 and 6.8, Cd in the presence of cysteine or glutathione at pH 7.9, and Pb in the presence of glutathione at pH 7.8. Plants were exposed to thiols or a control (water only) for 5 days with either daily addition of the thiol or a one-time initial addition of thiol followed by daily additions of water. Concentrated thiol solutions were prepared corresponding to the thiol application rate and applied to each pot in 10 mL portions distributed across the soil surface. Thiol solutions were pH adjusted as necessary with 10 mM NaOH. Pots were watered to field capacity after treatment.

Upon sampling, shoots were cut ½" above the soil surface and placed in paper bags to dry. Roots were first rinsed clean in deionized water and then rinsed for 10 minutes each in 4 successive solutions of 1 mM EDTA (adjusted to pH 7 with 10 mM NaOH). Samples were then placed in glass test tubes to dry.

Simultaneously, triplicate pots of 200 ± 20 g contaminated soil with plants as described above were setup to examine soil pore water concentrations of dissolved metal concentrations. Holes of about 1/8" were drilled in the plastic pots directly across from each other at the center height of the soil in each pot. Soil moisture samplers (Rhizosphere Research Products) were placed through these holes across the soil column. Pots were treated in the same manner as above with thiols. Pore-water samples were taken daily 1 day before and during treatments, 30 minutes after the treatment was applied, by drawing a vacuum on the pore water sampler using 10 mL syringes. The plunger was pulled out and a wooden retainer was propped in place to maintain the vacuum. Samples of pore water solution were collected after 1 hour, with a typical yield of about 5 mL.

Metal analysis

Dry *Z. mays* shoot samples were ground with a mortar and pestle and about 0.1 g was weighed into glass test tubes for digestion. Root samples were dried in glass test tubes as noted above. All samples were digested first with 5 mL of concentrated HNO_3 (70%; EMD Chemicals, Inc.) and heated at 90 ºC for 15 minutes, followed by the addition of 3 mL of H_2O_2 (30%; Mallinckrodt) and heating at 110 ºC for 10 minutes. Tissues samples were diluted as necessary and soil pore water samples were acidified to 5% HNO_3 and then analyzed by ICP-OES (Thermo Jarrell Ash ICAP 61). Pb and Cd tissue measurements were normalized to dry weights.

5.3 Results and Discussion

Soil pore water samples

The solubility of Pb and Cd in soil pore water increased immediately after the addition of either 10 mmole/kg cysteine or glutathione at all pH levels. In the case of each Pb soil treatment, cysteine at pH 6.2 and 6.8 and glutathione at pH 7.8, the initial increase in Pb solubility was temporary, and after the first days' treatment solubility decreased to background levels (Figures 5.1, 5.2, 5.3). This may be due to several factors as described in Chapter 4, including oxidation of the thiols, sorption of the thiols to the soil surface, biodegradation of the thiols, all limiting their ability to maintain metal solubility, or leaching of the Pb-thiol complex from the system. Under daily exposure to the cysteine treatment, Pb solubility varied at each pH tested. At pH 6.2, Pb solubility increased over 5 days, from about 20 ppm on day 1 to about 60 ppm on day 5 (Figure 5.1). On the contrary, Pb solubility decreased over time at pH 6.8, peaking around 70 ppm on day 1 and decreasing to below 10 on day 5 (Figure 5.2). These trends are likely due to more rapid oxidation of cysteine at a pH near 7 and above but less interaction with surfaces as the pH decreases (Chapter 4). After daily exposure of Hanshaw Pb soil at pH 7.8 to 10 mmole/kg glutathione, Pb pore water

concentrations increased to an average peak of 210 ppm on day 3 and then decreased to below 50 ppm by day 5 (Figure 5.3). This may be due to similar mechanisms of thiol loss as described for cysteine, and could also mean the majority of accessible Pb was solubilized and either leached from the soil or taken up by the plant.

While the average concentration of Cd in soil pore water at pH 7.9 under cysteine treatment went up over the first few days of thiol addition, it was typically not significantly different from the initial concentration on day 0 (Figure 5.4). Glutathione treatment did increase Cd solubility initially, but did not maintain that solubility over time (Figure 5.5). In all cases, the solubilized Pb and Cd concentrations were much lower than in the lab extractions (Chapter 4), which is expected because those samples were vigorously shaken. However, another contribution to reduced solubility over time may have been the formation of metal sulfides. In a previous pot experiment with the addition of 100 mmole/kg cysteine to Pb soil at pH 6.8, the soil in the bottom of the container was black. Although at these lower concentration thiol additions presented here, black particles were not visible to the naked eye, metal sulfides may still have formed after 5 days due to degradation and reduction of the thiols and formation of Pb or Cd sulfides in the water saturated soils.

Pb and Cd uptake in Zea mays

The uptake of Pb and Cd, based on measurements of root-associated metal, from the various pH soils under thiol treatment was never significantly higher than the control. In the Pb pH 6.2 soil, uptake into the roots or the shoots did not vary significantly between treatments, suggesting the thiol played no role in Pb uptake or that its role in uptake was similar to natural conditions at that pH (Figure 5.6). In both the Pb soil at the higher pH of 6.8 exposed to cysteine and Pb soil at pH 7.8

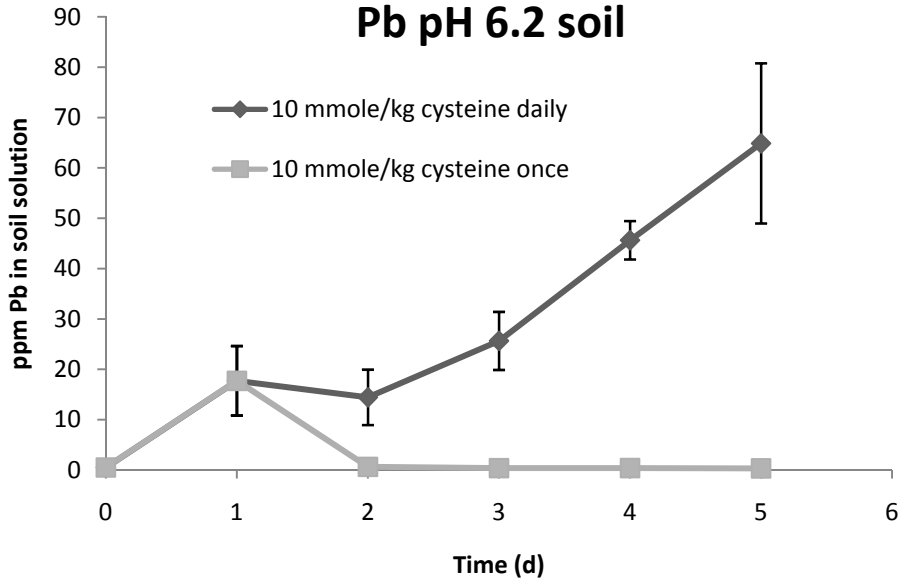

Figure 5.1 Soil pore water Pb concentration in Hanshaw Pb pH 6.2 soil exposed to a one time (day 1) or daily (day 1-5) addition of 10 mmoles/kg cysteine. Values are an average ± SD (n=3).

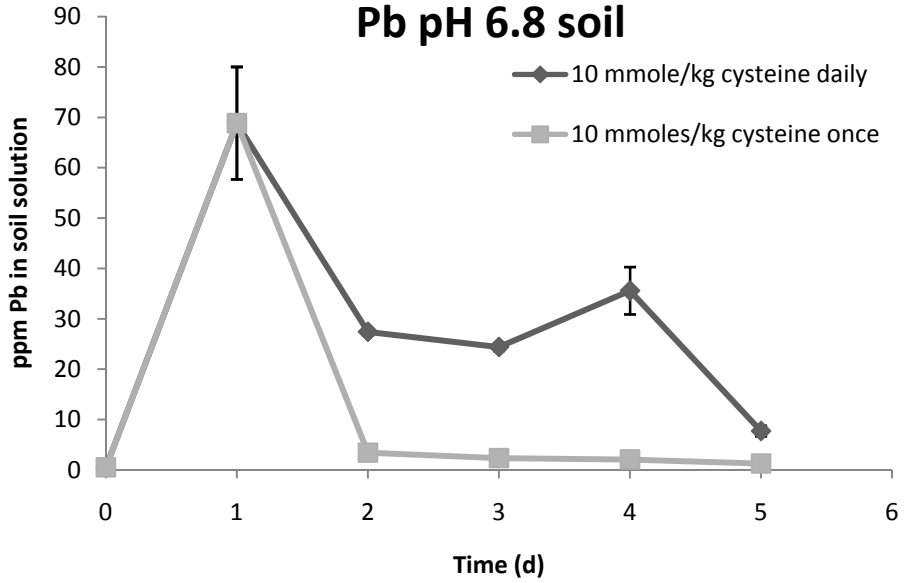

Figure 5.2 Soil pore water Pb concentration in Hanshaw Pb pH 6.8 soil exposed to a one time (day 1) or daily (day 1-5) addition of 10 mmoles/kg cysteine. Values are an average ± SD (n=3).

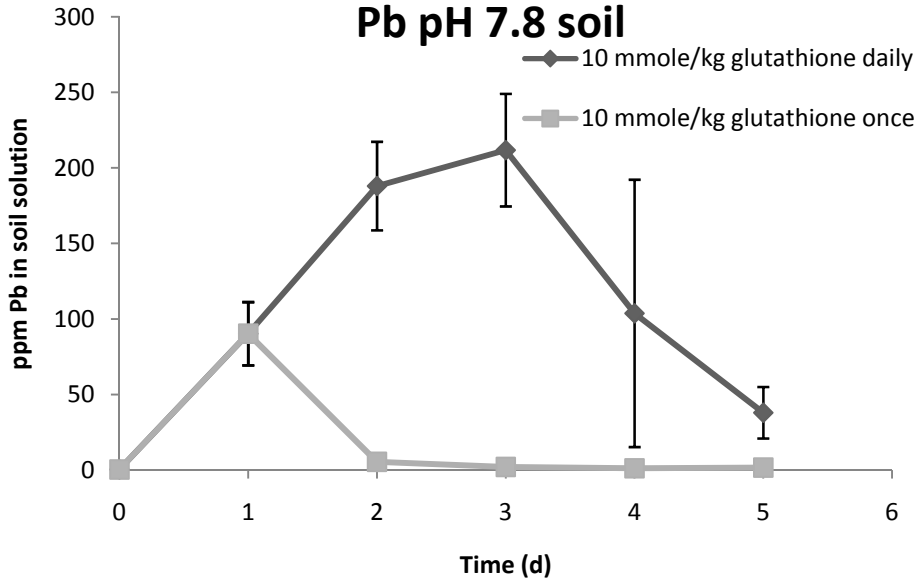

Figure 5.3 Soil pore water Pb concentration in Hanshaw Pb pH 7.8 soil exposed to a one time (day 1) or daily (day 1-5) addition of 10 mmoles/kg glutathione. Values are an average ± SD (n=3).

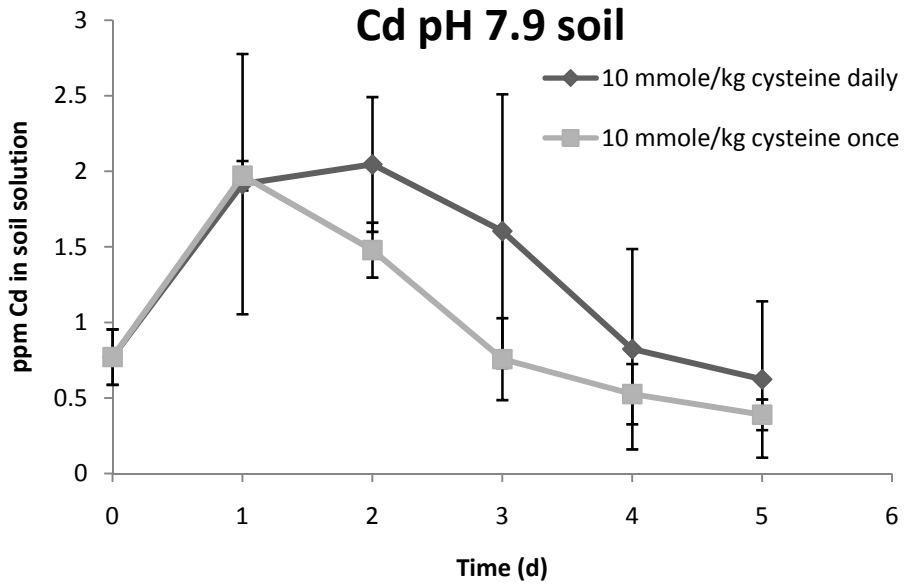

Figure 5.4 Soil pore water Cd concentration in Hanshaw Cd pH 7.9 soil exposed to a one time (day 1) or daily (day 1-5) addition of 10 mmoles/kg cysteine. Values are an average ± SD (n=3).

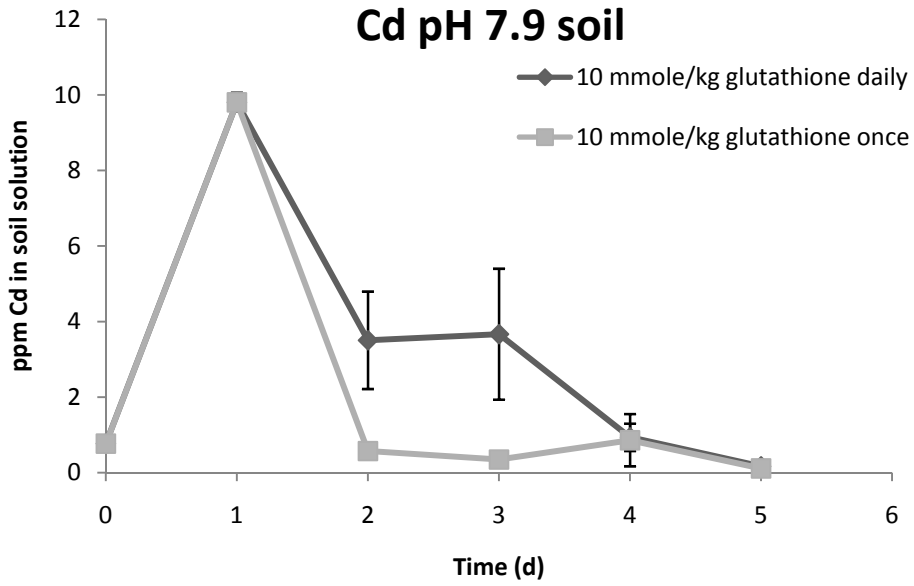

Figure 5.5 Soil pore water Cd concentration in Hanshaw Cd pH 7.9 soil exposed to a one time (day 1) or daily (day 1-5) addition of 10 mmoles/kg glutathione. Values are an average ± SD (n=3).

exposed to glutathione, Pb uptake into the roots was significantly lower than the control in all cases, suggesting the thiol had a role in limiting bioavailability and therefore uptake (Figure 5.7, 5.8). Also, uptake was higher after only a one time addition of thiol than with daily additions at both pH 6.8 with cysteine and pH 7.8 with glutathione, though not significantly in the latter case. The shoot concentrations generally followed the same patterns as the root concentrations, but were not significantly higher than the control.

The uptake of Cd from soils at pH 7.9 was different between cysteine and glutathione treatments. While daily additions of cysteine at low concentrations resulted in average root uptake greater than the control, one time additions did not, though again most values were not significantly different and would not be expected to be based on the low pore water concentrations of Cd observed in soil exposed to 10

mmole/kg cysteine treatments (Figure 5.4). Glutathione treatment resulted in either lower or about the same uptake as in control plants.

One confounding issue with root metal measurements in pot studies is the inability to remove all soil particles from the root surface even after rinsing with both water and EDTA solutions. This likely resulted in variable soil Pb associated with root measurements; however, since root Pb concentrations were lower after thiol treatment in both the pH 6.8 and 7.8 soils, the thiol may have leached Pb from soil particles associated with the root surface in those cases. Another potential reason for lower Pb and Cd uptake may be competition for uptake with other thiol complexes, such as with Fe, Ca, or Mg, all present at high concentrations in the soil, which also had elevated concentrations in the soil extractions.

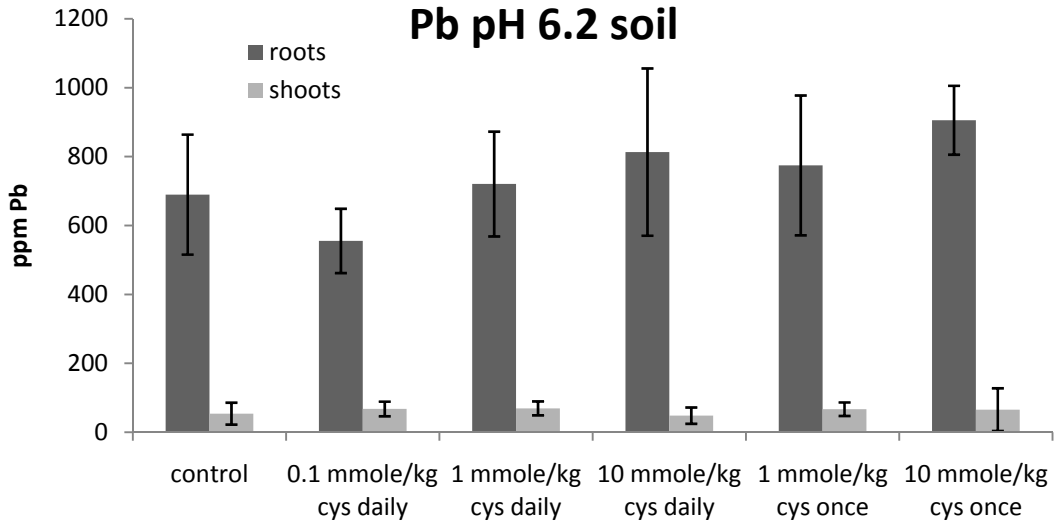

Figure 5.6 Root and shoot Pb concentration in *Z. mays* grown in Hanshaw Pb pH 6.2 soil exposed to a one time (first day) or daily additions of various cysteine (cys) concentrations. Values are an average ± SD (n=3).

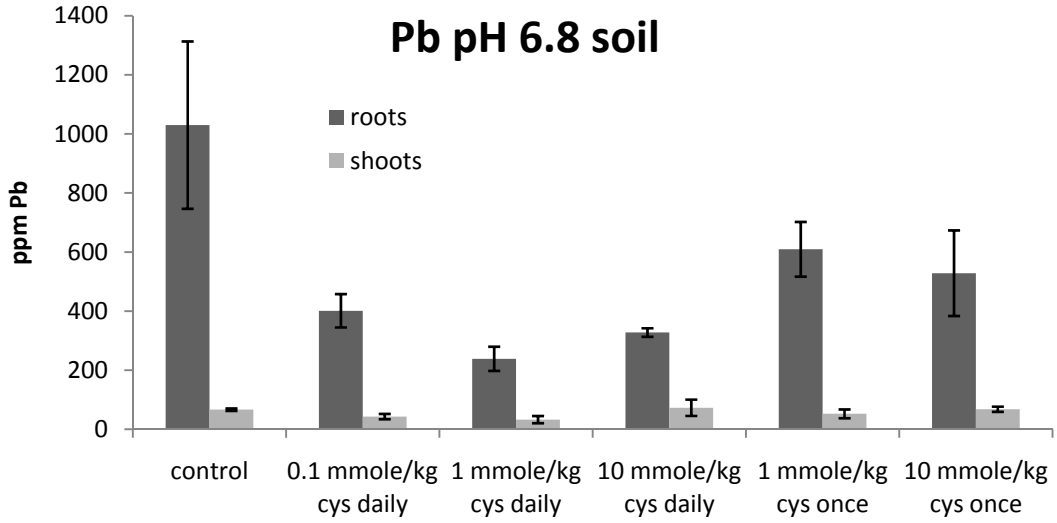

Figure 5.7 Root and shoot Pb concentration in *Z. mays* grown in Hanshaw Pb pH 6.8 soil exposed to a one time (first day) or daily additions of various cysteine (cys) concentrations. Values are an average ± SD (n=3).

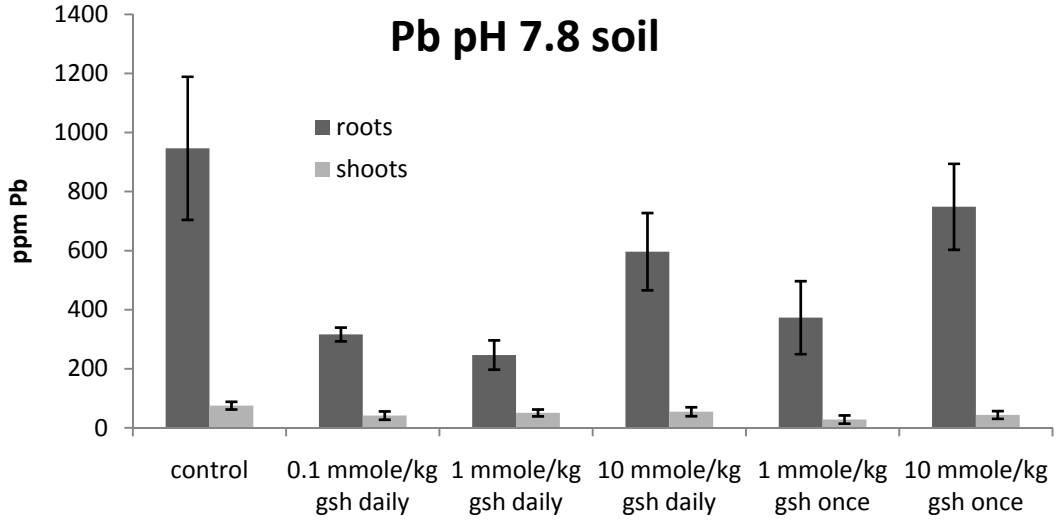

Figure 5.8 Root and shoot Pb concentration in *Z. mays* grown in Hanshaw Pb pH 7.8 soil exposed to a one time (first day) or daily additions of various glutathione (gsh) concentrations. Values are an average ± SD (n=3).

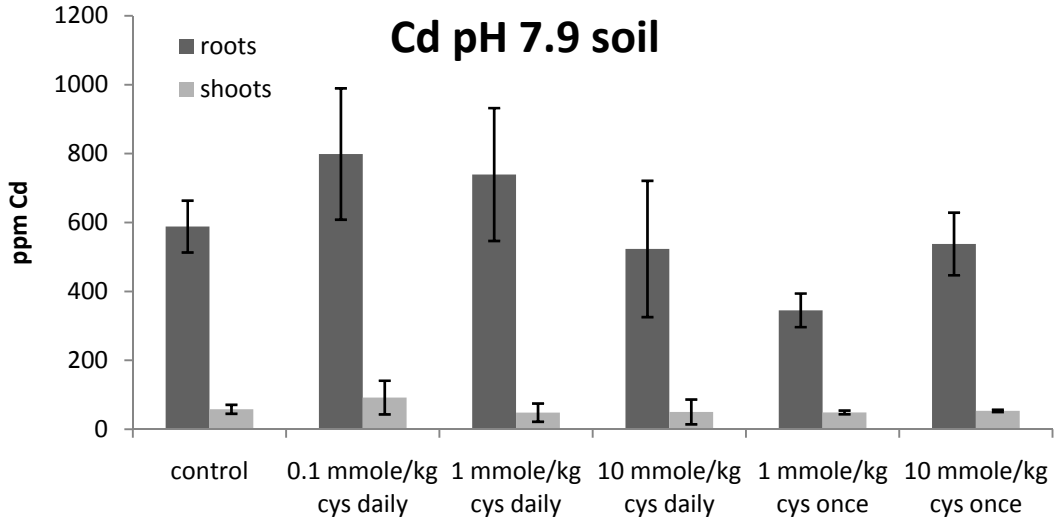

Figure 5.9 Root and shoot Cd concentration in *Z. mays* grown in Hanshaw Cd pH 7.9 soil exposed to a one time (first day) or daily additions of various cysteine (cys) concentrations. Values are an average ± SD (n=3).

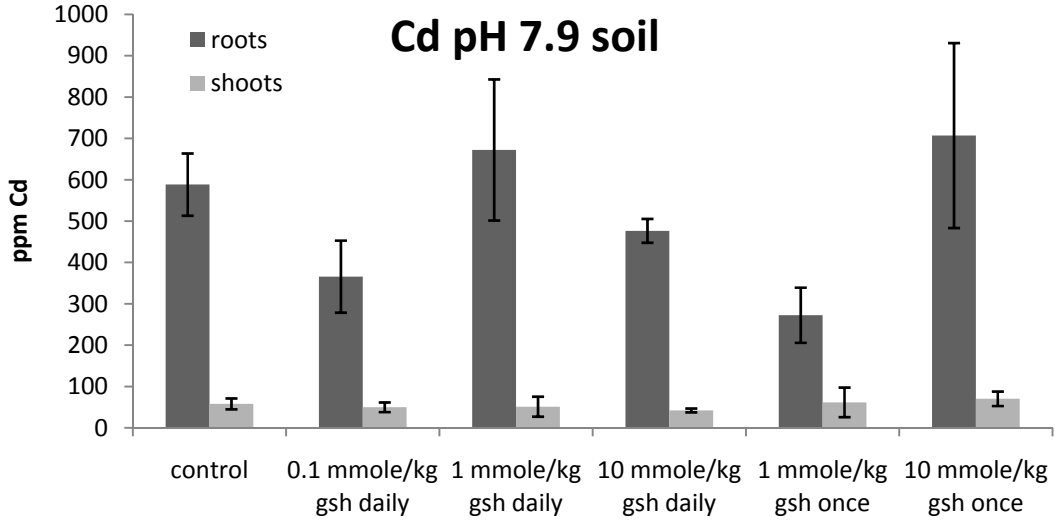

Figure 5.10 Root and shoot Cd concentration in *Z. mays* grown in Hanshaw Cd pH 7.9 soil exposed to a one time (first day) or daily additions of various glutathione (gsh) concentrations. Values are an average ± SD (n=3).

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Vogel-Mikuŝ, K., Drobne, D., Regvar, M. Zn, Cd, and Pb accumulation and arbuscular mycorrhizal colonization of pennycress *Thalaspi praecox* Wulf. (Brassicaceae) from the vicinity of a lead mine and smelter in Slovenia. *Environmental Pollution*, **2005**, *133*, 233-242.

CHAPTER 6

SUMMARY AND CONCLUSIONS

The uptake of metal-thiol complexes (e.g. Pb-glutathione or Pb-cysteine) was examined into *Brassica napus* and *Zea mays* root (Chapter 2). The mechanism is thought to be accidental transport of the metal-thiol complex through an amino acid transporter for Me-cysteine or through a peptide/glutathione transporter for Me-glutathione or Me-cysteine$_2$. Evidence includes the use of a biological transport inhibitor vanadate which inhibited transport by about 50%, competition for Pb-thiol uptake by free thiol species, and increased Pb transport after pre-exposure to thiols. Several experiments varied the speciation of Me-thiol complexes by altering the solution pH or altering the metal concentration, resulting in variable changes in uptake. However, altering solution speciation, particularly by changing pH, may alter biological activity as well as change the conformation and activity of the transport protein or alter the driving force (protons) across the membrane. While good evidence has been presented to support symplastic transport into the root cells, including the mechanistic studies in Chapter 2 and the long-term studies and electron microscopy in Chapter 3, more details and confirmation could be provided by conducting experiments with plasma membrane vesicles. Under those experimental conditions of nearly pure membranes, with no cell wall sorption interference and small volumes, several other transport inhibitors could be tested, proton gradients could be measured using fluorescent dyes, and both metal and thiol movement could be followed through the use of either stable or radioactive isotopes. Stable isotopes could also be used in whole-plant studies to confirm transport of thiols into plants and trace movement throughout the tissue.

Several experiments resulted in unexplained changes in transport. While we expected an amino acid transporter to play a role in Me-cysteine transport, inhibition

with increasing concentrations of amino acids, including acidic, neutral or basic amino acids, resulted in both large increases and decreases in Pb uptake (Appendix A). Consistently higher rates of Pb uptake resulted from cysteine pre-exposed plants treated simultaneously with methionine and cysteine, and in plants treated simultaneously with reduced and oxidized glutathione. Oxidized glutathione (GSSG) may activate an alternate transporter, but lower GSSG concentrations enhanced uptake to a greater degree. It is difficult to conjecture the role methionine or oxidized glutathione may play and further experimentation and clarification of the transport mechanism is needed. In addition, Pb uptake was examined in N-limited or S-limited *B. napus* (Appendix C). Only S-limited plants resulted in greater Pb uptake, again suggesting the role of a thiol transporter. Sulfur limiting the soil may be a strategy to accelerate Me-thiol uptake from soil systems.

In addition to Pb and Cd uptake, Ag uptake was also examined. Ag uptake was variable, but very high in the case of cysteine mediated uptake, perhaps due to transport of the smaller sized Ag-thiol complex more readily via an amino acid transporter (Appendix C). While this was not explored further, Ag and several other metals, including Hg, Ni, Zn should be examined for the potential of thiol-mediated uptake.

While there was Pb transport into root cells, particularly with glutathione, there was no translocation to shoots (Chapter 3). Experiments with several tDNA *Arabidopsis thaliana* mutants suggested a role for several glutathione/peptide transporters, including OPT7, OPT5, PDR12, and PTR3. Many more transporters, including members of these peptide transporter families as well as amino acid transporters that are currently unavailable as knockouts, could also be involved. In one case, the lack of OPT5 led to increased shoot translocation, but without detailed knowledge of the transport systems, it is difficult to deduce why. In order to

understand and in the future engineer a plant more tolerant and capable of root uptake and shoot translocation, knowledge of both root membrane and internal plant transport systems responsible for metal movement through plants is vital. The root vacuolar sequestration mechanism may be turned off or the mechanism of xylem loading may be upregulated, both of which would drive shoot translocation. It is difficult to measure internal movement of metals within cells, but electron microscopy tied to x-ray spectroscopy looks promising and will be pursued in the future to measure the distribution of Pb across cells following thiol treatments.

The application of both cysteine and glutathione to Pb or Cd contaminated soils results in a rapid increase in metal solubility which, in the presence of oxygen is short-lived in the case of cysteine but fairly stable in the case of glutathione (Chapter 4). Oxidation of cysteine interferes the most with its ability to solubilize metals, and is not controllable in the field. Glutathione may be more effective in natural systems. However, these thiols are quite expensive and initial pot studies did not show effective Pb or Cd uptake from soils (Chapter 5). Once the plant is engineered to maximize metal uptake, soil uptake may be more pronounced. Also, you may be able to engineer plants to exude glutathione, thus maintaining thiol concentrations around the active root zone and not wasting them by adding them to the entire soil volume.

Field application of this remediation strategy is a long way off, but there is hope for enhancing metal uptake with thiols. Alternative thiols may be designed that are more effective at both solubilizing metals under oxic conditions and transporting them across the plant membranes. There is more research needed on understanding the internal mechanisms of metal movement in plants; thiols likely play a large role, particularly in Pb, Cd, and other thiol-binding metals. Because the metals that require environmental remediation are not typically essential for the plant, it is important to

understand the plants' natural detoxification and shuttling pathways in order to alter or manipulate its ability and maximize uptake and shoot translocation.

APPENDIX A

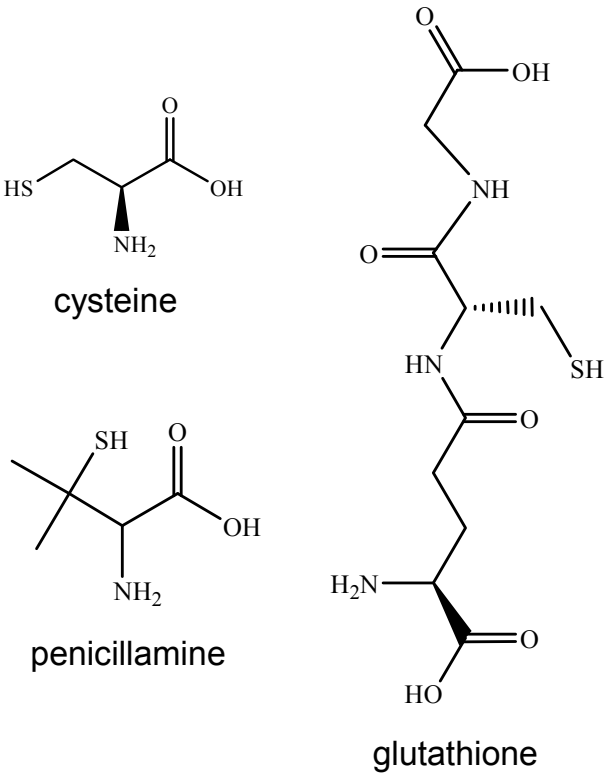

Figure A.1 Chemical structures of relevant molecules

Table A.1: Percent change in Pb uptake rate in *Brassica napus* due to variations in experimental conditions, including alternate ligands, substrates, or metals.

Change from initial experiment*	Ligand	Pb uptake (% control)
+ 10 μM GSSG	38 μM Glutathione	+ 453
+ 38 μM GSSG	38 μM Glutathione	+ 189
+ 100 μM GSSG	38 μM Glutathione	+ 209
+ 100 μM proline, pre-exposed to 1 mM cysteine	100 μM Cysteine	+ 40
+ 500 μM proline, pre-exposed to 1 mM cysteine	100 μM Cysteine	- 2[a]
+ 100 μM aspartate, pre-exposed to 1 mM cysteine	100 μM Cysteine	+ 82
+ 500 μM aspartate, pre-exposed to 1 mM cysteine	100 μM Cysteine	- 5[a]
+ 100 μM lysine, pre-exposed to 1 mM cysteine	100 μM Cysteine	+ 13[a]
+ 500 μM lysine, pre-exposed to 1 mM cysteine	100 μM Cysteine	+ 12[a]
+ 100 μM glycine, pre-exposed to 1 mM cysteine	100 μM Cysteine	- 12[a]
+ 500 μM glycine, pre-exposed to 1 mM cysteine	100 μM Cysteine	+ 23
+ 100 μM methionine, pre-exposed to 1 mM cysteine	100 μM Cysteine	+ 393
+ 200 μM methionine, pre-exposed to 1 mM cysteine	100 μM Cysteine	+ 254
+ 400 μM methionine, pre-exposed to 1 mM cysteine	100 μM Cysteine	+ 427
+ 800 μM methionine, pre-exposed to 1 mM cysteine	100 μM Cysteine	+ 521
pre-exposed to 1 mM lysine	100 μM Cysteine	- 36
pre-exposed to 1 mM aspartate	100 μM Cysteine	- 26[a]
pre-exposed to 1 mM methionine	100 μM Cysteine	+ 38

* as described in methods section

[a] not significantly different from the control

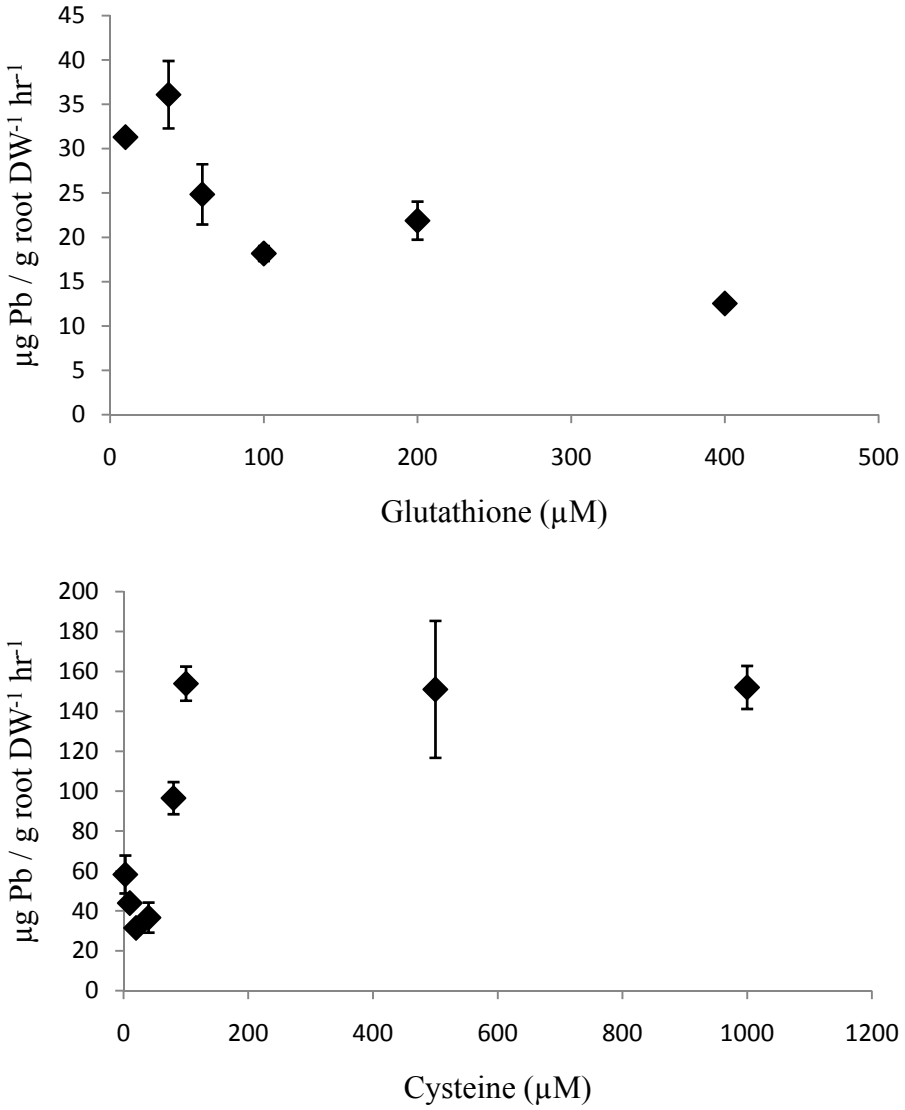

Figure A.2: Pb uptake rates into 1 mM thiol pre-exposed *B. napus* roots over varying solution concentrations of ligands glutathione or cysteine at a constant 1 µM Pb concentration. Values are ± SD (n=3)

APPENDIX B

SOIL EXTRACTION DATA

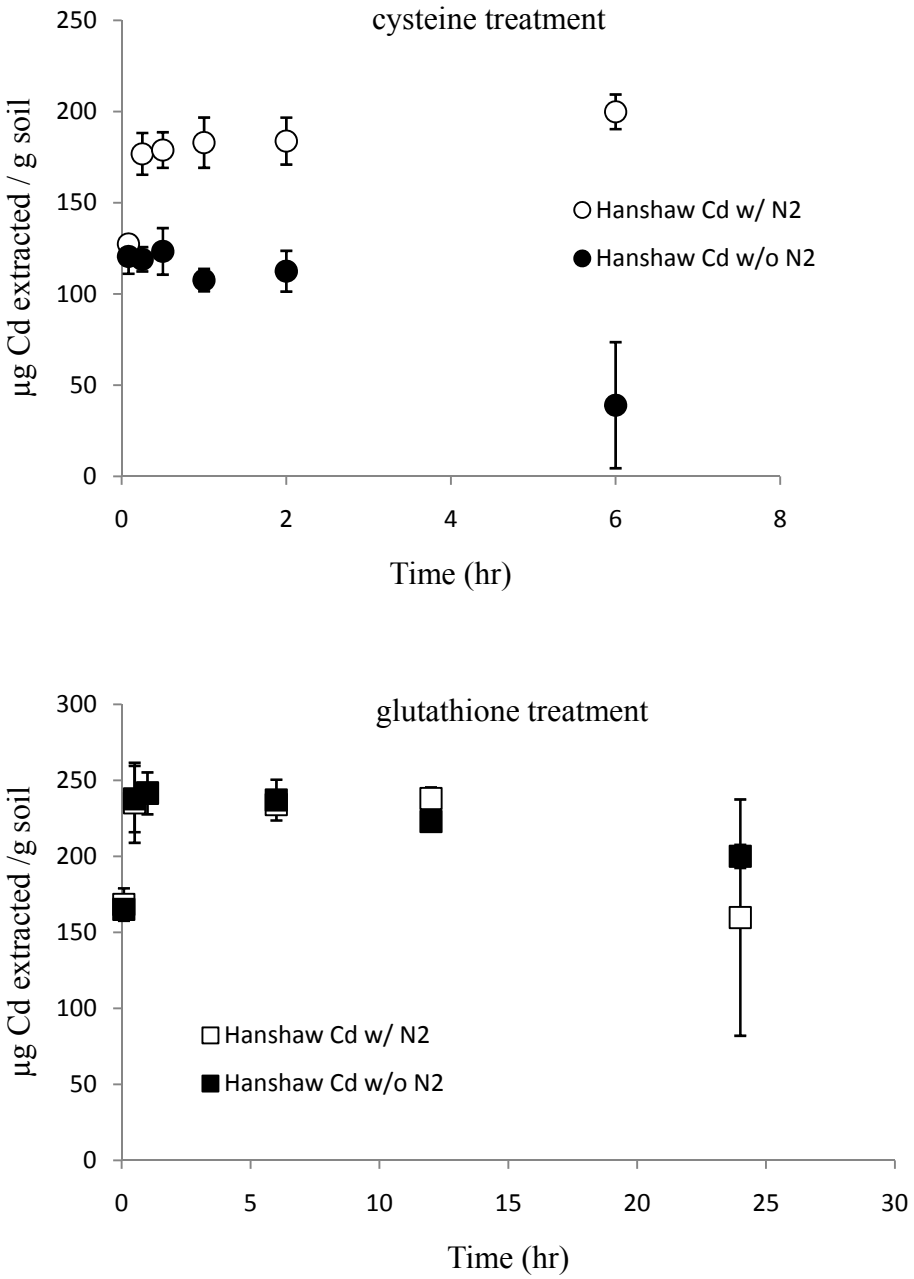

Figure B.1 Total Cd extracted over time over the course of 10 mM cysteine at pH 7 and 10 mM glutathione at pH 8 sterile Hanshaw Cd soil extractions with and without flushing solutions and headspace with N_2 gas. Error bars represent one standard deviation (n=3).

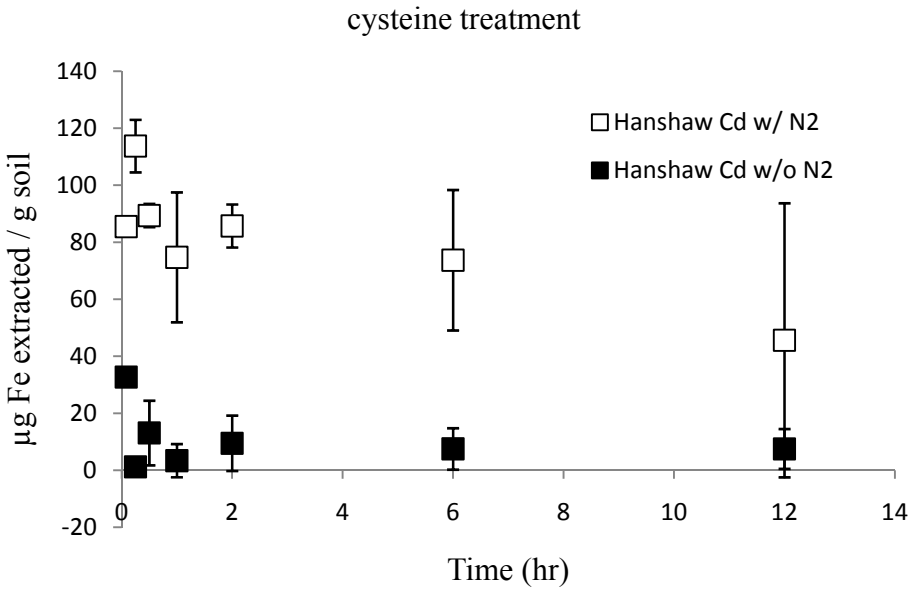

Figure B.2 Total Fe extracted over time over th e course of 10 mM cysteine at pH 7 sterile Hanshaw Cd soil extractions with and without flushing solutions and headspace with N_2 gas. Error bars represent one standard deviation (n=3).

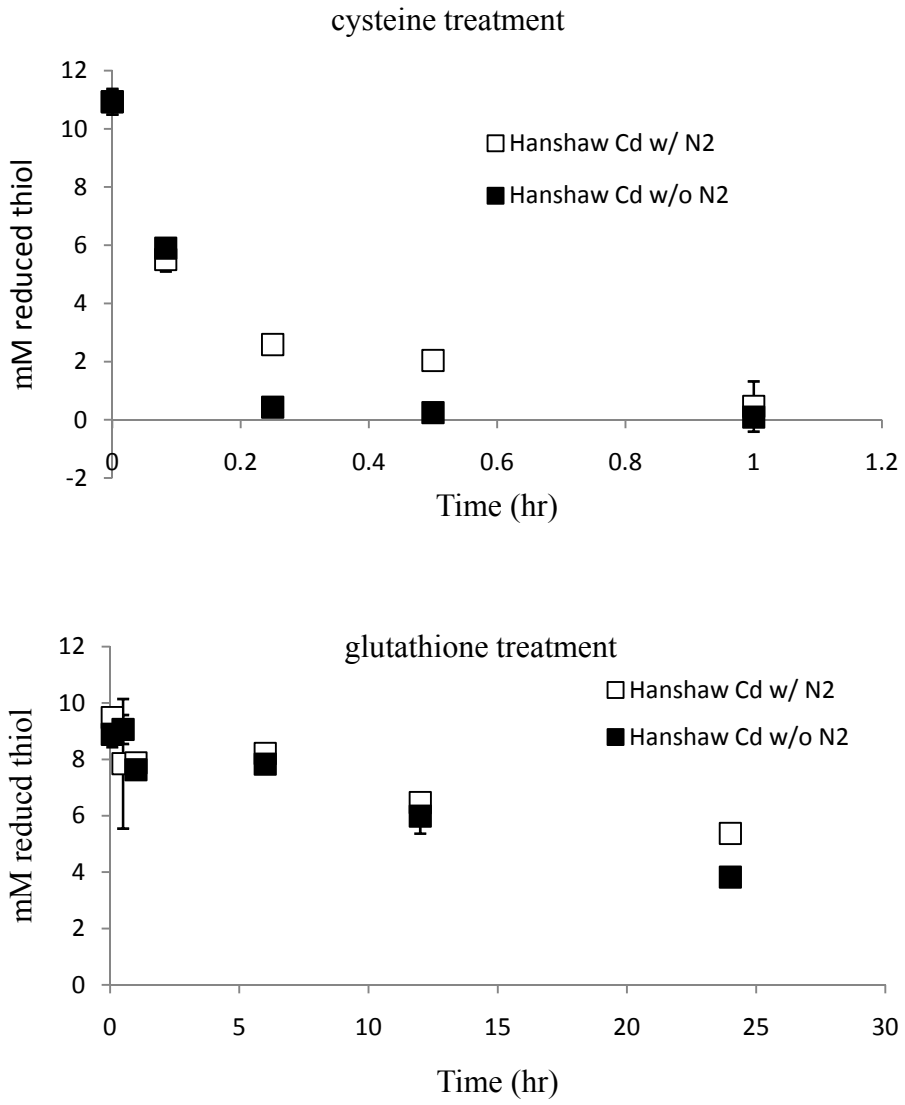

Figure B.3 Reduced thiol concentrations remaining in extraction solution over time over the course of 10 mM cysteine at pH 7 and 10 mM glutathione at pH 8 sterile Hanshaw Cd soil extractions with and without flushing solutions and headspace with N_2 gas. Error bars represent one standard deviation (n=3).

APPENDIX C

SHORT-TERM HYDROPONICS EXPERIMENTS

C.1 Introduction

The uptake of Pb into roots of *B. napus* and *Z. mays* mediated by cysteine or glutathione was shown in Chapter 2. In addition to the previous examination of Pb uptake under healthy plant conditions, the following experiments examine Pb uptake in *B. napus* under stressed conditions, namely N- or S-limitation. Both cysteine and glutathione are primary sulfur and nitrogen containing compounds in plants and uptake of these species from solution may be enhanced when the plant is N- or S-limited, which in turn may enhance the transport of Pb if uptake is due to co-transport through the same transporters. In addition, competition by Ag for transport via the same Me-thiol mechanism is examined.

C.2 Materials and Methods

Plant growth

B. napus (Quantum) was grown in the same manner as described above in Chapter 2. When plants were N- or S-limted, nutrient solutions were changed one week prior to the experiment to eliminate N or S from the media. The N-limited nutrient solution contained 1.2 mM KCl, 0.80 mM $CaCl_2 \cdot 2H_2O$, 2.14 mM KH_2PO_4, 2.4 mM $MgSO_4 \cdot 7H_2O$, 0.11 μM MoO_3, 5.3 μM H_3BO_3, 0.43 μM $ZnSO_4 \cdot 7H_2O$, 0.43 μM $MnSO_4 \cdot H_2O$, and 0.11 μM $CuSO_4 \cdot 5H_2O$, 4.0 μM $FeCl_3$, and 4.0 μM Na_3HEDTA (N-(2-hydroxyethyl)ethylenediaminetetraacetic acid). The S-limited nutrient media contained 1.2 mM KNO_3, 0.80 mM $Ca(NO_3)_2 \cdot 4H_2O$, 2.14 mM $NH_4H_2PO_4$, 2.4 mM $MgCl_2 \cdot 6H_2O$, 0.11 μM MoO_3, 10.7 μM KCl, 5.3 μM H_3BO_3, 0.43 μM $ZnNO_3 \cdot 6H_2O$, 0.43 μM $MnCl_2$, and 0.11 μM $CuNO_3 \cdot 3H_2O$, 4.0 μM $Fe(NO_3)_3 \cdot 9H_2O$, and 4.0 μM Na_3HEDTA (N-(2-hydroxyethyl)ethylenediaminetetraacetic acid).

Short-term metal hydroponic uptake experiments

Pb uptake experiments were conducted as described in Chapter 2. Pre-exposed plants were exposed to concentrations of 1 mM cysteine or glutathione for 6 hours prior to conducting the uptake experiment. When competition for uptake was examined with Ag in the experimental solution, Ag concentrations were 0.5, 1, or 2 μM in initial experiments in the presence of 2.8 μM cysteine or 72 μM glutathione. Higher concentration Ag experiments used concentrations of 1, 20, and 50 μM Ag in conjunction with either 100 μM cysteine and cysteine pre-exposed plants or 72 μM glutathione and non pre-exposed plants. All experimental solutions contained a background ion concentration, which for control or S-limited plants was the same as in Chapter 2, but for N-limited plants was altered and contained 1 mM $CaCl_2 \cdot 2H_2O$, 5 mM KCl, 1 mM $MgCl_2 \cdot 6H_2O$, 1 mM NaCl, and 1 mM HEPES, buffered at pH 7 (adjusted with 10 mM NaOH).

Metal analysis

Plant tissue was sampled and digested as described above in Chapter 2.

C.3 Results and Discussion

Short-term uptake under N- and S-limited conditions

The uptake of Pb in cysteine treated plants under S-limited conditions was significantly higher than the control by about 20 %, but significantly lower, by about 30 % under N-limited conditions (Table C.1). In the case of glutathione, Pb uptake was more than double under S-limited conditions, but not significantly different in the presence of glutathione. Pb uptake in the EDTA controls was low for all treatments.

A few different changes may play a role in the differences in Pb uptake under N- or S-limited conditions. In the case of S-limitation, the significant increase in Pb uptake under both cysteine and glutathione treatment may suggest a role in an upregulated amino acid or peptide transporter. A rye grass has been shown to take up

glycine from hydroponic solution even when other N sources are available (Thornton and Robinson 2005), but whether the transport mechanism is constitutive or induced is unknown. In the case of N-limitation, the plants were extremely unhealthy, with very stunted growth, purple leaves and very underdeveloped and browning root systems. The plant likely has less energy and fewer resources to devote to transporters which could play a role in the lower uptake seen in both cysteine and glutathione treatments.

Table C.1 Pb uptake rates in *B. napus* under control, S-limited, or N-limited conditions.

Growth treatment	Ligand treatment	Uptake rate (μg Pb/g root DW/hr)
Control	2.8 μM cysteine	32 ± 1
N-limited	2.8 μM cysteine	23 ± 2[a]
S-limited	2.8 μM cysteine	38 ± 2[a]
Control	72 μM glutathione	15 ± 3
N-limited	72 μM glutathione	12 ± 3
S-limited	72 μM glutathione	36 ± 4[a]
Control	1 μM EDTA	0.6 ± 0.2
N-limited	1 μM EDTA	3 ± 1[a]
S-limited	1 μM EDTA	3 ± 2

[a]Significantly different from control growth treatment ($p<.05$)

Competition for uptake by Ag

Pb uptake was examined in the presence of Ag, a competing thiol binding metal. Under cysteine treatment, the Pb-cysteine concentration was held constant, while the Ag-cysteine as well as the AgCl species concentrations varied. Uptake of Pb in the presence of Ag at all concentrations was about half the amount in the control (Figure C.1). The total moles of metal uptake (Pb + Ag) were about the same in the

presence of 0.5 μM Ag, but increased with increasing Ag concentration to almost double at 2 μM Ag. Uptake of Pb and Ag was also examined in cysteine pre-exposed *B. napus* in the presence of 100 μM cysteine (the concentration where the uptake rate was saturated in pre-exposed plants; Figure A.2). As Ag concentration increased, Pb uptake decreased slightly with increasing Ag concentration (Figure C.2). Ag uptake increased dramatically from about 2 μmoles/g in the presence of 1 μM Ag, to about 12 μmoles/g in the presence of either 20 or 50 μM Ag.

At low concentrations, if cysteine competes with Pb-cysteine uptake as was suggested in Chapter 2, Ag-cysteine may compete as well, resulting in the lower Pb uptake in the presence of Ag, though it is unknown why it does not compete to a greater degree at higher Ag concentrations. At the higher concentration cysteine with pre-exposed plants, the increase in Ag uptake and slight decrease in Pb uptake may also be due to competition, but it seems that Ag uptake has a much higher saturation point than Pb. Ag-cysteine is more likely to be accidentally transported compared to Pb-cysteine due to the size differences, which might explain the increasing transport of Ag with increasing concentration, for both pre-exposed and not.

A different response was seen with glutathione treatment, which during the experiments maintained nearly 100% metals bound to thiols across all the Ag concentrations. Total metal uptake (Pb + Ag) increased only slightly at 0.5 μM Ag, but by about 5-fold compared to the control with Ag concentrations of 1 to 50 μM. Uptake of Pb was about the same at 0.5 μM Ag, increased at 1 μM Ag by about 3-fold and slightly decreased as Ag concentration increased (Figure C.3). Uptake of Ag increased by about 2-fold from 0.5 to 1 μM Ag concentrations and stayed about the same at 2 and 50 μM Ag, but with a decrease at 20 μM Ag. The increased transport of Pb in the presence of Ag is puzzling, as is the increase, but relatively constant uptake of Ag even though the concentration of Ag-glutathione increases dramatically.

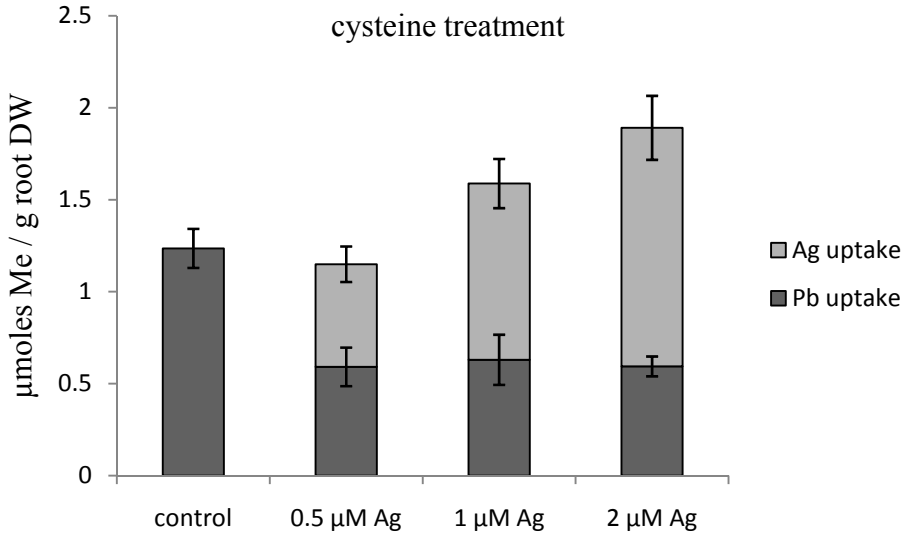

Figure C.1 Pb and Ag uptake in control (1 µM Pb) and 1 µM Pb in addition to various concentration Ag treated *Brassica napus* in the presence of 2.8 µM cysteine. Values are ± SD (n=3).

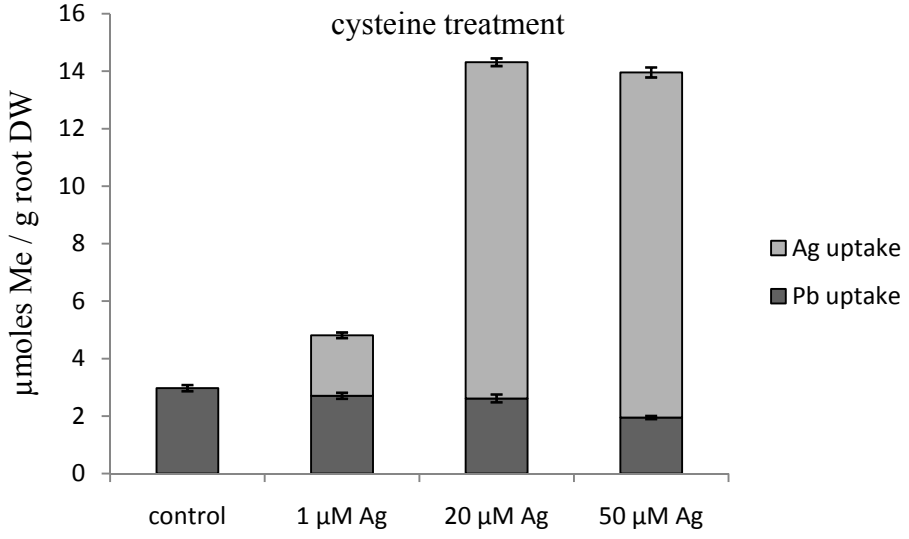

Figure C.2 Pb and Ag uptake in control (1 µM Pb) and 1 µM Pb in addition to various concentration Ag treated 1 mM cysteine pre-exposed *Brassica napus* in the presence of 100 µM cysteine. Values are ± SD (n=3).

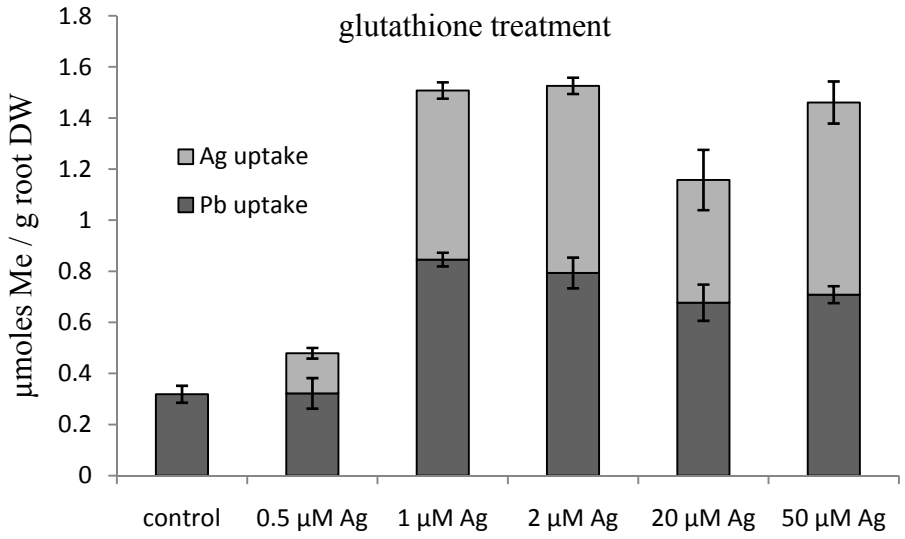

Figure C.3 Pb and Ag uptake in control (1 µM Pb) and 1 µM Pb in addition to various Ag concentration treated *Brassica napus* in the presence of 72 µM glutathione. Values are ± SD (n=3).

REFERENCES

Thornton, B., Robinson, D. Uptake and assimilation of nitrogen from solutions containing multiple N sources. *Plant, Cell and Environment*, **2005**, *28*, 813-821.