Metal response of Douglas-fir: a comparison of foliar metals and phytochelatin production in trees planted in soils amended with biosolids or metal salts

Katrina J. Mendrey

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

University of Washington
2013

Committee:
Dr. Sally L. Brown
Dr. Erica Cline
Peggy Leonard

Program Authorized to Offer Degree
School of Environmental and Forest Sciences
University of Washington

Abstract
Metal response of Douglas-fir: a comparison of foliar metals and phytochelatin production in trees planted in soils amended with biosolids or metal salts

Katrina J. Mendrey

Chair of the Supervisory Committee:
Sally L. Brown
School of Environmental and Forest Sciences

This study was designed to explore the relationship between metals in soil, foliar metal concentrations and phytochelatin (PC) production in Douglas-fir trees planted in soils amended with biosolids or metal salts. Both a greenhouse and field study were conducted to determine whether PC production could be correlated with increased foliar metal or soil metal concentrations. Two seedlings per pot were planted in one of seven greenhouse treatments. The control soil, a sandy loam forest soil, was amended with modern biosolids at rates of 20 Mg ha\(^{-1}\) or 40 Mg ha\(^{-1}\) or metal salts added to similar metal concentrations as biosolids treatments to represent low to moderate metal application rates. Two additional treatments using 100% historic biosolids and the control soil amended with metal salts represented a high metal application rate. The field study included trees amended with modern biosolids at a rate of 21 Mg ha\(^{-1}\) or 16 Mg ha\(^{-1}\) and corresponding trees of the same age that had not received biosolids applications. Soils in both studies were tested for total and \(\text{NH}_4\text{NO}_3\) extractable Cd, Cu, Pb and Zn, as well as pH, C, N, C:N and electrical conductivity. Tree foliage was analyzed for total foliar Cd, Cu, Pb and Zn, as well as phytochelatin, glutathione and cysteine content.

Metal concentrations in greenhouse soils ranged from 2.2-38.0 mg kg\(^{-1}\) Cd, 11-833 mg kg\(^{-1}\) Cu, 49-1361 mg kg\(^{-1}\) Pb and 33-726 mg kg\(^{-1}\) Zn depending on metal application rate. Total metal concentrations in field soils ranged from 3-4.8 mg kg\(^{-1}\) Cd, 15.0-41.0 mg kg\(^{-1}\) Cu, 65.0-79.5 mg kg\(^{-1}\) Pb and 40.0-99.0 mg kg\(^{-1}\) Zn. Greenhouse foliar metal concentrations were not significantly different among low and moderate metal application...
rates, however, foliar concentrations in high metal treatments were significantly higher than control with the exception of Pb. This followed a similar pattern as total soil metals, which varied only slightly among low and medium metal treatments, however, were significantly higher at high metal rates. No differences in foliar metal concentrations were observed in field samples. Foliar metal concentrations in either study were below toxic levels suggested by existing literature. PC production was not significant by any of the factors tested in the greenhouse study. In the field study, PC production was significantly higher in treatments without biosolids applications. In addition, no correlation was found between PC production and total soil metals or foliar metals in either the greenhouse or field study. These results indicate that foliar PC production in Douglas-fir should not be used as an indicator of metal-stress in trees exposed to similar concentrations of metals as those used in this study.
# Table of Contents

List of Figures ....................................................................................................................... v
List of Tables .......................................................................................................................... xii
Acknowledgments .................................................................................................................. xiv
Dedication ............................................................................................................................... xv

## Introduction

16

## CHAPTER ONE: Literature Review

21
Biosolids Quality and Regulation .......................................................................................... 21
Biosolids use in Forestry ........................................................................................................ 22
Natural and Anthropogenic Input of Metals to Soil .............................................................. 27
Metal Speciation in Soil and Biosolids .................................................................................. 29
Phytoavailability of Metals .................................................................................................... 33
Metal Stress in Plants ............................................................................................................ 36
Metal in Forest Ecosystems ..................................................................................................... 42
Phytochelatins ....................................................................................................................... 46
PC Field Studies and Applications in Forestry ...................................................................... 51

## CHAPTER TWO: Methods

54
Summary ............................................................................................................................... 54
Greenhouse Experiment Design ............................................................................................ 54
Treatments and Soils .............................................................................................................. 54
Mixing Soils .......................................................................................................................... 58
Douglas-fir Seedling Measurement and Planting ................................................................. 61
Tree Care and Monitoring .................................................................................................... 62
June Sampling ....................................................................................................................... 63
October Sampling ................................................................................................................ 64

Field Study: Hancock’s Snoqualmie Forest ......................................................................... 65

Soil Analysis .......................................................................................................................... 66
Total Metals Using Aqua Regia Digest ................................................................................... 66
NH₃NO₃ Extractable Metals ...................................................................................................... 67
Soil pH .................................................................................................................................... 67
Soil Electrical Conductivity (EC) .......................................................................................... 67
C/N Analysis ......................................................................................................................... 67

Plant Analysis ......................................................................................................................... 68
Foliar Metals Analysis ............................................................................................................ 68
Phytochelatin Extraction and HPLC Analyses ..................................................................... 68

## Statistical Analysis

69

## CHAPTER THREE: Results and Discussion

70
Greenhouse Study .................................................................................................................. 70
Total Soil Metals for Greenhouse Seedlings ......................................................................... 70
NH₃NO₃ Extractable Metals for Greenhouse Seedlings .......................................................... 71
Soil pH for Greenhouse Seedlings ........................................................................................ 71
Electrical Conductivity (EC) for Greenhouse Seedlings ....................................................... 73
C and N for Greenhouse Seedlings ....................................................................................... 74
Foliar Metals of Greenhouse Seedlings ............................................................................... 74
Phytochelatins (PC), Glutathione (GSH) and Cysteine (CYS) of Greenhouse Seedlings ..... 77
Biomass and Productivity of Greenhouse Seedlings ............................................................. 79
Mortality of Greenhouse Seedlings ...................................................................................... 80
Physical Appearance of Greenhouse Seedlings ................................................................. 81
Field Study ......................................................................................................................... 82
Total Metals for the Field Study ....................................................................................... 82
NH₃NO₃ Extractable Metals for Field Soils ...................................................................... 82
Carbon and Nitrogen for Field Soils ............................................................................... 83
pH for Field Soils .............................................................................................................. 83
Foliar Metals for Field Trees .......................................................................................... 84
Comparison of Field and Greenhouse Soil Characteristics and Foliar Metals ............. 84
Phytochelatins (PC), Glutathione (GSH) and Cysteine (CYS) for Field Trees ............ 85

Conclusion ......................................................................................................................... 86

Figures .................................................................................................................................. 90

Tables ..................................................................................................................................... 161

Bibliography ......................................................................................................................... 175
List of Figures

Figure 1.1: Range of Pb concentrations (mg kg\(^{-1}\)) in biosolids sampled between 1978-1999. In total 7,746 biosolids samples were sampled from 177 Pennsylvania wastewater treatment plants. As graphs demonstrate both concentration and variability across plants decreased during this time period. (Excerpted from Stehouwer and Wolf 1999).

Figure 1.2: Range of Cd concentrations (mg kg\(^{-1}\)) in biosolids sampled between 1978-1999. In total 7,746 biosolids samples were sampled from 177 Pennsylvania wastewater treatment plants. As graphs demonstrate both concentration and variability across plants decreased during this time period. (Excerpted from Stehouwer and Wolf 1999).

Figure 1.3: Results from work by McKenzie (1980) demonstrating adsorption of metals to goethite. Reported metal hydrolysis pK\(_a\) values for each metal were Pb (7.7), Cu (8), Zn (9.0), Co (9.7), Ni (9.9) and Mn (10.6). (Extracted from McKenzie, 1980 by Basta, et al. 2005).

Figure 1.4: Modes of metal adsorption onto functional groups of organic matter. Negatively charged sites necessary for chelation of metals by organic matter are influenced by pH (Extracted from Senesi, 1992 by Basta et al. 2005).

Figure 1.5: Steps involved in plant uptake of metals. 1. Metal must be released from soil through desorption or dissolution. 2. Free metal ions are transferred through diffusion or convection in soil solution. 3. Metals may be reabsorbed or precipitated on soil particles or 4. Absorbed by roots. (Adapted from McBride, 1994).

Figure 1.6: A. Differentiation of two –Glu-Cys linkages. B. The five families of \(\gamma\)-Glu-Cys peptides which scavenge and sequester metals in plants and yeasts. (Adapted from Rauser, 1995).

Figure 2.1: Lee Memorial Forest located in Snohomish County between SE 188th Street and SE 197th Street, between SR 9 and SR 522. Alderwood soil was collected near the 197th St SE entrance at the SW corner of the forest.

Figure 2.2: Charles Lathop Pack Experimental Forest is located at 9010 453rd St E, Eatonville, WA 98328. Historic biosolids used in this study were collected from a grassy area in the forest where the biosolids had been lagooned. The biosolids provided by King County in 1982 were later tested for Cd, Cu, Pb and Zn using the \textit{aqua regia} digest method and ICP-MS.

Figure 2.3a: Class B cake biosolids used to amend soils for Cake Rate 1 and Cake Rate 2 treatments. Class B cake was provided by King County. Application rates were equivalent to 20 Mg ha\(^{-1}\) (Cake Rate 1) and 40 Mg ha\(^{-1}\) (Cake Rate 2). Cake was 22% solids with metal concentrations (mg kg\(^{-1}\)) of Cd, Cu, Pb and Zn of 2.5, 500, 30 and 900, respectively, as reported by King County.

Figure 2.3b: Initial mixture of metal salts for high metal rate treatment (1.867g CdCl\(_2\), 69.720g CuSO\(_4\), 55.73g Pb(NO\(_3\))\(_2\), and 57.13g ZnSO\(_4\)). Due to precipitation of metals, metals were mixed separately in 1L of de-ionized water and added at a rate of 300ml solution to 10kg of soil.
**Figure 2.4**: (Upper Left): Alderwood soil for Cake Rate 1 portioned out by volume using a base measurement of 10kg soil.

**Figure 2.5**: (Upper Right): Cake Rate 2 pre-mixing. Reddish soil is Alderwood, darker amendment is Class B cake and light-colored granules are lime.

**Figure 2.6**: (Lower Left): Mixing soil on tarp. Each treatment was mixed in three batches then homogenized again before storage in five-gallon buckets. Order of mixing was from least contaminated (Control) to most contaminated (Metal Salts Historic Rate) treatments.

**Figure 2.7**: Tree height (cm) was measured from the base of each tree to the crown excluding any needles. Trees were measured throughout the eight-month growing period using the same method.

**Figure 2.8**: Root volume (ml) was measured by displacement of water using a 1000ml cylinder filled to 500ml with tap water. Seedling root balls were fully submerged in water and measurements were rounded to the nearest 5ml increment.

**Figure 2.9**: Metal Salts rate 1 seedlings labeled with Treatment ID (4) and letter (A-H) on pink flagging. In all, 56 trees (8 per treatment) were randomly selected from a group of 100. Trees were planted two per pot, for a total of eight trees per treatment (four pots per treatment).

**Figure 2.10**: Planted trees on greenhouse table prior to organization in random block design. Trees pictured in foreground are planted in historic biosolids. Trees were later moved outdoors as growing conditions in the greenhouse were not ideal for Douglas-fir seedlings.

**Figure 2.11**: Stressed trees were moved outside on April 2, 2012. Stress and premature death were noted in all treatments. Pictured trees are from Control, Metal Salts Historic Rate, Cake Rate 2 and Metal Salts Rate 2 treatments (left to right). One week after relocation fourteen trees from various treatments were identified as dead and replaced with trees from original 100. Thirteen additional days later five more dead trees were replaced as well. In total 20 trees were replaced.

**Figure 2.12**: Trees were covered with a shade cloth from July-September to protect seedlings from sun exposure. Seedlings were watered as needed, rotated weekly to avoid preferential shading with in random block design and measured approximately every thirty days to monitor growth.

**Figure 2.13**: First foliage sampling date was determined by bud-break and development of new foliage. By June 21, 2012 all living seedlings had formed new growth approximately 3-10cm long. Each sample was taken at random from new growth at different heights around seedling. PC samples included only needles, whereas full length of new growth was taken for foliar metal analysis. Seedling in foreground is from Treatment 6.

**Figure 2.14**: Upper Left: Removal of seedlings from pots. Roots had grown together in pots and were carefully separated for root sampling. Upper Right: Roots were rinsed with tap water prior to sampling of fine roots for PC analysis. Lower Left: Both fresh and dry
weight of trees were measured using a tray over a Salter scale consistent with original measurements.

**Figure 2.15**: Map of application area where five year-old trees were sampled. First biosolids application occurred in October 2011 at a rate of 15.9 Mg ha\(^{-1}\). Trees were sampled from section 25-08-25B (biosolids applied) and the area between application areas 26 and 25 where no biosolids were applied (control).

**Figure 2.16**: Brian Vrablick, Forestry Manager with King County Wastewater Treatment Division, pictured with five year-old Douglas-fir in biosolids application area. Understory vegetation was dominated by foxglove and fireweed. Trees were approximately 5-6 feet tall.

**Figure 2.17**: Map of application area where eight year-old trees were sampled. First biosolids application occurred in 2008 at 13 Mg ha\(^{-1}\). The second application was in March 2012 at 8 Mg ha\(^{-1}\). Trees were sampled from the northern portion of section 24-09-18A (biosolids applied). Control samples were taken from an area to the south of this section where no biosolids were applied.

**Figure 2.18**: Eight year-old trees in biosolids application area. Understory was primarily fireweed and foxglove though red alder and evergreen blackberry were also noted.

**Figure 2.19**: PC and foliar metal sampling from larger eight year-old trees. Samples of needles (PC samples) and full length on new growth were sampled from random locations around trees. Similarly, five randomly selected soil samples were taken from the upper horizons around base of the tree.

**Figure 3.1**: Mean total soil Cd (mg kg\(^{-1}\)) and standard error by treatment as determined by *aqua regia* digest. Total metals were not significant by date and means displayed here account for both March and October sampling dates. Significant differences in total Cd concentrations between control, low and medium rates were minimal compared to levels in high metal treatments. Cake treatments refer to treatments using modern Class B biosolids. Different letters above each mean indicate significant differences using Waller-Duncan post hoc tests (p<0.05).

**Figure 3.2**: Mean total soil Cu (mg kg\(^{-1}\)) and standard error by treatment as determined by *aqua regia* digest. Total metals were not significant by date and means displayed here account for both March and October sampling dates. Differences among control, low and medium metal rate treatments varied only slightly as compared to total Cu in high metal treatments. Different letters above each mean indicate significant differences using Waller-Duncan post hoc test (p<0.05).

**Figure 3.3**: Mean total soil Pb (mg kg\(^{-1}\)) and standard error by treatment as determined by *aqua regia* digest. Total metals were not significant by date and means displayed here account for both March and October sampling dates. As with other metals, minimum variation between total Pb values was seen at lower metal rates with increased Pb in both high metal treatments. Different letters above each mean indicate significant differences using Waller-Duncan post hoc test (p<0.05).

**Figure 3.4**: Mean total soil Zn (mg kg\(^{-1}\)) ± standard error for each treatment as determined by *aqua regia* digest. Total metals were not significant by date and means displayed here account for both March and October sampling dates. Total Zn was highest
for the Historic Biosolids treatment with minimal differences between low and medium rate treatments. Significant differences between treatments are indicated by different letters above each bar. Separation of means was determined using Waller-Duncan post hoc test (p<0.05).

**Figure 3.5:** Dilute salt (0.1 M NH$_3$NO$_3$) Cd (mg kg$^{-1}$) for soils extracted across both harvests. Means ± standard error are shown. Letters designate significant differences (Waller-Duncan post hoc test, p<0.05).

**Figure 3.6:** Dilute salt (0.1M NH$_3$NO$_3$) Cu (mg kg$^{-1}$) for soils extracted across both harvests. Means ± standard error are shown. Results are shown for each rate of metal addition. Metal source was not significant. Letters designate significant differences by rate (Waller-Duncan post hoc test, p<0.05).

**Figure 3.7:** Dilute salt (0.1M NH$_3$NO$_3$) Pb (mg kg$^{-1}$) for soils extracted across both harvests. Means ± standard error are shown. Results are shown for each rate of metal addition. Metal source was not significant. Letters designate significant differences by rate (Waller-Duncan post hoc test, p<0.05).

**Figure 3.8:** Dilute salt (0.1M NH$_3$NO$_3$) Zn (mg kg$^{-1}$) for soils extracted across both harvests. Means ± standard error are shown. Results are shown for each rate of metal addition. Metal source was not significant. Letters designate significant differences by rate (Waller-Duncan post hoc test, p<0.05).

**Figure 3.9:** Soil pH of greenhouse soils collected at planting in March. Significant differences between treatments could not be statistically analyzed as samples were collected as a composite by treatment.

**Figures 3.10:** pH of greenhouse soils in October at time of tree harvest. Letters indicate significant differences between treatments using the Waller-Duncan post hoc test (p<0.05).

**Figure 3.11:** Dilute salt (0.1M NH$_3$NO$_3$) extractable Zn versus pH for both harvests. There was no significant relationship between pH and extractable Zn (R=0.06).

**Figure 3.12:** Plant uptake of Zn plotted against pH. As with available Zn correlation between pH and uptake was low (R=0.14). Similar relationships were found for Cd, Cu and Pb.

**Figure 3.13:** Means ± standard error for foliar Cd as a function of rate of metal addition. Reported means include values from both harvests and from metals added to soils as salts and in biosolids. Different letters above means represent significant differences between rates (Waller-Duncan post hoc test p<0.05).

**Figure 3.14:** Means ± standard error for foliar Cu as a function of rate of metal addition. Reported means include values from both harvests and from metals added to soils as salts and in biosolids. Different letters above means represent significant differences between rates (Waller-Duncan post hoc test p<0.05).

**Figure 3.15:** Foliar Cu (mg kg$^{-1}$) means ± standard errors as a function of amendment type (p<0.017). Displayed means are the values for each type of amendment across both harvests and all rates of amendment addition.
Figure 3.16: Mean foliar Pb (mg kg\(^{-1}\)) ± standard errors as a function of harvest date (p<0.000). Displayed means represent the value for each harvest across all treatments. For foliar Pb, no other factors were significant with the exception of amendment type in October (p<0.033) when uptake was slightly elevated in biosolids treatments as compared to metal salt treatments. Letters represent significant differences between harvest dates.

Figure 3.17: Foliar Zn (mg kg\(^{-1}\)) means ± standard errors by harvest date and rate. Within each harvest, means with different letters are significantly different. Separation of means was determined using the Waller-Duncan post hoc test (p<0.05).

Figure 3.18: Foliar Zn (mg kg\(^{-1}\)) means ± standard errors as a function of harvest date and amendment type. Within each harvest, means with the same letter are not significantly different. Separation of means was determined using the Waller-Duncan post hoc test (p<0.05).

Figure 3.19: Phytocelatin (PC) concentrations in foliage for each treatment. Means ± standard error are shown. There were no statistically significant differences for any treatment.

Figure 3.20: Cysteine (CYS) (nmol SH eq/g FW) in foliage by treatment. Means ± standard error are shown. There were no statistically significant differences for any treatment.

Figure 3.21: Glutathione (GSH) concentrations (nmol SH eq/g FW) in foliage by treatment. Means ± standard error are shown. Significant differences were not observed by rate, amendment type or rate*amendment type.

Figure 3.22: Foliar Cd (mg kg\(^{-1}\)) and PC concentrations (nmol SH eq/g FW) in seedlings by treatment. No correlation was observed between PC production and metal concentrations in tissue for any of the tested metals.

Figure 3.23: Foliar Cu (mg kg\(^{-1}\)) and PC concentrations (nmol SH eq/g FW) in seedlings by treatment. No correlation was observed between PC production and metal concentrations in tissue for any of the tested metals.

Figure 3.24: Foliar Pb (mg kg\(^{-1}\)) and PC concentrations (nmol SH eq/g FW) in seedlings by treatment. No correlation was observed between PC production and metal concentrations in tissue for any of the tested metals.

Figure 3.25: Foliar Zn (mg kg\(^{-1}\)) and PC concentrations (nmol SH eq/g FW) in seedlings by treatment. No correlation was observed between PC production and metal concentrations in tissue for any of the tested metals.

Figure 3.26: Mean change in height by amendment type (p<0.038) reported as a ratio of final height:initial height measurements. Initial height (cm) was measured in March at planting and final height (cm) was measured at harvest in October. No separation of means was reported using Waller-Duncan post-hoc test (p<0.05).

Figure 3.27: Mean change in stem diameter by amendment type (p<0.000) reported as a ratio of final stem diameter:initial stem diameter measurements. Initial stem diameter (mm) was measured in March at planting and final stem diameter (mm) was measured at harvest in October using calipers. Letters indicate significant differences by amendment type. Separation of means was determined using the Waller-Duncan post hoc test (p<0.05).
Figure 3.28: Mean change in seedling volume by amendment type (p<0.000) reported as a ratio of final seedling volume: initial seedling volume measurements. Initial seedling volume (cm$^3$) was measured in March at planting and seedling volume (cm$^3$) was measured at harvest in October. Letters indicate significant differences by amendment type. Separation of means was determined using the Waller-Duncan post hoc test (p<0.05).

Figure 3.29: Total tree mortality by date. Tree mortality was significant in April only. Significance was based on amendment type (p<0.018) with higher mortality among biosolids treatments. No separation of means were reported by Waller-Duncan post-hoc test (p<0.05).

Figure 3.30: Comparison of treatments prior to harvest. From left to right: Control, Metal Salts Rate 1, Historic Biosolids, High Metal Salts Historic Rate, Metal Salts Rate 2, Cake Rate 1 (20 Mg ha$^{-1}$) and Cake Rate 2 (40 Mg ha$^{-1}$). Treatments using modern biosolids consistently had darker, fuller foliage then other treatments.

Figure 3.31: Photos of each tree were taken after removal from pots. The top tree was grown in Cake Rate 2 (40 Mg ha$^{-1}$) soil. The bottom tree was grown in the High Metal Salts Historic Rate treatment.

Figure 3.32: Mean soil Cd concentrations (mg kg$^{-1}$) ± standard error by treatment (p<0.000). The same letters above each mean indicate that values are statistically similar (p<0.05).

Figure 3.33: Soil Cu concentrations (mg kg$^{-1}$) ± standard error as a function of stand age/soil (p<0.000). Cu concentrations were elevated in the older stand and did not vary significantly by rate.

Figure 3.34: Soil Pb concentrations (mg kg$^{-1}$) ± standard errors as a function of stand age/soil (p<0.033). Pb concentrations did not vary by biosolids application rate (p<0.433).

Figure 3.35: Soil Zn concentrations (mg kg$^{-1}$) ± standard error for each treatment (p<0.000). Age/soil (p<0.018) was less significant then rate (p<0.000). Letters indicate significant differences between treatments. Separation of means was determined using Waller-Duncan post hoc test (p<0.05).

Figure 3.36: Extractable (0.01 M NH$_3$NO$_3$) soil Cd (mg kg$^{-1}$) for the two sites. Different letters above each column indicate significant differences.

Figure 3.37: Extractable (0.01 M NH$_3$NO$_3$) soil Cu (mg kg$^{-1}$) for each soil sampled. Biosolids application rate was not significant for Cu. Letters above columns indicate significant differences.

Figure 3.38: Extractable (0.01 M NH$_3$NO$_3$) soil Zn (mg kg$^{-1}$) for each sampled site (p<0.000). Letters above means indicate significant differences using Waller-Duncan post hoc test (p<0.05).

Figure 3.39: pH means +/-standard errors by treatment (p<0.000). Soil pH varied by both stand age and application rate. Letters above columns indicate significant differences. Separation of means was determined using Waller-Duncan post hoc test (p<0.05).
**Figure 3.40:** Mean foliar PC (nmol SH eq/g FW) +/- standard error by rate. Only foliar PC concentrations were significant by rate (p<0.047). Letters above means indicate significant differences (p<0.05).

**Figure 3.41:** Correlation between foliar Cd (mg kg⁻¹) and foliar PC (nmol SH eq/g FW). Of all metals, the relationship between plant uptake and foliar PC production was strongest for Cd (R=0.37).

**Figure 3.42:** Correlation between foliar Cu (mg kg⁻¹) and foliar PC (nmol SH eq/g FW). Similar to greenhouse results no correlation was observed between these variables (R=0.05).

**Figure 3.43:** Correlation between foliar Pb (mg kg⁻¹) and foliar PC (nmol SH eq/g FW). PC production varies regardless of metal uptake demonstrating lack of relationship between actual uptake and foliar PC production (R=0.03).

**Figure 3.44:** Correlation between foliar Zn (mg kg⁻¹) and foliar PC (nmol SH eq/g FW). (R=0.14). Further, the slope of the best fit line suggests a negative relationship between foliar Zn uptake and PC production.

**Figure 3.45:** Correlation between total soil Cd (mg kg⁻¹) and foliar PC (nmol SH eq/g FW), (R=0.02).

**Figure 3.46:** Correlation between total soil Cu (mg kg⁻¹) and foliar PC (nmol SH eq/g FW). No relationship was observed between these two variables (R=0.03).

**Figure 3.47:** Correlation between total soil Pb (mg kg⁻¹) and foliar PC (nmol SH eq/g FW). Little correlation was found between Foliar PC and Total Soil Pb (R=0.03).

**Figure 3.48:** Correlation between total soil Zn (mg kg⁻¹) and foliar PC (nmol SH eq/g FW), (R=0.16).
List of Tables

Table 1.1: Part 503 Land Application Pollutant Limits for Metals (Brobst, 1995, US EPA 1993). Limits were set based on those at highest risk to exposure (children) and using results from studies with metal sensitive crops including spinach and lettuce (Renner 2000).

Table 1.2: Comparison of metal concentration limits in Europe. Concentrations are in mg kg^{-1} and represent regulatory limits (Adapted from LeBlanc, et al 2008).

Table 1.3: Reductions in heavy metal concentrations reported for biosolids produced in Washington State. Biosolids with higher values (1983) were reported by Zasoski et al. (1983) and used to assess metal toxicity in early studies at Pack Forest. Lower values reported in 2012 were provided by King County.

Table 1.4: Maximum slope recommendations depending on groundcover for biosolids applications on forested land (Adapted from Henry, 1988 as cited in Harrison and Henry 2001).

Table 1.5: Recommended buffers (feet from water body) to protect streams from surface runoff when applying biosolids to forested land (Adapted from Henry, 1988 as cited in Harrison and Henry 2001).

Table 1.6: Natural range of means for metals in soils. All units are in mg kg^{-1}. (Adapted from McBride, 1994).

Table 1.7: Metal concentrations in various agricultural amendments. 1Values required for qualification of biosolids as High Quality in Table 3 of the CFR Part 503 rule (USEPA 1993). 2Values reported by King County 2012. 3Range of metal concentrations found in different manures (O'Connor, 2002). 4Mean metal concentrations of fertilizers (USEPA 1999).

Table 1.8: Physical response of plants to toxic concentrations of metals. (Adapted from Prasad, 1999).

Table 1.9: Summary of tissue concentrations at which decreased yield was reported in various studies using wheat and lettuce. Values reported here are summarized from a more detailed review by Macnicol and Beckett (1985) and represent the lower and upper range of values reported from several studies considering the effect of Cd, Cu and Zn on wheat and lettuce.

Table 1.10: Summary of reported foliar metal concentrations in various tree species. Values reported are not necessarily toxic but allow comparison of uptake by different species, metal forms and soil metal concentrations. All metal units are in mg kg^{-1}. *Indicates values reported for seepage water values as soil values were not reported. N/A indicates parameters not evaluated as part of the referenced study.

Table 2.1: Summary of seven treatments used in greenhouse experiment. Control Alderwood soil was used for all treatments with the exception of Treatment 6, which contained pure historic biosolids collected from Pack Forest. Metal salts were added as CdCl_{2}, CuSO_{4}, Pb(NO_{3})_{2} and ZnSO_{4}.
Table 2.2: Biosolids total metals and pH tested prior to potting soil mixes. Total metal values were used to calculate the amount of metals to add to control soil for the high metal salt treatment (Metal Salts Historic Rate).

Table 2.3: Comparison of metal concentrations (mg kg-1) in historic and modern biosolids used in this study, as well as EPA's Table 3 standards for biosolids land application in the United States (US EPA Part 503).

Table 2.4: Results from tests to determine lime application rate. pH values are reported as means of duplicates for each rate +/- standard deviation. Target pH for greenhouse study treatments was originally pH 5. Based on these results final lime application rates for the Alderwood soil and historic biosolids were 25 and 50 Mg ha-1 respectively.

Table 2.5: Number of trees in each treatment either replaced or dead by final harvest.

Table 3.1: Means ± standard errors for aqua regia total and 0.1M NH3NO3 extractable Cd, Cu, Pb and Zn (mg kg-1) for each treatment. Means are averaged across all replicates and harvests.

Table 3.2: Means ± standard error for pH, EC, C, N and C:N by treatment for March and October sampling dates. No standard error is reported for March samples as soil samples were taken as a composite for each treatment.

Table 3.3: Cd, Cu, Pb and Zn foliar concentrations (mg kg-1) by treatment and harvest date including ranges and standard errors. Significance of main effects varied by metal and are displayed in Figures 3.13 to 3.18. All metals were extracted using HNO3- and HCL acid.

Table 3.4: Means and standard errors for SH groups by treatment. No significant differences were observed between treatments based on rate or amendment type.

Table 3.5: Mean concentrations (mg kg-1) of total soil Cd, Cu, Pb and Zn ± standard errors for each treatment. Metal concentrations from the field sampling are similar to or slightly higher than greenhouse study control, modern biosolids and equivalent salt treatments.

Table 3.6: Mean plant available metals (mg kg-1) with standard errors for each site sampled. Lead was similar across all sites. Extractable Cd was higher in the older stand with a similar trend observed for Cu. Only Zn was significant by treatment (p<0.000).

Table 3.7: Means and standard errors for pH, EC, C, N and C:N for soils collected from the Hancock tree farm in King County, WA.

Table 3.8: Mean foliar metal concentrations (mg kg-1) +/- standard errors. Values were not significantly different by rate or amendment type.

Table 3.9: Mean foliar PC, GSH and CYS (SH eq. nmol/g FW) and standard errors. There was no significant difference between treatments for these groups. Only PCs were significant by biosolids application rate with highest concentrations seen in soils where no biosolids were applied.
Acknowledgments

This study was made possible through funding provided by King County in Washington State. Special thanks to my committee members Dr. Sally L. Brown, Dr. Erica Cline and Peggy Leonard. In addition I would like to thank Dongsen Xue and Dr. Jim Gawel for their professional assistance and guidance particularly in performing the necessary laboratory analyses. And of course my fellow students who assisted with collecting data and performing lab work including Rebecca Singer, Colton Miller, Amber Corfman and Dylan McCalmont.
Dedication

This thesis is dedicated to my family, especially my husband for his continued support in encouraging me to pursue my interests no matter how varied or where they take us. And of course my son, though you’ve yet to arrive, you were literally with me every step of the way providing the inspiration to finish in a timely fashion. I can’t wait to share this world with you.
Introduction

Biosolids are the solid, semi-solid or liquid by-product of municipal wastewater treatment. They are high in nutrients and fixed carbon and can be repurposed for multiple uses such as soil amendments as a substitute for synthetic fertilizer or as a soil conditioner, energy production or as an aggregate for construction materials including cement and bricks (Cline et al. 2012 and Wang et al. 2008). According to the last national biosolids survey 7,180,000 dry tons of biosolids were produced in 2004, of which 49% was land applied and 45% was land-filled (NEBRA 2007). The remaining 6% was either stored for long-term use or not reported (NEBRA 2007). As our population grows and more wastewater is treated, there will be a need to identify alternative uses for biosolids. However, due to concerns over the presence of potentially harmful compounds such as heavy metals in biosolids, the various potential uses are controversial and carefully studied.

Currently the most cost-effective and productive use of biosolids is land application (Wang et al. 2008). The biosolids produced through anaerobic digestion are high in essential nutrients including N, P, S and other required plant micronutrients. All of these are essential for plant growth and have limited natural availability. For example, the only sources of phosphorous are mining and manures, and estimates of available reserves indicate that at our current rate of consumption they will be depleted in the next century (Wang et al. 2008). Preserving and reusing these vital nutrients and resources is essential to agricultural production. In certain regions such as the Pacific Northwest, soils are young and relatively low in organic matter and nutrients. Application of biosolids as fertilizers to these soils may elicit a more significant plant response than use of synthetic fertilizers (Henry et al. 2007). Anaerobic digestion of sewage sludges into biosolids is also relatively cost-effective when compared with more energy consumptive measures such as pyrolysis and incineration.

In response to growing demands for repurposing biosolids the EPA conducted a risk assessment of biosolids land application and set exposure limits to ensure that the
environment and human safety were protected. These risk assessments formed the basis for federal quality standards for biosolids in 40 CFR Part 503, known simply as Part 503. It serves as the official standard in the U.S. for the processing of biosolids and for land application, incineration and surface disposal. Since the standard went into effect in February 1993, advances in technology and pre-treatment regulations have resulted in the production of biosolids that are significantly lower in potentially hazardous contaminants, in particular metals, than current regulatory requirements (Brown et al. 1998). For example, a wastewater treatment facility in Washington State reported decreases in biosolids metal levels upwards of 84% between 1981 and 1993 (Henry and Cole, 1997). The public does not universally accept the land application of biosolids. Some authors have suggested that a potentially more popular option would be application for forest management and production of biofuels as opposed to application for food production (Wang et al. 2008). Others argue that forest application has greater potential for public exposure due to recreational uses of forest land.

Several studies have indicated that biosolids can increase productivity and be safely used in agricultural production without increasing risk of human exposure to Cd and Pb (Kukhier et al. 2010; Sukkariyah et al. 2010; Zerzghi et al. 2005 and 2009; Brown et al. 1998). In the case of trees grown for timber production, trees showed a growth response and no signs of metal stress in studies using biosolids with much higher metal concentrations (Wang et al. 2006; Prescott and Blevins 2005; Harrison et al. 2002; Henry et al. 1994; Zasoski, et al 1983). These older studies used historic biosolids with metal concentrations 2 to 20 times those in soils amended with modern biosolids depending on metal (Table 1.3). Despite these elevated metal concentrations, the authors of these studies noted no negative impacts on tree growth. These studies, however, do not provide foliar metal uptake data.

A breadth of research exists to demonstrate the safety and benefits of using biosolids to increase productivity and remediate contaminated soils. Much of the recent research has
focused on the effects of using biosolids over the long-term. Long-term studies have shown that despite reductions in organic carbon, uptake of metals into plant tissue have not increased (Sukkariyah et al. 2010; Gaskin et al. 2003). Studies investigating the effects of biosolids used as fertilizers have shown positive correlations with increased yields for commercial tree plantations. However, these results have been paired with caveats such as decreased wood density (Wang et al. 2006; and Prescott and Blevins 2005).

A recent study by Cline et al. (2012) considered the productivity and metal uptake of Douglas-fir (*Pseudostoga menziesii*) in soils amended with biosolids, for both historic high metal materials and biosolids typical of what are currently produced. The study was conducted using soils and Douglas-fir trees treated with biosolids 25 years ago at the University of Washington’s Charles Lathrop Pack Experimental Forest (Pack Forest), as well as fresh biosolids from the City of Tacoma applied to seedlings in a separate greenhouse study. The greenhouse study included a control soil from the field study site, historic biosolids collected from the field site, the control soil mixed in a 1:1 ratio with historic biosolids-amended soils, and the control soil amended with 8% (160 Mg ha\(^{-1}\)) and 18% (360 Mg ha\(^{-1}\)) fresh cake, dewatered biosolids processed to meet Class B standards for pathogen reduction. Trees were grown for six weeks. Metal concentrations in the fresh cake were 4.7 mg g\(^{-1}\) Cd, 490.1 mg g\(^{-1}\) Cu, 120.4 mg g\(^{-1}\) Pb, and 762 mg g\(^{-1}\) Zn. Metal concentrations in historic biosolids and 18% fresh cake soils, respectively, were 5.4 and 2.8 mg g\(^{-1}\) for Cd, 88 and 66 mg g\(^{-1}\) for Cu, 114 and 50 mg g\(^{-1}\) for Pb, and 185 and 126 mg g\(^{-1}\) for Zn, with the 50% historic and the 8% fresh treatments intermediate between the control soil and the 100% historic biosolids and 16% fresh cake soils, respectively. Concentrations of elements increased significantly relative to the control soil in all the historic biosolids treatments for all four metals, but for the fresh cake only the 16% treatment showed significant increases in Cu and Zn, and there was no significant increase in Cd and Pb.

Changes in phytochelatins (PC), cysteine (CYS) and glutathione (GSH) levels were measured as biological indicators of metal stress, which were hypothesized to reflect a
physiological response to metal uptake (Cline et al. 2012). Biomass measurements, as well as PC, CYS and GSH levels in roots and needles of trees were then used to evaluate the trees response to biosolids. PC, GSH and CYS production was not significant elevated in field trees grown in high metal biosolids and differed in the greenhouse trial only to a limited degree with lower metal rates. Treatments using historic biosolids, with higher metal concentrations, did not produce elevated levels of these groups compared to control. PC levels in older field trees were comparatively higher than those produced in roots and foliage of greenhouse seedlings. Based on significant increases in PCs of seedlings with 8% fresh cake and GSH and CYS with 18% fresh cake, the authors suggested that seedlings were experiencing metals stress primarily when exposed to fresh biosolids. Seedlings treated with fresh biosolids also demonstrated significantly decreased growth compared to the control and there was no reported difference in biomass production of field trees (Cline et al. 2012). The soils amended with fresh biosolids (pH 4.9) showed lower pH compared to controls (pH 5.5), which could have contributed to the lower growth rates. Foliar metal uptake by trees was not measured in either the field or greenhouse study, leading the authors to suggest that future studies should examine the role of soil pH and directly measure foliar metal uptake (Cline et al. 2012).

The growth inhibition of greenhouse seedlings from fresh biosolids observed by Cline et al. (2012) is inconsistent with results of most field studies, although see Zasoski et al. (1983) and Berry (1985) for exceptions. Studies regarding field application of biosolids to forestland have generally demonstrated increased yield from use of biosolids particularly in the case of Douglas-fir (Harrison et al. 2002; and Henry et al. 1994; Brockway 1986; Chapman-King et al. 1986). On the other hand previous studies have shown that application of biosolids to young seedlings can hinder growth. This seems to be more an issue of competition with understory vegetation then related directly to biosolids (Zasoski et al. 1983; Bledsoe and Zasoski, 1981). In a greenhouse study, Bledsoe and Zasoski (1981) reported increased mortality in seedlings of western red cedar (Thuja plicata) compared to
other conifers potted in pure biosolids, but the authors noted that red cedar was the only bare-root stock used. Seedling survival of all species in a 3:1 mixture of soil and biosolids was 99-100%. Based on this they concluded mortality may have been due to the moist poorly aerated conditions of the undiluted biosolids.

Despite this, biosolids applied in the field are typically not applied to young seedlings due to increased understory vegetation and increased competition. Given the age of the seedlings used in this study, biosolids application rates 8-18 times typical current rates, the absence of tissue analysis for total metals, and the short duration of the greenhouse experiment, application of these results to evaluating the use of biosolids in the field should be made with caution.

This follow-up study was conducted to more closely examine the relationship between phytochelatins, metals and the role of biosolids as a soil amendment. Extending upon the previous study, this study compares PC levels to total metals found in foliage when metals are added as both metal salts and with biosolids. To address possible impacts of changes in soil pH, the pH was adjusted to hold it constant for all soil treatments. In addition, rates of biosolids application were adjusted to reflect more realistic applications, and the length of the study was extended to allow for multiple samplings of foliage. Further, samples were also taken from trees planted in fields with more recent biosolids applications.
CHAPTER ONE: Literature Review

Biosolids Quality and Regulation

Prior to pretreatment regulations promulgated in the late 1980s, heavy metal concentrations in biosolids were much higher than today. Enforcement of the regulations and the adoption of pretreatment regulations that restrict industrial discharge into municipal wastewater systems have resulted in decreased metal concentrations in biosolids (Stehouwer and Wolf 1999; Brown et al. 1998). For example, a wastewater treatment facility in Washington State reported decreases in biosolids metal levels upwards of 84% between 1981 and 1993 (Henry and Cole 1997). Similarly, Stehouwer and Wolf (1999) found that from 1978-1997 median concentrations of Cd, Cr, Cu, Pb, Ni, and Zn decreased in a survey of 7,746 biosolids samples from 177 Pennsylvania treatment plants. The authors noted that all elements with the exception of Zn and Cu decreased by over 50%. The variability of metal concentrations also decreased, indicating that the trend was widespread across treatment plants. This trend is shown for Pb and Cd in Figures 1.1 and 1.2.

Along with such changes in pretreatment processes, the US EPA began conducting risk assessments in 1984 to develop standards for biosolids quality and long-term application that would be protective of both human and environmental health. The assessment considered 14 various pathways of exposure to biosolids contaminants, with a highly exposed individual (HEI) or organism as the endpoint for each pathway. HEIs included adult humans, herbivores, soil organisms, soil predators, plants, and a human child ingesting biosolids directly. Depending on metal, this included children and individuals with high exposures to biosolids based on proximity to biosolids applications and sources of food and water (Renner 2000; US EPA 1993). The assessment also considered sensitive crop species such as lettuce and spinach (Renner 2000). For the final standards for biosolids quality, EPA chose the most limiting pathway, that is, the one that resulted in the lowest numerical limit for a given pollutant.
This assessment culminated in the 40 CFR Part 503 Rule, which went into effect in February 1993. Metal concentrations for ceiling limits (maximum allowable concentrations) in biosolids, as well as cumulative and annual pollutant loading rates for land application are summarized in Table 1.1. Most biosolids in the U.S. are regulated under the Table 3 standards. According to Stehouwer and Wolf (1999) in their review of Pennsylvania biosolids, Cu would be the first metal to reach long-term application limits but would require 282 years to reach this limit if biosolids were applied at a typical agronomic rate of 4.5 tons acre\(^{-1}\).

The 503 standards are considered to be protective because the risk assessment process used highly conservative assumptions and worst-case exposure data that tended to overestimate risk. For example, risk was evaluated not for the average person but for a highly exposed individual: someone who ate 60% of their food from a home garden with acid soils that had been amended at the maximum cumulative rate for pollutants (US EPA 1995). Despite these seemingly conservative standards, the U.S. allows considerably higher loading rates of metals than countries in Europe (Table 1.2) where assessments were based on assumed risk to soil microbes and the precautionary principle as opposed to calculated risk to humans, plants, livestock and wildlife (Renner 2000; US EPA 1993). This has resulted in criticisms of U.S. standards, which are seen by some as not conservative enough to protect soil health. In addition, the U.S. risk assessment has been criticized for not taking into account regional factors that could affect metal mobility such as soil depth, acidity and various agricultural practices (Renner 2000).

**Biosolids use in Forestry**

Given the public's uncertainties regarding exposures to contaminants in biosolids the application of biosolids for food production is not universally accepted. Some authors have suggested that a potentially more popular option would be application for forest management and production of biofuels (Wang et al. 2008). Others argue that forestry application has potential for overlap with recreational users increasing risk to the public.
The Pacific Northwest has a long history of biosolids use in forestry and is a leader in developing methods and standards for safe forest application of biosolids. This role has been supported by early recognition of the importance of wastewater treatment and a regional focus on timber production.

In 1958, spurred by efforts to restore Lake Washington, Seattle citizens passed a levy tax measure to restructure the municipality’s wastewater treatment system. In the 1960s, treatment facilities along the lake were decommissioned to reduce nutrient loading to the lake, reducing algal blooms and risk to humans from recreational exposures. In addition to diverting discharge of effluent from the lake to Puget Sound, the measure also led to construction of new primary and secondary treatment facilities allowing for production of biosolids (Harrison and Henry 2001).

Land application of biosolids was seen as an opportunity to improve the overall quality of Northwest soils, which tend to be low in nutrients and water holding capacity, both of which can be improved by biosolids application (Harrison and Henry 2001). Forest soils also have high infiltration rates reducing potential for nutrient run-off, an environmental concern when applying fertilizers. Further, given the amount of forestland compared to agricultural land surrounding Seattle, the focused efforts for recycling the biosolids on forest land application (Harrison and Henry 2001).

In 1973, with financial support from the Municipality of Metropolitan Seattle (now King County), the University of Washington College of Forest Resources (now the School of Environment and Forest Sciences), began a research program at the UW’s Charles Lathrop Pack Demonstration Forest (Pack Forest) to explore land application of biosolids for timber production. The primary objectives of this program were to evaluate beneficial use of biosolids as a nutrient-rich soil amendment capable of enhancing tree growth, identify environmental concerns, and identify methods of applying biosolids to forestland as well as projecting operational costs associated with applications (Henry et al. 1994).
The program at Pack Forest continued for over two decades. A majority of the research was conducted on Douglas-fir stands, a prominent timber crop in the region. Initial research used high application rates of biosolids (up to 500 Mg ha\(^{-1}\)) resulting in growth increases ranging from 60-2000% (Harrison and Henry 2001). Experiments examined not only growth but also application methods and timing. Many studies found that applications to seedlings resulted in higher mortality as a result of competition from understory growth limiting the benefits of such applications (Harrison and Henry 2001 and Zasoski et al. 1983). In addition, various tree species were evaluated for responses to biosolids applications, with results indicating that Douglas-fir responded particularly well when compared with western redcedar (\textit{Thuja plicata}) and western hemlock (\textit{Tsuga heterophylla}) (Henry et al. 1994; Zasoski et al 1983; Bledsoe and Zasoski 1981). These studies were also conducted with biosolids containing metal concentrations 2-20 times higher than those produced today depending on metal concentrations (Table 1.3).

Due to concerns regarding nitrate leaching to groundwater, not metal contamination, application rates have since been restricted. Follow up studies using rates closer to 20 Mg ha\(^{-1}\) still showed increased growth, particularly for Douglas-fir trees (Harrison et al. 2002). Increased productivity is well documented in studies outside of the Northwest as well (Wang et al. 2006; Prescott and Blevins 2005).

While decreased wood density has been noted in timber grown with biosolids, this seems to be a negligible trade off due to accelerated growth and does not hinder economic gains associated with faster yields. In a study by Cole et al. (1984), the authors found that the specific gravity of biosolids-grown wood is still adequate for sale and is similar to wood grown on higher productivity sites. In addition Sonne et al. (2004) found that the long-term financial gains from Douglas-fir grown with biosolids and some thinning was up to 155% compared to control even when log grade was taken into account.

In addition to examining the effects of biosolids on tree growth and wood quality other studies at Pack Forest examined the environmental impact of biosolids applications
particularly regarding metal bioavailability. Given historically higher concentrations of metals in biosolids during this time, such experiments represent a worst-case scenario. For example, biosolids used in an experiment by Zasoski et al. (1983) examining growth responses of different tree species treated with biosolids indicate that metals in historic biosolids were up to 20 times higher than modern day biosolids (Table 1.3). These concentrations are similar to those found in biosolids taken from Pack Forest for use in this study. Despite these relatively high metal concentrations, the authors still noted increased growth with application of biosolids particularly among more mature stands.

In summarizing metals research from Pack Forest, Harrison and Henry (2001) noted that trace metals were not identified below 20cm in the soil and little movement was found in terms of plant uptake or in wildlife. Additionally the authors noted that risk of trace metal exposure to humans was low due to minimal food availability in such sites as compared to exposures from agricultural applications of biosolids. The authors suggested that current standards set forth by the EPA (Table 1.1) were appropriate for ensuring the ecological health of forestland.

These results are congruent with other studies showing that metal mobility and bioavailability, particularly in the case of biosolids added to forest soil, is limited despite the low pH and high porosity of these soils (Wang et al. 2010; Chang et al. 1986; Harris and Urie 1986). According to Chang et al. (1986) in a review of trace element behaviors in land application of biosolids, trace elements tend to accumulate in leaf litter and A horizons of forest soils. This was confirmed by Wang et al. (2010) in their study of biosolids applications at a Pinus radiata plantation in New Zealand. The authors noted only at the highest biosolids application rate (600 kg N/ha) did Cu, Pb and Zn concentrations in soil and leaf litter increase. Cu, Pb and Zn concentrations in leaf litter were 61.3, 8.8 and 69.8 mg kg\(^{-1}\) respectively whereas deeper in the soil profile (.25-.5 meters) concentrations were reduced to 4.9, 4.0 and 29.3 mg kg\(^{-1}\) Cu, Pb and Zn. Despite this increase, metal levels were still at levels considered very low for soils. Wang et al. (2010) also reported that
metals were concentrated in leaf litter and no negative impact to tree health was reported. Harris and Urie (1986) noted similar results when metals in biosolids were applied to an aspen grove. Metals were immobilized in the top 5cm of soil 3-5 years after application.

Consideration as to how biosolids are applied is important in interpretation of these results. As biosolids are typically surface applied such results suggest that only solubilized metals are able to move into the soil profile and become available for plant uptake via roots. This accumulation in the surface layer can result in higher metal exposure for soil microbes and invertebrates that populate this environment. Multiple studies have shown higher concentrations of Cd in livers and kidneys of wildlife including deer mice and meadow voles on sites where biosolids have been applied (Chang et al 1986; and Haufler and West 1986; Anderson 1982). In the case of meadow voles, short-term effects of such exposure were not observed. As previously suggested by the lack of mobility of metals within the soil profile, this exposure may also be limited by metal species of less bioavailable form at the soil surface. In addition, it has been suggested that increased access to nutrient rich foods provided by plant growth in biosolids can outweigh the risk from metal exposure (Harrison and Henry 2001; Haufler and West 1986). Further, such studies were conducted during a period when biosolids contained far higher metal concentrations than today (Table 1.3).

A review of the literature from this time period, however, indicates that metal analysis was mostly limited to soil, with only one study found reporting foliar metals in tree seedlings (Bledsoe and Zasoski 1981). The greenhouse study conducted by Bledsoe and Zasoski (1981) at the University of Washington’s Arboretum examined nutrient and growth response of six common Northwest tree species to biosolids, fertilizer and compost amended soils. Tree species observed included Douglas-fir, Sitka spruce (Picea sitchensis), western red cedar, ponderosa pine (Pinus phyla), grand fir (Abies grandis), black cottonwood (Populus trichocarpa) and Lombardy poplar (Populus nigra var. italica). Treatments included a forest soil, a 4:1 sawdust to biosolids mixture, a 3:1 soil to biosolids mixture and a compost of unspecified feedstock. Metals in the biosolids and biosolids soil treatments
were 2400 mg kg\(^{-1}\) Zn, 970 mg kg\(^{-1}\) Cu and 37 mg kg\(^{-1}\) Cd and 210 mg kg\(^{-1}\) Zn, 95 mg kg\(^{-1}\) Cu and 3 mg kg\(^{-1}\) Cd respectively. Soil pH was not reported.

Trees were grown in pots for two growing seasons. Above and below ground biomass measurements, as well as foliar nutrient and metal concentrations were analyzed. Results from these analyses indicated increased foliar nutrients and biomass in trees grown in the biosolids-amended soil. In addition, poplar trees took up significantly more metal than conifers. Only zinc uptake was significantly higher for biosolids treatments than control with all other metals remaining in low concentrations. Foliar metal results from some of the trees used in this study are summarized in Table 1.10. Based on results of this study, the authors concluded that trees grew best on biosolids amended soils despite heavy metals.

Based on such assessments, over the course of the Pack Forest research program several parameters for the safe and effective application of biosolids were developed. Research addressed not only appropriate application rates for maximizing tree growth but also methods for protecting environmental quality. Such methods included identifying maximum slopes for biosolids application (Table 1.4) and use of buffers near streams (Table 1.5) (Henry, 1988). Application rates and methods developed in this program informed the Process Design Manual of the US EPA and Best Management Practices of the Washington DOE, the primary regulatory documents used to protect water quality, soil health and wildlife pertaining to biosolids applications (Henry et al 1994).

**Natural and Anthropogenic Input of Metals to Soil**

Under natural conditions, heavy metals are stored as mineral components of parent rocks. Weathering of these parent materials provides inputs to soils establishing natural background levels. These minerals may be present in various forms including carbonates, phosphates, oxides and hydroxides or as in the case of the chalcophilic Cu, Cd, Zn and Pb as sulfides (He et al. 2005; Dube et al. 2000; McBride 1994). Table 1.6 shows average background levels for Cd, Zn, Cu and Pb in soils worldwide and in the U.S. (McBride 1994).
Some important sources of metals include basaltic igneous rocks, which contain higher concentrations of Cu, Zn, Cr, Co and Ni, as well as sedimentary rocks particularly shales containing Cu, Zn, Mn, Pb and Cd (He et al. 2005). Limestone and sandstone on the other hand contain far lower concentrations of metals. While most of these inputs are from weathering, atmospheric inputs can occur from volcanic activity, forest fires and as the product of wind erosion (Fergusson 1990).

Many of the heavy metal inputs to soils, however, are the result of anthropogenic activities. These can occur in the form of mining and smelting of metal ores, land application of industrial byproducts, emissions including historic auto emissions, and through uses of pesticides, fungicides and fertilizers including manures, synthetic fertilizers, biosolids and composts. Atmospheric sources include combustion (historically for Pb) and incineration of municipal solid waste or coal for energy production. These inputs tend to have short residence times in the atmosphere and do not travel great distances; however, this depends on the size of the particulate and can range from hours to days with travel up to several hundred kilometers (Fergusson 1990). Atmospheric inputs tend to be higher in industrialized countries (Allen et al. 1995).

Of much greater quantity, however, are inputs from direct deposition such as runoff from mine tailings, landfills or streets, as well as the application of agricultural products including pesticides, herbicides, fungicides and fertilizers which may contain Cu, Cd, Zn, or Pb. The former will be highly localized and would be expected based on industrial activities. Previously unregulated contamination due to these processes was severe, but modern regulations have reduced levels of contamination.

Though pollution from many of these sources has been reduced through regulations such as the Clean Water Act of 1972, metals are still applied to soils directly through fertilizers and agricultural products. While land applied organic residuals can be much lower in metals than other sources, their proper use and regulation is still important to protecting environmental and human health. Currently, among these substances, only metals in
biosolids are regulated by both the EPA and state specific standards (US EPA 1999). Metal mobility and accumulation as a result of using these products will depend on several factors including metal concentrations in amendments and site specific criteria such as soil pH, texture and the type of crop to which the amendment is applied. Table 1.7 provides a comparison of metal concentrations in some common agricultural fertilizers, biosolids and manures.

**Metal Speciation in Soil and Biosolids**

Many studies have indicated that while several factors contribute to metal mobility in soils and resulting leaching or uptake by plants the most important factor is pH (Ahmad et al. 2005; Basta et al. 2005; Dube et al. 2000; Allen et al. 1995; McBride 1994). Depending on media pH and the chemical makeup of the soil, metals in soil will be soluble or will bind with Fe, Al and Mn oxides and/or organic matter or precipitate with phosphates or carbonates. Metals bound to these functional groups have varying degrees of solubility; however, are considered less available for plant uptake than those in free ionic form.

These same principles apply to metals in biosolids, which are rich in both Fe and Mn oxides as well as phosphates and organic matter. Recent studies, particularly those using spectroscopic methods, have shown that the inorganic fraction of biosolids may be more important in metal binding then the organic fraction (Brown et al. 2012; Donner et al. 2011; Hetterachchi, et al. 2006; Basta, et al. 2005; Brown et al. 1998; Mahler et al. 1987). Such properties of biosolids can serve as a mechanism for immobilizing metals including Cd, Zn and Pb in contaminated soils (Brown et al., 2003; Li et al. 2001; Chaney et al. 1999).

In addition, depending on processing methods, biosolids may have elevated pH as compared to amended soils (Heemsbergen et al. 2010; Basta et al. 2005; Chang et al. 1986). This can reduce the availability of certain metals depending on pH levels. For example, while Pb is largely unavailable until pH levels drop below 3.2, Cd, Pb and Zn can all precipitate with carbonates becoming more soluble, at pH levels above 6-7 (McBride 1994; Zasoski and Edmonds 1986).
As a measure of hydrogen ion activity, pH influences whether there are available sites for adsorption of metals onto oxides or organic matter. As pH increases more negatively charged sites become available for adsorption of cations like Cu, Zn, Pb and Cd. The following chemical reaction demonstrates how pH influences the binding of such cations to hydroxides (Basta et al. 2005):

\[
\text{Fe-OH} + M^+ = \text{Fe-OM} + H^+
\]

As pH decreases there is increased “competition” between metals and hydrogen as well as Al\(^{3+}\) for binding sites (Ying et al. 2005; McLean et al. 1992). Work by McKenzie (1980) has shown that binding of cations by oxides can approach nearly 100% at higher pH values (Basta et al. 2005). The point at which adsorption of metal cations increases is called the adsorption edge and is inversely related to the pK\(_a\) values of the metal hydrolysis reaction; however, particular soil mineral properties can influence this as well (Sparks, 2003 as cited in Basta et al. 2005; Kinniburgh and Jackson 1981). These relationships are demonstrated for Pb, Cu, Zn, Co, Mn and Ni adsorbed by goethite in Figure 1.3.

In the case of organic matter, metals are chelated by various forms of organic acids in the soil formed during decomposition or released from plant roots (He et al. 1995). Chelates as defined by Adriano (2001) occur when “two or more functional groups of a single ligand are coordinated to a metal ligand.” Spectroscopic studies have shown metals bind with various functional groups including phenols, COOH, and thiol-SH (Basta et al. 2005). Figure 1.4 models various bonds between metals and forms of organic matter. As pH increases more negatively charged sites become available for adsorption of metal cations to these surfaces. Despite this, pH is less of a factor in the capacity of organic matter to form bonds with metals than is the case for hydroxides (Basta et al. 2005).

Which functional groups a metal attaches to depends on if the metal is a soft or hard acid (Basta et al. 2005; Essington, 2004; Pearson 1968). For example, cadmium, a soft acid, binds to soft base groups such as thiols. Hard acids such as Fe\(^{3+}\) will bind with hard bases including OH- and COO- groups. Intermediate acids including Cu, Zn and Pb bind
with a larger range of both weak and strong bases. However, many studies have shown that Cu tends to bind more readily with organic matter than other metals (Yuan et al. 2009; Yang et al. 2008; Chaignon, 2003; McBride 1994; Bates 1978).

In the case of precipitation, metals in soil solution interact with hydroxides, sulfides, phosphates and carbonates to form precipitates of varying solubility (Miretzky et al. 2007; Basta et al. 2005; Sigel 1984). Similar to other sorption processes precipitation increases with increasing pH. The solubility of the resulting mineral depends on the precipitate formed (Basta et al. 2005).

Which metal speciation dominates in biosolids has been explored by several studies (Basta et al. 2005; Hettiarachchi et al. 2006; Hettiarachchi et al. 2003; Li et al. 2001; Chaney et al. 1999; McBride 1995; Beckett et al. 1979). Recent spectroscopic studies allowing better analysis of biosolids have suggested that the inorganic portion may play a more important role than previously thought (Hettiarachchi et al. 2006). Further speciation remains dependent both on metal and characteristics of the biosolids itself (Brown et al. 2012; Donner et al. 2011; and Hettiarachchi et al. 2006).

For example, a spectroscopic study by Hettiarachchi et al. (2006) demonstrated that Fe hydroxides are particularly important binding sites for Cd, Cr and Zn in biosolids. The authors used μ-XANES and μ-XRF technologies to analyze metal speciation in two biosolids, a composted limed biosolids and a biosolids digested in an Imhof tank and dried on a sand bed. Analysis was performed before and after digestion of samples to remove carbon in order to determine if metals were associated with Fe hydroxides or organic matter.

The results of the spectroscopic analysis indicated that Cd, Cr and Zn were all correlated with Fe before and after digestion; however, Cu seemed to be bound more to organic matter. In the case of Pb the correlation was weaker for the composted biosolids, but the authors suggested this may have been due to the poor distribution of Pb within that particular sample. Another study by Brown et al. 2012 using similar spectroscopic techniques did find associations of Pb with Fe and phosphate (pyromorphite). Schenkel and
Ryan (2004) also used spectroscopic analysis to evaluate Pb in soils amended with P. The authors found that 1-16% of Pb in biosolids samples was bound as pyromorphite when additional P was added as triplesuperphosphate. The authors suggested these low values could be due to coatings of organic matter on pyromorphite crystals or Pb bound to organic matter preventing further formation of pyromorphite. However, as supported by Hettiarachchi et al. (2000 and 2003) the authors believed as organic matter was decomposed Pb would become bound as pyromorphite over time.

In another study by Donner et al. (2011), the authors analyzed three anaerobically digested fresh cake biosolids and three biosolids from a stockpile. Spectroscopic analysis was performed with XAS techniques, including both X-ray Absorption Near Edge Spectroscopy (XANES) and Extended X-ray Absorption Fine Structure Spectroscopy (EXAFS). The authors found that while Zn was associated with Fe hydroxides, Cu was less so, leading the authors to hypothesize that Cu may be associated with organic matter. In addition, samples from the fresh cake showed more associations with sulfide groups then from the older biosolids samples. The authors suggested that as these biosolids aged speciation of these metals may have changed. They also noted that due to the heterogeneity of samples it was difficult to conclusively determine dominant species in the case of either metal.

Both Hettiarachchi et al. (2006) and Donner et al. (2011) also found stronger correlations between metals under consideration and Fe minerals, then these metals and Mn oxides present in samples. Further while Donner et al. (2011) noted that metals bound to organic matter or sulfides would likely eventually be released through decomposition and oxidation, Hettiarachchi et al. (2006) contended these metals would be adsorbed to available sites on Fe minerals as supported by previous studies (Hettiarachchi et al. 2000 and 2003). Both authors agreed this would lead to the long-term immobilization of metals that has been observed in previous studies of biosolids amended soils (Kukier et al. 2010; Zerzghi et al. 2005; Brown et al. 1996).
While metal speciation and pH are important factors influencing metal availability in a soil system, metal concentration and soil texture can also play an important role. For example, metal mobility studies using biosolids have found that metal movement increases in sandy textured soils and with increasing metal concentration (Qi, et al 2010; Yuan et al. 2009; Yang et al. 2008). Such results infer that application rate (metal concentration), pH, metal type and soil texture are all important considerations when determining application rates.

**Phytoavailability of Metals**

Beginning in the rhizosphere, metals can be mobilized for plant uptake due to changing pH and redox conditions influenced directly by plants and microbes or due to changing conditions in the soil matrix as previously discussed. In the case of direct mobilization of metals by plants and microbes, this is largely due to the release of organic acids, or through microbial respiration and decomposition. In order for metals to become bioavailable, however, they must be converted into a bioavailable form. In the case of plants this involves desorption from complexes, diffusion in soil solution and precipitation on or absorption by plant roots as displayed in Figure 1.5 (McBride 1994). Factors influencing whether plants take up available metals include: diffusion, rates of transpiration (mass flow), root growth, temperature, nutrient status of soil, plant species and presence of other ions (Greger 1999; Allen et al. 1995).

Metals, if soluble and available, are usually taken up in the rhizosphere by roots. Depending on the metal, uptake may either occur across the root surface or through the apical region (Greger 1999). Foliar accumulation can also occur through leaf cuticles in the case of atmospheric deposition of metals (Marschner 1986). Typically though metals are taken up via roots from the soil through ion uptake channels. There are specific mechanisms for different ions, namely required nutrients. Some studies have also shown that mycorrhizal fungi associated with plant roots may protect trees including conifers from
taking up metals, particularly Al (Jentschke and Godbold 2000; Cumming and Weinstein 1990).

Once metals are taken up by plants, they tend to accumulate in roots followed by shoots, leaves and lastly fruit, though this can vary by metal and plant (Greger 1999; Jung et al 1996; Brallier et al. 1994). For example, in the case of conifers, a similar pattern has been shown for non-essential metals such as Cd and Pb; however, essential micronutrients such as Cu have been reported to accumulate in new growth of needles and young tissue (Aznar et al. 2009; Rothpfeffer and Karltun 2007; Truby 1995). According to Rothpfeffer and Karltun (2007) factors contributing to translocation include ion solubility and charge, xylem or sap pH, concentration gradients, as well as cell wall quality. On a cellular level, accumulation typically occurs in the cell wall, cytosol or vacuole (Rausser 1999). According to Greger (1999) 75-90% of metals found in plants are bound to cell walls in the roots. Accumulation in the root is largely a function of natural barriers in the root preventing translocation such as Casparian strips in root epidermis.

Metals that enter the root xylem can be transported elsewhere in the plant driven by transpiration. These metals usually enter through the root tip where Casparian strips do not occur. The final location of metals in the plant also depends on the plant species and metal. For example, in the tree F. andustifolia, Cd was found in the inner cortex while Cu was found on the cortex cell walls (Arduini et al. 1996). Lead on the other hand tends to accumulate in roots more than elsewhere in the plant likely due to its ionic radius, lack of similarity to plant nutrients and resulting relative immobility (Kocjan et al. 1996).

Just as in the soil, mobilization of metals for plant uptake is heavily influenced by pH, metal speciation, and metal concentration. In the case of biosolids, many studies have shown that metals do not travel far through the soil, particularly when it is maintained at a near neutral pH (Qi et al 2010; Yuan et al. 2009; Yang et al 2008; Stenhouwer, et al 2005; Brown et al 1997 and 1998; Brallier, et al 1994; Chang et al 1986; Harris and Urie 1986). This immobility is indicative of insoluble metal species, which are also less likely to be taken
up by plants then metal salts. However, as metal concentration increases, available sites for binding are reduced resulting in bioavailable forms of metals (Brown et al. 1998).

In a study considering uptake of Cd by lettuce, Brown et al. (1998) found that pH, metal speciation and Cd concentration were important factors in determining Cd phytoavailability in lettuce amended with biosolids and metal salts. Lettuce was grown on plots amended with 100 Mg ha\(^{-1}\) of two biosolids with respective Cd concentrations of 13.4 and 210 mg kg\(^{-1}\). In addition, a control soil was amended with the equivalent concentration of Cd as biosolids treatments added as CdCl\(_2\). The pH values for the treatments were adjusted to 5.5 (low) and 6.5 (high). The biosolids had been added to soils 13 to 15 years prior and only 16% of the organic carbon applied with biosolids in the original study was remaining allowing for evaluation of the effects of C oxidation on metal availability overtime.

Cd uptake by lettuce was significantly higher in the metal salt treatments than in the biosolids treatments. This is consistent with several other studies, which demonstrated that metals added as metal salts tend to be more available than those added with biosolids (Brown et al., 1998; Hooda and Alloway, 1993; Bell et al., 1991; Mahler et al., 1987; Gaynor and Halstead, 1976; Cunningham et al., 1975a, 1975b, 1975c). Lettuce grown on higher metal biosolids plots had higher tissue concentrations than those grown in low Cd biosolids treatments and control which were not significantly different, indicating the role of metal concentration on uptake. Further, differences in uptake were more pronounced for lower pH treatments and there were no significant differences in uptake between the original study and the one conducted 15 years later.

The role of pH in the mobility of metals between different plant species was also demonstrated by Braillier et al (1994) in a study considering the effects of liming on the availability of Cd, Cu, Ni and Zn in a soil treated with biosolids. The authors observed a negative relationship between increasing soil pH and bioavailable metals in lettuce, cabbage, potato tuber and tomato fruit. In addition to changes in pH, uptake by the plants
was significantly different, with lettuce and cabbage consistently demonstrating higher levels of metal in tissue than potato tubers (peeled) and tomato fruit.

Similarly, in a study conducted by Jung, et al (1996) near a lead-zinc mine in Korea, the authors found that plants in gardens contained metals; however, this varied by metal concentration (proximity to mine), pH and plant species in the following order: tobacco leaves > spring onions > soybean leaves > red pepper = corn grain. In addition the authors found that the ratio of metals in the soil to metals in plants varied by metal demonstrating that different metals have different rates of phytoavailability as well. According to the results of this study, bioavailability as a ratio of soil concentration to uptake followed in the order of Zn > Cd > Cu > Pb.

These relationships can be explained by a variety of factors including plant type, nutrient demands and increased complexation of metals due to changing pH (Lambers et al. 2008; Ahmad et al. 2005). Nutrient status of the plant is particularly important in the case of Cd uptake. Cd is chemically similar to Zn, a required plant nutrient. In circumstances where Zn is limited plants may take up Cd in its place, however, this is limited to circumstances in which the ratio of Zn to Cd is less than 100:1 (Basta et al. 2005). Due to the high ratio of Zn to Cd in most biosolids Cd uptake is not an issue.

**Metal Stress in Plants**

The harmful nature of a metal can be direct or indirect as metals may interfere with several processes important to a plant's nutrition and survival. Physiological and morphological responses to these effects include reduced biomass and root growth, chlorosis, oxidative tissue damage, interference with photosynthesis, ATP processing and nutrient uptake, as well as, reduced water use efficiency (Lambers et al. 2008; Menon et al. 2007; Ahmad et al. 2005; Prasad 1999; Allen et al. 1995; Kahle 1992). Indirect effects involve reduced nitrogen fixation due to harmful effects on microbes and complexation with P, limiting uptake of this nutrient (Ahmad et al. 2005; Prasad 1999; Allen et al. 1995; Kahle
et al. 1992; Lamersdorf et al. 1991). Physical presentations of these toxic effects are listed in Table 1.8.

Experiments measuring metal stress and toxicity have drawn on these physiological and morphological responses to make conclusions regarding metal toxicity. Plant leaf metal concentrations are a widely accepted measurement of phytotoxicity (Burton 1983; Beckett and Davis 1977). However, inferring universal toxic thresholds based on plant tissue is impractical as these values are highly variable depending on plant organ, species and genotype, as well as, age and growth stage. For example, seeds and seedlings tend to be more sensitive to heavy metals than older plants, making results from greenhouse studies difficult to apply to actual scenarios in a forest (Lamersdorf et al. 1991). In addition Kahle et al. (1992) noted that Cu has been shown to limit seed germination though this is more pronounced for deciduous trees than conifers. Despite this, some suggested ranges for Cu and Zn toxicity are 25-40 mg kg\(^{-1}\) and 500-1500 mg kg\(^{-1}\) respectively in plant dry matter (Chaney et al. 1989). It is important to note that Cu and Zn are also micronutrients necessary for plant growth. In the case of Douglas-fir trees grown for commercial Christmas tree sales, suggested deficiency values of these nutrients in tree foliage are <3 mg kg\(^{-1}\) Cu and 10 mg kg\(^{-1}\) Zn (Hart et al. 2004).

More specific toxic threshold values are available for cultivated and highly studied species. Some of these values are reported in Table 1.9 and represent those collected by Macnicol and Beckett (1985) in a review of literature for a variety of crop and test plants. However, application of such results should be performed with caution for the reasons previously stated.

While such ranges give room for interpretation, evaluating such thresholds is further complicated in the case of forest ecosystems where plant diversity is far greater both in terms of ecological biodiversity and genetic variation within a species. For example, in a study by Burton et al. (1983) the authors suggested upper critical concentrations in leaf tissue of Sitka spruce seedlings grown in nutrient solutions spiked with metal chloride salts
to be 4.8, 5.8, 19, 88 and 226 mg kg\(^{-1}\) Cd, Ni, Pb, Cu and Zn respectively. For comparison, a study considering the effect of metal pollution on Norway spruce in soils of Northern Germany, suggested Pb needle concentrations of 2-5 mg kg\(^{-1}\) were correlated with a risk to tree vitality based on observations of root elongation in trees grown on forest soils contaminated by anthropogenic sources (Lamersdorf et al. 1991). Further Bledsoe and Zasoski (1981) noted that seedlings planted in soils amended with biosolids with total Cd concentration of 3 mg kg\(^{-1}\), had foliar Cd values of 4.0 mg kg\(^{-1}\) yet still had higher yields than those planted in control soils.

Higher thresholds for Zn toxicity have been reported for other forest trees. For instance, in a study allowing comparison of uptake from high and low metal biosolids, Gaulke et al. (2006), reported Zn foliage concentrations in red alder of 249 mg kg\(^{-1}\) in low metals biosolids (Zn soil concentration of 283.2 mg kg\(^{-1}\)) and 279 mg kg\(^{-1}\) in high metals biosolids (Zn soil concentration of 1759.6 mg kg\(^{-1}\)) treatments with no corresponding signs of metal phytotoxicity. Soil pH in the high metal treatment ranged from 4.1-4.6 indicating a potential for high phytoavailability or Zn. Further, Jeyakumar et al. (2010), found that poplars grown in treatments containing various concentrations of Cu and Zn in biosolids spiked with metal salts did not show signs of phytotoxicity at leaf concentrations of 9.4-38.4 mg kg\(^{-1}\) Cu and 169.2-1408.5 mg kg\(^{-1}\) Zn; however, trees grown in high metal treatments (78 and 334 mg kg\(^{-1}\) Zn) did show signs of stress including yellowing and withering of leaves with leaf concentrations of 3417.8 and 3755 mg kg\(^{-1}\) Zn. Poplars, however, are known to have high metal tolerance levels and accumulate metals quickly due to rapid biomass production (Jeyakumar et al. 2010). In addition, deciduous trees have been reported to accumulate more metals in foliage than conifers grown under similar conditions (Bledsoe and Zasoski 1981). As demonstrated by these results, plant uptake and toxic concentrations of metals vary widely and no absolute values exist even amongst members of a particular species.
However, caution should also be taken when extrapolating potential for metal toxicity and uptake by plants based solely on soil metal concentrations. Table 1.10 provides a summary of foliar metal uptake in several tree species at varying soil metal concentrations. A comparison of these values demonstrates that total soil metal concentration and plant tissue metal concentrations may not be sufficient to define potentially toxic levels. Additional considerations should include some evaluation of phytoavailability of the metals in question. Variability in uptake and toxicity will likely also be observed based on plant species, age and plant organ. For instance, with the exception of metals in shoots of Sitka spruce grown in soils amended with metal salts, uptake of Cd and Pb remains relatively low and consistent in foliage of various trees treated with biosolids. However, in the case of similar soil Cd and Pb concentrations, plant uptake of these elements by Sitka spruce was dissimilar to uptake in leaves of red alder; the primary differences likely being the result of a combination of plant species, soil pH, metal form, and plant tissue analyzed.

Further differences can be demonstrated between species in the case of Sitka spruce, Douglas-fir and western hemlock trees planted in similar soils (Bledsoe and Zasoski 1981). While the former two species showed similar Zn uptake, 270 and 240 mg kg\(^{-1}\) respectively, needle concentration for Zn in western hemlock were only 96 mg kg\(^{-1}\). In addition, these same species planted in soils amended with biosolids to a Zn concentration of 210 mg kg\(^{-1}\) demonstrated lower uptake than Douglas-fir and Scots pine planted in soils with lower concentrations of Zn contaminated by a nearby zinc smelter (Van Nevel et al. 2010; Bledsoe and Zasoski 1981). All of these differences demonstrate the importance of considering soil pH, metal form, plant species and plant tissue when drawing conclusions about metal toxicity. More importantly, they show that we have no real understanding of what phytotoxic metal concentrations of these metals are for different trees.

These same principles apply to measuring toxicity based on other methods including measurement of root mass and length. This has been suggested as a better method of determining metal stress as often roots are often more affected by metals than above

As mentioned, toxicity of metals to plants is highly variable depending on the plant species and metals in question. It is well known that some species of plants have adapted to withstand high levels of metals either within their habitat or in their tissue. These plants are referred to as phytoaccumulators. This trait is particularly true of herbaceous plants and less so for trees though exceptions exist particularly among poplar and willow species (Jeyakumar et al. 2010; Melezeck et al. 2009; Kahle et al. 1992).

For plants that are sensitive to metal toxicity, certain metals can be more toxic then others or may interact with one another. While results and literature reviews to this end have suggested various orders of toxicity it is generally accepted that Hg, Pb, Cu, Cd, Zn, Ni and As can all be toxic at various concentrations if taken up by the plant (Shaw et al. 1999). In an experiment evaluating root elongation as a sign of metal toxicity, spruce seedlings grown in metal salt solutions were found to be affected by metals in the order Hg>Pb>Cd>Zn when concentrations of each metal were added at 0.1-15 mM, 0.5-2.0 mM, 5-60 mM, 30-60 mM respectively (Godbold et al. 1987). Tissue concentrations were not reported. A more general review of the literature by Kahle, et al (1992), suggested the order to be Cd>Cu>Pb>Zn.

Such generalizations from metal salt laboratory experiments, however, must be reviewed with caution. As previously stated, metal availability and uptake in the soil will be influenced by metal concentration, speciation and the presence of other plant nutrients. For these reasons, Hg and Pb are rarely in concentrations and forms potentially toxic to plants in soils. In contrast, Cd, Zn and Cu tend to pose a greater threat to plant health due to their relative level of availability and in the case of Zn and Cu due to their dual role as both
a nutrient and potential toxin. Further, due to its prevalence in the environment, Zn is the metal most likely to cause phytoxicity particularly when soil pH is below 5 (Chaney 1993).

Inhibitory and synergistic relationships between other metals and plant nutrients has been shown in particular for Cd and Zn as well as Pb and P. In the case of Zn and Cd, a Zn to Cd ratio of greater than 100:1 has been reported to limit Cd uptake (Basta et al. 2005). In their review of such studies considering the relationship between Zn and Cd uptake, Welch and Norval (1999), reported Cd uptake in Zn deficient environments may be the result of weak root-cell plasma membranes. Zn is an important nutrient in sustaining the integrity of such surfaces to prevent abnormal uptake of various elements. Due to the high ratio of Zn to Cd in most biosolids Cd uptake is not an issue. In addition, Mg and Ca have also been reported to impede Cd uptake (Kahle et al. 1992).

Anions such as phosphate on the other hand may hinder uptake by binding with metals. This has been a suggested mechanism for metal immobilization in the presence of P and Pb. The precipitation of Pb with P results in pyromorphite the least bioaccessible form of Pb (Hetteriachchi 2000). Like galena (lead sulfide), one of the most common forms of Pb, pyromorphite is insoluble across a range of pH levels limiting uptake of Pb by plants. For example, Rolfe (1973) found decreased uptake of Pb in loblolly pine and yellow poplar seedlings grown with 190ppm phosphate. Similarly, several studies have reported the high presence of P in biosolids as a mechanism for limiting Pb mobility and plant uptake (Scheckel and Ryan 2004; Brown et al. 2003; Hetteriachchi et al. 2000).

In addition, Larcheveque et al. (2006) suggested immobilization of Cu and Zn by P could be a reason for limited metal uptake by Quercus ilex, Pinus halepensis and Pinus pinea seedlings, in a study considering the bioavailability of metals in biosolids. The experiment measured foliar metal concentrations in seedlings of three tree species after two years of growth in soils amended with 20kg and 40kg of composted biosolids per seedling. Their results indicated no significant difference between foliar metals in the three
treatments for all seedlings tested. Reported Cd, Cu, Pb and Zn concentrations in the biosolids used were 0.77, 122, 65 and 266 mg kg\(^{-1}\) respectively.

Such a relationship, however, could lead to P deficiencies depending on the metal concentration, indirectly hindering tree development. Phosphorus applied with biosolids, however, is typically supplied in excess particularly compared to metals. Therefore, any potential for nutrient deficiencies are limited under such circumstances.

In summary, while metals can interfere with several important processes in plants, at what concentration a particular metal becomes toxic is highly variable depending on various plant and soil factors. Values derived from greenhouse studies or laboratory experiments using metal salts and cultivated plants should be interpreted with caution when evaluating metals in forest ecosystems and metals applied as biosolids. This is particularly true for metals applied with biosolids as the inherent properties of such an amendment are known to limit metal availability particularly the presence of Fe and Mn oxides, phosphorous, organic matter and the potential to elevate pH.

Some studies have suggested that methods of evaluating biosolids-amended soils for potential phytotoxicity should not be based on total soil metal concentrations. Preferable methods suggested by these studies include soil extractable metals or isotope dilution methods depending on the metal to be tested (Jeyakumar et al. 2010; Heemberger et al. 2009). Further, it has been noted that use of the bioconcentration factor, the ratio of metals in plant tissue to metals in soil, is a more useful value for reporting metal toxicity thresholds then simply tissue concentrations (Jeyakumar et al. 2010; Heemberger et al. 2009).

**Metals in Forest Ecosystems**

While most research has focused on metal uptake by agricultural crops, increasing metal pollution from mining, coal and smelting processes, as well as land application of biosolids has spurred interest in metal toxicity to forest ecosystems. Forest soils tend to be lower in nutrients and more acidic than other soils. While conifers are often adapted to
these environments their tolerance for heavy metals has been the subject of several studies regarding the effects of metals on forest ecosystems (Menon et al. 2007; Larcheveque et al. 2006; McLaren et al. 2006; Arduini et al. 1998; Kahle et al. 1992; Godbold et al. 1991; Lamersdorf et al. 1991; Brockway et al. 1983; Burton 1983, 1984 and 1986).

Of particular concern has been whether more acid forest soils can compound the effects of metal accumulation in such environments by making metals more mobile. In a study by Menon et al. (2007), the authors compared growth, fine root biomass and water use efficiency of *Picea abies*, *Salix viminalis*, *Populus tremula* and *Betula pendula* grown on acidified (pH 4.2) and calcareous soils (pH 7.4) contaminated with non-ferrous smelter dust containing 654 g kg\(^{-1}\) Zn, 65 g kg\(^{-1}\) Cu, 12 g kg\(^{-1}\) Pb and 270 mg kg\(^{-1}\) Cd. The authors, however, did not measure plant uptake of metals. Their results indicated that while *P. abies* did demonstrate reduced fine root biomass and water use efficiency on both soils, overall growth response was better than the other tree species examined. Further, confirming common knowledge that acidity can enhance metal bioavailability, the observed reductions in fine root biomass and water use efficiency were higher in trees grown in acid soils compared to calcareous soils.

Inhibition of root and shoot growth due to metal exposure was also reported by Burton et al. (1984) in Sitka spruce seedlings. The authors reported shoot yield of Sitka spruce seedlings grown for 100 days in acidic peaty soils (pH 3.3-3.92) spiked with 0.1-16 mg kg\(^{-1}\) Cd, 5-400 mg kg\(^{-1}\) Pb and 0.5-32 mg kg\(^{-1}\) Cu was only inhibited by Cd at increasing concentrations. Further toxicity based on reduced root and shoot yield was only noted in trees grown at the highest Pb concentration of 400 mg kg\(^{-1}\). In a field study measuring Pb and Cd uptake by Norway spruce trees where metal deposition from industrial processes had occurred, Lamersdorf et al. (1991) reported Cd and Pb uptake by trees decreasing in tree parts moving further from the rhizosphere. In addition, the authors noted that Cd may accumulate more in the roots as exhibited by higher Cd concentrations in roots than humus.
layer. In contrast, Pb was reported to have similar fine root/humus concentrations, but Pb concentrations decreased markedly in above ground biomass.

Other studies have shown conifers exposed to metals including Cu, Cd and Pb exhibit other signs of metal stress. For example in a study exposing four-week-old seedlings of *Pinus pinea L.*, *Pinus pinaster Ait.* and *Fraxinus angustifolia* to increasing concentrations of CuSO$_4$ (0.012-5 mM) and CdSO$_4$(0.0-5 mM) added to nutrient solutions, Arduini et al. (1998) found that chlorophyll content decreased with increasing metal treatments for all species. They also reported decreased biomass in trees but only at the upper Cu concentrations for the pine trees. In addition, Mn and Zn concentrations in roots were inhibited by increasing Cu and Cd, but uptake of Mg and Ca varied significantly by species leading the authors to conclude that such differences in species tolerance should be considered in efforts to restore such forests.

While all of these studies focus on uptake of metals in the form of salts, particularly those deposited from pollution, other studies have focused on the potential for heavy metal toxicity in forests treated with biosolids. Considering that metals added in composts tend to be in low concentrations and relatively immobile, many of these studies have concluded that heavy metals added with biosolids do not interfere with plant growth especially when the benefits of compost and fertilization are considered. One such study by Larcheveque et al. (2006), found that biosolids used to restore a site damaged by fire increased seedling survivability. The authors applied 20kg and 40kg of biosolids to each seedling. Reported Cd, Cu, Pb and Zn concentrations in the biosolids used were 0.77, 122, 65 and 266 mg kg$^{-1}$ respectively. After two years of growth, the authors reported that Cd, Cr, Ni and Pb concentrations in both soils and seedlings were not significantly higher than the control. Cu and Zn concentrations in soils were higher in biosolids amended soils but not seedlings. Further, the authors noted that biosolids application rates would be limited not by metals but by high P concentrations.
Of particular concern to the use of biosolids in the forest industry has been the effect of metals on mycorrhizal relationships and nitrogen fixation. Mycorrhizas have been shown to be vary in their tolerance to metals while Pb and Cd have been reported to interfere with production of nitrate reductase activity (Jeyakumar 2010; Lambers 2008; Gaulke et al. 2006; Kahle 1993).

Gaulke et al. (2006) explored this relationship using two biosolids (high and low metals) applied at 250, 500 and 1000 Mg ha\(^{-1}\) to soils growing red alder (Alnus rubra Bong.) to evaluate both metal toxicity to trees and Frankia, the bacterial endosymbiont responsible for N fixation by red alder. Metal concentrations in treatments ranged from 1.2-32.5 mg kg\(^{-1}\) Cd, 28.3-623 mg kg\(^{-1}\) Pb, and 109.5-1759.6 mg kg\(^{-1}\) Zn depending on treatment. The biosolids used included high metals biosolids (45 mg kg\(^{-1}\) Cd, 958 mg kg\(^{-1}\) Pb, and 2623 mg kg\(^{-1}\) Zn) produced 25 years previously and modern biosolids containing lower metal concentrations (0.8 mg kg\(^{-1}\) Cd, 20 mg kg\(^{-1}\) Pb, and 160 mg kg\(^{-1}\) Zn). Soil pH was 4.1-4.7 for biosolids treatments and 5.1 for control.

Parameters evaluated included rate of N fixation, foliar metals and tree biomass. Results from this study indicated no significant difference between treatments and nitrogen fixation. Further, foliar metals were undetectable for Cd and Pb where as Zn concentrations did increase with increasing application rates. Even at the highest reported foliar Zn concentration of 279 mg kg\(^{-1}\), no signs of toxicity were present. In addition, biomass in both high and low metal treatments was higher than the control, however, biomass decreased with increasing metals in the high metal treatments.

These results are in agreement with other biosolids studies. For instance, Bledsoe and Zasoski (1981) observed that seedlings of Douglas-fir, Sitka spruce, western red cedar, ponderosa pine, grand fir, black cottonwood, and Lombardy poplar, grown in pots with a 3:1 mixture of soil to biosolids exhibited 2-5 times increased biomass production despite increased metal levels in the soil. Reported metals in control soil and 3:1 soil to biosolids treatment were 46 mg kg\(^{-1}\) Zn, 18 mg kg\(^{-1}\) Cu and >0.025 mg kg\(^{-1}\) Cd and 2400 mg kg\(^{-1}\)
Zn, 970 mg kg\(^{-1}\) Cu and 37 mg kg\(^{-1}\) Cd respectively. Foliar samples further indicated increased N uptake in the biosolids treatment and only elevated Zn uptake among metals tested.

Similarly, Brockway et al. (1983) found increased N uptake in poplar, Douglas-fir and loblolly pine planted with biosolids, suggesting that heavy metals in biosolids were not interfering with uptake of this nutrient. While metal concentrations in soils are not provided, this evaluation was conducted during a period when biosolids contained higher levels of metals than modern biosolids. This would suggest that metals from biosolids applied under current standards and regulations would also not hinder N uptake and growth. Other studies demonstrating the growth benefits of biosolids offer similar conclusions (Harrison et al. 2002; Henry et al. 1994; Brockway 1986; Chapman-King et al. 1986).

As a comparison of these studies demonstrates, consideration of metal form in evaluating metal availability and toxicity is important. Table 1.11 provides a summary of metal uptake in various trees planted with metal salts and biosolids. While studies using metal salt solutions similar in form to metals from anthropogenic inputs may be useful for interpreting the effects of industrial pollution, these studies should not be directly applied to evaluation of metals applied with biosolids. Further, greenhouse and seedling studies are not always indicative of tree responses in field scenarios due to age of trees, climatic factors and changes in soil dynamics. Given that biosolids are rarely applied to seedlings and contain metals in less bioavailable forms, comparisons to studies using metal salts and seedlings should be made with caution when examining the risk of metals in biosolids to forest trees.

**Phytochelatins**

Phytochelatins (PCs) are bioindicators of metal stress produced by plants in response to free ions of certain metals. Their role in the plant is to protect plant cells from oxidative stress and interference with other enzymes including Rubisco and nitrate reductase (Hirata et al. 2005; Prasad 1999; Rauser 1995 and 1999; Kneer and Zenk 1992; Thumann et al
1992). For this reason, they have been suggested as a possible measurement of metal
tolerance in plants and metal stress in environmentally degraded ecosystems. However,
inconsistent results from in vivo and in vitro studies have led some to question whether
such applications of PC measurements are appropriate (Prasad 1999; de Knecht et al. 1994;

Phytochelatins were first identified in the early 1980s as a sub-group of
metallothioneins (MT), cysteine (Cys) rich molecules involved in the chelation of metals.
There are three groups of MT all of which provide binding sites for metals on thiol groups
attached to polypeptide chains. MT-I is found in species of the animal kingdom where as
MT-II is reported in sea urchins, plants, fungi, algae and nematodes (Rauser 1999). Both
these groups were identified early on as being genetically encoded. MT-IIIIs, now commonly
referred to as PCs, are found in fungi, algae and higher plants (both dicots and monocots).
Originally PCs were not believed to have any associated genetic code. In 1999, however,
three individual research groups confirmed that some yeasts exhibited a gene associated
with PC synthase production, this has since been confirmed for other higher plants such as

PCs are formed from three amino acids: Glu, Cys and Gly. These amino acids form
chains of y-Glu-Cys repeats linked with a y-carboxylamide bond. Most commonly they end
with the amino acid Gly and repeat 2-5 times though they may form chains as long as 11
bonds. The length of the chain may determine how effective the PC is in transporting
metals and over all stability of the PC at low pH (Prasad 1999).

There are five families of PCs based on chain length and terminal amino acid as
shown in Figure 1.6 (Rauser 1995). PCs are built from glutathione (GSH), which has a
similar structure to PCs (y-Glu-Cys-Gly). This theory is based both on the structure of
 glutathione AND also observations that GSH concentrations decrease when PCs are
produced. In the absence of GSH, PCs are not formed (Rauser 1995). The effectiveness of
PCs role in preventing metal toxicity is complicated by the dual role of GSH, which is also
involved in plant response to oxidative stress. For example, reduction in GSH from PC production prompted by Cu has been shown to induce oxidative stress in *Silene cucubalus*, a metal tolerant species (De Vos et al. 1992).

Transport mechanisms for sequestration of metal-PC complexes in vacuole are yet to be proven, however, work by Ortiz et al. 1995, as well as, Salt and Rauser 1995 suggest that PCs are formed in the cytoplasm (Prasad 1999). From here they complex with metals and may be transported in an ATP-dependent fashion to the vacuole. In the vacuole, lower pH conditions lead to dissolution of the bond and recycling of PC. This ability to efficiently transport PC-metal complexes may be another indicator of metal tolerance and an explanation for why some studies have shown lower PC concentrations in plants with higher metal tolerance (Prasad 1999).

PCs are found predominately in roots and to a lesser extent in shoots and foliage (Cobbett 2000; Rauser 1999), although this pattern was reversed in the Douglas-fir seedlings studied by Cline et al. (2011). This is consistent with metal accumulation, which follows a similar pattern due to metal exposures usually originating in the rhizosphere. Metals deposited on leaves can also enter plants through leaf cuticles (Greger 1999).

A study by Gawel et al. (2001) suggests that direct uptake through foliage may result in elevated foliar PC concentrations. The authors tested this hypothesis in a forested area near a smelting operation. Foliar samples of naturally occurring *Populus alba* and *Betula papyrifera* located at varying distances from the smelter were analyzed for PC production. These were compared to seedlings potted in uncontaminated soils, with peat moss filters placed to prevent metals deposition reaching the roots. At the end of the experiment, metals deposition was measured in the peat moss filters. Graphically depicted metal concentrations in peat were approximately 0-540 nmol Cu/g peat and 15-115 nmol Ni/g peat. Elevated PC levels were correlated with increased aerial deposition of Cu and Ni. As this was also the case for the seedlings that were potted to prevent exposure to soil metals, the authors concluded that metal exposure was occurring through foliar uptake.
Foliar and soil metals were not directly measured, but the highest PC levels were observed at the distance from the newly constructed 600 m tall “superstack” that had been predicted to have maximal atmospheric deposition but relatively low soil metal concentrations. These conclusions, however, are difficult to prove without the support of foliar metal concentrations or soil metal concentrations neither of which were analyzed.

Regardless of uptake mechanism, in order for a metal to be effectively chelated, it must first activate the enzyme PC synthase. Then chelation of the metal by the PCs synthesized must occur followed by transport and sequestration of the metal in the vacuole where the metal may be further complexed with sulfide or organic acids (Cobbett 2000). This initial activation of PC synthetase has been demonstrated predominately with Cd and to a lesser extent Ag, Bi, Cu, Hg, Ni, Sn, Sb, Te, W, Zn, SeO$_4^{2-}$, SeO$_3^{2-}$, and AsO$_4^{3-}$ (Prasad 1999). The order of potency depends on plant species, cells tested and metal concentrations (Cobbett 2000; Prasad 1999; Rauser 1995).

For example, PC response to metals in cell suspension cultures of *Rauvolfia serpentina* was shown to occur in the following order Hg$\gg$Cd, As, Fe$>$Cu, Ni$>$Sb, Au$>$Sn, Se, B$>$Pb, Zn (Grill et al. 1987). Another suggestion for root cultures of *Rubia tinctorum* indicated PC response could be attributed to the following metals in decreasing order Hg$\gg$Ag$>$Cd$>$As$>$Cu$>$Pd$>$Se$>$Ni$>$Pb$>$Zn$>$In$>$Ga (Maitani et al. 1996). On the other hand, Schat et al. (2002) showed PC activation in roots of non-metallicolous and metallicolous varieties of *Silene vulgaris, Holcus lanatus, Agrostis castellana, Thlaspi caerulescens*, followed in the order of As/Cd/Cu$>$Zn$>$Ni/Co in plant roots. PC accumulation, the authors noted, was consistently lower in metal tolerant varieties with the exception of those exposed to As. Activation of PC synthase in tobacco leaves was reported to occur with Cd, Ag, Cu, Pb, Zn and Hg with the later three metals resulting only in a weak response (Chen et al. 1997). Additionally, in a review of literature by Cobbett (2000) the author reported that Zn and Ni have not been shown to significantly increase activation of PC synthetase *in vitro*.
Complexation and sequestration is also limited to certain metals where again Cd is the most researched and only metal reported to readily complex with PCs (Rauser 1995). Evidence also exists for PC-metal complexes involving Cu, Ag, Zn and to a lesser extent Hg and Pb (Schat et al. 2002; Maitani et al. 1996; Kneer and Zenk 1992; Schat and Kalff 1992; Grill et al. 1987). However, Rauser (1995) and Cobbett (2000) both caution that this relationship is highly dependent on plant species and note that most early studies focused on in vitro responses of plant cells. Rauser (1995) further reported that complexation has really only been shown extensively for Cd in vivo and in some cases Cu.

According to Prasad (1999) evidence that PCs are involved in metal homeostasis and indicative of metal tolerance are based on studies of algae, tomatoes and other herbaceous plants and cell cultures, which demonstrate that metal tolerant mutants produce more PCs and uptake more metals than non-tolerant types (Huang and Goldsborough 1988; Huang et al. 1987; Jackson et al. 1984). It has also been shown that metals taken up in these mutant plants are predominately complexed with PCs. Conversely non-tolerant plant cells bind little metal to PCs and show more signs of stress including reduced growth and premature death (Jackson et al. 1984; Huang et al 1987; Huang and Goldsborough 1988; Delhaize et al. 1989).

Casting doubts on this role of PCs are other studies demonstrating that tolerant plants produce no more PCs than non-tolerant plants and in some cases less despite increased metal uptake (de Knecht et al. 1994; de Vos et al. 1992; Schat and Kalff 1992). Schat and Kalff (1992), noted that this may be due in part to experiments designed to subject plants to equal concentrations of metals despite differences in tolerance. The authors attempted to address this by determining whether PC production was significantly different for copper tolerant and non-tolerant Silene vulgaris when exposed to Cu concentrations specific to plant tolerance. The authors found that PC production was proportional to metal tolerance. From this experiment, Schatt and Kalff concluded that variance in PC production was not necessarily an indicator of metal tolerance but a response
to metal exposure. In a similar study by de Knecht et al. (1992) the authors found that non-tolerant lines of *S. vulgaris* exposed to the same level of Cd as tolerant lines produced the same ratio of PC-SH:Cd initially, but after three days the ratio was higher in sensitive plants. The authors suggested that their results indicated that PC production is not necessarily a mechanism of increasing tolerance to Cd.

However, such results are further convoluted by the short lifespan of PCs, particularly in metal tolerant plants. Some suggest that reduced PCs in such circumstances may be the result of faster shuttling of metals by PCs and prompt dissolution of PCs once the job has been completed (Prasad 1999). Despite such efficiencies, if PCs are in fact indicators of metal stress, PCs should be continually activated by the presence of metals in high concentrations. Further the ability of some metals to induce PC production but not be complexed offers little evidence that plants infer much physiological protection from PC synthesis alone (McLaughlin and Wang 2006). As a review of the literature offers no non-metal related theories for PC production, their role in plant metal tolerance or stress response remains controversial. For this reason, it is important to consider not only PC production but also metal uptake in plant tissue when evaluating plant responses to metals, particularly in field studies.

**PC Field Studies and Applications in Forestry**

PCs are relatively short-lived and, as reported by Gawel et al. (2001 and 1996), production varies seasonally, being limited to periods of new growth. This makes field sampling for these compounds difficult. For this reason, most studies have been limited to controlled *in vitro* analysis of cells as previously discussed. Recent advances in fluorometric techniques, however, have allowed for detection of PCs at significantly lower levels than previously possible (Gawel et al. 2004). This development has provided opportunities to test PCs in environments where metal contamination and toxicity is of concern.

For example, in one of only a few field studies of PCs, Gawel et al. (1996) found that PC concentrations in declining red spruce were higher than balsam fir, a species not
reported to be in decline. Further, PC concentrations increased in areas of increased forest decline by both elevation and by region. Forest decline was defined in this study by the concentration of standing dead trees present, whereas healthy trees were defined as those with less than 10% foliage loss or discoloration. The authors hypothesized that the source of metal exposure prompting PC production was from atmospheric deposition. In addition to comparing PC production between tree species, samples from several sites across the Northeast were taken from healthy red spruce trees located in areas of varying forest decline. Elevated PC concentrations in healthy trees were correlated with increasing forest decline (P< 0.001). However, while the authors contend that PCs in all stands are elevated indicating metal stress, typical values of PC for red spruce not under potential metal stress were not available to allow comparison of PC production. In addition, soil and foliar metals were not measured, which would have allowed for more direct comparisons of metal exposure and PC production.

Nevertheless, the response seen under field conditions is supported by in vitro studies of PC production in red spruce by Thangavel et al. (2007). Results from this study demonstrated that PC (n=2) concentrations increased 2-4 times higher than control when red spruce tissue was exposed to increasing concentrations of Cd and to a lesser degree in a cell suspension solution. These results led the authors to conclude that PCs in red spruce might be an early indicator of metal stress in forest ecosystems.

Other applications for testing potential metal stress in forests have demonstrated correlations between elevated environmental metal concentrations and increased PC production in trees (Gawel et al 2001 and 2004). As with previously mentioned studies, these studies do not include measurements of metal concentrations in foliage. Studies in this regard are limited, however, particularly in relation to analysis of PC production induced by metals applied with biosolids.

The previously cited study by Cline et al. (2012) that examined the response of Douglas-fir seedlings exposed to metals from biosolids, in both a greenhouse environment,
as well as in a forest ecosystem, was the first study to analyze the effect of biosolids on PC production. Furthermore, a review of the literature indicates that no other studies have measured PC production in Douglas-fir trees.

As mentioned, the correlation between soil metals and PC production in this study was somewhat inconsistent, in that PC production was sometimes lower in treatments with higher concentrations of metals. Furthermore, elevated PC production was noted between treatments with similar concentrations of metals. While Cline et al. (2012) hypothesized that this may have been a result of differences in metal availability caused by aging of biosolids and lower pH values of soils treated with fresh cake, this does not explain why PCs may have been produced at higher levels in the 8% fresh cake treatment compared to the treatment using 18% fresh cake in which pH values were similar. In addition, these results are controversial in that conifers including Douglas-fir have been shown to be tolerant of metal salt and biosolids concentrations close to or above those reported in this study (Van Nevel 2010; Burton 1984; Bledsoe and Zasoski 1981), although these studies did not measure PC production.

The objective of the study described in the following sections is to build off the previous study by Cline et al. (2012) to further examine the role of PCs as a response of Douglas-fir to metal exposure. This lengthened study measured metal uptake in tree foliage as well as PC production in foliage and roots of seedlings. In addition, treatments included biosolids applications that were intermediate to those used in the previous study and were more typical of modern application rates.
CHAPTER TWO: Methods

Summary

This study was designed to compare metal uptake in foliage of Douglas-fir (*Pseudotsuga menziesii*) trees to tree phytochelatin (PC) production for trees grown in soils amended with metals added as salts or in a biosolids matrix at different concentrations. A second aim of the study was to compare the phytoavailability of metals added to soils as metal salts and in a biosolids matrix. The study included seedlings planted at the University of Washington’s Center for Urban Horticulture (CUH) in Seattle, WA in a replicated study and sampling of older trees at the Snoqualmie Forest, managed by Hancock Natural Resources Group, near Snoqualmie, WA. Greenhouse seedlings were grown over an eight-month period in pots containing soils amended with biosolids or metal salts of similar metal concentrations. Metal exposure of field trees resulted from use of biosolids for fertilization. Both field and greenhouse soils were analyzed for total metals, plant available metals, pH, total C/N and electrical conductivity. Foliage samples collected from both greenhouse (June) and field trees (July) were tested for total metals and PC (n=2), Cysteine(CYS) and Glutathione(GSH) concentrations (nmol/g fresh weight). Yield measures were also made on the trees in the greenhouse study.

Greenhouse Experiment Design

Treatments and Soils

Seven treatments were used in the greenhouse study (Table 2.1). These treatments were defined by amendment with biosolids or metal salts (CdCl$_2$, CuSO$_4$, Pb(NO$_3$)$_2$ and ZnSO$_4$). Treatments included a control, three biosolids treatments using two rates of Class B dewatered biosolids (referred to as "cake" in this document) pure historic biosolids and three metal salts treatments to match metal concentrations of soils amended with biosolids. All soils were adjusted with lime to a target pH of 5.

Soils used for the greenhouse study included an Alderwood soil (Loamy-skeletal, isotic, mesic Aquic Dystroxserepts), collected from Lee Memorial Forest near Maltby, WA and historic
biosolids collected from Charles Lathrop Pack Forest near Eatonville, WA (NRCS). The Alderwood soil was used as the control soil and as the base soil for treatments amended with Class B cake and metal salts. Biosolids was applied at two rates of 20 Mg ha\(^{-1}\) (Cake Rate 1) and 40 Mg ha\(^{-1}\) (Cake Rate 2). Historic biosolids were used alone and represented the highest rate of metal application. Metal salts were added to control soil at rates equivalent to metals in biosolids treatments. These treatments are summarized in Table 2.1. Further descriptions of various components are provided below.

Alderwood Soil

The Alderwood soil series is a forest soil formed in glacial drift. The upper A and B horizons are well-drained gravelly sandy-loam above a densic C horizon of glacial till breaking to gravely sandy loam at around 100cm depth (NRCS). The soil used for this experiment was collected from the top 50cm (mostly A horizon) of mineral soil at Lee Memorial Forest (Figure 2.1) in Snohomish County, Wa.

Residential communities and a quickly urbanizing community surround the experimental forest. It is used for recreation, but some field experiments exist to evaluate forest productivity and off trail use is discouraged (UW, 2013). The facility is 160 acres and consists of predominately Douglas-fir and red alder (UW, 2013). Other vegetation includes Western hemlock, Western red cedar, swordfern (Polystichum munitum), Oregon grape (Mahonia aquifolium) red huckleberry (Vaccinium parvifolium) and salal (Glauberia shallon). Nine five-gallon buckets of soil were collected approximately 90 meters from a pedestrian trail about 1/2 km from the forest entrance. Understory vegetation in this area consisted primarily of salal, sword fern and some red huckleberry.

The soil was stored at CUH where it was homogenized for later use. All nine buckets were emptied onto a large tarp and thoroughly mixed by hand using a shovel and pitch fork. Large rocks were removed and soil was placed back in original buckets used for transport. Soil pH was measured on dried sieved soil (<2mm) with water added at a 1:1 volume ratio.
Soils were allowed to equilibrate for 30min after water addition prior to measurement of pH measured on a Denver Instruments Model 220 meter. Soil pH was 3.77.

**Historic Biosolids**

Historic biosolids were collected from Charles Lathrop Pack Forest near Enumclaw, Wa (Figure 2.2). Currently the 4,300 acre forest is used by UW as a demonstration and research center for sustainable forest management. Trails are open to the public for hiking, horseback riding and hunting.

Biosolids, generated in King County WA, were stored in a lagoon at the experimental forest in 1982. The biosolids were collected from beneath a thick layer of grass, which easily peeled away from the soil. After 3 decades in the lagoon, the biosolids were soil like in appearance and did not show signs of anaerobic conditions (mottling). Eight five-gallon buckets were collected and later homogenized by hand at CUH. After collection historic biosolids were tested for pH and total metals using *aqua regia* digest as described in total metals soil analysis (McGrath and Cunliffe 1985 and ISO standard 11466). The pH was 3.01 measured using a 2:1 ratio of water to soil. Metal concentrations were measured at this time using a composite sample gathered from several of the buckets. Reported values (mg kg\(^{-1}\)) of Cd, Cu, Zn and Pb were 48, 1172, 1446 and 999 respectively. These results with standard deviations are summarized in Table 2.2.

**Class B Biosolids Cake**

Modern day biosolids, provided by King County, was used to amend Alderwood soils for seedling planting (Figure 2.3). The biosolids are treated to reach Class B pathogen reduction using anaerobic digestion. The metals concentrations in the biosolids meet US EPA requirements for land application to a variety of crops, including food crops (US EPA 1993). These biosolids are used to fertilize commercial Douglas-fir plantations, wheat and other agricultural crops, and as a feedstock for compost (King County 2012). In forest applications, biosolids are typically applied to meet the nitrogen needs of the trees and forest understory at rates ranging from 6.75-14 Mg ha\(^{-1}\) (Brian Vrablick, personal...
communication). King County's biosolids are dewatered by centrifuge to 20-26% solids. These biosolids will be referred to as Class B cake for the remainder of this paper. For this study biosolids were added to soils at 20 metric tons ha$^{-1}$ (Cake Rate 1) and 40 metric tons ha$^{-1}$ (Cake Rate 2). Metal concentrations (mg kg$^{-1}$) of the biosolids reported by King County for Cd, Cu, Pb and Zn were 2.5, 500, 30 and 900 respectively. Table 2.3 provides a comparison between these concentrations and those in the historic biosolids as well as EPA regulatory limits.

**Metal Salts**

Metal salts (CdCl$_2$, CuSO$_4$, Pb(NO$_3$)$_2$ and ZnSO$_4$) were added to the Alderwood soil to reach total metal concentrations similar to the metal concentrations in the biosolids-amended treatments. Target metal concentrations for each metal salt treatment were calculated using reported metal concentrations for Class B cake and historic biosolids as determined by aqua regia digest.

Based on biosolids’ metal values, liquid solutions of CdCl$_2$, CuSO$_4$, Pb(NO$_3$)$_2$ and ZnSO$_4$ were mixed in order to amend soils for metal salt treatments. To match the two rates of Class B cake a metal solution of 2.8 mg CdCl$_2$, 6.0 mg CuSO$_4$, 34 mg Pb(NO$_3$)$_2$ and 1.53 g ZnSO$_4$ was mixed with 500ml of water. 50ml of this solution added to 10kg of soil was equivalent to the metals in the Cake Rate 1 (20 Mg ha$^{-1}$) treatment using the lower rate of biosolids addition. 100ml of the solution added to 10kg of soil was equivalent to the metals for Cake Rate 2 (40 Mg ha$^{-1}$).

Matching the significantly higher metal concentrations in the historic biosolids required metal salts to be mixed separately to avoid precipitation of metals from solution (Figure 2.4). The following amounts of metal salts were mixed with 1 L of de-ionized water and added at a rate of 300 ml of solution to 10kg of soil: 1.867 g CdCl$_2$, 69.72 g CuSO$_4$, 55.73 g Pb(NO$_3$)$_2$, and 57.13g ZnSO$_4$. 1ml HNO$_3$ was added to the solution containing ZnSO$_4$ to completely dissolve the salt.
**Lime**

Soil pH for all treatments was adjusted to a target value of 5, limiting pH as a complicating factor. ‘Lilly Miller Super Sweet lime’ (86% CaCO$_3$) was used to adjust soil pH. The rate of lime application was determined by adding lime to 50g dry weight samples of biosolids and control soil at the equivalent rate of 10, 20 and 40 Mg ha$^{-1}$ and 5, 10 and 20 Mg ha$^{-1}$ respectively. Duplicate samples for each rate were tested. Soil was then mixed with 50ml of de-ionized water and placed on a shaker for 24 hours. The pH of each sample was measured. Results from these test batches established how much lime would be required to bring the soil and historic biosolids to pH 5 (Table 2.4). Treatments with Alderwood soil were then amended with lime at a rate of 25 Mg ha$^{-1}$. Historic biosolids was amended with 50 Mg ha$^{-1}$ of lime.

**Mixing Soils**

Soils were prepared seven days prior to planting with seedlings. Percent solids for both the historic biosolids (64%) and Alderwood soil (69%) were previously calculated by drying a 100g sample of soil in a 105°C oven for 24 hours. For each treatment, soils were portioned out by volume using a baseline measurement of 10kg dry weight, the approximate weight of soil to fill each pot. Amendments were added to each 10 kg subsample on a dry weight basis. This was repeated 3x to mix enough soil for four pots for each treatment (Figure 2.4). Appropriate amounts of lime, cake and metal solution were added to portioned soils (Figure 2.5). Control, low, medium metal rate and historic biosolids treatments as well as batch 1 of the Metal Salts Historic Rate treatment were mixed on a tarp (Figure 2.6). Batches 2 and 3 of the Metal Salts Historic Rate treatment were mixed in buckets as the metal salt solution made the soil too muddy to effectively mix on the tarp. This ensured that none of the metal solution was lost in transfer to a bucket. For each treatment, the three batches were then combined into two five-gallon buckets for storage, additional mixing and incubation until potting. Each bucket was labeled with colored tape to identify the treatment.
To limit cross contamination between treatments, soils were mixed starting with the least contaminated soil (Control) and ending with the most contaminated (Metal Salts Historic Rate). Following are specific steps for each treatment including photographs of the process (Figures 2.4-2.6):

**Control: Alderwood Soil**

Alderwood forest soil (control) was measured by first weighing 10kg of soil in a five-gallon bucket to identify associated volume. All subsequent measurements of this control soil were then measured to this volume. Soil was spread on a tarp and large rocks were removed. 77.45 g (equivalent to 25 Mg ha\(^{-1}\)) of lime was sprinkled over the soil. Soil was mixed thoroughly to homogenize. All three batches were combined into two five-gallon buckets.

**Cake Rate 1 (20 Mg ha\(^{-1}\)): Alderwood Soil + Class B Cake equivalent to 20 Mg ha\(^{-1}\)**

Control soil was measured and treated with lime as described above. Class B Cake was measured by volume to approximately 500 g wet weight or 110g dry weight to reflect appropriate rate. The cake was sprinkled over the soil and incorporated along with the lime addition. This was repeated three times and combined into two five-gallon buckets.

**Cake Rate 2 (40 Mg ha\(^{-1}\)) : Alderwood Soil + Class B Cake equivalent to 40 Mg ha\(^{-1}\)**

Alderwood soil was measured and treated with lime and cake as described above, with 1 kg wet (220 g dry) cake added for the higher application rate. This was repeated three times and combined into two five-gallon buckets.

**Metal Salts Rate 1: Alderwood Soil + Low Rate Metal Salt Solution (metal concentration equivalent to metals in Cake Rate 1)**

Alderwood soil was measured and treated with lime as described above. 50 ml of the metal salt solution previously described was diluted with water to 1L and poured over the soil. Soil was then mixed to homogenize. The moist soil became muddy with the additional water. For the next two batches, the 50 ml metal solution was diluted to 500ml. The three batches were combined into two five-gallon buckets.
Metal Salts Rate 2: Alderwood Soil + Medium Rate Metal Salt Solution (metal concentration equivalent to metals in Cake Rate 2)

Alderwood soil was measured and treated with lime as described above. 100 ml of concentrated metal salt solution was diluted with water to 500ml and poured over the soil. Soil was then mixed to homogenize. This was repeated three times and combined into two five-gallon buckets.

Historic Biosolids

Historic biosolids were measured out by volume to 10kg as previously described for Alderwood soil as the percent solids was slightly different. 144.6 g (equivalent of 50 Mg ha⁻¹) of lime was added to the biosolids to adjust the pH. The soil was then mixed on the tarp as previously described for the previous treatments. This was repeated three times and combined into two five-gallon buckets.

Metal Salts Historic Rate: Alderwood Soil + High Rate Metal Salt Solution (metal concentration equivalent to metals in historic biosolids)

Control soil was measured and treated with lime as described above. The metal solution for the first batch was mixed by combining 300ml each of the associated four concentrated solutions of Cd, Cu, Pb and Zn salts (previously described). This was accomplished by mixing 200ml of each solution in a 1L volumetric flask and 100ml of each solution in a 500ml volumetric flask. However, the Pb caused some of the metals salts to precipitate and when the full quantity of solution was poured onto the limed soil it was too muddy to effectively mix on the tarp. Subsequently, metals for batches 2 and 3 were mixed by adding 300ml of Cd, Cu and Zn to a 1L volumetric and then adding 300 ml Pb separately. Each solution was then poured over the soil. Batches 2 and 3 were limed on the tarp and then the metal solution was incorporated after the soils had been transferred to a 5 gallon bucket. To allow for additional thorough mixing, each batch was kept in the associated bucket rather then being combined into just two five-gallon buckets. Separate buckets
were later combined after some drying and homogenized on a tarp as with other treatments.

Between initial mixing and planting of trees, soils were intermittently mixed, leached and allowed to equilibrate. Soils were remixed three and five days after initial mixing to ensure mixtures were homogenous. After the last mixing, each treatment was portioned into pots. Pots were lined with a fiberglass screen to prevent soil from falling out of pots during leaching and watering. 2” strips of screen were cut for each pot and taped in place with electrical tape.

Soils in each pot were leached by running water through the pots until water drained from the bottom of the pot. This was repeated two times. This was done to remove excess salts from the pots prior to planting. Pots were stored on a greenhouse table and allowed to dry for three days prior to planting trees. The experiment was set up with four pots or replicates per treatment. All mixing and preparation for potting took place at the CUH greenhouse.

**Douglas-fir Seedling Measurement and Planting**

One hundred bare-root Douglas-fir (*Pseudotsuga menziesii*) seedlings were purchased from King County’s 2012 spring native plant sale. Seedlings were two years old at the time of purchase and were grown in seed zone 2-2-1 at an elevation of approximately 500 feet. Seedlings were stored in a temperature-controlled cooler at CUH for three weeks prior to planting.

Seedlings were planted in soils on March 16, 2012, one week after initial soil mixing. 56 seedlings (two per pot) were randomly chosen from the group of 100 trees. Prior to planting, tree wet weight (g), height (cm), base diameter (mm) and root volume (ml) were measured. Tree wet weight was measured on a Salter digital scale. To ensure accurate measurements, trees were measured on a tray so no part of the tree was resting on the table (as pictured for dry weight measurements in Figure 2.14). Height of above ground portion was measured from the base of the tree just above the highest root to the tip of the
stem, not including needles (Figure 2.7). Initially base diameter was measured using measuring tape wrapped around the base of the tree just above the highest root. Base diameters were measured again thirteen days later using calipers to collect more accurate measurements. Root volume representing below ground biomass was measured by displacement of water in a 1000ml cylinder (Figure 2.8). The cylinder was filled with water to 500ml and roots were submerged in the water. The recorded value was the amount of water displaced by the roots rounded to the nearest 5ml increment. After each tree was measured, water was added to the cylinder to keep the original depth at 500ml.

Trees were chosen randomly from the group of 56 and assigned a number corresponding to the treatment they would be planted in (1-7), as well as a letter (A-H) for identification of individual trees (Figure 2.9). Tree I.D., weight, height and diameter were recorded on flagging and tied to each tree. Two trees were then randomly selected from each treatment group and planted two per pot (Figure 2.10). Each treatment was replicated in four times based on the number of pots used. At this time, a composite soil sample representing grab samples from each pot was collected for each treatment for subsequent soil analysis. Pots were then placed in random block design on a greenhouse table at the CUH greenhouse. Greenhouse temperatures are maintained at 68-72F and sodium lights are used from 8am-10pm. As the soil was still close to saturated, trees were not watered for three days after planting.

Experimental Design

Each treatment was replicated four times based on the number of pots used. Each pot contained two trees, for a total of 8 trees per treatment. Each tree was sampled separately for foliar metals and foliar and root PC, CYS and GSH concentrations. Pots were placed in a random block design throughout the course of the study.

Tree Care and Monitoring

On April 2, 2012, seventeen days after planting, the trees were moved outdoors as the greenhouse was determined to be too warm for the trees. Many were beginning to
show signs of stress including mottling on foliage fading from yellow to brown and some needle drop (Figure 2.11). There was no correlation between the observed stress and the soil treatments. Treatment 3 did have higher mortality than the other treatments. This may have been due to the enhanced water holding capacity of the soil or ammonia toxicity from the higher biosolids application rate.

One week after being moved outdoors, the trees were examined and dead trees were slated for replacement with trees remaining from the original group of 100 trees. Fourteen dead trees were removed from their pots cut at the root and stored in paper bags for possible analysis. New trees were measured using the same methods as before. Replaced trees were identified using the same original ID followed by a 2. Thirteen days later, another five trees were identified as dead and replaced using the same methods as before. These trees were identified using the same ID followed by 2B. No additional trees were replaced after this time; however, five more trees died between planting and harvest. Replaced and dead trees are summarized by treatment in Table 2.5.

Trees were watered as needed and monitored for bud break to determine appropriate sampling date. Additional height and base diameter measurements were taken approximately every thirty days on June 28, August 1, August 30 and November 8. Trees were rotated weekly to avoid any possible preferential shading. Watering regime increased later in the summer months and a shade cloth was placed over the trees from July-September to protect trees from sun exposure (Figure 2.12).

**June Sampling**

On June 21, 2012, tree foliage was sampled for PC analysis and foliar metal analysis (Figure 2.13). For PC samples, needles of new growth were taken at random from eight different locations on each tree. Needles were placed immediately in cryovials in a Styrofoam cooler packed with dry ice. The samples were transported to UW Tacoma and stored in a -80 C freezer prior to extraction of PC, CYS and GSH for HPLC analysis.
Metal samples were taken by removing three samples of new growth from each tree. Samples were placed in labeled paper bags and subsequently dried in a 105 C oven for 24 hours. Dried foliage was stored in original paper bags prior to acid digestion.

June Sampling

On June 21, 2012, tree foliage was sampled for PC analysis and foliar metal analysis (Figure 2.13). For PC samples, needles of new growth were taken at random from eight different locations on each tree. Needles were placed immediately in cryovials in a Styrofoam cooler packed with dry ice. The samples were transported to UW Tacoma and stored in a -80 C freezer prior to extraction of PC, CYS and GSH for HPLC analysis.

October Sampling

On October 29, 2012, trees were moved indoors for final sampling. Trees were removed from pots, photographed and placed according to treatment in five-gallon buckets that had been filled with cool water (Figure 2.14). Samples were then taken for root and foliar PC analysis and for foliar metal analysis. Foilar samples for both metals and PC analysis were taken as previously described for June samples. PC root samples were collected from 1cm portions of fine roots lightly cleaned with tap water (Figure 2.14). All PC samples were placed in cryovials and immediately stored in coolers containing dry ice. PC samples were later transported to UW Tacoma for storage in a -80C freezer.

Tree biomass measurements were taken as previously described, including height (cm), weight (g), base diameter (mm) and root volume (ml). Trees were subsequently cut at the top of the root ball and placed in paper bags for drying at 60C for 48 hours in drying ovens. Final dry weight measurements of tree above and below ground biomass were recorded (Figure 2.14).
Soil samples were also taken at this time. After trees were removed, approximately 20g of soil from each pot were placed in a Ziplock bag. Soil samples were labeled by the ID of the trees grown in the corresponding pot. Soil samples were taken to Bloedel Hall and air dried for storage prior to analysis for total metals, plant available metals, pH, EC and C/N.

**Field Study: Hancock’s Snoqualmie Forest**

In order to evaluate the relationship between PC production and metal uptake in mature Douglas-fir trees grown for timber production, field samples were taken from trees at Hancock’s Snoqualmie Forest near Snoqualmie, WA. Stands of eight and five year-old trees growing on Klaus soil (medial over sandy or sandy-skeletal, amorphic over mixed, mesic, ortstein Andic Durorthods) similar to the Alderwood series were identified (NRCS). These stands included areas where biosolids had been applied and others where no biosolids application had occurred allowing for collection of control and amended samples for each stand. Biosolids are surface applied at these sites using a side-cast spreader.

Trees in the five year-old stand were treated with one biosolids application of 15.9 Mg ha\(^{-1}\) in October 2011 (Figure 2.15 and 2.16). There was an area in the center of this stand where no biosolids application had occurred, though trees were planted at the same time. Trees in the eight year-old stand were treated with biosolids twice (Figure 2.17 and 2.18). Biosolids were applied at 13 Mg ha\(^{-1}\) in 2008 and received a second application of 8 Mg ha\(^{-1}\) in March 2012. Control samples were taken from a strip of trees between two application areas where no biosolids had been applied. The two stands are about 12 km apart in areas with similar slope and aspect. Foxglove (*Digitalis purpurea*) and fireweed (*Epilobium angustifolium*), as well as some evergreen blackberry (*Rubus laciniatus*) dominated the understory at both sites. Red alder (*Alnus rubra*) was noted as well particularly amongst the older trees.

Five trees from each stand and control areas were sampled for PC and foliar metals analysis (Figure 2.19). Eight pinches of needles from new growth were selected randomly from different heights on each tree. Needles were immediately placed in cryovials and
stored in a Styrofoam cooler containing dry ice. PC samples were later transported to UW Tacoma for storage in a -80°C freezer. Five samples of new growth were randomly cut with scissors from different areas of each tree and placed in paper bags labeled with a number corresponding to the sampling site (1-20). Foliar samples used for metal analysis were later dried at 105°C for 24 hours in drying ovens.

Soil samples of the A horizon were also collected from around the base of each tree. Soils were sampled by clearing the duff and organic matter then taking five samples from the mineral horizon at random intervals around each tree. These samples were then combined as a composite sample labeled with a number corresponding to each foliar sample from the same tree (1-20). Soils were stored in plastic bags and later dried for long-term storage at Bloedel Hall.

**Soil Analysis**

All soils were air-dried and sieved using a 2mm sieve. Soils were stored at Bloedel Hall until subsequent analysis in December-February 2013. Soils were tested for pH, EC, C/N, total metals and plant available metals. Duplicate, blanks and known standards were routinely used in analysis. All analyses were conducted using laboratory space and instruments associated with the School of Environment and Forest Resources Biogeochemistry Soils Laboratory located in Bloedel Hall.

**Total Metals Using Aqua Regia Digest**

Total metals analysis was conducted using the aqua regia digest method (ISO standard 11466). For greenhouse and field samples, approximately 5g of dry soil was dissolved in 2.5ml concentrated HNO3- and 7.5ml concentrated HCl and cooked to dryness. Samples were then refluxed in 10ml of 3N HCl and subsequently filtered using #40 and #42 Whatman filter papers in combination. Samples were brought to volume in 25ml volumetric flasks using 0.1N HCL. Samples were analyzed for total Cd, Cu, Pb and Zn using ICP-MS. The Natural Matrix Certified Reference Material Metals was used for all soils with the exception of the historic biosolids originally tested to determine appropriate metal salt
additions. This standard is certified to contain 2.9-3.3 mg kg\(^{-1}\) Cd, 8.6-16.2 mg kg\(^{-1}\) Cu, 298-531 mg kg\(^{-1}\) Pb and 28.7-62.6 mg kg\(^{-1}\) Zn. Recovery from these standards was within these ranges. The Environmental Resource Associates Trace Metals in Soil (Log No D046540) standard was used when testing the historic biosolids. This standard is certified to contain 58.4-84.8 mg kg\(^{-1}\) Cd, 55.3-79.1 mg kg\(^{-1}\) Cu, 95.1-141 mg kg\(^{-1}\) Pb and 229-339 mg kg\(^{-1}\) Zn. Recovery from these standards was within certified ranges.

**NH\(_3\)NO\(_3\) Extractable Metals**

Plant available metals in soils were measured using a dilute salt extract (Brown et al. 2003). 25 ml of 0.01 M NH\(_3\)NO\(_3\) solution was added to 5 g of air-dried soil. Samples were placed on a side-to-side shaker for one hour and filtered through #40 Whatman filter paper. ICP-MS was used to analyze samples for Cd, Cu, Pb and Zn. NBS Standard Reference Material 1571 made from orchard leaves was used as the standard for foliar metals. Certified ranges for this standard are 0.11 +/- 0.1 mg kg\(^{-1}\) Cd, 12 +/- 1 mg kg\(^{-1}\) Cu, 45 +/- 3 mg kg\(^{-1}\) Pb and 25 +/- 3 mg kg\(^{-1}\) Zn. Recovery from these standards was within certified ranges.

**Soil pH**

Soil pH was measured using a 2:1 ratio of de-ionized water to dry soil. After addition of water, samples were stirred and allowed to rest for 30 minutes prior to pH measurement. A SymHong SP70P pH meter was used to perform all measurements.

**Soil Electrical Conductivity (EC)**

Soil EC was measured using a 5:1 ratio of de-ionized water to dry soil. Samples were stirred twice at 0 minutes and 30 minutes then tested for EC at 60 minutes. An Orion Conductivity Salinity Model 140 meter was used to measure EC.

**C/N Analysis**

Percent total carbon and nitrogen were measured with a Perkin Elmer CHN analyzer model 2400. Previously sieved samples were ground using a mortar and pistol. Ground samples were weighed to between 30-40 µg.
Plant Analysis

Foliar Metals Analysis

Plant foliage was analyzed for total metals using a method adapted from the Association of Official Agricultural Chemists (AOAC) method 3.014(a). Previously dried 1-2g samples of tree foliage were ashed at 480°C for 16 hours (Figure 2.20). Ashed material was cooked to dryness for approximately one hour with 2 ml concentrated HNO₃. Samples were then refluxed with 10ml of 3N HCl for two hours. Digested samples were filtered with #40 Whatman filter paper and brought to 25ml in volumetric flasks with 0.1 N HCl. Analysis for Cd, Cu, Pb and Zn was performed using ICP-MS.

Phytochelatin Extraction and HPLC Analyses

All PC (n=2) and its precursors glutathione (GSH) and cysteine (CYS), were quantified using a method modified from Thangavel et al. (2007) and Wei et al. (2003), which involves derivatization with a fluorescent tag (monobromobimane), HPLC separation, and fluorometric detection. Analysis was performed at UW Tacoma’s Chemistry Laboratory in the Environmental Sciences Building. Extraction of peptides from plant tissue was performed prior to HPLC separation using full plant tissue sample (0.13-0.5mg wet weight). Sample was ground for five minutes in 5ml of 0.01 M metanesulfonic acid at 15,000 rpm using a Brinkmann Instruments’ Polytron Homogenizer PT3000. Each sample was decanted into a 1.5-ml eppendorf tube and centrifuged at 16,000g for 30 minutes under refrigeration. Samples were then filtered through 0.2 µm Nalgene syringe filters and stored in the refrigerator for further analyses using HPLC.

HPLC analysis was performed using a Shimadzu controller CBM 20A, Shimadzu pumps LC-20AB, Shimadzu autosampler SIL-10AF and Shimadzu fluorometer RF-10AXL. Output data was interpreted using EZStart 7.3 SP1 software. To prepare samples for HPLC analysis, 100 µL of each previously extracted sample was prepared with 84 µL of buffer solution containing 100 mM sodium borate and 10 mM diethylenetriaminepentaacetic acid and 25 µL of 20 mM TCEP. Samples were agitated and allowed to react for 10min prior to
addition of 30 µL of 50 mM monobromobimane (mBBr) dissolved in acetonitrile. Samples were then placed in the dark and allowed to react for 30 minutes. Prepared samples were transferred to the HPLC autosampler and analyzed for nmol/g fresh weight glutathione (GSH), Cysteine (CYS) and PC (n=2).

**Statistical Analysis**

Statistical analysis was performed in IBM SPSS Statistics 19 for Mac. Data was examined for outliers and normal distribution using histograms and box plots. No outliers were identified; however, data was skewed. All variables with the exception of pH were log transformed using log10 to reduce kurtosis and skewness. Main effects of harvest date, amendment type and rate were examined using ANOVA (p<.05, α=0.05) for differences in soil properties, plant uptake, SH-group production and biomass. Separation of means were determined using Waller-Duncan.
CHAPTER THREE: Results and Discussion

This study was designed to explore the relationship between metals in soils, foliar metal uptake and phytochelatin production in Douglas-fir trees planted in soils amended with biosolids or metal salts. Greenhouse treatments included seven soils amended with biosolids and metal salts across a range of low and high metal concentrations. An additional field study was conducted to compare foliar metal uptake and phytochelatin production in trees planted in soils with typical metal concentrations and biosolids applications.

Greenhouse Study

Total Soil Metals for Greenhouse Seedlings

Total soil metals for the control, modern biosolids and high metal historic biosolids are reported in Table 2.3. Control soils were elevated in Cd (3.0 mg kg\(^{-1}\)) and Pb (58.2 mg kg\(^{-1}\)) as compared to typical ranges reported in North America (Table 1.6, McBride, 1994). The modern biosolids meet EPA standards for unrestricted land application based on metal concentrations as set forth by CFR Part 503 (Table 1.1). Historic biosolids, while typical for the time period in which they were produced, would not meet the current standards for unrestricted application and are above current ceiling application limits for many metals.

Total soil metals of individual treatments are reported in Table 3.1. There were no significant changes in total soil metals between the March and October soil sampling dates. As expected, significant differences were seen between treatments for Cd, Cu, Pb and Zn (p<0.000). Significance was greater by rate (all metals p<0.000), than by amendment type (Cd p<0.035, Cu p<0.003, Pb p<0.025, Zn p<0.001). Small variations in metal concentrations were observed between control, low (20 Mg ha\(^{-1}\)) and medium (40 Mg ha\(^{-1}\)) metal application rates. For all metals, concentrations in the control soils were higher than in the low and in some cases medium metal salt treatments. The magnitude of differences varied by metal; however, metal concentrations for high metal application rates for both salt and historic biosolids were significantly higher than all other treatments for all metals (Figures 3.1-3.4). For all metals (Cd, Cu, Pb, and Zn), concentrations in the control, 20 and
40 Mg ha\(^{-1}\) modern biosolids treatments and corresponding metal salt treatments were similar.

**NH\(_3\)NO\(_3\) Extractable Metals for Greenhouse Seedlings**

March soil samples were collected for each treatment as a composite sample combining small grab samples from each pot within a treatment. Therefore variations within March sampling groups could not be statistically analyzed. Available metals, however, were similar for soils collected from both the March and October soil sampling dates (Table 3.1). All extractable metals represent a comparatively small fraction of total metals (Table 3.1).

Extractable Cu, Pb, and Zn varied based on rate of metal addition rather than the source of the metals (Figures 3.5-3.8). The availability of all three metals was similar to total metal concentrations as control, low and medium application rates were more similar than availability in higher rate treatments. Rate (p<0.000), amendment type (p<0.000) and rate*amendment type (p<0.000) were significant for Cd. Extractable Cd was highest in the high metal salts treatment in comparison to historic biosolids (Figure 3.5). Total Cd in these treatments were similar (38.4 mg kg\(^{-1}\) Cd in the metal salt and 35.4 mg kg\(^{-1}\) in the high metal biosolids). The higher extractability of Cd in the metal salt treatment may be due to different forms of Cd having different availability. Several other studies have made similar observations when comparing availability of metals in biosolids and metal salts (Cunningham et al., 1975a, 1975b, 1975c; Gaynor and Halstead, 1976; Mahler et al., 1987; Bell et al., 1991; Hooda and Alloway, 1993; Brown et al., 1998). Increased availability in metal salt versus biosolids amended soils was only observed for Cd.

**Soil pH for Greenhouse Seedlings**

Original pH values for the control soil and historic biosolids were 3.77 and 3.01 respectively. The pH for both the control and historic biosolids was adjusted with CaCO\(_3\) at 25 and 50 Mg ha\(^{-1}\) respectively prior to tree planting. Soil pH ranged from 5.26 to 6.69 depending on sampling date and treatment (Table 3.2 and Figure 3.9). For the March
sampling, pH varied from 5.26 in the Cake Rate 1 (20 Mg ha\(^{-1}\)) treatment to 6.69 in the Historic Biosolids treatment. March soil samples were collected for each treatment as a composite sample combining small grab samples from each pot within a treatment. Therefore variations within March sampling groups could not be statistically analyzed. In October samples were taken from each pot allowing statistical analysis within this sampling group.

In October, soil pH values across the different treatments were statistically significant. As with the March sampling, the pH of all of the treatments for both dates fell within a relatively narrow range. For the 2\(^{nd}\) harvest, the variability in soil pH had narrowed with pH values ranging from 5.86 in the Historic Biosolids treatment to 6.4 in the Control treatment. At this time, the pH in both high metal treatments were similar to each other and lower than all other treatments.

Despite these similarities between sampling dates and narrow ranges in pH values, variations in pH between sampling dates were significant (p<0.000) and interactions were observed between sampling date and both amendment type and rate (p<0.000). With the exception of the historic biosolids treatment, the pH increased between March and October for all treatments. Less variation between treatments was seen in October soils than March (Figure 3.9 and 3.10). Further, pH of metal and biosolids treatments within high and low rate categories were statistically similar and only slight variations were seen between medium rates and control. Differences in pH between treatments and overtime are likely due to the organic matter and clay content of the different soils, which can buffer the effect of lime on pH (Brady and Weil 1996). In addition, while the method used for pH calibration allowed for maximum contact with the soil, it did not take into account organic matter degradation possibly resulting in higher pH levels than originally planned.

While pH has been shown as a primary factor in metal availability there was no correlation found between pH and extractable metals (Figure 3.11). This was likely due to the small range in pH values between treatments. In addition, all pH values of the
treatments were neutral limiting the availability of these metals as most metals become more available at lower pH levels or those above neutral (McBride 1994). This range, however, depends on the metal. For example, while Pb is largely unavailable until pH levels drop below 3.2, Cd, Pb and Zn can all precipitate with carbonates, becoming more soluble at pH levels above 6-7 (McBride, 1994 and Zasoski and Edmonds 1986).

The effect of pH on metal solubility has also been reported to be greater in the case of metals bound to hydroxides than organic matter (Basta et al. 2005). As Cu tends to bind more readily to organic matter, pH may be less influential on Cu availability than other heavy metals. Spectroscopic studies evaluating metal species in biosolids have suggested that Zn and Cd and in some cases Pb are largely bound to hydroxides while Cu is associated with organic matter (Brown et al. 2012; Donner et al. 2011 and Hettiarachchi et al. 2006). These observations suggest that variations in pH would influence Cd, Pb and Zn availability more than Cu; however, no correlation was observed between pH and metal availability in this study (Figure 3.11).

**Electrical Conductivity (EC) for Greenhouse Seedlings**

Electrical conductivity varied based on sampling date (p<0.000). Overall conductivities in March and October were 732 ± 439 mS cm⁻¹ and 126 ± 47 mS cm⁻¹ respectively. The EC of the soils collected in March was similar across all treatments, however, this could not be statistically analyzed as samples were collected as composites by treatment. EC decreased between sampling dates for all treatments. This would be expected, as regular watering likely flushed out the excess salts in the soil from biosolids and metal salt addition. In October, EC on average was higher in the treatments that included biosolids than control and salt treatments. Conductivity was also higher in the high metal treatments in comparison to the control and lower metal biosolids and salt treatments. EC results are reported in Table 3.2.
C and N for Greenhouse Seedlings

Total carbon and nitrogen, as well as the C:N ratio, were consistent across both sampling dates. However, total C and N varied based on biosolids application rate and type of amendment (metal salt or biosolids). In addition, total carbon and nitrogen did not vary consistently by amendment type and rate (p<0.000). Carbon and N were highest in the historic biosolids treatment with values of 188 g kg\(^{-1}\) C and 22 g kg\(^{-1}\) N in March and 170 g kg\(^{-1}\) C and 20 g kg\(^{-1}\) N in October. The other biosolids treatments also had higher C and N values compared to other treatments. As would be expected, total C and N in the Control and metal salts treatments were similar. The C:N ratio was lowest for the historic biosolids treatments followed by both current biosolids treatments. This suggests that the historic biosolids treatment still had excess plant available N. The control treatment had the highest C:N ratio followed by the salt treatments. Nitrogen was added in salt treatments as PbN0\(^3\). This could explain the narrower C:N ratio in salt treatments. C and N results are reported in Table 3.2.

Foliar Metals of Greenhouse Seedlings

As with extractable metals, there was no correlation between pH and foliar metal uptake in Douglas-fir needles (Figure 3.12). The impact of soil treatment on foliar metal concentration varied for the different metals. For example, plant Cd was consistent across both harvests (p<0.577). Source of metals (amendment type p<0.312) was also not significant for foliar Cd. Rate was significant (p<0.000) with higher foliar Cd concentrations for trees grown in the two high metals treatments (Figure 3.13 and Table 3.3). In addition, a trend was observed at higher rates, in which Cd uptake did increase in high metal treatments between June and October sampling dates, however, this increase was not found to be significant. These results are in contrast to extractable Cd, which indicated higher metal availability of Cd added as salts compared to biosolids.

Cadmium concentrations at control, low and medium metal application rates were low ranging from 0.21 mg kg\(^{-1}\) in control treatments to 0.32 mg kg\(^{-1}\) in both Cake Rate 1
(20 Mg ha\(^{-1}\)) and Cake Rate 2 (40 Mg ha\(^{-1}\)) treatments in October samples. Higher metal applications resulted in October foliar Cd concentrations of 4.2 mg kg\(^{-1}\) in the historic biosolids treatment and 2.6 mg kg\(^{-1}\) in high rate metal salts treatments. All of these values fall within the wide range of those reported by other studies (Table 1.11).

Overall, foliar Cu concentrations were consistent across both harvests (p<0.193). Both rate and source of metals did impact foliar Cu concentrations (p<0.001 and (p<0.017) (Figure 3.14). Foliar Cu concentrations were higher both at high metal application rates and in biosolids treatments compared with Control and metal salts. While harvest date and rate*amendment type were not statistically significant, a trend was observed in which mean foliar Cu concentrations increased between June and October for plants grown in the control, low and medium metal rates. In contrast, foliar Cu decreased in high metal treatments from the first to the second harvest.

As with Cd, these results are not congruent with extractable Cu, which did not indicate a difference in metal extractability based on the source of the metal. Previous studies have indicated that extractable Cu may not be the best indicator of phytoavailable Cu. In a study comparing availability of soil metal pools to plant uptake in lettuce, radish and rye grass grown in contaminated urban soils, Sauve et al. (1996), observed that free ionic Cu\(^{2+}\) was a better predictor of Cu uptake then total soil Cu or exchangeable Cu measures.

For all treatments, the Cu uptake observed in this trial was minimal compared to values reported in the literature. For example, Sauve et al. (1996) reported plant tissue concentrations of Cu ranging from 8.1-82.6 mg kg\(^{-1}\) in radish, lettuce and rye grass grown in urban contaminated soils with total Cu concentrations of 32-640 mg kg\(^{-1}\). In addition, foliar Cu concentrations below 3 mg kg\(^{-1}\) have been suggested as an indication of Cu deficiency in Douglas-fir trees cultivated for Christmas trees (Hart et al. 2004). While mean foliar Cu concentrations were above this threshold, some individual seedlings in Control,
biosolids (20 and 40 Mg ha\(^{-1}\)) and the equivalent metal salt treatments, foliar Cu concentrations were below 3 mg kg\(^{-1}\) (Table 3.3).

Harvest date was the most significant factor for foliar Pb (p<0.000). Foliar Pb concentrations decreased between the June and October sampling dates (Figure 3.15-3.16 and Table 3.3). No other factors were significant with the exception of amendment type in October (p< 0.033) when foliage in biosolids treatments, particularly at lower rates, contained higher concentrations of Pb than metal salt treatments. Extractable soil Pb did not follow a similar trend as availability increased by increasing metal addition, regardless of the metal source.

Foliar uptake of Zn varied by harvest date (p<0.000), as well as metal addition rate and source of metal (p<0.00)(Figures 3.17-3.18). These effects were consistent across the different rates of metal addition and the sources of the metals. Tree uptake of Zn increased between June and October sampling dates for all treatments. This trend was most pronounced in the Historic Biosolids treatment, in which mean foliar concentrations tripled between June (45 ± 8.8 mg kg\(^{-1}\)) and October (150 ± 21 mg kg\(^{-1}\)). Uptake within this treatment was also much higher than foliar Zn concentrations for trees grown in the equivalent metal salt treatment. Plants grown in the high metal salt treatment had foliar Zn concentrations that were similar to trees grown in the Cake Rate 2 (40 Mg ha\(^{-1}\)) treatment (Table 3.2). High variability in Zn uptake was also observed for trees grown in the same treatment. This variability is likely more common in wild type trees such as Douglas-fir in comparison to agronomic crops (Table 3.3).

This issue in assessing Zn uptake is also demonstrated by comparing metal uptake values of this study to those reported in the literature (Table 1.11). For instance, in a similar study using Douglas-fir seedlings grown in biosolids amended soils with total Zn and Cd concentrations of 210 mg kg\(^{-1}\) and 3 mg kg\(^{-1}\), plant uptake in needles was 270 mg kg\(^{-1}\) Zn and 4 mg kg\(^{-1}\) Cd. In this study, Zn soil concentrations for high metal biosolids were 726 mg kg\(^{-1}\) and mean foliar Zn concentration was 150 mg kg\(^{-1}\) Zn. In this study, foliage of
trees planted in similar soil Cd concentrations contained 0.21-0.27 mg kg\(^{-1}\) Cd. These differences may be a result of time as trees in the previous study were sampled after two growing seasons. In addition, trees planted in soils with similar total Cd concentrations (3-4 mg kg\(^{-1}\)) had plant Cd values of <1.0 to 14.4 demonstrating once again that a range of factors are involved in uptake besides simply soil metal concentrations.

Rate was the most significant factor for Cu, Zn and Cd. While ranges were high for all metals within treatments, differences in mean uptake by treatment is minimal in the case of Cu and Pb and more pronounced for Zn and Cd. This is particularly the case for Zn and Cd in seedlings grown in high metal treatments.

**Phytochelatins (PC), Glutathione (GSH) and Cysteine (CYS) of Greenhouse Seedlings**

For this study, both phytochelatins (PC) and related compounds, (GHS and CYS) were measured in plant foliage collected for each harvests. Data on plant concentrations of these compounds for the first harvest are presented here. There were no significant differences in foliar PC, GHS, and CYS concentrations as a function of rate, amendment type or the interaction between these variables (rate*amendment type interaction) (Figures 3.19-3.21). No correlations were observed between metal concentrations in foliage phytochelatin production (Figures 3.22-3.25). Phytochelatin production varied by treatments independently of soil metal concentrations. For foliage collected during the first harvest, there was a trend for elevated PC production in Cake Rate 1 (20 Mg ha\(^{-1}\)) and Metal Salts Rate 2 treatments as compared to the Historic Biosolids and Metal Salts Historic Rate treatments. Phytochelatin production in the two high metal treatments was similar to the Control. The absence of a relationship between PC, GSH and CYS and plant metal uptake may indicate that for Douglas-fir trees grown in this study, foliar PC production was not a direct response to metal exposure or stress.

As previously reported, differences in foliar concentrations between treatments were most pronounced for Zn and Cd. Both metals were highest in foliage planted in the high
metal treatments. If Zn and Cd induce PC production, it would be expected that these high metal rate treatments would have higher PC levels than other treatments. Previous studies have correlated stress associated with elevated Pb, Cu, Cd and Zn with increased PC production (Schat et al. 2002; Prasad 1999; Chen et al. 1997; Maitani et al. 1996; Kneer and Zenk 1992; Schat and Kalff 1992; Grill et al. 1987). Increased PC production based on metal exposure, however, has been observed primarily with Cd and Cu and to a lesser degree Zn and Pb (Cobett 2000; Chen et al. 1997; Rauser 1995). The order of potency depends on plant species, cells tested and metal concentrations (Cobbett 2000; Prasad 1999; Rauser 1995).

In this study, no such relationship could be confirmed. Further, within each treatment there were large standard error values. This indicates that PC production in this study was not well-regulated by the trees and did not seem to be consistent based on metal exposure. In the only previous study of PC production by Douglas-fir, Cline et al. (2012) also saw no induction of PC in foliage of seedlings grown in historic and fresh biosolids-amended soils; instead, significantly elevated PC was observed only in roots. Root PC was not determined in the June sampling due to the potentially destructive nature of the sampling, but will be determined in future work for the October sample date. This will allow for a direct comparison to the results of the study by Cline et al. (2012).

Cysteine (CYS) and GSH, the precursors to PC, were also measured. No significant differences were observed between treatments based on rate and amendment type. Concentrations of these compounds were also uncorrelated with metal concentrations in the soil and foliar metal uptake. For example, mean CYS concentrations were similar for all treatments except Metal Salts Rate 2 where they were comparatively elevated. In the case of GSH, concentrations varied amongst treatments with the highest mean values found in Cake Rate 2, Metals Salts Rate 2 and Metal Salts Historic Rate treatments. Again, high variability within treatments resulted in overlap in standard errors and limited statistical significance as a result of treatment.
The results of this study do not seem to suggest a strong relationship between relative concentrations of GSH, CYS and PC. As GSH and CYS are the precursors to PC, it has been suggested that increased PC production may result in reduced GSH concentrations (Ric de Vos et al. 1992). This could be a potentially threatening reaction as GSH is an important scavenger of free radicals, which can be produced upon exposure to metals leading to oxidative stress in cells (Ric de Vos et al. 1992). Counter to this argument is the suggestion that PCs are longer chains of GSH and CYS, and thus thought to be highly effective metal chelators as compared to GSH and CYS alone therefore overcoming the benefits of reduction in free radicals from GSH (Joszeak 2012).

In previous work examining PC production in Douglas-fir planted with biosolids, Cline et al. (2012) noted that PC production did not have a significant impact on GSH concentrations. The authors suggested that this may have been due to the relatively low PC levels as compared to GSH concentrations which were 10-20 times higher. In this study, similarly, there seems to be no strong relationships between higher PC levels and higher or lower GSH or CYS concentrations as all levels are fairly variable both within and between treatments.

Many of the studies suggesting metals induce PC production have been limited to in vitro environments using cell cultures, rather than whole plant experiments. Further, many studies allowing comparison by plant species focus on specific metal-tolerant and non-tolerant species with only one experiment involving Douglas-fir trees (Cline et al. 2012). Given the multitude of factors involved in phytochelatin production including metal and plant species, metal concentration and cell tissue analyzed, it is difficult to draw conclusions regarding what factors result in elevated PC production.

**Biomass and Productivity of Greenhouse Seedlings**

Changes in seedling biomass over the course of the study were evaluated based on ratios of final to initial root volume (ml), height (cm), stem diameter (mm) and seedling volume (cm$^3$) measurements. The later measurement was calculated using the following
allometric equation: \( (\pi) \times (\text{base diameter})^2 \times \text{height}/2 \) (Cline et al. 2012; den Driessche 1992). The effect of rate, amendment type and the interaction of rate and amendment type were insignificant for all variables with the exception of initial height:final height \((p<0.038)\), initial stem diameter:final stem diameter \((p<0.000)\) and initial seedling volume:final seedling volume \((p<0.001)\) by amendment type. In all cases seedlings in biosolids treatments were more productive.

Differences, however, were relatively small. For example, while mean results indicate similar changes in height for both control and salt treatments for the duration of the trial, the increase in growth for the biosolids treatments though statistically significant, was not large (Figure 3.26). Increased seedling volume and stem diameter, on the other hand was greater, particularly between metal salts treatments and biosolids (Figures 3.27 and 3.28).

The relatively small changes observed in this experiment may be a consequence of the short duration of the study. Other studies have demonstrated that nutrients in biosolids can result in increased productivity despite the presence of metals (Harrison and Henry 2001; Henry et al. 1994; Zasoski, et al 1983; Bledsoe and Zasoski 1981). Harrision and Henry (2001) reported increased yield from biosolids applications to be 60%-2000%. Such a range demonstrates the variety of factors likely involved in biomass production of forest trees including light, water, nutrients, tree species and soil properties.

**Mortality of Greenhouse Seedlings**

After initial planting in the greenhouse several trees in various treatments began showing signs of stress (Figure 2.11). Trees were moved outdoors as greenhouse was thought to be too hot for Douglas-fir seedlings. One week after being moved outdoors, the trees were examined and dead trees were slated for replacement with trees remaining from the original group of 100 trees. Fifteen dead trees were replaced at this time. Thirteen days later, another five trees were identified as dead and replaced using the same methods as before. No additional trees were replaced after this time; however, five more trees died
between planting and harvest. Replaced and dead trees are summarized by treatment in Table 2.5.

Mortality was tested for significant differences by date as well as rate, amendment type and rate amendment type interaction for initial mortality (all trees replaced in April) and final mortality (trees identified as dead in October). Results indicate that the only significant effect was amendment type (p<0.018) for initial trees replaced April (Figure 3.29). In this instance, biosolids treatments had a higher mortality rate than control and salt treatments. This may have been due to the increased water holding capacity of biosolids as soils were wet when trees were planted or ammonia toxicity. Metal toxicity did not seem to be a factor in increased mortality as trees in higher metal treatments did not experience higher mortality than those at lower rates. No separation of means were reported by Waller-Duncan post-hoc test (p<0.05).

**Physical Appearance of Greenhouse Seedlings**

No quantitative measures of metal stress based on physical characteristics were collected. Qualitative observations of foliage, however, did indicate that trees in the high metal treatments may have been stressed compared to trees in lower salt and modern biosolids treatments. Such observations included chlorosis of needles and loss of foliage particularly from lower branches. As shown in Figures 3.30 and 3.31, treatments using modern biosolids were generally darker green in color and fuller compared to higher metal treatments. Control trees were not as dark green as those with biosolids applications but they appeared fuller than higher metal treatments in terms of foliage and branching.

Uptake of Cd, Cu and Zn were significantly higher in these treatments than control, low and medium metal treatments. This may have contributed to the changes in the physical appearance. PC production on the other hand was lower in these treatments compared to others with lower metal concentrations in soils and foliage. Differences in physical appearance may also be attributed to higher N values and added nutrients in the fresh cake treatments.
**Field Study**

**Total Metals for the Field Study**

Total metal concentrations in field soils were analyzed to assess the significance of biosolids application rate and stand age, which is representative of soil differences between stands. Only Cd and Zn were significant by age/soil (Cd \( p<0.000 \), Zn \( p<0.000 \)) and rate (Cd: \( p<0.031 \), Zn: \( p<0.018 \)) (Figures 3.30 and 3.33). Age/soil was more significant for Cd and Zn than rate. Cu (\( p<0.000 \)) and Pb (\( p<0.033 \)) were only significant by age/soil not by rate (Figures 3.33 and 3.34). This suggests that the soil factors were more important than biosolids applications and that soil is the source of the metals rather than the biosolids. Both control soils and those with biosolids applications have similar and in some cases higher total metal concentrations as compared to control, low and medium metal rate greenhouse soil treatments (Table 3.1 and 3.5). With the exception of Cu in the five-year-old stand with no biosolids applied and Zn in both five-year-old stands, metal concentrations are in the upper range or above those reported naturally in the United States (Table 1.6, McBride 1994).

**NH\(_3\)NO\(_3\) Extractable Metals for Field Soils**

Ammonia nitrate (0.1M) was also used to measure extractable metals for field soils. Extractable Cu and Cd varied for each site sampled (Cu and Cd \( p<0.000 \)) but not as a result of biosolids application (Cd \( p<0.910 \), Cu \( p<0.091 \)) (Figures 3.36 and 3.37). Extractable Cu and Cd concentrations were higher in soils of the eight-year-old stands than the five-year-old stands. Extractable Pb was similar for both soils and both biosolids application rates (Table 3.6). Extractable Zn was highest in the eight-year-old stand with biosolids application (21 Mg ha\(^{-1}\)). Only Zn was significant by both age (\( p<0.017 \)), rate (\( p<0.000 \)) and thus treatment (\( p<0.000 \)) (Figure 3.38). These values are similar to those reported for greenhouse trees with the exception of high metal treatments and Zn values (Table 3.6).
**Carbon and Nitrogen for Field Soils**

Total carbon was higher in the five-year-old stand than in the eight-year-old stand. In the younger stand the portion of the site that had not received biosolids had higher total C and N than the portion that had received biosolids. The opposite was true for the older stand. This may be the result of the higher cumulative biosolids loading at the site. Soil C and N (g kg\(^{-1}\)) were significant by age (C \(p<0.000\), N \(p<0.000\)) but only N was significant by rate (\(p<0.005\)). C:N ratio was significant only by age (\(p<0.002\)) with higher ratios in five-year-old stands. Treatment means for C, N and C:N are listed in Table 3.5.

**Electrical Conductivity (EC) for Field Soils**

Soil EC was significant by rate (\(p<0.002\)) only. Stands with biosolids applications had higher EC values compared to those without, however, all values were low. Treatment means are reported in Table 3.7.

**pH for Field Soils**

As expected of Northwest forest soils, soil pH was acidic. The eight-year-old stand with biosolids application (21 Mg ha\(^{-1}\)) had the lowest pH (3.7) followed by the five-year-old stands, which had statistically similar pH values. The eight-year-old stand without biosolids application had the highest pH value (4.6). The range of pH values represented here are relatively small. Soil pH was significant by rate, age and thus treatment (\(p<0.000\)). Mean pH values by treatment are displayed in Figure 3.39 and Table 3.7.

The soil pH does not seem to be a factor for Cd, Cu or Pb availability. Metal availability for Cd and Cu is significant by stand age while no factors are significant for Pb availability. The extractable Zn concentration was significantly higher in the eight-year-old stand with biosolids application (21 Mg ha\(^{-1}\)), in comparison to all other sites. The pH at this site was also low (3.7). Given this increase in availability and lower pH value one might expect higher metal uptake and PC production in these trees compared to others; however, this was not the case for Zn or any other metals.
Foliar Metals for Field Trees

The effect of biosolids application rate and soil type on metal uptake by field trees was examined. None of these variables explained the variability in foliar tissue concentrations of Cd, Cu, Pb and Zn uptake. Foliar metal means and standard error by treatment are reported in Table 3.8.

Comparison of Field and Greenhouse Soil Characteristics and Foliar Metals

A non-statistical comparison of the results from the greenhouse and field tree sampling values demonstrates both similarities and differences based on soil metal concentration, greenhouse foliage sampling date and treatment. Foliar Cd was similar in field trees compared to greenhouse seedlings for all treatments for both June and October sampling dates. Extractable soil Cd was also similar between field soils and control, low and medium metal rate greenhouse soils. Foliar copper in the field trees was higher in comparison to most greenhouse treatments in October with the exception of high metals treatments in which values were similar. Extractable Cu in the greenhouse treatments was similar for control, low and medium metal rates but higher in high metal rate treatments than all field samples. The range of foliar Cu concentrations in both cases was relatively narrow, varying from 3.0-6.0 mg kg\(^{-1}\) in greenhouse treatments and 3.8-5.7 mg kg\(^{-1}\) in field trees. While Pb concentration for foliage collected from the greenhouse at the first sampling in June was elevated compared to field trees, values in October were similar to field trees. This is consistent with extractable Pb which was similar between control, low and medium metal rate treatments and field samples. Foliar zinc was higher in field trees compared to control, low and medium rate treatments for the first greenhouse sampling. In October greenhouse samples, Zn concentration in foliage was elevated compared to field trees in all but Metal Salts Rates 1 and 2. For all metals with the exception of Zn, extractable soil metals were similar between control, low and medium metal rate greenhouse soils and field soils despite slightly higher total soil metals in field samples. Extractable Zn, in contrast, was higher in field soils than greenhouse soils. These observations demonstrate
that once again foliar metals are dependent on a variety of factors including possibly tree age and/or length of metal exposure.

**Phytochelatins (PC), Glutathione (GSH) and Cysteine (CYS) for Field Trees**

Foliar PC, GSH and CYS were examined for statistical differences between biosolids application rate and stand age. No effects were observed for GSH and CYS, however, foliar PC levels were slightly reduced for trees that had received biosolids applications in comparison to the control trees (p<0.047) (Figure 3.40). Trees in stands with no biosolids applied had higher PCs in foliage than those with biosolids application. Phytochelatin production was lowest in stands with 21 Mg ha$^{-1}$ biosolids application.

As with the greenhouse results, there was no correlation between foliar metals and PC production for Cu, Pb and Zn (Figures 3.42 to 3.44). There appears to be a stronger relationship between foliar Cd and foliar PC production (R<0.37), though the correlation is still weak and even weaker in the case of foliar PC production and total Cd concentrations in the soil (Figures 3.41 and 3.45). Previous studies have shown that Cd is the strongest inducer of PC in laboratory experiments, followed by Cu (Rauser, 1995). No correlation, however, was observed for foliar PC concentrations and total soil metals including Cd (Figures 3.45 to 3.48), demonstrating the importance of testing foliar metals when examining the role of PC in response to metal stress.

Further, for the field sampling, total and available metals varied based on site, not as a result of biosolids application rate. For the younger stand, there was some indication of an increase in soil Cu and Zn as a result of biosolids application. For the older stand, the control soil generally had higher total metals than the biosolids amended portion of the site. Extractable Zn was highest in the eight-year-old (21 Mg ha$^{-1}$) stand in comparison to other stands. Extractable concentrations of Cu, Cd, and Pb did not vary considerably for the different sites. In addition, metal uptake was not significantly different between biosolids application rates or stand ages. If there was a direct correlation between these variables
and PC production, one would assume a similar pattern in significant differences in PC results.

This field study demonstrates that, for this site, moderate applications of modern biosolids have not resulted in consistently elevated soil metals; instead, the control soil in some cases had higher metal concentrations than the amended soils. While this is reassuring support for the safety of modern biosolids, at least where metals are concerned, it does not allow for direct comparison with other studies with higher soil metals. In the more mature Douglas-fir stand treated with historic biosolids studied by Cline et al. (2012), soil metals were consistently higher than those seen in the current study, with 5.4 mg kg$^{-1}$ Cd, 71 mg kg$^{-1}$ Cu, 118 mg kg$^{-1}$ Pb, and 160 mg kg$^{-1}$ Zn. In that study, foliar PCs were consistently, but not significantly, elevated compared to the control stand. Other studies of conifer PC production have not directly measured soil metals (e.g., Gawel et al. 1996, 2001, and 2004), and no previous studies have measured foliar metals at the same time as PCs and their precursors. The results of this field study demonstrate that for Douglas-fir growing in soils with only moderate levels of soil metals total soil metals are a poor predictor of both foliar metals and PC production. Therefore, based on these results, it is advisable to measure foliar metals directly rather than relying on measurement of total soil metals.

**Conclusion**

This study was designed to explore the relationship between metal uptake and phytochelatin production. This was evaluated based on plant response to metals depending on metal concentration, availability and form. Plant response was evaluated based on foliar metals, PC production, and biomass measurements, as well as observations of physical appearance. Differences in metal availability between treatments were considered based on available soil metal results and plant uptake.

In the greenhouse study, metal uptake of Cd, Cu and Zn did increase based on metal rate but significant differences were only seen at high metal loading rates. Differences in metal availability between metal salts and biosolids treatments were observed, although
metal rate was a stronger factor in metal availability and foliar uptake than amendment
type. For example, metal salts were only more available in the case of soil Cd, whereas
amendment type was not a significant factor in foliar Cd concentrations. In contrast, foliar
metal values were higher in biosolids treatments for both Cu, Zn and to a lesser degree Pb.

The field study did not allow for comparison of uptake by metal form; however,
significant differences in total and available metals were observed, allowing for comparisons
based on metal concentration. Soil type was the primary factor in these differences with
soil from the eight-year-old stands containing higher metals than five-year-old stands
regardless of biosolids application rates. Despite differences in metal concentrations, there
were no significant differences in foliar metal concentrations among the treatments. This is
likely a result of the minimal range in metal concentrations within the field samples. Metal
concentrations in all field soils were similar to greenhouse control, low and medium rate
treatments, which also did not have significantly different foliar metal values.

No correlation was observed in either study between soil or foliar metals and foliar
PC, CYS or GSH concentrations. Only PC concentrations in the field study were significantly
affected by biosolids application rate, with plant PC concentration decreasing with higher
biosolids application rates. Biosolids application rate was not a significant factor in foliar
metal, availability or total soil concentrations. While the literature suggests that PC
production is a result of exposure to certain metals including Cd, Cu, Pb and Zn, the results
of this study suggest no correlation between metal levels in soil or foliage and PC production,
at least within this range of metal concentrations. In addition, stress factors involved in
elevated metal uptake, including changes in root volume, biomass and physical appearance,
could not be attributed to changes in foliar PC concentrations. Therefore, foliar PC
production does not appear to be a useful measure of metal stress for Douglas-fir.

A direct comparison of these results with those from Cline et al. (2012) might
provide insight into the role of phytochelatins in metal stress response of Douglas-fir trees.
As previously discussed, Cline et al. (2012) found no significant elevation of foliar PCs in
greenhouse seedlings grown in biosolids-amended soils, but did see elevated root PC production in treatments amended with 8% (160 Mg ha\(^{-1}\)) fresh biosolids, and these levels were higher than all other treatments including those with significantly higher metal concentrations. The authors hypothesized that this increased PC production may have been due to higher metal availability in this low metal treatment due to a lower pH value when compared to control soils (4.9 vs. 5.5, respectively). This hypothesis could not be fully explored, however, as foliar metals and extractable soil metals were not tested. While PC production in this study was not significantly correlated with foliar metals, PC production was elevated in some of the treatments with lower total and extractable metal concentrations, although these differences were not significant (Figure 3.19). In this study the soil pH was adjusted to above 5 (ranging from 5.26 to 6.69 depending on treatment) and was not identified as a factor in foliar metal uptake or metal availability in the soil (Figures 3.11 and 3.12).

Further, Cline et al. (2012) suggested that the increased root PC production was a result of metal stress. This conclusion was largely based on decreased biomass production in biosolids treatments in combination with increased root PC production as compared to control. In contrast, this study found biomass production was significantly higher in biosolids treatments based on initial and final measurements of seedling height, stem diameter and seedling volume. In addition, even in treatments with higher soil metal concentrations, PC production in the previous study was lower than values reported by this study in treatments with lower metal concentrations. These results suggest that either there is no consistent relationship between total soil or foliar metals and foliar PC production in Douglas-fir, or the levels in both studies did not reach the critical threshold required for induction of foliar PCs in Douglas-fir. Examination of root PCs should further clarify this question.

While total soil metal concentration seems to be the most significant factor in predicting foliar metal levels, the wide range of values between treatments may reflect the
high variability among genetically diverse species such as Douglas-fir. This variability is also observed in the case of foliar PC production, which does not correlate well with foliar or soil metal concentrations. Therefore, foliar PC measures should not be used to draw conclusions regarding metal stress or exposure in Douglas-fir. In addition, these results indicate that foliar metals should be measured along with PC production, rather than total soil metals alone, when evaluating the response of trees to heavy metals.
Figures 1.1 and 1.2: Range of Pb and Cd concentrations (mg kg$^{-1}$) in biosolids sampled between 1978-1999. In total 7,746 biosolids samples were sampled from 177 Pennsylvania wastewater treatment plants. As graphs demonstrate both concentration and variability across plants decreased during this time period. (Excerpted from Stehouwer and Wolf 1999).
Figure 1.3: Results from work by McKenzie (1980) demonstrating adsorption of metals to goethite. Reported metal hydrolysis pKₐ values for each metal were Pb (7.7), Cu (8), Zn (9.0), Co (9.7), Ni (9.9) and Mn (10.6). (Extracted from McKenzie, 1980 by Basta, et al. 2005).
Figure 1.4 Modes of metal adsorption onto functional groups of organic matter. Negatively charged sites necessary for chelation of metals by organic matter are influenced by pH (Extracted from Senesi, 1992 by Basta et al. 2005).
Figure 1.5 Steps involved in plant uptake of metals. 1. Metal must be released from soil through desorption or dissolution. 2. Free metal ions are transferred through diffusion or convection in soil solution. 3. Metals may be reabsorbed or precipitated on soil particles or 4. Absorbed by roots. (Adapted from McBride, 1994).
Figure 1.6 A. Differentiation of two –Glu-Cys linkages. B. The five families of γ-Glu-Cys peptides which scavenge and sequester metals in plants and yeasts. (Adapted from Rauser, 1995).
Figure 2.1 Lee Memorial Forest located in Snohomish County between SE 188th Street and SE 197th Street, between SR 9 and SR 522. Alderwood soil was collected near the 197th St SE entrance at the SW corner of the forest.
Figure 2.2 Charles Lathop Pack Experimental Forest is located at 9010 453rd St E, Eatonville, WA 98328. Historic biosolids used in this study were collected from a grassy area in the forest where the biosolids had been lagooned. The biosolids provided by King County in 1982 were later tested for Cd, Cu, Pb and Zn using the *aqua regia* digest method and ICP-MS.
Figure 2.3a Class B cake biosolids used to amend soils for Cake Rate 1 and Cake Rate 2 treatments. Class B cake was provided by King County. Application rates were equivalent to 20 Mg ha\(^{-1}\) (Cake Rate 1) and 40 Mg ha\(^{-1}\) (Cake Rate 2). Cake was 22% solids with metal concentrations (mg kg\(^{-1}\)) of Cd, Cu, Pb and Zn of 2.5, 500, 30 and 900, respectively, as reported by King County.
Figure 2.3b Initial mixture of metal salts for high metal rate treatment (1.867g CdCl₂, 69.720g CuSO₄, 55.73g Pb(NO₃)₂, and 57.13g ZnSO₄). Due to precipitation of metals, metals were mixed separately in 1L of de-ionized water and added at a rate of 300ml solution to 10kg of soil.
Figure 2.4 (Upper Left): Alderwood soil for Cake Rate 1 portioned out by volume using a base measurement of 10kg soil.

Figure 2.5 (Upper Right): Cake Rate 2 pre-mixing. Reddish soil is Alderwood, darker amendment is Class B cake and light-colored granules are lime.

Figure 2.6 (Lower Left): Mixing soil on tarp. Each treatment was mixed in three batches then homogenized again before storage in five-gallon buckets. Order of mixing was from least contaminated (Control) to most contaminated (Metal Salts Historic Rate) treatments.
Figure 2.7 Tree height (cm) was measured from the base of each tree to the crown excluding any needles. Trees were measured throughout the eight-month growing period using the same method.
Figure 2.8 Root volume (ml) was measured by displacement of water using a 1000ml cylinder filled to 500ml with tap water. Seedling root balls were fully submerged in water and measurements were rounded to the nearest 5ml increment.
Figure 2.9 Metal Salts rate 1 seedlings labeled with Treatment ID (4) and letter (A-H) on pink flagging. In all, 56 trees (8 per treatment) were randomly selected from a group of 100. Trees were planted two per pot, for a total of eight trees per treatment (four pots per treatment).
Figure 2.10  Planted trees on greenhouse table prior to organization in random block design. Trees pictured in foreground are planted in historic biosolids. Trees were later moved outdoors as growing conditions in the greenhouse were not ideal for Douglas-fir seedlings.
Figure 2.11 Stressed trees were moved outside on April 2, 2012. Stress and premature death were noted in all treatments. Pictured trees are from Control, Metal Salts Historic Rate, Cake Rate 2 and Metal Salts Rate 2 treatments (left to right). One week after relocation fourteen trees from various treatments were identified as dead and replaced with trees from original 100. Thirteen additional days later five more dead trees were replaced as well. In total 20 trees were replaced.
Figure 2.12 Trees were covered with a shade cloth from July-September to protect seedlings from sun exposure. Seedlings were watered as needed, rotated weekly to avoid preferential shading with in random block design and measured approximately every thirty days to monitor growth.
Figure 2.13 First foliage sampling date was determined by bud-break and development of new foliage. By June 21, 2012 all living seedlings had formed new growth approximately 3-10cm long. Each sample was taken at random from new growth at different heights around seedling. PC samples included only needles, whereas full length of new growth was taken for foliar metal analysis. Seedling in foreground is from Treatment 6.
Figure 2.14 Upper Left: Removal of seedlings from pots. Roots had grown together in pots and were carefully separated for root sampling. Upper Right: Roots were rinsed with tap water prior to sampling of fine roots for PC analysis. Lower Left:
Both fresh and dry weight of trees were measured using a tray over a Salter scale consistent with original measurements.

Figure 2.15 Map of application area where five year-old trees were sampled. First biosolids application occurred in October 2011 at a rate of 15.9 Mg ha\(^{-1}\). Trees were sampled from section 25-08-25B (biosolids applied) and the area between application areas 26 and 25 where no biosolids were applied (control).
Figure 2.16 Brian Vrablick, Forestry Manager with King County Wastewater Treatment Division, pictured with five year-old Douglas-fir in biosolids application area. Understory vegetation was dominated by foxglove and fireweed. Trees were approximately 5-6 feet tall.
Figure 2.17 Map of application area where eight year-old trees were sampled. First biosolids application occurred in 2008 at 13 Mg ha$^{-1}$. The second application was in March 2012 at 8 Mg ha$^{-1}$. Trees were sampled from the northern portion of section 24-09-18A (biosolids applied). Control samples were taken from an area to the south of this section where no biosolids were applied.
Figure 2.18 Eight year-old trees in biosolids application area. Understory was primarily fireweed and foxglove though red alder and evergreen blackberry were also noted.
Figure 2.19  PC and foliar metal sampling from larger eight year-old trees. Samples of needles (PC samples) and full length on new growth were sampled from random locations around trees. Similarly, five randomly selected soil samples were taken from the upper horizons around base of the tree.
Figure 3.1. Mean total soil Cd (mg kg$^{-1}$) and standard error by treatment as determined by *aqua regia* digest. Total metals were not significant by date and means displayed here account for both March and October sampling dates. Significant differences in total Cd concentrations between control, low and medium rates were minimal compared to levels in high metal treatments. Cake treatments refer to treatments using modern Class B biosolids. Different letters above each mean indicate significant differences using Waller-Duncan post hoc tests (p<0.05).
Figure 3.2. Mean total soil Cu (mg kg\(^{-1}\)) and standard error by treatment as determined by *aqua regia* digest. Total metals were not significant by date and means displayed here account for both March and October sampling dates. Differences among control, low and medium metal rate treatments varied only slightly as compared to total Cu in high metal treatments. Different letters above each mean indicate significant differences using Waller-Duncan post hoc test (p<0.05).
Figure 3.3 Mean total soil Pb (mg kg$^{-1}$) and standard error by treatment as determined by $aqua$ $regia$ digest. Total metals were not significant by date and means displayed here account for both March and October sampling dates. As with other metals, minimum variation between total Pb values was seen at lower metal rates with increased Pb in both high metal treatments. Different letters above each mean indicate significant differences using Waller-Duncan post hoc test (p<0.05).
Figure 3.4  Mean total soil Zn (mg kg$^{-1}$) ± standard error for each treatment as determined by *aqua regia* digest. Total metals were not significant by date and means displayed here account for both March and October sampling dates. Total Zn was highest for the Historic Biosolids treatment with minimal differences between low and medium rate treatments. Significant differences between treatments are indicated by different letters above each bar. Separation of means was determined using Waller-Duncan post hoc test (p<0.05).
Figure 3.5 Dilute salt (0.1 MNH₃NO₃) Cd (mg kg⁻¹) for soils extracted across both harvests. Means ± standard error are shown. Letters designate significant differences (Waller-Duncan post hoc test, p<0.05).
Figure 3.6 Dilute salt (0.1M NH$_3$NO$_3$) Cu (mg kg$^{-1}$) for soils extracted across both harvests. Means ± standard error are shown. Results are shown for each rate of metal addition. Metal source was not significant. Letters designate significant differences by rate (Waller-Duncan post hoc test, p<0.05).
Figure 3.7 Dilute salt (0.1M NH$_3$NO$_3$) Pb (mg kg$^{-1}$) for soils extracted across both harvests. Means ± standard error are shown. Results are shown for each rate of metal addition. Metal source was not significant. Letters designate significant differences by rate (Waller-Duncan post hoc test, p<0.05).
Figure 3.8 Dilute salt (0.1M NH$_3$NO$_3$) Zn (mg kg$^{-1}$) for soils extracted across both harvests. Means ± standard error are shown. Results are shown for each rate of metal addition. Metal source was not significant. Letters designate significant differences by rate (Waller-Duncan post hoc test, p<0.05).
Figure 3.9 Soil pH of greenhouse soils collected at planting in March. Significant differences between treatments could not be statistically analyzed as samples were collected as a composite by treatment.
Figures 3.10  pH of greenhouse soils in October at time of tree harvest. Letters indicate significant differences between treatments using the Waller-Duncan post hoc test ($p<0.05$).
Figure 3.11 Dilute salt (0.1M NH₃NO₃) extractable Zn versus pH for both harvests. There was no significant relationship between pH and extractable Zn (R=0.06).
Figure 3.12 Plant uptake of Zn plotted against pH. As with available Zn correlation between pH and uptake was low (R=0.14). Similar relationships were found for Cd, Cu and Pb.
Figure 3.13 Means ± standard error for foliar Cd as a function of rate of metal addition. Reported means include values from both harvests and from metals added to soils as salts and in biosolids. Different letters above means represent significant differences between rates (Waller-Duncan post hoc test p<0.05).
Figure 3.14 Means ± standard error for foliar Cu as a function of rate of metal addition. Reported means include values from both harvests and from metals added to soils as salts and in biosolids. Different letters above means represent significant differences between rates (Waller-Duncan post hoc test p<0.05).
Figure 3.15 Foliar Cu (mg kg\(^{-1}\)) means ± standard errors as a function of amendment type (p<0.017). Displayed means are the values for each type of amendment across both harvests and all rates of amendment addition.
Figure 3.16 Mean foliar Pb (mg kg$^{-1}$) ± standard errors as a function of harvest date ($p<0.000$). Displayed means represent the value for each harvest across all treatments. For foliar Pb, no other factors were significant with the exception of amendment type in October ($p<0.033$) when uptake was slightly elevated in biosolids treatments as compared to metal salt treatments. Letters represent significant differences between harvest dates.
Figure 3.17 Foliar Zn (mg kg⁻¹) means ± standard errors by harvest date and rate. Within each harvest, means with different letters are significantly different. Separation of means was determined using the Waller-Duncan post hoc test (p<0.05).
Figure 3.18 Foliar Zn (mg kg\textsuperscript{-1}) means ± standard errors as a function of harvest date and amendment type. Within each harvest, means with the same letter are not significantly different. Separation of means was determined using the Waller-Duncan post hoc test (p<0.05).
Figure 3.19 Phytocelatin (PC) concentrations in foliage for each treatment. Means ± standard error are shown. There were no statistically significant differences for any treatment.
Figure 3.20 Cysteine (CYS) (nmol SH eq/g FW) in foliage by treatment. Means ± standard error are shown. There were no statistically significant differences for any treatment.
Figure 3.21 Glutathione (GSH) concentrations (nmol SH eq/g FW) in foliage by treatment. Means ± standard error are shown. Significant differences were not observed by rate, amendment type or rate*amendment type.
Figure 3.22 Foliar Cd (mg kg$^{-1}$) and PC concentrations (nmol SH eq/g FW) in seedlings by treatment. No correlation was observed between PC production and metal concentrations in tissue for any of the tested metals.
Figure 3.23 Foliar Cu (mg kg$^{-1}$) and PC concentrations (nmol SH eq/g FW) in seedlings by treatment. No correlation was observed between PC production and metal concentrations in tissue for any of the tested metals.
Figure 3.24 Foliar Pb (mg kg\(^{-1}\)) and PC concentrations (nmol SH eq/g FW) in seedlings by treatment. No correlation was observed between PC production and metal concentrations in tissue for any of the tested metals.
Figure 3.25 Foliar Zn (mg kg$^{-1}$) and PC concentrations (nmol SH eq/g FW) in seedlings by treatment. No correlation was observed between PC production and metal concentrations in tissue for any of the tested metals.
Figure 3.26  Mean change in height by amendment type ($p<0.038$) reported as a ratio of final height:initial height measurements. Initial height (cm) was measured in March at planting and final height (cm) was measured at harvest in October. No separation of means was reported using Waller-Duncan post-hoc test ($p<0.05$).
Figure 3.27 Mean change in stem diameter by amendment type (p<0.000) reported as a ratio of final stem diameter:initial stem diameter measurements. Initial stem diameter (mm) was measured in March at planting and final stem diameter (mm) was measured at harvest in October using calipers. Letters indicate significant differences by amendment type. Separation of means was determined using the Waller-Duncan post hoc test (p<0.05).
Figure 3.28 Mean change in seedling volume by amendment type (p<0.000) reported as a ratio of final seedling volume: initial seedling volume measurements. Initial seedling volume (cm$^3$) was measured in March at planting and seedling volume (cm$^3$) was measured at harvest in October. Letters indicate significant differences by amendment type. Separation of means was determined using the Waller-Duncan post hoc test (p<0.05).
Figure 3.29 Total tree mortality by date. Tree mortality was significant in April only. Significance was based on amendment type (p<0.018) with higher mortality among biosolids treatments. No separation of means were reported by Waller-Duncan post-hoc test (p<0.05).
Figure 3.30 Comparison of treatments prior to harvest. From left to right: Control, Metal Salts Rate 1, Historic Biosolids, High Metal Salts Historic Rate, Metal Salts Rate 2, Cake Rate 1 (20 Mg ha\(^{-1}\)) and Cake Rate 2 (40 Mg ha\(^{-1}\)). Treatments using modern biosolids consistently had darker, fuller foliage than other treatments.
Figure 3.31 Photos of each tree were taken after removal from pots. The top tree was grown in Cake Rate 2 (40 Mg ha\textsuperscript{-1}) soil. The bottom tree was grown in the High Metal Salts Historic Rate treatment.
Figure 3.32 Mean soil Cd concentrations (mg kg$^{-1}$) ± standard error by treatment $(p<0.000$. The same letters above each mean indicate that values are statistically similar $(p<0.05)$. 
Figure 3.33 Soil Cu concentrations (mg kg$^{-1}$) ± standard error as a function of stand age/soil (p<0.000). Cu concentrations were elevated in the older stand and did not vary significantly by rate.
Figure 3.34 Soil Pb concentrations (mg kg\(^{-1}\)) ± standard errors as a function of stand age/soil (p<0.033). Pb concentrations did not vary by biosolids application rate (p<0.433).
Figure 3.35 Soil Zn concentrations (mg kg$^{-1}$) ± standard error for each treatment (p<0.000). Age/soil (p<0.018) was less significant than rate (p<0.000). Letters indicate significant differences between treatments. Separation of means was determined using Waller-Duncan post hoc test (p<0.05).
Figure 3.36  Extractable (0.01 M NH$_3$NO$_3$ ) soil Cd (mg kg$^{-1}$) for the two sites. Different letters above each column indicate significant difference.
Figure 3.37 Extractable (0.01 M NH$_3$NO$_3$) soil Cu (mg kg$^{-1}$) for each soil sampled. Biosolids application rate was not significant for Cu. Letters above columns indicate significant differences.
Figure 3.38 Extractable (0.01 M NH₃NO₃) soil Zn (mg kg⁻¹) for each sampled site (p<0.000). Letters above means indicate significant differences using Waller-Duncan post hoc test (p<0.05).
Figure 3.39  pH means +/-standard errors by treatment (p<0.000). Soil pH varied by both stand age and application rate. Letters above columns indicate significant differences. Separation of means was determined using Waller-Duncan post hoc test (p<0.05).
Figure 3.40 Mean foliar PC (nmol SH eq/g FW) +/- standard error by rate. Only foliar PC concentrations were significant by rate (p<0.047). Letters above means indicate significant differences (p<0.05).
Figure 3.41 Correlation between foliar Cd (mg kg⁻¹) and foliar PC (nmol SH eq/g FW). Of all metals, the relationship between plant uptake and foliar PC production was strongest for Cd (R=0.37).
Figure 3.42 Correlation between foliar Cu (mg kg\(^{-1}\)) and foliar PC (nmol SH eq/g FW). Similar to greenhouse results no correlation was observed between these variables (R=0.05).
Figure 3.43 Correlation between foliar Pb (mg kg$^{-1}$) and foliar PC (nmol SH eq/g FW). PC production varies regardless of metal uptake demonstrating lack of relationship between actual uptake and foliar PC production (R=0.03).
Figure 3.44 Correlation between foliar Zn (mg kg\(^{-1}\)) and foliar PC (nmol SH eq/g FW), \((R=0.14)\). Further, the slope of the best fit line suggests a negative relationship between foliar Zn uptake and PC production.
Figure 3.45 Correlation between total soil Cd (mg kg\textsuperscript{-1}) and foliar PC (nmol SH eq/g FW), (R=0.02).
Figure 3.46 Correlation between total soil Cu (mg kg\(^{-1}\)) and foliar PC (nmol SH eq/g FW). No relationship was observed between these two variables (R=0.03).
Figure 3.47 Correlation between total soil Pb (mg kg⁻¹) and foliar PC (nmol SH eq/g FW). Little correlation was found between Foliar PC and Total Soil Pb (R=0.03).
Figure 3.48 Correlation between total soil Zn (mg kg$^{-1}$) and foliar PC (nmol SH eq/g FW), (R=0.16).
### Tables

#### PART 503 LAND APPLICATION POLLUTANT LIMITS FOR METALS

<table>
<thead>
<tr>
<th>Pollutant</th>
<th>Ceiling Concentration Limits (Cd mg kg(^{-1}))</th>
<th>Cumulative Pollutant Loading Rates (kg ha(^{-1}))</th>
<th>&quot;High Quality&quot; Pollutant Concentration Limits (mg kg(^{-1}))</th>
<th>Annual Pollutant Loading Rates (kg ha(^{-1}) yr(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arsenic</td>
<td>75</td>
<td>41</td>
<td>41</td>
<td>2.0</td>
</tr>
<tr>
<td>Cadmium</td>
<td>85</td>
<td>39</td>
<td>39</td>
<td>1.9</td>
</tr>
<tr>
<td>Copper</td>
<td>4300</td>
<td>1500</td>
<td>1500</td>
<td>75</td>
</tr>
<tr>
<td>Lead</td>
<td>840</td>
<td>300</td>
<td>300</td>
<td>15</td>
</tr>
<tr>
<td>Mercury</td>
<td>57</td>
<td>17</td>
<td>17</td>
<td>0.85</td>
</tr>
<tr>
<td>Molybdenum</td>
<td>75</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Nickel</td>
<td>420</td>
<td>420</td>
<td>420</td>
<td>21</td>
</tr>
<tr>
<td>Selenium</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>5.0</td>
</tr>
<tr>
<td>Zinc</td>
<td>7500</td>
<td>2800</td>
<td>2800</td>
<td>140</td>
</tr>
</tbody>
</table>

Table 1.1 Part 503 Land Application Pollutant Limits for Metals (Brobst, 1995, US EPA 1993). Limits were set based on those at highest risk to exposure (children) and using results from studies with metal sensitive crops including spinach and lettuce (Renner 2000).

<table>
<thead>
<tr>
<th>Country</th>
<th>Year</th>
<th>Cd mg kg(^{-1})</th>
<th>Cu mg kg(^{-1})</th>
<th>Pb mg kg(^{-1})</th>
<th>Zn mg kg(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>European Directive</td>
<td>1986</td>
<td>20-40</td>
<td>1000-1750</td>
<td>750-1200</td>
<td>2500-4000</td>
</tr>
<tr>
<td>Germany</td>
<td>2007</td>
<td>2.5</td>
<td>700</td>
<td>120</td>
<td>1500</td>
</tr>
<tr>
<td>Italy</td>
<td>2008</td>
<td>20</td>
<td>1000</td>
<td>750</td>
<td>2500</td>
</tr>
<tr>
<td>Netherlands</td>
<td>1995</td>
<td>1.25</td>
<td>50</td>
<td>100</td>
<td>300</td>
</tr>
</tbody>
</table>

Table 1.2 Comparison of metal concentration limits in Europe. Concentrations are in mg kg\(^{-1}\) and represent regulatory limits (Adapted from LeBlanc, et al 2008).
<table>
<thead>
<tr>
<th>Metal</th>
<th>Concentration Reported in 1983 mg kg(^{-1})</th>
<th>Concentration Reported in 2012 mg kg(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lead</td>
<td>1200</td>
<td>30</td>
</tr>
<tr>
<td>Copper</td>
<td>900</td>
<td>50</td>
</tr>
<tr>
<td>Zinc</td>
<td>2000</td>
<td>900</td>
</tr>
<tr>
<td>Cadmium</td>
<td>50</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Table 1.3 Reductions in heavy metal concentrations reported for biosolids produced in Washington State. Biosolids with higher values (1983) were reported by Zasoski et al. (1983) and used to assess metal toxicity in early studies at Pack Forest. Lower values reported in 2012 were provided by King County.

<table>
<thead>
<tr>
<th>SEASON</th>
<th>VEGETATIVE COVER</th>
<th>SLOPE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wet</td>
<td>Good</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Poor</td>
<td>8</td>
</tr>
<tr>
<td>Dry</td>
<td>Good</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Poor</td>
<td>15</td>
</tr>
</tbody>
</table>

Table 1.4 Maximum slope recommendations depending on groundcover for biosolids applications on forested land (Adapted from Henry, 1988 as cited in Harrison and Henry 2001).
<table>
<thead>
<tr>
<th>Type of water body</th>
<th>Application Method</th>
<th>Continuously flowing</th>
<th>Small tributary</th>
<th>Ephemeral</th>
<th>Ditches</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface applied</td>
<td>Undisturbed buffer</td>
<td>60’</td>
<td>30’</td>
<td>15’</td>
<td>10’</td>
</tr>
<tr>
<td></td>
<td>Disturbed buffer</td>
<td>60’</td>
<td>60’</td>
<td>30’</td>
<td>15’</td>
</tr>
<tr>
<td>Injected or incorporated</td>
<td></td>
<td>30’</td>
<td>30’</td>
<td>15’</td>
<td>10’</td>
</tr>
</tbody>
</table>

Table 1.5 Recommended buffers (feet from water body) to protect streams from surface runoff when applying biosolids to forested land (Adapted from Henry, 1988 as cited in Harrison and Henry 2001).

<table>
<thead>
<tr>
<th>Metal</th>
<th>Worldwide mg kg(^{-1})</th>
<th>United States mg kg(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cd</td>
<td>0.06-1.1</td>
<td>N/D</td>
</tr>
<tr>
<td>Cu</td>
<td>6-80</td>
<td>14-29</td>
</tr>
<tr>
<td>Pb</td>
<td>10-84</td>
<td>17-26</td>
</tr>
<tr>
<td>Zn</td>
<td>17-25</td>
<td>34-84</td>
</tr>
</tbody>
</table>

Table 1.6 Natural range of means for metals in soils. All units are in mg kg\(^{-1}\). (Adapted from McBride, 1994).

<table>
<thead>
<tr>
<th>Soil Amendment</th>
<th>Cd mg kg(^{-1})</th>
<th>Cu mg kg(^{-1})</th>
<th>Pb mg kg(^{-1})</th>
<th>Zn mg kg(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biosolids 503 Table 3(^1)</td>
<td>39</td>
<td>1500</td>
<td>300</td>
<td>2800</td>
</tr>
<tr>
<td>Biosolids King County(^2)</td>
<td>2.5</td>
<td>1000</td>
<td>50</td>
<td>1200</td>
</tr>
<tr>
<td>Manures(^3)</td>
<td>.25-.2.5</td>
<td>36-465</td>
<td>7.6-46</td>
<td>150-656</td>
</tr>
<tr>
<td>Phosphate (P2O(_5))(^4)</td>
<td>65</td>
<td>56.6</td>
<td>12.2</td>
<td>240.2</td>
</tr>
<tr>
<td>NPK for P(^6)</td>
<td>30.6</td>
<td>31.4</td>
<td>216.5</td>
<td>233.6</td>
</tr>
<tr>
<td>NPK for N(^4)</td>
<td>5</td>
<td>41.3</td>
<td>31.8</td>
<td>204.5</td>
</tr>
</tbody>
</table>

Table 1.7 Metal concentrations in various agricultural amendments. \(^1\)Values required for qualification of biosolids as High Quality in Table 3 of the CFR Part 503 rule (USEPA 1993). \(^2\)Values reported by King County 2012. \(^3\)Range of metal concentrations found in different manures (O’Connor, 2002). \(^4\)Mean metal concentrations of fertilizers (USEPA 1999).
<table>
<thead>
<tr>
<th>METAL</th>
<th>PHYSICAL RESPONSE OF PLANT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cadmium</td>
<td>Brown margin to leaves, chlorosis, necrosis, curled leaves, brown stunted roots, reddish veins and petioles, reduction in growth, purple coloration</td>
</tr>
<tr>
<td>Copper</td>
<td>Chlorosis, yellow coloration, purple coloration of the lower side of the mid rib, less branched roots, inhibition of root growth</td>
</tr>
<tr>
<td>Lead</td>
<td>Dark green leaves, stunted foliage, increased amounts of shoots</td>
</tr>
<tr>
<td>Zinc</td>
<td>chlorosis, stunting, reduction of root elongation</td>
</tr>
</tbody>
</table>

Table 1.8 Physical response of plants to toxic concentrations of metals. (Adapted from Prasad, 1999).

<table>
<thead>
<tr>
<th>Metal</th>
<th>Plant</th>
<th>Upper Critical Level mg kg⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cd</td>
<td>Wheat</td>
<td>4-43</td>
</tr>
<tr>
<td></td>
<td>Lettuce</td>
<td>10-95</td>
</tr>
<tr>
<td>Cu</td>
<td>Wheat</td>
<td>11-18</td>
</tr>
<tr>
<td></td>
<td>Lettuce</td>
<td>8-23</td>
</tr>
<tr>
<td>Zn</td>
<td>Wheat</td>
<td>108-500</td>
</tr>
<tr>
<td></td>
<td>Lettuce</td>
<td>150-530</td>
</tr>
</tbody>
</table>

Table 1.9 Summary of tissue concentrations at which decreased yield was reported in various studies using wheat and lettuce. Values reported here are summarized from a more detailed review by Macnicol and Beckett (1985) and represent the lower and upper range of values reported from several studies considering the effect of Cd, Cu and Zn on wheat and lettuce.
<table>
<thead>
<tr>
<th>Metal Source</th>
<th>Tree Species, Organ</th>
<th>Soil pH</th>
<th>Soil Total Cd mg kg&lt;sup&gt;-1&lt;/sup&gt;</th>
<th>Soil Total Cu mg kg&lt;sup&gt;-1&lt;/sup&gt;</th>
<th>Soil Total Pb mg kg&lt;sup&gt;-1&lt;/sup&gt;</th>
<th>Soil Total Zn mg kg&lt;sup&gt;-1&lt;/sup&gt;</th>
<th>Plant Total Cd mg kg&lt;sup&gt;-1&lt;/sup&gt;</th>
<th>Plant Total Cu mg kg&lt;sup&gt;-1&lt;/sup&gt;</th>
<th>Plant Total Pb mg kg&lt;sup&gt;-1&lt;/sup&gt;</th>
<th>Plant Total Zn mg kg&lt;sup&gt;-1&lt;/sup&gt;</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biosolids</td>
<td>Douglas-fir, needles</td>
<td>N/A</td>
<td>3</td>
<td>95</td>
<td>N/A</td>
<td>210</td>
<td>4</td>
<td>5.3</td>
<td>5.3</td>
<td>270</td>
<td>Bledsoe and Zasoski 1981</td>
</tr>
<tr>
<td>Zinc Smelters</td>
<td>Douglas-fir, leaf litter</td>
<td>4.7-4.4</td>
<td>0.33-1.88</td>
<td>N/A</td>
<td>N/A</td>
<td>25-117</td>
<td>&lt;1.0</td>
<td>N/A</td>
<td>N/A</td>
<td>295</td>
<td>Van Nevel 2010</td>
</tr>
<tr>
<td>Biosolids</td>
<td>Western hemlock, needles</td>
<td>N/A</td>
<td>3</td>
<td>95</td>
<td>N/A</td>
<td>210</td>
<td>3.9</td>
<td>5.2</td>
<td>3.1</td>
<td>96</td>
<td>Bledsoe and Zasoski 1981</td>
</tr>
<tr>
<td>Biosolids</td>
<td>Sitka spruce, needles</td>
<td>N/A</td>
<td>3</td>
<td>95</td>
<td>N/A</td>
<td>210</td>
<td>&lt;.025</td>
<td>7.1</td>
<td>5.8</td>
<td>240</td>
<td>Bledsoe and Zasoski 1981</td>
</tr>
<tr>
<td>Metal Salts</td>
<td>Sitka spruce, shoot</td>
<td>3.3-3.92</td>
<td>4</td>
<td>8</td>
<td>100</td>
<td>N/A</td>
<td>14.4</td>
<td>36</td>
<td>71.7</td>
<td>N/A</td>
<td>Burton et al. 1984</td>
</tr>
<tr>
<td>Zinc Smelters</td>
<td>Scots pine, leaf litter</td>
<td>4.5-4.8</td>
<td>0.33-2.34</td>
<td>N/A</td>
<td>N/A</td>
<td>21-142</td>
<td>1.08</td>
<td>N/A</td>
<td>N/A</td>
<td>321</td>
<td>Van Nevel 2010</td>
</tr>
<tr>
<td>Biosolids</td>
<td>Red alder, foliage</td>
<td>5.3</td>
<td>4</td>
<td>N/A</td>
<td>50.2</td>
<td>283.2</td>
<td>&lt;.06</td>
<td>N/A</td>
<td>&lt;1.2</td>
<td>249</td>
<td>Gaulke et al. 2006</td>
</tr>
<tr>
<td>Biosolids</td>
<td>Red alder, foliage</td>
<td>4.6</td>
<td>32.5</td>
<td>N/A</td>
<td>623</td>
<td>1759.6</td>
<td>&lt;.07</td>
<td>N/A</td>
<td>&lt;1.3</td>
<td>274</td>
<td>Gaulke et al. 2006</td>
</tr>
<tr>
<td>Industrial</td>
<td>Norway spruce, needles</td>
<td>3.3-4.1*</td>
<td>2.1-4.7*</td>
<td>N/A</td>
<td>2.1-41.6*</td>
<td>N/A</td>
<td>0.2-0.4</td>
<td>N/A</td>
<td>2-5</td>
<td>N/A</td>
<td>Lamersdorf et al. 1991</td>
</tr>
</tbody>
</table>

Table 1.10 Summary of reported foliar metal concentrations in various tree species. Values reported are not necessarily toxic but allow comparison of uptake by different species, metal forms and soil metal concentrations. All metal units are in mg kg<sup>-1</sup>. *Indicates values reported for seepage water values as soil values were not reported. N/A indicates parameters not evaluated as part of the referenced study.
<table>
<thead>
<tr>
<th>Treatment ID</th>
<th>Metal Application Rate</th>
<th>Amendment Type</th>
<th>Treatment Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>None</td>
<td>None</td>
<td>Alderwood Soil</td>
</tr>
<tr>
<td>Cake Rate 1 (20 Mg ha(^{-1})) Low</td>
<td>Class B Dewatered Biosolids</td>
<td>Alderwood Soil + Class B Biosolids</td>
<td></td>
</tr>
<tr>
<td>Cake Rate 2 (40 Mg ha(^{-1})) Medium</td>
<td>Class B Dewatered Biosolids</td>
<td>Alderwood Soil + Class B Biosolids</td>
<td></td>
</tr>
<tr>
<td>Metal Salts Rate 1 Low</td>
<td>Metal Salt</td>
<td>Alderwood Soil + Metal Salts to equivalent metals in Cake Rate 1 (20 Mg ha(^{-1}))</td>
<td></td>
</tr>
<tr>
<td>Metal Salts Rate 2 Medium</td>
<td>Metal Salt</td>
<td>Alderwood Soil + Metal Salts to equivalent metals in Cake Rate 2 (40 Mg ha(^{-1}))</td>
<td></td>
</tr>
<tr>
<td>Historic Biosolids High</td>
<td>Historic Biosolids</td>
<td>Historic Biosolids from Pack Forest</td>
<td></td>
</tr>
<tr>
<td>Metal Salts Historic Rate High</td>
<td>Metal Salt</td>
<td>Alderwood Soil + Metal Salts to equivalent metals as Historic Biosolids</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2.1** Summary of seven treatments used in greenhouse experiment. Control Alderwood soil was used for all treatments with the exception of Treatment 6, which contained pure historic biosolids collected from Pack Forest. Metal salts were added as CdCl\(_2\), CuSO\(_4\), Pb(NO\(_3\))\(^2\) and ZnSO\(_4\).
BIOSOLIDS TOTAL METALS AND pH

<table>
<thead>
<tr>
<th></th>
<th>Cd mg kg⁻¹</th>
<th>Cu mg kg⁻¹</th>
<th>Pb mg kg⁻¹</th>
<th>Zn mg kg⁻¹</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>48±2</td>
<td>1172±46</td>
<td>1446±66</td>
<td>999±40</td>
<td>3.01</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.2 Biosolids total metals and pH tested prior to potting soil mixes. Total metal values were used to calculate the amount of metals to add to control soil for the high metal salt treatment (Metal Salts Historic Rate)

<table>
<thead>
<tr>
<th>Biosolids Type</th>
<th>Cd mg kg⁻¹</th>
<th>Cu mg kg⁻¹</th>
<th>Pb mg kg⁻¹</th>
<th>Zn mg kg⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Historic Biosolids</td>
<td>48±2</td>
<td>1172±46</td>
<td>1446±66</td>
<td>999±40</td>
</tr>
<tr>
<td>Modern Class B Cake</td>
<td>2.5</td>
<td>500</td>
<td>30</td>
<td>900</td>
</tr>
<tr>
<td>EPA Regulatory Limits*</td>
<td>39</td>
<td>1500</td>
<td>300</td>
<td>2800</td>
</tr>
</tbody>
</table>

Table 2.3 Comparison of metal concentrations (mg kg⁻¹) in historic and modern biosolids used in this study, as well as EPA's Table 3 standards for biosolids land application in the United States (US EPA Part 503).

<table>
<thead>
<tr>
<th>Soil</th>
<th>Lime Application Mg ha⁻¹</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Historic Biosolids</td>
<td>0</td>
<td>3.01</td>
</tr>
<tr>
<td>Historic Biosolids</td>
<td>10</td>
<td>3.7±0.05</td>
</tr>
<tr>
<td>Historic Biosolids</td>
<td>20</td>
<td>3.9±0.22</td>
</tr>
<tr>
<td>Historic Biosolids</td>
<td>40</td>
<td>4.8±0.11</td>
</tr>
<tr>
<td>Alderwood</td>
<td>0</td>
<td>3.77</td>
</tr>
<tr>
<td>Alderwood</td>
<td>5</td>
<td>4.6±0.12</td>
</tr>
<tr>
<td>Alderwood</td>
<td>10</td>
<td>4.9±0.01</td>
</tr>
<tr>
<td>Alderwood</td>
<td>20</td>
<td>4.9±0.33</td>
</tr>
</tbody>
</table>

Table 2.4 Results from tests to determine lime application rate. pH values are reported as means of duplicates for each rate +/- standard deviation. Target pH for greenhouse study treatments was originally pH 5. Based on these results final lime application rates for the Alderwood soil and historic biosolids were 25 and 50 Mg ha⁻¹ respectively.
Table 2.5 Number of trees in each treatment either replaced or dead by final harvest.

![Table](image)

Table 3.1 Means ± standard errors for *aqua regia* total and 0.1M NH₃NO₃ extractable Cd, Cu, Pb and Zn (mg kg⁻¹) for each treatment. Means are averaged across all replicates and harvests.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Metals rate</th>
<th>March</th>
<th></th>
<th>March</th>
<th></th>
<th>March</th>
<th></th>
<th>March</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>pH</td>
<td>EC μS cm⁻¹</td>
<td>C g kg⁻¹</td>
<td>N g kg⁻¹</td>
<td>C:N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>Control</td>
<td>5.91</td>
<td>269</td>
<td>44</td>
<td>1.6</td>
<td>28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cake Rate 1 (20 Mg ha⁻¹)</td>
<td>Low</td>
<td>5.26</td>
<td>979</td>
<td>52</td>
<td>3.5</td>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cake Rate 2 (40 Mg ha⁻¹)</td>
<td>Medium</td>
<td>5.57</td>
<td>1415</td>
<td>371</td>
<td>2.7</td>
<td>14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metals Rate 1</td>
<td>Low</td>
<td>6.17</td>
<td>425</td>
<td>48</td>
<td>2.1</td>
<td>23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metals Rate 2</td>
<td>Medium</td>
<td>6.13</td>
<td>356</td>
<td>37</td>
<td>1.8</td>
<td>21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Historic Biosolids</td>
<td>High</td>
<td>6.69</td>
<td>562</td>
<td>188</td>
<td>22</td>
<td>8.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metal Salts Historic Rate</td>
<td>High</td>
<td>5.79</td>
<td>1117</td>
<td>38</td>
<td>1.9</td>
<td>19</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Metals rate</th>
<th>October</th>
<th></th>
<th>October</th>
<th></th>
<th>October</th>
<th></th>
<th>October</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>pH</td>
<td>EC μS cm⁻¹</td>
<td>C g kg⁻¹</td>
<td>N g kg⁻¹</td>
<td>C:N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>Control</td>
<td>6.4 ± 0.02</td>
<td>95 ± 2b</td>
<td>42 ± 0.4</td>
<td>2.0 ± 0</td>
<td>21 ± 0.30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cake Rate 1 (20 Mg ha⁻¹)</td>
<td>Low</td>
<td>6.33 ± 0.03</td>
<td>107 ± 0.03b</td>
<td>47 ± 0.6</td>
<td>2.7 ± 0</td>
<td>17 ± 0.25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cake Rate 2 (40 Mg ha⁻¹)</td>
<td>Medium</td>
<td>6.16 ± 0.03</td>
<td>147 ± 8.6c</td>
<td>61 ± 2</td>
<td>4.0 ± 0.2</td>
<td>16 ± 0.72</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metals Rate 1</td>
<td>Low</td>
<td>6.3 ± 0.01</td>
<td>88 ± 3.5a</td>
<td>43 ± 1.4</td>
<td>2.1 ± 0</td>
<td>20 ± 0.38</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metals Rate 2</td>
<td>Medium</td>
<td>6.36 ± 0.02</td>
<td>72 ± 4.1a</td>
<td>43 ± 1.6</td>
<td>2.0 ± 0</td>
<td>21 ± 0.61</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Historic Biosolids</td>
<td>High</td>
<td>5.86 ± 0.04</td>
<td>176 ± 6.0c</td>
<td>170 ± 10</td>
<td>20 ± 0.1</td>
<td>8.8 ± 0.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metal Salts Historic Rate</td>
<td>High</td>
<td>5.95 ± 0.04</td>
<td>196 ± 25c</td>
<td>40 ± 1.7</td>
<td>1.9 ± 0.01</td>
<td>21 ± 0.21</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.2 Means ±standard error for pH, EC, C, N and C:N by treatment for March and October sampling dates. No standard error is reported for March samples as soil samples were taken as a composite for each treatment.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Metal Rate</th>
<th>Range</th>
<th>Foliar Cd June</th>
<th>Foliar Cd October</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Control</td>
<td>0.09-0.35</td>
<td>0.21 ± 0.02</td>
<td>0.21 ± 0.03</td>
</tr>
<tr>
<td>Cake Rate 1</td>
<td>Low</td>
<td>0.12-0.54</td>
<td>0.23 ± 0.04</td>
<td>0.32 ± 0.07</td>
</tr>
<tr>
<td>Cake Rate 2</td>
<td>Medium</td>
<td>0.13-0.54</td>
<td>0.24 ± 0.04</td>
<td>0.32 ± 0.05</td>
</tr>
<tr>
<td>Metals Rate 1</td>
<td>Low</td>
<td>0.01-0.56</td>
<td>0.20 ± 0.03</td>
<td>0.27 ± 0.07</td>
</tr>
<tr>
<td>Metals Rate 2</td>
<td>Medium</td>
<td>0.01-0.90</td>
<td>0.25 ± 0.05</td>
<td>0.26 ± 0.11</td>
</tr>
<tr>
<td>Historic Biosolids</td>
<td>High</td>
<td>0.31-14</td>
<td>1.2 ± 0.64</td>
<td>4.2 ± 1.6</td>
</tr>
<tr>
<td>Metals Historic Rat High</td>
<td>High</td>
<td>0.60-6.4</td>
<td>1.7 ± 0.40</td>
<td>2.6 ± 0.84</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Metal Rate</th>
<th>Range</th>
<th>Foliar Cu June</th>
<th>Foliar Cu October</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Control</td>
<td>2.6-7.5</td>
<td>3.3 ± 0.15</td>
<td>4.4 ± 0.62</td>
</tr>
<tr>
<td>Cake Rate 1</td>
<td>Low</td>
<td>1.8-6.7</td>
<td>3.0 ±0.57</td>
<td>5.7 ± 0.40</td>
</tr>
<tr>
<td>Cake Rate 2</td>
<td>Medium</td>
<td>2.2-6.3</td>
<td>3.4 ± 0.41</td>
<td>5.3 ± 0.22</td>
</tr>
<tr>
<td>Metals Rate 1</td>
<td>Low</td>
<td>2.3-4.5</td>
<td>3.3 ± 0.23</td>
<td>3.3 ± 0.21</td>
</tr>
<tr>
<td>Metals Rate 2</td>
<td>Medium</td>
<td>2.0-5.5</td>
<td>3.4 ± 0.39</td>
<td>3.7 ± 0.26</td>
</tr>
<tr>
<td>Historic Biosolids</td>
<td>High</td>
<td>3.0-14</td>
<td>6.0 ± 1.3</td>
<td>5.5 ± 0.45</td>
</tr>
<tr>
<td>Metals Historic Rat High</td>
<td>High</td>
<td>3.2-13</td>
<td>5.6 ± 1.3b</td>
<td>4.5 ± 0.27</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Metal Rate</th>
<th>Range</th>
<th>Foliar Pb June</th>
<th>Foliar Pb October</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Control</td>
<td>0.01-5.9</td>
<td>3.0 ± 0.57</td>
<td>1.2 ± 0.60</td>
</tr>
<tr>
<td>Cake Rate 1</td>
<td>Low</td>
<td>0.99-6.7</td>
<td>4.3 ± 1.3</td>
<td>3.4 ± 0.66</td>
</tr>
<tr>
<td>Cake Rate 2</td>
<td>Medium</td>
<td>1.9-4.8</td>
<td>3.7 ± 0.52</td>
<td>2.8 ± 0.26</td>
</tr>
<tr>
<td>Metals Rate 1</td>
<td>Low</td>
<td>0.01-7.9</td>
<td>5.45 ± 0.55</td>
<td>1.9 ± 0.53</td>
</tr>
<tr>
<td>Metals Rate 2</td>
<td>Medium</td>
<td>0.01-5.2</td>
<td>3.5 ± 0.49</td>
<td>0.9 ± 0.53</td>
</tr>
<tr>
<td>Historic Biosolids</td>
<td>High</td>
<td>0.01-15</td>
<td>6.4 ± 1.4</td>
<td>1.8 ± 0.59</td>
</tr>
<tr>
<td>Metals Historic Rat High</td>
<td>High</td>
<td>0.50-19</td>
<td>6.4 ± 2.4</td>
<td>2.2 ± 0.56</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Metal Rate</th>
<th>Range</th>
<th>Foliar Zn June</th>
<th>Foliar Zn October</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Control</td>
<td>8.8-60</td>
<td>12 ± 1.1</td>
<td>33 ± 6.4</td>
</tr>
<tr>
<td>Cake Rate 1</td>
<td>Low</td>
<td>9.7-60</td>
<td>14 ± 2.6</td>
<td>38 ± 5.7</td>
</tr>
<tr>
<td>Cake Rate 2</td>
<td>Medium</td>
<td>15-96</td>
<td>17 ± 0.92</td>
<td>52 ± 8.0</td>
</tr>
<tr>
<td>Metals Rate 1</td>
<td>Low</td>
<td>9.6-31</td>
<td>13 ± 0.76</td>
<td>20 ± 2.6</td>
</tr>
<tr>
<td>Metals Rate 2</td>
<td>Medium</td>
<td>9.2-52</td>
<td>13 ± 1.3</td>
<td>26 ± 4.4</td>
</tr>
<tr>
<td>Historic Biosolids</td>
<td>High</td>
<td>26-268</td>
<td>45 ± 8.8</td>
<td>150 ± 21</td>
</tr>
<tr>
<td>Metals Historic Rat High</td>
<td>High</td>
<td>13-116</td>
<td>25 ± 5.3</td>
<td>67 ± 11</td>
</tr>
</tbody>
</table>

Table 3.3. Cd, Cu, Pb and Zn foliar concentrations (mg kg⁻¹) by treatment and harvest date including ranges and standard errors. Significance of main effects varied by metal and are displayed in Figures 3.13 to 3.18. All metals were extracted using HNO₃ and HCL acid.
Foliar SH Groups (nmol SH eq/g FW)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Metal Rate</th>
<th>PC</th>
<th>GSH</th>
<th>CYS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Control</td>
<td>6.2±3.21</td>
<td>63.8±13.8</td>
<td>8.69±1.61</td>
</tr>
<tr>
<td>Cake Rate 1</td>
<td>Low</td>
<td>9.64±5.31</td>
<td>68.8±19.4</td>
<td>11.3±0.873</td>
</tr>
<tr>
<td>Cake Rate 2</td>
<td>Medium</td>
<td>2.84±0.478</td>
<td>131±51.4</td>
<td>12.3±2.31</td>
</tr>
<tr>
<td>Metals Rate 1</td>
<td>Low</td>
<td>6.51±2.70</td>
<td>87.2±23.0</td>
<td>11.0±1.30</td>
</tr>
<tr>
<td>Metals Rate 2</td>
<td>Medium</td>
<td>10.4±3.77</td>
<td>109±27.2</td>
<td>25.9±16.0</td>
</tr>
<tr>
<td>Historic Biosolids</td>
<td>High</td>
<td>5.79±2.38</td>
<td>62.4±14.9</td>
<td>11.2±0.609</td>
</tr>
<tr>
<td>Metal Historic Rate</td>
<td>High</td>
<td>7.69±3.25</td>
<td>127±34.3</td>
<td>10.2±1.60</td>
</tr>
</tbody>
</table>

Table 3.4 Means and standard errors for SH groups by treatment. No significant differences were observed between treatments based on rate or amendment type.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cd</th>
<th>Cu</th>
<th>Pb</th>
<th>Zn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Five-Year-Old Stand (16 Mg ha⁻¹)</td>
<td>3.5 ± 0.3</td>
<td>25 ± 5.7</td>
<td>65 ± 4.6</td>
<td>51 ± 6.4</td>
</tr>
<tr>
<td>Five-Year-Old Stand (0 Mg ha⁻¹)</td>
<td>3 ± 0.18</td>
<td>15 ± 1.1</td>
<td>65 ± 4.7</td>
<td>40 ± 4.5</td>
</tr>
<tr>
<td>Eight-Year-Old Stand (21 Mg ha⁻¹)</td>
<td>4 ± 0.24</td>
<td>35 ± 3.7</td>
<td>71 ± 1.4</td>
<td>75 ± 7.2</td>
</tr>
<tr>
<td>Eight-Year-Old Stand (0 Mg ha⁻¹)</td>
<td>4.8 ± 0.12</td>
<td>41 ± 1.3</td>
<td>79.5 ± 5.3</td>
<td>99 ± 4.1</td>
</tr>
</tbody>
</table>

Table 3.5 Mean concentrations (mg kg⁻¹) of total soil Cd, Cu, Pb and Zn ± standard errors for each treatment. Metal concentrations from the field sampling are similar to or slightly higher than greenhouse study control, modern biosolids and equivalent salt treatments.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cd mg kg⁻¹</th>
<th>Cu mg kg⁻¹</th>
<th>Pb mg kg⁻¹</th>
<th>Zn mg kg⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Five-Year-Old Stand (16 Mg ha⁻¹)</td>
<td>0.02 ± 0.00</td>
<td>0.078 ± 0.02</td>
<td>0.20 ± 0.04</td>
<td>0.70 ± 0.07</td>
</tr>
<tr>
<td>Five-Year-Old Stand (0 Mg ha⁻¹)</td>
<td>0.02 ± 0.00</td>
<td>0.18 ± 0.02</td>
<td>0.21 ± 0.03</td>
<td>0.88 ± 0.07</td>
</tr>
<tr>
<td>Eight-Year-Old Stand (21 Mg ha⁻¹)</td>
<td>0.07 ± 0.01</td>
<td>0.19 ± 0.01</td>
<td>0.20 ± 0.01</td>
<td>2.6 ± 0.45</td>
</tr>
<tr>
<td>Eight-Year-Old Stand (0 Mg ha⁻¹)</td>
<td>0.06 ± 0.00</td>
<td>0.26 ± 0.01</td>
<td>0.20 ± 0.02</td>
<td>0.52 ± 0.10</td>
</tr>
</tbody>
</table>

Table 3.6 Mean plant available metals (mg kg⁻¹) with standard errors for each site sampled. Lead was similar across all sites. Extractable Cd was higher in the older stand with a similar trend observed for Cu. Only Zn was significant by treatment (p<0.000).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>pH</th>
<th>EC uS cm⁻¹</th>
<th>C g kg⁻¹</th>
<th>N g kg⁻¹</th>
<th>C:N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Five-Year-Old Stand (16 Mg ha⁻¹)</td>
<td>4.1</td>
<td>124 ± 20</td>
<td>109 ± 3.2</td>
<td>4.6 ± .22</td>
<td>24 ± 0.88</td>
</tr>
<tr>
<td>Five-Year-Old Stand (0 Mg ha⁻¹)</td>
<td>4.0</td>
<td>77 ± 12</td>
<td>133 ± 15</td>
<td>6.3 ± 0.72</td>
<td>21 ± 0.73</td>
</tr>
<tr>
<td>Eight-Year-Old Stand (21 Mg ha⁻¹)</td>
<td>3.7</td>
<td>155 ± 23</td>
<td>72 ± 8.1</td>
<td>4.7 ± 0.34</td>
<td>15 ± 0.68</td>
</tr>
<tr>
<td>Eight-Year-Old Stand (0 Mg ha⁻¹)</td>
<td>4.6</td>
<td>65 ± 1.7</td>
<td>51 ± 2.0</td>
<td>3.0 ± 0.23</td>
<td>17 ± 1.0</td>
</tr>
</tbody>
</table>

Table 3.7 Means and standard errors for pH, EC, C, N and C:N for soils collected from the Hancock tree farm in King County, WA.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cd  mg kg⁻¹</th>
<th>Cu  mg kg⁻¹</th>
<th>Pb  mg kg⁻¹</th>
<th>Zn  mg kg⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Five-Year-Old Stand (16 Mg ha⁻¹)</td>
<td>0.35 ± 0.05</td>
<td>3.8 ± 0.29</td>
<td>2.3 ± 0.29</td>
<td>24 ± 1.8</td>
</tr>
<tr>
<td>Five-Year-Old Stand (0 Mg ha⁻¹)</td>
<td>0.24 ± 0.2</td>
<td>5.1 ± 0.44</td>
<td>1.9 ± 0.40</td>
<td>20 ± 1.1</td>
</tr>
<tr>
<td>Eight-Year-Old Stand (21 Mg ha⁻¹)</td>
<td>0.23 ± 0.04</td>
<td>5.0 ± 0.56</td>
<td>1.7 ± 0.22</td>
<td>21 ± 1.7</td>
</tr>
<tr>
<td>Eight-Year-Old Stand (0 Mg ha⁻¹)</td>
<td>0.23 ± 0.03</td>
<td>5.7 ± 0.34</td>
<td>2.6 ± 0.90</td>
<td>22 ± 2.2</td>
</tr>
</tbody>
</table>

Table 3.8 Mean foliar metal concentrations (mg kg⁻¹) +/- standard errors. Values were not significantly different by rate or amendment type.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>PC</th>
<th>GSH</th>
<th>CYS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SH eq. nmol/g FW</td>
<td>SH eq. nmol/g FW</td>
<td>SH eq. nmol/g FW</td>
</tr>
<tr>
<td>Five-Year-Old Stand (16 Mg ha⁻¹)</td>
<td>2.7 ± 1.6</td>
<td>91 ± 40</td>
<td>8.1 ± 0.89</td>
</tr>
<tr>
<td>Five-Year-Old Stand (0 Mg ha⁻¹)</td>
<td>2.6 ± 1.0</td>
<td>39 ± 13</td>
<td>6.7 ± 1.1</td>
</tr>
<tr>
<td>Eight-Year-Old Stand (21 Mg ha⁻¹)</td>
<td>0.69 ± 0.22</td>
<td>26 ± 10</td>
<td>6.5 ± 0.54</td>
</tr>
<tr>
<td>Eight-Year-Old Stand (0 Mg ha⁻¹)</td>
<td>4.7 ± 1.8</td>
<td>37 ± 7.8</td>
<td>5.6 ± 0.75</td>
</tr>
</tbody>
</table>

Table 3.9 Mean foliar PC, GSH and CYS (SH eq. nmol/g FW) and standard errors. There was no significant difference between treatments for these groups. Only PCs were significant by biosolids application rate with highest concentrations seen in soils where no biosolids were applied.
Bibliography


King County Department of Natural Resources and Parks. 2012. Biosolids Plan 2012-2016. King County, Seattle, WA.


NRCS Soil Series Descriptions online at: https://soilseries.sc.egov.usda.gov/OSD_Docs/A/ALDERWOOD.html
     https://soilseries.sc.egov.usda.gov/OSD_Docs/K/KLAUS.html


