PHYSIOLOGY AND CARBON ALLOCATION OF TWO CO-OCCURRING POPLAR

SPECIES (*POPULUS DELTOIDES* AND *POPULUS TREMULOIDES*) IN AN URBAN

BROWNFIELD

by

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A Dissertation submitted to the

Graduate School-Newark

Rutgers, The State University of New Jersey

in partial fulfillment of the requirements

for the degree of

Master of Science

Graduate Program in Biology

written under the direction of

Dr. Karina V.R. Schäfer

and approved by

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Newark, New Jersey

May, 2016

ABSTRACT OF THE DISSERTATION

Physiology and carbon allocation of two co-occurring poplar species (*Populus deltoides* and *Populus tremuloides*) in an urban brownfield

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Phytoremediation is a technique to reclaim heavy metal-contaminated soils which has increasingly gained interest, and an understanding of plant physiological responses to heavy metals is critical to success of its implementation. However, the majority of studies documenting heavy metal impact on plant functioning have been performed in laboratory or greenhouse settings. We tested the hypothesis that increasing loads of heavy metals in soil reduce photosynthesis and biomass production in trees growing along a soil metal contamination gradient in a naturally re-vegetated urban brownfield. Gas exchange and leaf composition were recorded and compared for *Populus deltoides* and *Populus tremuloides* growing in low and high soil metal load areas, while biomass was recorded and compared for both species in low, medium and high soil metal load areas. Heavy metals were found to have minimal impact on photosynthesis of either species. In terms of biomass, only total branch weight (TBW) and leaf area (LA) differed significantly between metal loads, though the difference was largely attributable to variation in diameter at breast height (DBH). Furthermore, TBW and LA values for *P. deltoides* did not decrease with

increasing metal load. Metal load had minimal effect on the relationship between tree age and DBH, and no effect on relationships of tree age and height, LA, or total tree mass, respectively. Significant differences between metal loads were found for $\delta^{15}N$ (isotopic nitrogen ratio). However, $\delta^{15}N$ and leaf nitrogen content (% N) also differed significantly between species, despite no differences in total leaf nitrogen between species or metal loads indicating that both species were able to obtain similar amounts of N, regardless of metal load, which may be related to mycorrhizae. While short-term water use efficiency (WUE) and nitrogen use efficiency were unaffected by metal load, long-term WUE was, though *P. tremuloides* enhanced and *P. deltoides* reduced long-term WUE in high metal load. While these findings do not indicate that heavy metals play no significant role for plants living in metal-contaminated environments, heavy metal impacts on plants are likely altered in the field due to site- and species-specific factors such as nutrient availability and mycorrhizae.

Preface

This thesis is submitted for the degree of Master of Biology at Rutgers, The State University of New Jersey. The research described here was conducted under the supervision of Dr. Karina V. R. Schäfer in the Department of Biology, Rutgers - Newark. This work is to the best of my knowledge original, except where acknowledgements and references are made to previous work.

Acknowledgements

The author would like to acknowledge and thank F. Adedeji, B. Awundaga, J. Chunchi, I. Cocuzza, A. Cullen, M. DeFreitas, D. Hanna, T. Khan, N. Murray, J. Riera, A. Salisbury, K. StAnge, M. Tejeda, B. Tsai, J. Wright, P. Salcedo, the Ecophysiology class of 2010 and the Aim High Academy of 2011 for field and laboratory assistance. The author would also like to thank Karina V.R. Schäfer, Dirk Vanderklein, and Frank Gallagher for important contributions to fieldwork and advice in thesis editing. The author would also like to thank Daniel Bunker for time in considering and reviewing this thesis.

Dedications

This work is dedicated to my mother who provided endless support and encouragement, and taught me that I could accomplish anything if taken one step at a time and that knowledge is something worth pursuing for its own intrinsic value. This work is also dedicated to my older brother, Joseph Radwanski, the layman scientist, who provided technical support and most importantly, laughter during the toughest times.

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Introduction

Numerous areas across the globe are suffering from heavy metal contamination (McGrath et al. 2001; Lone et al. 2008; Meers et al. 2010). Heavy metals pose a serious threat due to their non-biodegradable nature, difficulty of removal, health risks, and buildup in water and soils (Giller et al. 1998; Mendola et al. 2002; Lone et al. 2008). In the United States there are over 450,000 brownfields, and nearly 80% of Superfund sites and over 50 million cubic meters of soil are contaminated with heavy metals (Dermont et al. 2008; EPA 2012). It has been estimated that brownfields in the U.S. alone account for the loss of more than 205,000 ha of valuable land that could be used for agriculture, pasture, or forests (Lone et al. 2008). As such, phytoremediation has become widely recognized as a cost-effective, efficient, eco-friendly, and *in situ* applicable method for remediating metal-contaminated soils (Saier and Trevors 2010; Wang and Jia 2010; Vithanage et al. 2012; Ali et al. 2013). Species with traits that include fast growth, extensive root systems, and large biomass production have become a focus of many phytoremediation studies (Pulford and Watson 2003; Jabeen et al. 2009; Seth 2012). Among these plants, members of the genus, *Populus*, are known to comprise a large number of species that are able to grow in metal-contaminated soils (Wang and Jia 2010).

Many studies on the effects of heavy metals on plant function have been carried out in laboratory or greenhouse settings (Giachetti and Sebastiani 2006; Hermle et al. 2006; Dos Santos Utmazian et al. 2007; Hermle et al. 2007; Robinson et al. 2007; Ali et al. 2013). Considering that such cases do not reflect field conditions, and that others have identified field conditions that affect plant responses to heavy metals (Vangronsveld et al. 2009),

further work is needed to expand our knowledge of plant functioning in response to heavy metal contamination in the field.

Studies that have examined the impact of heavy metals on many plant species have found negative effects on photosynthesis and biomass production. Leaf damage, alterations in plant cell structure, and damage to both photosynthetic and water-conducting tissues have been reported in plants dealing with heavy metal contamination (Baryla et al. 2001; Hermle et al. 2007). In response to treatments with copper (Cu), nickel (Ni), and cadmium (Cd), *Populus deltoides* and *Populus euramericana* undergo oxidative stress, leading to damaged chlorophyll molecules followed by reductions in photosynthetic capacity (Trudić et al. 2012). Young trees of *Populus x euramericana* suffered from reduced foliage, impaired gas exchange, and decreased photosynthesis rates in response to zinc (Zn) treatments (Di Baccio et al. 2003). Young trees of *Populus tremula* experienced decreases in transpiration, diameter growth, foliage area, fine root biomass, aboveground biomass, and leaf area, resulting in lower photosynthetic ability when grown with topsoil treated with Cu, Zn, Cd, and lead (Pb) (Hermle et al. 2006; Menon et al. 2007). When grown in soils treated with Cd, Zn, and Cu, two-year-old seedlings of *Populus canadensis* only reached 75% of their biomass production compared to individuals of the same species and age growing in untreated soil (Wang and Jia 2010). Mature trees of *Betula populifolia* displayed reduced productivity when growing in soils with higher Zn levels (Gallagher et al. 2008a). Mature trees of *P. deltoides* did not significantly differ in terms of photosynthetic functioning between low and high soil metal concentrations, though it was found that trees of *P. deltoides* that were of similar size, tended to be younger when growing on soils with lesser metal contamination (Renninger et al. 2013). Older trees may

simply have greater ability to tolerate heavy metals due to their larger size which conveys greater number of water- and nutrient-conducting, as well as photosynthetic tissues, which may enhance the ability to offset negative impacts of heavy metals.

The purpose of this study is to examine and compare the effects of heavy metals on photosynthesis and biomass production in *Populus deltoides* (USDA 2015a) and *Populus tremuloides* (USDA 2015b) growing in a naturally re-vegetated brownfield in New Jersey, which contains a soil metal contamination gradient. Here, it is hypothesized that *P. deltoides* and *P. tremuloides* will exhibit a higher photosynthetic capacity in sites containing lower soil metal contamination compared to sites with higher soil metal contamination. As a result, productivity for both species is expected to be higher in sites with lower soil metal contamination. Trees of both species growing in highly metal-contaminated soils are expected to have higher leaf dark respiration compared to trees growing in lesser metal-contaminated soils because more energy is required in order to maintain leaf structural and photosynthetic integrity in a heavy metal soil environment. Similarly, transpiration is expected to be lower for trees in sites with higher soil metal contamination in order to tolerate metal-induced water stress. Photosynthetic and intrinsic water use efficiency should then be higher for trees growing in sites of higher soil metal contamination due to lower transpiration or lower stomatal conductance, respectively.

This study will provide further knowledge regarding the effects of heavy metals on plant physiological function in an actual brownfield, which contains many processes and interactions that affect plant responses, which cannot be recreated in laboratory or greenhouse settings. In addition to increased field knowledge, a comparison of the

performance of *P. deltoides* and *P. tremuloides* growing in metal-contaminated areas will be useful in future phytoremediation strategies for heavy metal-contaminated soils.

Materials and Methods

Study site and species selection

The study was carried out in Liberty State Park (LSP), which is located in Jersey City, New Jersey, U.S.A. LSP originally existed as an intertidal mudflat and salt marsh before undergoing a land use change and being filled with waste from New York City in order to be made usable as a rail yard for the Central Railroad of New Jersey (CRRNJ) (Gallagher et al. 2008b). The area was used by the CRRNJ until 1967, leaving the area abandoned (NJDEP 2009). Later, the area was purchased with government funds and upon the completion of cleanup efforts, the area was officially opened as a public park in 1976 (NJDEP 2009). LSP consists of approximately 490 ha, and while the park has been largely restored, a 102 ha area still exists that is fenced off and closed to public access (NJDEP 2001; Renninger et al. 2013) because of its classification as a brownfield due to soil heavy metal contamination (Gallagher et al. 2008a; 2008b). Specifically, the area has been found to be contaminated with arsenic (As), Cr, Cu, Pb, vanadium (V), and Zn, of which soil concentrations of As, Cr, Pb, V, and Zn are considered to be above normal for New Jersey soils (Sanders 2003; Gallagher et al. 2008b). Previous soil sampling done in 2005 indicated that soil contamination was highly heterogeneous across the area (Gallagher et al. 2008a; 2008b). Despite the presence of metal-contaminated soils, the area has been naturally re-vegetated, with *Betula populifolia* Marsh. (35%), *Populus deltoides* W. Bartram ex Marshall (16%), and *Populus tremuloides* Michx. (14%) representing the dominant species in the hardwood areas (Gallagher et al. 2008a; 2008b; Renninger et al. 2013).

Within this 102 ha fenced off area, six sites were chosen for this study, each representing areas of high, medium, or low soil metal contamination. All six sites were selected from areas of successional northern hardwood plant assemblages as established by Gallagher et al. (2008b). Using the total soil metal load map (Gallagher et al. 2008a) and the site designations created by Gallagher et al. (2008a), sites 41 and 48 were selected as areas of low soil metal load (LML) and sites 14/16 and 25 as areas of high soil metal load (HML) in order to examine heavy metal effects on gas exchange and leaf composition. Sites 11, 41, and 48 were selected to represent LML, site 18 to represent medium soil metal load (MML), and finally, sites 25 and 14/16 to represent HML for analysis of tree biomass. In an effort to fill knowledge gaps, the species of interest for this study were *P. deltoides* and *P. tremuloides*, as previous studies conducted at the same study site have focused on *B. populifolia* (Gallagher et al. 2008a) and *P. deltoides* (Renninger et al. 2013), but have not focused on *P. tremuloides* nor a comparison between *P. deltoides* and *P. tremuloides*.

Photosynthesis measurements

Photosynthetic measurements were taken during the last week of each month during the period of June, 2013 to September, 2013, and from the last week of each month during the period of May, 2014 to September, 2014, using a LiCor 6400 XT (LiCor Biosciences, Inc., Lincoln, NE, USA). Measurements were made each day from the morning to the afternoon, during peak photosynthetic activity, while avoiding the presence of early morning dew on leaves and the natural closure of stomata in the late afternoon. Measurements were taken from one leaf for each of 5 trees of *P. deltoides* and 5 trees of *P. tremuloides* at sites 41, 48, and 25. At site 14/16, however, due to a lack of presence of *P. deltoides*, measurements were taken for *P. tremuloides* only. Samples were taken by

cutting branches, located in open areas of the canopy, off of the trees and then keeping and cutting them underwater so as to avoid stomatal closure due to water stress. Leaves of the cut branches were measured within 90 minutes after each branch cutting. Assimilation-carbon dioxide response (A/C_i) curves and assimilation-light response curves were made for each leaf measured. The A/C_i curves were constructed by setting a constant photosynthetically active irradiance level (PAR) to 1500 μmol m^{-2} s^{-1} and altering the CO_2 concentration in the leaf chamber from 50 ppm to 1500 ppm. Light response curves were constructed by setting a constant CO_2 concentration in the leaf chamber to 400 ppm, and then varying PAR from 0 μmol m^{-2} s^{-1} to 2000 μmol m^{-2} s^{-1}.

The data from the A/C_i curves were used to calculate the maximum carboxylation rate as limited by Rubisco (V_{cmax} in μmol m^{-2} s^{-1}), maximum electron-transport-limited carboxylation rate (J_{max} in μmol m^{-2} s^{-1}), carboxylation rate as limited by triose-phosphate use (TPU in μmol m^{-2} s^{-1}), mesophyll conductance (g_m in μmol m^{-2} s^{-1} Pa^{-1}), carboxylation efficiency (CE in μmol CO_2 m^{-2} s^{-1} ppm^{-1}), and the CO_2 compensation point (CCP in ppm). Using the A/C_i Curve Fitting Utility Version 2007.1 (Sharkey et al. 2007a; 2007b), a best fit model was used to minimize the difference of the sum of squares between measured and modeled data for the Rubisco-limited, electron-transport-limited, and TPU-limited section of the A/C_i curve to estimate V_{cmax}, J_{max}, and TPU, respectively. The model also provided output with an estimate of mesophyll conductance, g_m, whereby g_m is estimated from the observed A/C_i data as described in Sharkey et al. (2007a). Final values of V_{cmax}, J_{max}, TPU, and g_m were taken as the respective values scaled by the model to a leaf temperature of 25°C to avoid comparison of values at different observed leaf temperatures.

In order to calculate carboxylation efficiency and CO_2 compensation points, the A/C_i data were plotted in Sigmaplot 11.0 (Sysstat Software Inc., San Jose, CA, USA) for each measured leaf. A linear regression was fit through the initial, linear section of the A/C_i curve, roughly from 50 to 200 ppm CO_2. The linear equation of the regression was then used to calculate the slope (carboxylation efficiency), and x-intercept (CO_2 compensation point).

The data from light response curves were used to calculate the maximum rate of net photosynthesis (A_{max} in $\mu mol\ CO_2\ m^{-2}\ s^{-1}$), quantum yield ($\Phi$ in $\mu mol\ CO_2\ \mu mol^{-1}$ photons), dark respiration rate (R_D in $\mu mol\ CO_2\ m^{-2}\ s^{-1}$), and light compensation point (LCP in $\mu mol\ photons\ m^{-2} s^{-1}$) for each measured leaf. As per Lobo et al. (2013), multiple models were used to best fit the original, observed light response data and the model with the lowest sum of the squares of the errors (SSE) was used to estimate A_{max}, Φ, R_D, and LCP for each measured leaf. Three light response curves were calculated for each leaf, using 3 different models as outlined in Lobo et al. (2013) and based on work by Webb et al. (1974), Prioul & Chartier (1977), and Ye (2007). The 3 models included an exponential curve-fitting model which utilized equation 8 in Lobo et al. (2013), a non-rectangular hyberbola-fitting model which utilized equation 6 in Lobo et al. (2013), and the final model based on Ye (2007), which utilized equation 11 in Lobo et al. (2013). Overall, for light response data from 2013 and 2014, the accepted, best-fit models were represented largely by the Ye model (63%) and the non-rectangular hyperbola model (35%), with only 2% of cases where the exponential model was considered the best fit.

Transpiration (Transp in mmol H_2O), the ratio of intercellular CO_2 to ambient CO_2 (C_i/C_{ainst}), photosynthetic water use-efficiency (WUE in mmol CO_2 mmol^{-1} H_2O), defined

as photosynthetic carbon assimilation/transpiration, and intrinsic water use efficiency (iWUE in μmol CO_2 $mmol^{-1}$ H_2O), defined as photosynthetic assimilation/stomatal conductance, were estimated from A/C_i and light response curves from points where PAR was 1500 μmol m^{-2} s^{-1} and CO_2 concentrations were 400 ppm for each leaf.

Leaf composition

Leaves that were used for photosynthesis measurement were collected from five trees of *P. deltoides* and *P. tremuloides* in each of the four study sites, with the exception of site 14/16, which did not contain *P. deltoides*, in order to calculate leaf carbon (C) and nitrogen (N) concentrations, as well as C and N isotopic ratios. The leaves were first dried at 60°C for at least 48 hours in a convection oven (Thermo Scientific Precision 3050 Series premium oven, Thermo Fisher Scientific, USA) then ground into a fine powder, placed in tin capsules, and sent out to the University of California's Davis Stable Isotope center located in Davis, CA, USA, for analysis of leaf C and N concentrations (C and N in %), C:N ratios, isotopic C ratios ($\delta^{13}C$), and isotopic N ratios ($\delta^{15}N$). Nitrogen concentrations (%) were scaled to a per unit leaf area by multiplying N concentration by the leaf mass per area measured from the respective collected leaves. Total leaf N (TLN) was calculated by multiplying leaf N concentration by total leaf weight. Average N concentration was determined from leaf composition data categorized by species and site, and then multiplied by the total leaf weight of trees harvested for biomass analysis that were classified as the same species and site. In the case of *P. tremuloides* growing in site 11, which was not represented in leaf composition data, N concentrations were averaged from *P. tremuloides* from sites 41 and 48, since sites 41, 48, and 11 are all classified as LML sites, and have similar amounts of heavy metals that do not exceed a certain total soil metal load (Gallagher

et al. 2008a), and then multiplied by the total leaf weight of *P. tremuloides* from site 11. Trees from site 18 were excluded, as leaf N concentration was not determined for trees at MML sites. Additionally, nitrogen use efficiency (NUE in μmol CO_2 g^{-1} N s^{-1}) was calculated by dividing the net photosynthesis rate, which was estimated from A/C_i and light response curves from points where PAR was 1500 μmol m^{-2} s^{-1} and CO_2 concentrations were 400 ppm for each leaf, by the concentration of N, scaled per unit leaf area, in each of the measured leaves. Carbon isotopic discrimination (Δ in ‰) was also calculated from the C isotope data using equation 4 in Renninger et al. (2014) based on work by Farquhar et al. (1989). Isotopic intrinsic water use efficiency ($iWUE_{isotope}$) was calculated using equation 5 in Renninger et al. (2014) based on work by Farquhar et al. (1989). The isotopic ratio of intercellular CO_2 to ambient CO_2 (C_i/$C_{a, isotope}$) was calculated using the following equation based on Farquhar et al. (1989):

$$Ci/Ca, isotope = \frac{(\Delta - 4.4)}{(27 - 4.4)} \tag{1}$$

where Δ is in ‰, 4.4 is the diffusive discrimination of ^{13}C in air through the stomata, and 27 is the discrimination of Rubisco to ^{13}C.

Biomass harvest and aboveground growth

The harvest protocol for *P. deltoides* was previously reported in Renninger et al. (2013). In addition, twenty-two trees of *P. tremuloides* were harvested in the spring of 2010 and summer of 2011 to comprise a sufficiently broad diameter at breast height range from 0.85 cm to 14.7 cm and a sample size of 5 to 7 in each metal loading site, with total sample size of 22 per species being typical for felled tree studies (Arias et al. 2011; Suchomel et al. 2012; Karlik and Chojnacky 2014). Additionally, the branch diameter was

recorded from every branch taken from each harvested tree in 2011. *P. deltoides* was harvested in sites 41, 18, and 25, while *P. tremuloides* was harvested in sites 11, 18, 48, 14/16, and 25. Trees were cut at 10 cm from the ground after recording the diameter at breast height. The main axis of the fallen tree was cut into one-meter segments and fresh weight of each of the one-meter segments was recorded using scales in the field. From the base of each one-meter segment, one slice of the bottom of the stem (cookie) was collected and fresh weight of the cookie was recorded. For each of the one-meter segments, branch diameters of all the branches were recorded. All the branches and leaves were labeled and were collected in separate bags. All the bags with branches, leaves, and cookies for individual trees were dried in a convection oven (at 60°C, 6 days) in the laboratory (Thermo Scientific Precision 3050 Series premium oven, Thermo Fisher Scientific, USA). The ratio of the dry to fresh weight of the cookies was used to estimate the dry weight of the one-meter segments of tree stem previously recorded in the field. Ring widths of each tree cookie were measured along two perpendicular radii to the nearest 0.1 mm. This allowed for determination of the age and height of each tree, as well as overall growth pattern. Tree height was divided into three segments (bottom, middle, and top) for selecting the number of leaves to estimate leaf area (LA). The numbers of leaves selected for scanning were proportional to the number of branches on each segment. Total leaf weight (TLW) was estimated by adding the dry weight of all the leaves of all the branches from an individual tree. Total branch weight (TBW) was determined by adding the dry weight of all the branches. To calculate the LA for individual trees, leaves collected from a tree in the field were scanned on the same day in the laboratory using a commercial scanner and analyzing the images using Image J (http://rsbweb.nih.gov/ij/). Specific leaf area (SLA) was

calculated as the ratio of LA per unit of leaf dry mass. Canopy level SLA was then used to determine total leaf area by multiplying it with total leaf mass of each canopy level. Total aboveground tree mass (TTM), was estimated by adding the dry weight of leaves, branches, and total stem mass.

As the large *P. tremuloides* trees (diameter at breast height > 7cm) were harvested during the leafless stage in the spring of 2010, ten branches from all sites where the trees were harvested, were collected in the summer of 2013 and a branch diameter to leaf mass and branch diameter to leaf area relationship generated (with an R^2 of 0.62 and P<0.0001, respectively), given that there is a clear, allometric relationship between branch diameter, LA, and leaf mass (Hunt et al. 1999; McDowell et al. 2002; Jiao-Lin and Kun-Fang 2009) based largely on pipe model theory (Shinozaki et al. 1964). These relationships, separated for each metal load, were used to calculate each individual tree leaf biomass and LA for the trees harvested previously.

Statistical analysis

For all statistical evaluations of gas exchange, leaf composition, and biomass data, data were combined from 2013 and 2014, and 2010 and 2011. Means and standard errors were calculated for gas exchange and leaf composition variables for *P. deltoides* and *P. tremuloides* growing in LML and HML. A linear mixed effects model was created in Matlab R2014b (The Mathworks Inc, Natick, MA) to determine if means differed between metal load and/or species. Model parameters included two independent, fixed effects, which were metal load and species, with both metal load and species nested within sites. An interaction effect between species and metal load was also included. The month and year of measurement for each tree was included as a single random effect. Analysis of

variance (ANOVA) used within the model was set to indicate significant difference at α =0.05. Adjusted R^2 values of the model varied from 0.07 to 0.32 and from 0.25 to 0.50 for the gas exchange and leaf composition data, respectively.

Similar procedure was used to analyze biomass and TLN means, with the following differences. In order to improve fit to the model, all values were log transformed (log, base 10). Model parameters additionally included diameter at breast height (DBH) and age as independent, fixed effects, and another interaction term between species and age. Adjusted R^2 values of the model varied from 0.77 to 0.97.

In order to investigate the effects of heavy metals on allometric growth patterns, the relationships between tree age and DBH, height (H), or LA, as well as between DBH and total tree mass (TTM), were plotted for *P. deltoides* and *P. tremuloides* growing in LML, MML, and HML. Using aoctool in Matlab R2014b, linear regressions were performed to fit separate lines to the data of tree age versus DBH, height, or LA, and DBH versus TTM. Due to the nonlinear relationships between tree age and the aforementioned variables, data were log transformed (log, base 10) before regression analysis. Regressions were followed by an analysis of covariance (ANCOVA) to determine if slopes differed at α =0.05. For the ANCOVA, tree age was designated as the explanatory variable, and DBH, height, or LA as the response variable. For DBH versus TTM, DBH and TTM were the explanatory and response variables, respectively. All variables were categorized by species and metal load. Tukey's HSD test was employed if the ANCOVAs signified differences in the slopes and/or y-intercepts at α =0.05.

Results

Gas exchange parameters

The V_{cmax}, TPU, g_m, and CE did not differ significantly between metal load or species (**Table 1**). Also, J_{max} did not differ significantly between metal load (p=0.88), but did differ between species (p=0.04). In both LML and HML sites, J_{max} was greater for *P. deltoides* compared to *P. tremuloides* (by approximately 24% and 17%, respectively) (**Table 1**). The CCP differed significantly between trees due solely to the effects of metal load (p=0.05), but not between species (p=0.69). Additionally, a significant (p=0.008) interaction effect between metal load and species was found. *P. deltoides* had a greater CCP compared to *P. tremuloides* in both LML and HML, but was 63% greater in HML and only 11% greater in LML (**Table 1**). In HML, *P. deltoides* displayed a greater CCP when compared to LML (by roughly 23%), while *P. tremuloides* displayed a greater CCP in LML than HML (by approximately 19%) (**Table 1**).

The A_{max}, Φ, and R_D did not differ significantly between metal loads or species. The LCP differed significantly between species (p=0.02), but not metal load (p=0.13). In both LML and HML, the LCP was greater for *P. deltoides* compared to *P. tremuloides* (by roughly 25% and 50%, respectively) (**Table 1**).

The C_i/C_{ainst}, WUE, and iWUE did not differ between metal loads or species. However, transpiration differed significantly between species (p<0.001), but not metal load (p=0.13). Moreover, a significant (p=0.04) interaction effect between metal load and species was found. *P. tremuloides* preferred to restrict transpiration more so than *P. deltoides* in both LML and HML, with lower transpiration by roughly 23% and 4%, respectively (**Table 1**).

Leaf composition

Species differed significantly (p<0.0001) in leaf carbon content [C (%)], though metal load had no effect (p=0.93). In both LML and HML, *P. tremuloides* contained greater leaf C content (by roughly 6% and 3%, respectively) compared to *P. deltoides* (**Table 2**). Isotopic C ratios ($\delta^{13}C$), carbon isotopic discrimination ratios (Δ‰), isotopic ratios of intercellular CO_2 to ambient CO_2 ($C_i/C_{a,\ isotope}$), and isotopic intrinsic water use efficiency ($iWUE_{isotope}$) all differed significantly between metal load (p<0.001), but not between species (p=0.83), and a significant (p<0.0001) interaction effect between species and metal load was found. *P. deltoides* displayed a reduced $\delta^{13}C$ (by roughly 3%) and $iWUE_{isotope}$ (by roughly 11%), while Δ‰ and $C_i/C_{a,\ isotope}$ were greater (by roughly 6%) when growing in HML compared to LML (**Table 2**). However, *P. tremuloides* displayed a greater $\delta^{13}C$ and $iWUE_{isotope}$ (by roughly 2%) and reduced Δ‰ and $C_i/C_{a,\ isotope}$ (by roughly 2%) when growing in HML compared to LML (**Table 2**).

Leaf nitrogen content [N (%)] differed significantly between species (p=0.001), but not between metal load (p=0.45). In both LML and HML, *P. tremuloides* contained greater N content in the leaves (by approximately 10% and 11%, respectively) compared to *P. deltoides* (**Table 2**). In the case of isotopic N ratios ($\delta^{15}N$), trees differed significantly between species (p<0.0001) and metal load (p<0.0001). A significant (p<0.0001) interaction effect between species and metal load was also found in the comparison of $\delta^{15}N$. In both LML and HML, *P. deltoides* contained a greater $\delta^{15}N$ (by approximately 93% and 19%, respectively) compared to *P. tremuloides* (**Table 2**). *P. deltoides* displayed a greater $\delta^{15}N$ (by roughly 89%) when growing in LML compared to HML (**Table 2**). *P. tremuloides* showed the opposite response, with a greater $\delta^{15}N$ (by approximately 23%)

when growing in HML, compared to LML, sites (**Table 2**). Despite differences in leaf N composition, NUE and TLN did not differ significantly between species or metal load. However, leaf C:N ratios differed significantly between species (p=0.03), with *P. deltoides* having greater leaf C:N compared to *P. tremuloides* in both LML and HML, by roughly 6% and 10%, respectively (**Table 2**).

Biomass and allometric relationships

The DBH of trees did not differ significantly between species (p=0.57) or metal load (p=0.059). The mean age (in years) of *P. deltoides* in LML, MML, and HML was 8.6±0.95, 6.5±1.4, and 12.1±1.5, respectively (data not shown). The mean age (in years) of *P. tremuloides* in LML, MML, and HML was 11.2±1.6, 7.8±2.1, and 5.5±0.62, respectively (data not shown). In terms of tree height, TBW, TLW, LA, and TTM, significant differences were discovered to be due largely to the effect of DBH (p<0.0001 in all cases).

Some significant differences between trees, in terms of biomass, were found to be due to effects other than DBH. TBW did differ significantly between species and metal load (p=0.04). In LML and HML, *P. deltoides* displayed a greater TBW compared to *P. tremuloides*, where the TBW was double and ninefold that of *P. tremuloides* in LML and HML, respectively (**Table 3**). Only in the MML sites, was TBW of *P. tremuloides* greater than that of *P. deltoides* (by roughly 28%) (**Table 3**). Furthermore, *P. deltoides* displayed the largest TBW in HML, being roughly 63% and fourfold greater compared to growing in LML and MML, respectively (**Table 3**). *P. tremuloides* displayed the greatest TBW in LML, being approximately 6% and threefold greater compared to when growing in MML and HML, respectively (**Table 3**). LA of trees did not differ significantly between species

(p=0.98), but did differ significantly between metal load (p=0.03). For both *P. deltoides* and *P. tremuloides*, LA was greatest in LML, compared to MML and HML. For *P. deltoides*, LA was greater in LML compared to MML and HML by roughly threefold and 36%, respectively (**Table 3**). For *P. tremuloides*, LA was greater in LML compared to MML and HML by roughly 43% and threefold, respectively (**Table 3**).

Trees displayed a significant difference in the relationship between tree age and DBH, with statistically different slopes between metal load categories (p=0.02), however, the two study species did not differ in the relationship between tree age and DBH within HML, MML, or LML (**Fig. 1**). However, *P. deltoides* from LML differed significantly from *P. deltoides* in MML sites with statistically different slopes (p=0.03). In particular, *P. deltoides* generally displayed a greater DBH at a given age when growing in LML, compared to MML, after roughly age 10 (**Fig. 1**). Trees did not differ in the relationship between tree age and height, with statistically similar slopes (p=0.16) (**Fig. 2**). Furthermore, trees did not differ significantly in the relationship between tree age and LA, with statistically similar slopes (p=0.10) (**Fig. 3**). Trees also did not differ significantly in the relationship between DBH and TTM, with statistically similar slopes (p=0.28) and y-intercepts (p=0.28) (**Fig. 4**).

Discussion

Metal load was not found to have a significant effect, with the exception of the CCP, on the gas exchange performance of either of the two study species (**Table 1**). Such findings are not entirely unusual as others have reported finding no apparent effects of heavy metals on photosynthesis from trees growing in the same brownfield (Renninger et al. 2013), though non-field studies have reported reduced rates of photosynthesis in plants

exposed to heavy metals (Romanowska et al. 2002; Di Baccio et al. 2003; Hermle et al. 2006; Menon et al. 2007). However, our findings could be expected to differ since natural processes and interactions occurring in the field could offset heavy metal effects.

In the case of the CCP, *P. deltoides* experienced a greater CCP when growing in HML, which agreed with the study hypothesis, though not because CO_2 assimilation was lower or R_D was higher, as neither differed between metal loads (**Table 1**). Therefore, the higher CCP of *P. deltoides* in HML is not indicative of negative, metal-induced effects on CO_2 uptake. However, greater maintenance demand for non-leaf tissues, such as roots, may be present (Kozhevnikova et al. 2007; Demchenko et al. 2013) as a response to metal-induced water stress. Neither transpiration, or mesophyll conductance, differed between metal loads (**Table 1**), so *P. deltoides* in HML may have enhanced root maintenance or growth in order to tolerate heavy metal-induced water stress. However, instantaneous and intrinsic WUE did not differ, and isotopic intrinsic WUE, though different between metal loads, was actually lower for *P. deltoides* in HML, rather than LML, (**Table 2**) and thus the premise that increasing metal load imposes greater water stress on plants is unsubstantiated.

Differences in C allocation, which differed between species but not metal load, as indicated by leaf C:N while TLN did not differ (**Table 2**), could reflect differences in maintenance demand. Indeed, *P. deltoides* had greater biomass, as indicated by TBW, in HML while *P. tremuloides* had a smaller TBW in HML (**Table 1, 3**), which would reflect a lower maintenance requirement. Additionally, *P. tremuloides* averaged half the age in HML, compared to LML, while *P. deltoides* was roughly 3 years older on average, in HML, and it is reasonable that maintenance respiration increases with increasing age or size.

Higher root maintenance and growth could also be a factor influencing respiration in response to nutrient acquisition and mycorrhizal colonization (Birhane et al. 2012).

As with photosynthetic performance, biomass was predicted to be greater for both species in LML. However, biomass, in terms of tree height, TBW, TLW, LA, and TTM, were largely explained by differences in tree DBH (**Table 3**). Metal load only significantly affected the TBW and LA, yet both study species did not display trends in TBW and LA that decreased with increasing metal load (**Table 3**). Therefore, the claim that biomass should be greater as soil metal contamination declines is not entirely substantiated.

Allometric relationships did not differ significantly, with the exception of DBH versus age (**Fig. 1**). Interestingly, *P. deltoides* in LML displayed a greater DBH at a given age than when growing in MML, suggesting that growth rate declines could be due to heavy metals, possibly requiring greater resource allocation to maintenance than growth. However, this difference was seen at around age 10, and *P. deltoides* growing in MML actually obtained greater DBH at a given age than those growing in LML before age 10 (**Fig. 1**). Additionally, no differences were found in the relationship between DBH and age for *P. deltoides* growing in LML and HML, while *P. tremuloides* did not differ across metal load at all.

Nutrient availability can strongly influence heavy metal effects on plants (Silva et al. 2010; Zhang et al. 2011). Additionally, nutrient availability and uptake in trees is closely tied to mycorrhizal associations, which also affect C allocation, as there is a C cost to the host plant (Allen et al. 2003). Furthermore, mycorrhizal associations can increase trees' tolerance to heavy metals (Lingua et al. 2008; Zhang et al. 2010). Members of the genus, *Populus*, are able to establish relationships with arbuscular, ectomycorrhizal, and

endomycorrhizal fungi (Vozzo and Hacskaylo 1974; Lukac et al. 2003; Gehring et al. 2006). Examination of the fungal community of *P. deltoides* in the field revealed eight different phyla of fungi (Shakya et al. 2013), including an endomycorrhizal fungus that has been reported elsewhere to form parasitic relationships with insects and transfer the N directly to host plants (Behie et al. 2012). *P. tremuloides* and *P. deltoides* associate with *L. bicolor* (Desai et al. 2014; Labbé et al. 2014) though extent of colonization could differ (Tschaplinski et al. 2014). Only *P. deltoides* has been reported to form endomycorrhizal associations (Vozzo and Hacskaylo 1974; Behie et al. 2012). Considering that δ^{15}N differed between species and metal load (**Table 2**), the two study species may be getting N from different sources. δ^{15}N patterns may then differ between *P. deltoides* and *P. tremuloides*, and between metal load, due to different mycorrhizal associations that differ in N uptake performance and heavy metal tolerance. However, since TLN did not differ between species or metal load (**Table 2**), there may be differences in acquisition of other nutrients such as phosphate, based on differing mycorrhizal associations. Indeed, recent examination of ectomycorrhizae along a metal contamination gradient in LSP revealed that mycorrhizal fungal species diversity is similar across soils of varying metal load, yet species composition changes (J. M. Evans et al., unpublished), suggesting that in LSP, mycorrhizal fungal species do have differing levels of metal tolerance. Naturally, *P. deltoides* and *P. tremuloides* in LSP will experience differences in productivity associated with changes in available and compatible fungal symbionts across varying soil metal load, which could explain differing biomass patterns in the two study species found here. While alterations in δ^{15}N values may be related to changes in the amount of N transferred from fungus to plant (He et al. 2003), it is not yet clear how and if the type of mycorrhizal

colonization affects $\delta^{15}N$ values (Michelsen et al. 1998; He et al. 2003). However, the situation is more complex as information emerges on the occurrence of common mycorrhizal networks (CMNs) (van der Heijden and Horton 2009; Gorzelak et al. 2015) which can interconnect different plant individuals, including those of different life stages (Beiler et al. 2010) and species (He et al. 2003). Furthermore, plant-to-plant transfers of C, nutrients, and water have been reported to occur through CMNs such that seedlings can benefit from being a part of the same CMN as mature trees (van der Heijden and Horton 2009; Gorzelak et al. 2015) and individuals that contribute less C to a CMN can gain greater benefits in terms of nutrient return compared to individuals that contribute more C to the same CMN (Walder et al. 2012). Differences in leaf N content may also be due to differences in the forms of N found in the soil at the study site as well as N form preferences of fungal symbionts. Templer et al. (2007) found that species differed significantly in $\delta^{15}N$ values, which could have been due to differences in preferences for ammonium or nitrate. Indeed, it has been found that *P. tremuloides* accumulated higher ^{15}N when $^{15}NH_4NO_3^-$, rather than $NH_4^{15}NO_3^-$, was provided (Woo-Jung et al. 2005). Others have found that *P. tremuloides* seedlings can utilize both ammonium and nitrate, though certain mycorrhizal associations may be required (Siemens et al. 2011).

Conclusions

Contrary to the study hypothesis, soil metal load did not generally affect photosynthetic and growth performance of *P. deltoides* or *P. tremuloides* growing in Liberty State Park, and when metal load was significant, photosynthesis and growth performance did not necessarily decline as metal load increased. Nutrient status and mycorrhizal associations may be ameliorating factors in Liberty State Park, though further

study is required to understand the impact of these factors. Further studies incorporating ecosystem approaches that consider interactions between plants, soil biota, resource presence, and heavy metals, are required to improve understanding of heavy metal impact on plant growth in sites of heavy metal contamination.

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Appendix A

Table 1 Comparison of gas exchange performance of *Populus deltoides* and *Populus tremuloides* across a metal contamination gradient in Liberty State Park.

	LML[a]		HML[a]		P-values*		
	PD[a]	PT[a]	PD	PT	*S*	*ML*	*S:ML*
V_{cmax}	65.2(5.7)	61.9(4.8)	83.0(16.0)	77.4(6.9)	0.43	0.14	0.91
J_{max}	101.1(6.9)	81.5(3.8)	102.5(19.1)	87.7(3.2)	**0.04**	0.88	0.74
TPU	7.7(0.52)	5.9(0.32)	7.3(1.4)	6.1(0.18)	0.051	0.75	0.70
g_m	11.1(2.4)	14.5(2.1)	9.3(2.5)	8.9(1.7)	0.25	0.85	0.39
CE	0.056(0.004)	0.079(0.015)	0.092(0.034)	0.069(0.007)	0.49	0.15	0.23
CCP	68.3(4.9)	61.2(5.5)	83.7(13.7)	51.4(3.1)	0.69	**0.05**	**0.008**
A_{max}	10.7(0.59)	10.6(0.59)	10.1(1.00)	9.4(0.54)	0.72	0.53	0.74
Φ	0.069(0.001)	0.069(0.003)	0.063(0.002)	0.068(0.003)	0.94	0.14	0.26
R_D	1.7(0.09)	1.46(0.16)	1.8(0.18)	1.43(0.11)	0.08	0.78	0.75
LCP	27.2(1.7)	21.7(2.3)	33.0(3.9)	22.0(1.4)	**0.02**	0.13	0.21
Transp	3.1(0.17)	2.4(0.17)	2.6(0.19)	2.5(0.14)	**<0.001**	0.13	**0.04**
C_i/C_{ainst}	0.68(0.02)	0.61(0.01)	0.69(0.03)	0.62(0.01)	0.15	0.62	0.79
WUE	3.9(0.25)	4.4(0.14)	3.5(0.32)	4.3(0.14)	0.55	0.48	0.81
iWUE	64.5(5.5)	83.3(3.4)	63.8(8.6)	81.3(3.2)	0.09	0.75	0.82

Values are means and standard errors, and are defined as follows: Maximum carboxylation rate as limited by Rubisco (V_{cmax}; μmol m^{-2} s^{-1});

maximum electron-transport-limited carboxylation rate (J_{max}, μmol m^{-2} s^{-1}); carboxylation rate as limited by triose-phosphate use (TPU, μmol

m^{-2} s^{-1}); mesophyll conductance (g_m, μmol m^{-2} s^{-1} Pa^{-1}); carboxylation efficiency (CE, μmol CO_2 m^{-2} s^{-1} ppm^{-1}); CO_2 compensation point (CCP,

Appendix A

ppm); maximum rate of net photosynthesis (A_{max}, µmol CO_2 m^{-2} s^{-1}); quantum yield (Φ, µmol CO_2 µmol^{-1} photons); dark respiration rate (R_D,

µmol CO_2 m^{-2} s^{-1}); light compensation point (LCP, µmol photons m^{-2} s^{-1}); transpiration (Transp,

mmol H_2O); C_i/C_{ainst}, ratio of intercellular CO_2 to ambient CO_2; photosynthetic water use efficiency (WUE, mmol CO_2 mmol^{-1} H_2O); intrinsic

water use efficiency (iWUE, µmol CO_2 mmol^{-1} H_2O)

*P-values indicate significant difference at α=0.05 (in bold) between gas exchange performance of species (S), metal load (ML), and for an

interaction between S and ML (S:ML) based on a linear mixed effects model; n =65, 70, 33, and 72 for PD LML, PT LML, PD HML, and PT

HML, respectively

[a]LML, low soil metal concentration; HML, high soil metal concentration; PD, *Populus deltoides*; PT, *Populus tremuloides*

Appendix A

Table 2 Comparison of leaf composition between *Populus deltoides* and *Populus tremuloides* across a soil metal contamination gradient in Liberty State Park.

	LML[a]		HML[a]		P-values*		
	PD[a]	PT[a]	PD	PT	S	ML	S:ML
C	45.6(0.17)	48.3(0.24)	45.6(0.53)	47.0(0.31)	**<0.0001**	0.93	0.051
N	2.2(0.06)	2.5(0.06)	2.2(0.09)	2.5(0.06)	**0.001**	0.45	0.75
TLN	0.05(0.03)	0.01(0.008)	0.04(0.02)	0.005(0.002)	0.29	0.86	0.61
C:N	21.0(0.74)	19.8(0.46)	21.3(1.0)	19.4(0.43)	**0.03**	0.43	0.27
$\delta^{13}C$	-29.9(0.14)	-30.0(0.10)	-30.9(.23)	-29.7(0.15)	0.83	**<0.001**	**<0.0001**
$\delta^{15}N$	-0.17(0.14)	-2.3(0.07)	-1.5(0.12)	-1.8(0.12)	**<0.0001**	**<0.0001**	**<0.0001**
Δ (‰)	17.9(0.15)	18.0(0.10)	19.0(0.24)	17.7(0.16)	0.83	**<0.001**	**<0.0001**
$C_i/C_{a,isotope}$	0.60(0.006)	0.60(0.004)	0.64(0.01)	0.59(0.007)	0.83	**<0.001**	**<0.0001**
$iWUE_{isotope}$	99.5(1.6)	98.9(1.2)	88.3(2.7)	102.3(1.8)	0.83	**<0.001**	**<0.0001**
NUE	6.2(0.39)	5.9(0.40)	6.1(0.82)	6.1(0.37)	0.52	0.98	0.72

Values are means and standard errors, and are defined as follows: Leaf carbon concentration (C, %); leaf nitrogen concentration (N, %); total leaf nitrogen content (TLN, kg); C:N, leaf carbon to nitrogen ratio; $\delta^{13}C$, isotopic carbon ratio; $\delta^{15}N$, isotopic nitrogen ratio carbon isotopic discrimination (Δ, ‰); $C_i/C_{a,isotope}$, isotopic ratio of intercellular CO_2 to ambient CO_2; $iWUE_{isotope}$, isotopic intrinsic water use efficiency (μmol CO_2 mmol^{-1} H_2O); nitrogen use efficiency (NUE, μmol CO_2 g^{-1} N s^{-1})

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*P-values indicate significant difference at α=0.05 (in bold) in leaf composition between species (S), metal load (ML), and for an interaction between species and metal load (S:ML), respectively, based on a linear mixed effects model; n = 65, 70, 33, and 72 for PD LML, PT LML, PD HML, and PT HML, respectively, except for TLN, where n = 6, 4, 7, and 11 for PD LML, PT LML, PD HML, and PT HML, respectively

[a]LML, low soil metal concentration; HML, high soil metal concentration; PD, *Populus deltoides*; PT, *Populus tremuloides*

Appendix A

Table 3 Comparison of biomass parameters between *Populus deltoides* and *Populus tremuloides* across a soil metal contamination gradient in Liberty State Park.

|	LML[a]		MML[a]		HML[a]		P-values*					
	PD[a]	PT[a]	PD	PT	PD	PT	*DBH*	*S*	*ML*	*Age*	*S:M*	*S:A*
DBH	8.8(3.3)	7.0(1.9)	4.0(1.0)	4.9(1.8)	8.9(2.8)	3.6(0.9)	n/a	0.57	0.059	**<0.001**	0.72	0.55
H	7.2(1.6)	7.7(1.2)	4.8(0.6)	6.4(1.8)	8.0(1.4)	5.1(1.0)	**<0.0001**	0.58	0.50	0.89	0.46	0.07
TBW	6.8(5.2)	3.4(2.1)	2.5(1.9)	3.2(2.1)	11.1(7.9)	1.2(0.42)	**<0.0001**	**0.04**	**0.04**	0.54	0.06	0.06
TLW	2.6(1.6)	0.69(0.33)	0.78(0.52)	0.49(0.26)	1.9(0.99)	0.21(0.09)	**<0.0001**	0.059	0.11	0.94	0.55	0.93
LA	34.1(21.6)	8.6(3.9)	10.8(7.0)	6.0(3.2)	25.0(12.5)	2.6(1.0)	**<0.0001**	0.98	**0.03**	0.52	0.26	0.79
TTM	30.2(21.3)	13.5(8.8)	9.4(7.5)	13.1(8.6)	35.6(22.6)	4.4(2.2)	**<0.0001**	0.74	0.07	0.09	0.69	0.94

Values are means and standard errors, and are defined as follows: Diameter at breast height (DBH, cm); tree height (H, m); total branch weight

(TBW, kg); total leaf weight (TLW, kg); leaf area (LA, m^2); total tree mass (TTM, kg)

*P-values under 'DBH', 'S', 'ML', and 'Age', 'S:M', and 'S:A' indicate significant difference at α=0.05 (in bold) between means due to DBH,

species (S), soil metal load (ML), age, and for interactions between species and metal load (S:M) and species and age (S:A), respectively, based

on a linear mixed effects model; n = 6, 4, 7, 7, 7, and 11 for PD LML, PT LML, PD MML, PT MML, PD HML, and PT HML, respectively

[a]LML, low soil metal concentration; MML, medium soil metal concentration; HML, high soil metal concentration; PD, *Populus deltoides*; PT,

Populus tremuloides

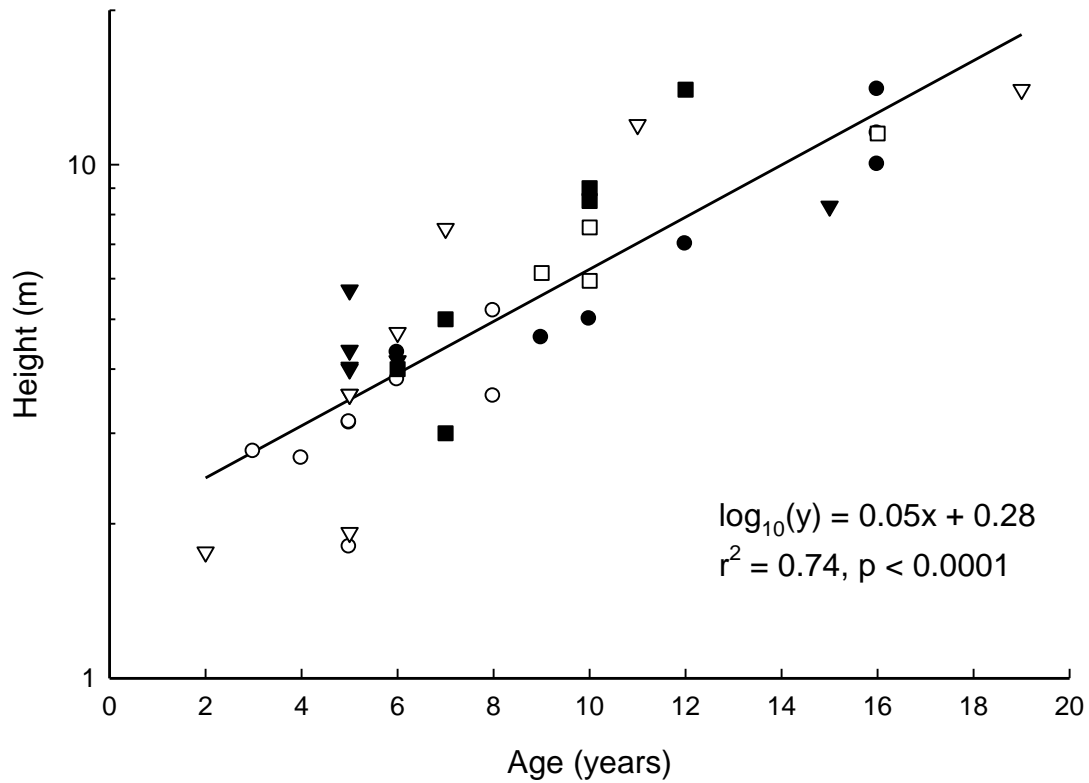Figure 1 Relationship between diameter at breast height (DBH) and tree age for *P. deltoides* (PD) and *P. tremuloides* (PT) in high soil metal load (HML), medium soil metal load (MML), and low soil metal load (LML).
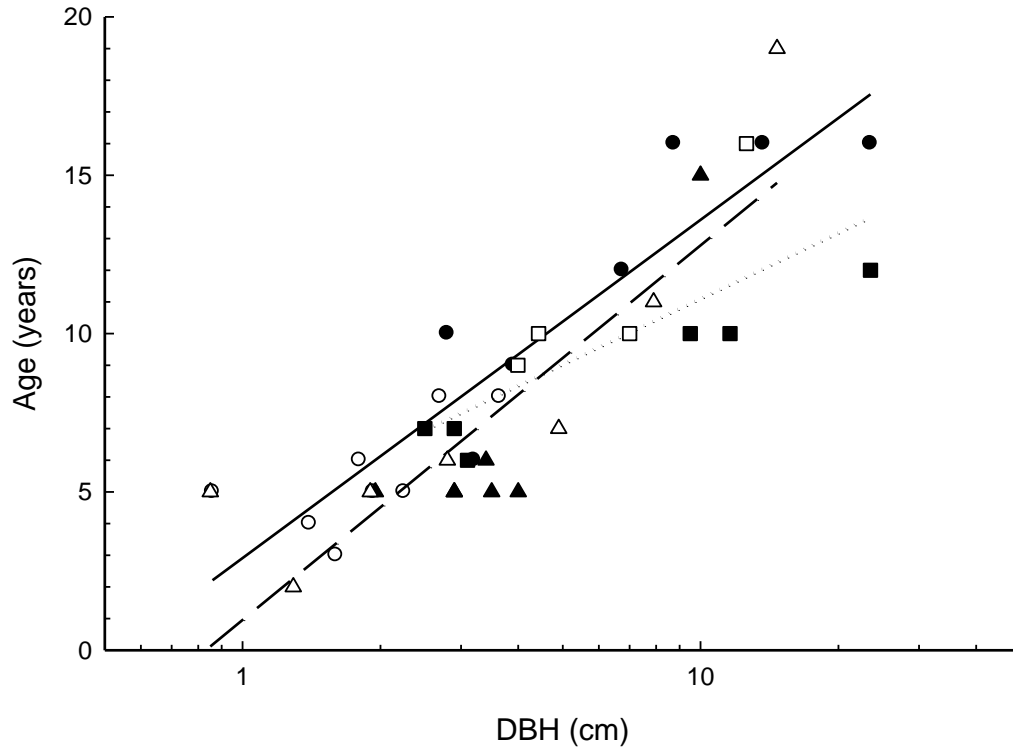
Closed symbols represent *P. deltoides* and open symbols represent *P. tremuloides*. HML is indicated by circles and solid lines, MML is indicated by triangles and dashed lines, and LML is indicated by squares and dotted lines. Separate linear regressions were fitted to each soil metal load category with both species combined as species did not differ within the same metal load category: y=10.6x+2.9 ($r^2 = 0.85$, P < 0.0001) for PD and PT in HML (n=7 and 8, respectively), y=11.8x+0.95 ($r^2 = 0.73$, P < 0.0001) for PD and PT in MML (n=7 and 7, respectively), and y=6.9x+4.2 ($r^2 = 0.61$, P = 0.007) for PD and PT in LML (n=6 and 4, respectively). Separate linear regressions (not shown) were calculated for PD

in MML and PD in LML as PD differed significantly between MML and LML, where

y=15.3x-1.9 (r^2 = 0.80, P = 0.005) for PD in MML (n=6) and y=5.6x+4.1 (r^2 = 0.94, P =

0.001) for PD in LML (n=4). Note logarithmic scale used on x-axis.

Figure 2 Relationship between tree age and height for *P. deltoides* (PD) and *P. tremuloides* (PT) in high soil metal load (HML), medium soil metal load (MML), and low soil metal load (LML).

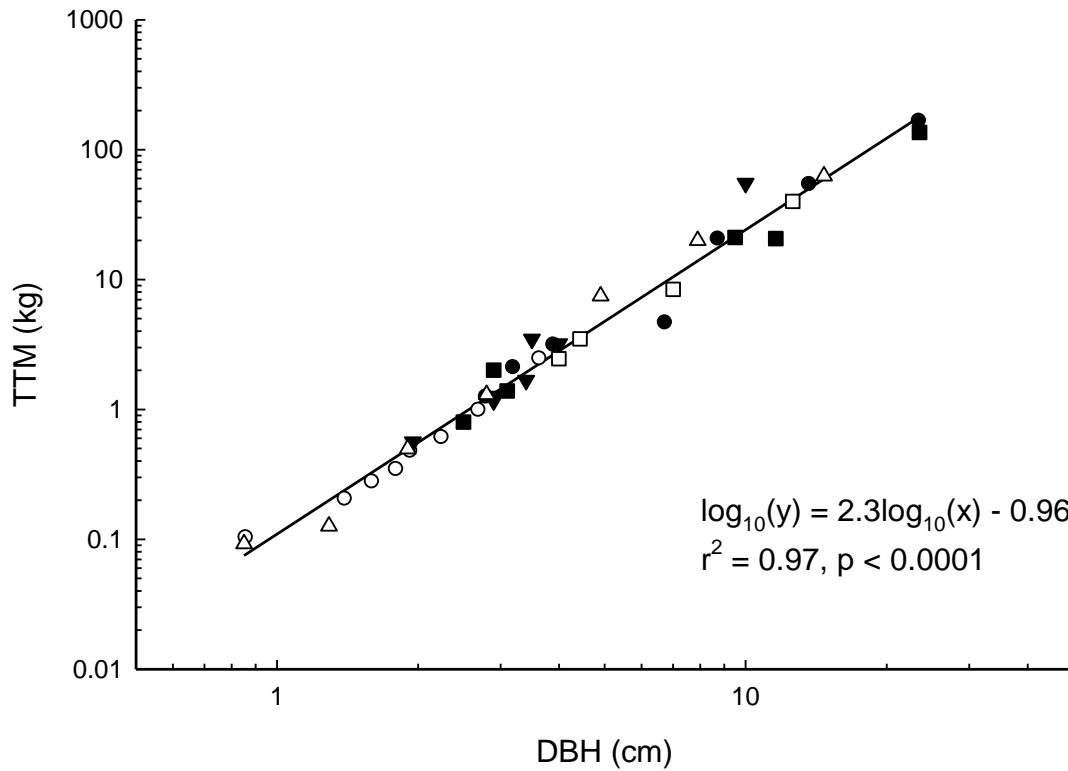$$\log_{10}(y) = 0.05x + 0.28$$
$$r^2 = 0.74, p < 0.0001$$

Squares represent LML, triangles represent MML, and circles represent HML, while closed symbols indicate *P. deltoides* and open symbols indicate *P. tremuloides*. For PD in HML, PT in HML, PD in MML, PT in MML, PD in LML, and PT in LML, n = 7, 8, 7, 7, 6, and 4, respectively. A single linear regression was applied to all data points in the plot. Note logarithmic scale used on y-axis.

Figure 3 Relationship between tree age and leaf area for *P. deltoides* and *P. tremuloides* in high soil metal load (HML), medium soil metal load (MML), and low soil metal load (LML).
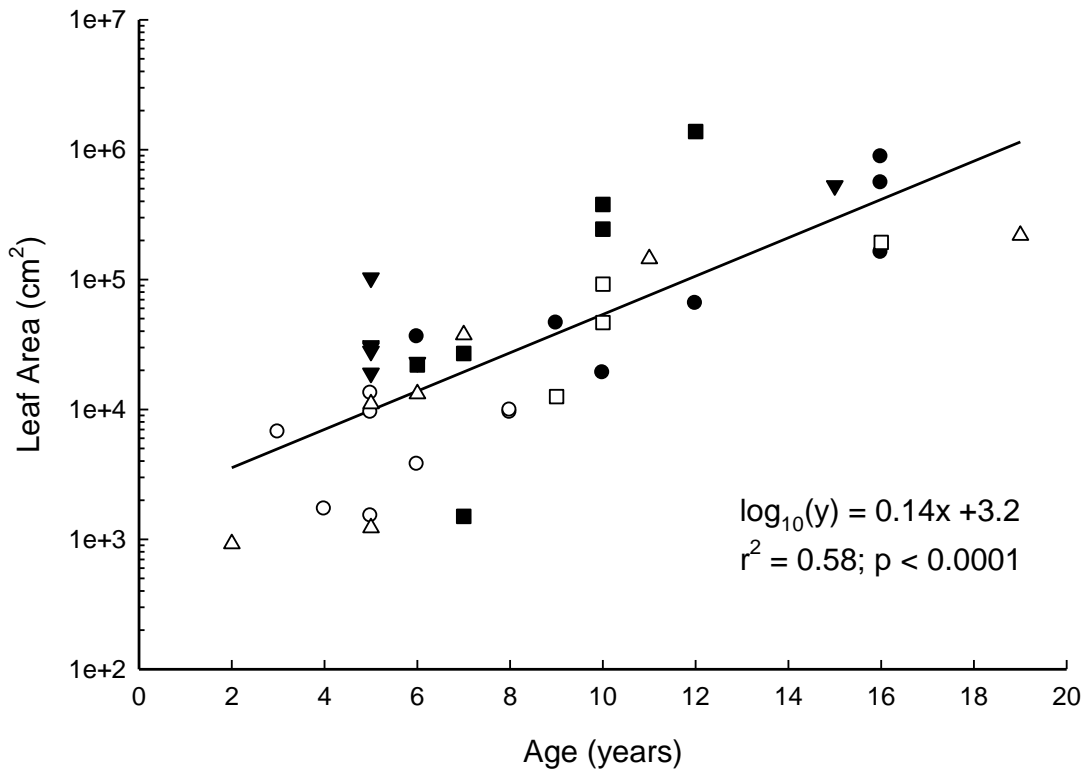
Squares represent LML, triangles represent MML, and circles represent HML, while closed shapes indicate *P. deltoides* and open shapes indicate *P. tremuloides*. For PD in HML, PT in HML, PD in MML, PT in MML, PD in LML, and PT in LML, n = 7, 8, 7, 7, 6, and 4, respectively. A single linear regression was applied to all data points in the plot. Note logarithmic scale used on y-axis.

Figure 4 Relationship between diameter at breast height (DBH) and total tree mass (TTM)

for *P. deltoides* and *P. tremuloides* in high soil metal load (HML), medium soil metal load

(MML), and low soil metal load (LML).

$$\log_{10}(y) = 2.3\log_{10}(x) - 0.96$$
$$r^2 = 0.97, p < 0.0001$$

Squares represent LML, triangles represent MML, and circles represent HML, while closed

shapes indicate *P. deltoides* and open shapes indicate *P. tremuloides*. For PD in HML, PT

in HML, PD in MML, PT in MML, PD in LML, and PT in LML, n = 7, 8, 7, 7, 6, and 4,

respectively. A single linear regression was applied to all data points in the plot. Note

logarithmic scale used on x- and y-axis.