URBANIZATION IMPACTS ON RED MAPLE (Acer rubrum L.)

IN DECIDUOUS FORESTS EMBEDDED IN CITIES

by

Covel Rashique McDermot

A dissertation submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Plant and Soil Sciences

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ABSTRACT

The urban forest should be viewed as living 'public health' and an environment 'bio-recording' infrastructure in urban environments. This infrastructure is extremely vital to achieving 'greener' cities, as the globe becomes more urbanized. However, plant data supporting urban plants as environmental mitigating infrastructures are sparse, inconsistent, and not long-term in approach. Moreover, the lack of data on the physiology, biochemistry, morphology, and long-term stress adaptation of plants in urban environment is a current gap in urban plant-ecology research. Cities are a natural 'open lab', and city size can be a proxy for multiple cooccurring impacts on plants that determine productivity and acclimation to better assess plant effectiveness. The FoRests Among Managed Ecosystem (FRAME) network was used to investigate urbanization (e.g., city size) impacts on the physiobiochemistry and morphology of red maple (Acer rubrum L.) in urban forests in a small (Newark, DE) and large (Philadelphia, PA) city. In addition, a manipulated field experiment was used to decipher above- and below-ground urbanization impacts on the physio-morphology of red maple (A. rubrum) saplings in urban forests. I measured health indicating pigments, nutrient ions, stress signaling metabolites, and morphological traits that can integrate multiple stress impacts and provide insight into urban forest trees' ability to effectively mitigate urban conditions. I found greater chlorophyll, free amino acids and spermine, nutrient ions (N, P, K), and stomatal size in red maples in Philadelphia forests relative to Newark forests suggesting these traits

are good indicators and integrators of multiple co-occurring stress impacts on plant growth, productivity, and acclimation in the large city. The results indicate cities as a surrogate and red maple as a biomonitor for urbanization impacts can be a good model system to study urban plant physiology and long-term stress mitigation-adaptation. The findings showing greater physio-biochemical and morphological response in the large city suggest theoretical models that do not account for physiological acclimation are overestimating deciduous forest tree mitigating potential in urban environments.

Keywords: Acclimation, *Acer rubrum* L., Amino acids, Biomonitor, Chlorophyll, City size, Heavy metal, Nitrogen deposition, Nutrients, Polyamine, Stomate size, Subcanopy soil, Urban forests, Urban heat island, Urbanization

INTRODUCTION

1.1 Background

Globally, approximately 2% of the earth's land surface is now urbanized and more than 50% of the global community now inhabits urban environments; this is expected to increase to 70% by 2050, compared to 10% at the beginning of the 20th century (Yule et al. 2015; UN 2014). Interestingly, more than 3% of the U.S. land area is already urbanized and it is expected to increase to about 8% by 2050 (Nowak and Walton, 2005). In addition, more than 80% of the population already lives in cities and associated suburban areas in the U.S. (UN 2014). While the urban land area is a relatively small proportion of total land area, the high population leads to dense urban living that can lead to proportionally greater ecological and environment ramifications, such as altered materials (e.g., nutrients) and energy fluxes, water limitation, and elevated pollution at fine and large scales. Thus, there is a need to explore the characteristics and impacts of urbanization on ecosystem function.

Urbanization is a fast-growing, unplanned experiment that has reconfigured landscape surfaces and transformed materials, water, and energy resulting in altered environmental conditions that mimic global environmental change, such as altered climate conditions, altered biogeochemical cycling, and biotic invasions in urban ecosystems (McDonnell and Pickett, 1990; Grimm et al. 2008). Urban environments are characterized by higher temperatures due to extensive impervious surface cover (urban heat island effect; Idso et al. 2001, 2002), altered precipitation (Ma et al. 2012; Ahn and Steinschneider, 2019), point and non-point source pollution (e.g., nutrients, heavy metals, salts and nitrogen deposition; Lovett et al. 2000, Pouyat and

McDonnell, 1991; Manta et al. 2002; Pouyat et al. 1997; Trammell et al. 2011), and non-native biotic invasion (Trammell et al. 2012). Cities experience differing magnitudes of urban intensity, therefore, city size (i.e., metro-scale) can be used as a surrogate or natural 'open lab' for classifying differing magnitude of urbanization impacts and urbanization gradients (Oke 1973; Farrell et al. 2015; Lahr et al. 2018). In addition, the urban ecosystems and organisms can be excellent bio-monitors for investigating simultaneous and cumulative global change impacts on the sustainability and resilience of cities and associated greenspaces.

Urban forests and trees are extremely vital to the livelihood, sustainability, and resilience for achieving 'greener' cities (Colding and Barthel, 2013). This living green infrastructure provides well-needed ecosystem services (e.g., carbon sequestration; Cheng 2015; Velasco et al. 2016), environmental benefits (e.g., cleaner air; Xing and Brimblecombe, 2019), recreational activities (e.g., hiking; Smardon 1998), and public health eco-therapies (e.g., depression management; Smardon 1998). The urban forests and trees should be viewed as a living 'public health' and environment 'bio-recording' infrastructure in urban environments. Interestingly, the issues and challenges that confront urban environments can have local-, regional- and global-scale impacts, yet these poorly maintained ecosystems are largely human influenced. Therefore, the reality of 'greener cities' can only be achieved through indigenous knowledge and projects that involve a diverse network of natural scientists, health scientists, social scientists, engineers, and non-scientists (e.g., citizenry). Importantly, to address these scales of impacts and prepare for global projections in urban and suburban landscapes, a robust multidisciplinary approach coupled with transdisciplinarity is necessary to

contribute towards a growing trend of understanding in urban ecology and moving the field towards ecology "for" the city (Schröder et al. 2019).

Our understanding of the combined and successive impacts of multiple cooccurring global change factors on trees and forests is still nascent in real-world settings (i.e., *in situ*) due to the difficulty in conducting long-term manipulated experiments mimicking global change. However, previous research has shown inconsistent tree response to urbanization such as enhanced and suppressed tree growth (Gregg et al. 2003, Sonti et al. 2019). In addition, tree species responses and long-term adaptation to urban conditions in urban forests have received very little attention in urban-plant ecology research (Calfapietra et al. 2015). Furthermore, studying city size (i.e., metro-scale) and site-scale (i.e., above- and belowground) impacts can be used to answer questions about tree physiology, biochemistry, morphology, survival and adaptability, and identify indicators to greater urban intensities on the ecological processes in urban forests, a current gap in urban-plant ecology research.

Metro-scale impacts may overrule some direct site-scale conditions and drive response patterns in trees and the edaphic environment. Additionally, the interactive effect of metro-scale impacts and site-scale conditions can also drive tree response patterns and ecosystem processes due to differing above- and belowground complexities (Figure 1.1). Some metro-scale abiotic aboveground (e.g., elevated N deposition, temperature, CO₂, oxidize gases, and heavy metals, and altered precipitation) factors that directly impact plants eventually move to soils (e.g., direct soil impact, litterfall input, root reabsorption) and become direct or indirect belowground drivers of soil properties overriding some direct belowground (e.g., N

mineralization rate, SOM, soil C/N ratio, soil pH, bulk density, nutrient/heavy metal biogeochemistry, soil moisture) influences that shape trees to urban conditions (Figure 1.1). Finally, there is the need to conduct long-term *in situ* tree physiology, biochemistry, and morphology studies in forests embedded in cities to investigate plant response to metro-scale and site-scale impacts on above- and below-ground drivers of change, a current gap in to urban plant-ecology research.



Figure 1.1 Experiment approach to investigate urbanization and site-scale (aboveand below-ground) impacts on the physio-biochemistry and morphoanatomy of *in situ* adult red maple (*Acer rubrum*) trees, and role of above- and below-ground impacts on the physio-morphology of red maple saplings in temperate deciduous forests embedded in a small (Newark, DE) and large (Philadelphia, PA) city.

1.2 Dissertation Summary

1.2.1 Urbanization and Site-level Impacts on the Physio-biochemistry of Red Maple Trees in Temperate Deciduous Forests

It is now widely accepted that trees in urban environments respond to the altered urban conditions (Gregg et al. 2003; Livesley et al. 2016; Lahr et al. 2018; Steele and Wolz, 2019), largely a contribution by anthropogenic influences. A number of studies have measured leaf physiology (such as chlorophyll, carotenoids), biochemistry (e.g., amino acids, polyamines), nutrition (e.g., N, P, K, Mg, Ca, Fe, Mn, Zn, Cu), morphology (e.g., leaf area, leaf thickness, stomatal density, stomatal size) and heavy/toxic metals (e.g., Na, Al, Mn, Cu, Zn, Co, Cr, As, Pb, Se) in trees in contrasting environments (e.g., Minocha et al. 2000; Pal et al. 2002; Vallano and Sparks, 2013; Falxa-Raymond et al. 2014; Gao et al. 2016; Du et al. 2017; Molnár et al. 2018; Sonti et al. 2019). However, *in situ* field studies investigating the physiology and long-term adaptation of plants living in urban environments experiencing differing magnitudes of urban intensity are sparse (Calfapietra et al. 2015) and plant responses reported are usually inconsistent (e.g., Gregg et al. 2003, Lahr et al. 2018; Molnár et al. 2018; Sonti et al. 2019). Furthermore, there are few studies investigating *in situ* leaf physio-biochemistry of plants in forests embedded in cities. Moreover, understanding the physio-biochemistry response of forest tree species to altered urban conditions can provide insights into the capacity of urban forest trees and urban trees to mitigate, acclimate, and indicate urban pollution impacts in urban environments. In Chapter 2, I examine leaf chlorophyll, carotenoids, free amino acids, free polyamines, nutrients, and heavy metals of red maple trees (Acer rubrum) in forests in a small (e.g., Newark, DE) and a large city (e.g., Philadelphia, PA) experiencing differing magnitudes of urban intensities (i.e. population, house, developed land use and land cover,

impervious surface). I hypothesized that red maples in Philadelphia forests compared to Newark forests will have higher foliar pigments, nutrients, and heavy metals, and more enriched δ^{15} N isotope due to greater nutrient and metal inputs, urban heat island, and elevated atmospheric CO₂ that create stronger above- and below-ground controls with respect to city size leading to stress acclimation (Figure 1.1). I also hypothesized higher foliar polyamines and amino acids, and nutrients (K, P, Zn) in trees in Philadelphia forests relative to Newark forests would indicate elevated stress mitigating tolerance to the cumulative impacts of a large city.

1.2.2 Use of a Manipulative Field Experiment to Investigate the Role of Metro-Scale and Site-scale Impacts on Red Maple (*Acer rubrum*) Physiology and Morphology in Urban Forests

Use of manipulative field experiments exploiting saplings as biomonitors and soil translocation in urban forests can allow for studies across urban gradients to better understand *in situ* tree response in urbanized settings. In *Chapter 3*, I utilized a manipulative field experiment to comprehend and elucidate the role of metro-scale impacts and site-level (e.g., aboveground and belowground) conditions that drive physiology and morphology of plants in urban forests. Several studies have used reciprocal soil transplant experiments (Sun et al. 2013) and potted saplings/trees experiments (Gregg et al. 2003) in the field to demonstrate the impact of complex belowground and aboveground influences on the physiology and morphology of plants to key environmental factors. If the transplant experiment can be successfully implemented in the field for plant physiology and morphology research, then this will offer a model approach to understand the role of city impacts on above- and below-ground conditions that influence the physiology and morphology of plants.

drivers controlling soil processes and plants ability to adapt to the local environmental conditions. To my knowledge, there is no study utilizing this experimental design to compare city size impacts on aboveground and belowground conditions that influence red maples, a widely adapted species. In Chapter 3, I present a study using the transplant experimental approach with red maple saplings (*A. rubrum*) as biomonitors for above- and below-ground urban conditions, in a small (e.g., Newark, DE) and a large (e.g., Philadelphia, PA) city.

1.2.3 Urbanization Impacts on Leaf Morpho-anatomy Traits in Red Maple Trees in Temperate Deciduous Forests

Leaf morphology and anatomy of trees can change with pollution, yet little is known about how multiple global change factors, such as climate and pollution, will influence leaf morpho-anatomy. Leaf morpho-anatomy responses in trees are a good indicator of local site conditions and regional scale climate impacts (Gray et al. 2000; Pal et al. 2002; Miller-Rushing et al. 2009; Pourkhabbaz et al. 2010; Urban et al. 2017). Therefore, leaf structural traits of plants growing in highly urbanized areas can be an ideal study system for understanding how multiple environmental changes influence plant anatomy and morphology, which is an important determinant of plant health and function. In *Chapter 4* of this dissertation, I investigated metro-scale impacts of urban heat island, reactive N deposition inputs, and elevated CO₂ on leaf anatomy and morphology of red maple trees growing in urban forests. My goal is to compare metro-scale impacts on leaf morpho-anatomy traits in red maple (*Acer rubrum*) trees in deciduous forests in a small (Newark, DE) and a large (Philadelphia, PA), in an attempt to complement the physio-biochemical study in *Chapter 2*. I measured fresh leaf weight, dry leaf weight, specific leaf area, leaf thickness, leaf dry

matter content, leaf water content as morphology traits, and upper epidermal thickness, palisade mesophyll thickness, spongy mesophyll thickness, stomata density, and stomate length and width as anatomy traits.

Chapter 2

RED MAPLE (Acer rubrum L.) TREES DEMONSTRATE ACCLIMATION TO URBAN CONDITIONS IN DECIDUOUS FORESTS

2.1 Abstract

Urbanization drives important shifts in environment conditions, such as urban heat island (UHI) and nutrient loads, with high potential impact on plant function. Our goal was to compare stress mitigating capacity in red maple (Acer rubrum L.) trees in deciduous forests embedded in a small (Newark, DE, US) and a large (Philadelphia, PA, US) city. The study was conducted in the FoRests Among Managed Ecosystems (FRAME) network on seventy-nine mature red maple trees spanning ten urban forests in Newark and Philadelphia. We hypothesized that red maples in forests in Philadelphia compared to Newark will have higher foliar pigments, nutrients, and stress-inducible elements, more enriched δ^{15} N isotopes, and increased free polyamines and amino acids in response to multiple cumulative stimulating and stress-inducible impacts at the city level. Our results indicate red maples are much better acclimated to multiple stress factors in forests in Philadelphia where there is a greater magnitude of urban intensity. Red maples showed higher concentrations of foliar chlorophyll, %N, δ^{15} N and nutrient elements in Philadelphia forests than in Newark forests. Similarly, lower foliar magnesium and manganese, and higher foliar zinc, cadmium, lead, and aluminum reflected the altered soil biogeochemistry in Philadelphia forests. Accumulation patterns of foliar free amino acids, polyamines, and K⁺ ions in red maples in Philadelphia forests revealed a reshuffle in cellular metabolite-nutrient

metabolism pathways responsible for physiological acclimation. Our results validate the study as a model approach for studying 'urban plant physiology' and utilizing urban trees as a biomonitor of the 'urban pollution' impacts on urban forests. The results from this study suggest nutrient loads and UHI overrule potential stress causing pollutants by amino acid-polyamine-induced acclimation. Our study provides a framework for determining whether trees respond to complex urban environments through dampened physiology or acclimation.

2.2 Introduction

The magnitude of urbanization across cities alters abiotic conditions that can influence tree physio-metabolism, which has global implications for urban forest health (Gregg et al. 2003; Falxa-Raymond et al. 2014; Pretzsch et al. 2017) as cities across the world continue to expand (Argüeso et al. 2014). Cities are an ideal 'natural experiment' for assessing the cumulative impacts of current global change (Grimm et al. 2008; Farrell et al. 2015; Calfapietra et al. 2015; Lahr et al. 2018). Forest trees are subjected to increased atmospheric reactive nitrogen (Nr) deposition inputs (Nadelhoffer et al. 1999; Craine et al. 2009; Vallano and Sparks, 2013), greater carbon dioxide (CO₂) concentrations (Stitt and Krapp, 1999; Ziska et al. 2004), and higher temperatures (Moser et al. 2017; Founda and Santamouris, 2017) that have the potential to stimulate growth. However, increased soil heavy metal concentrations (Pouyat et al. 1991; Manta et al. 2002) and reduced soil moisture (Livesley et al. 2016) due to urban heat island have the potential to stress trees in urban forests. Markert (1992) has established the element content of 'reference plant' for inorganic chemical fingerprinting that identify threshold levels of elements. However, Krämer (2010) has reported many stress-inducible metals having no reported threshold deficiency levels,

but critical toxicity levels have been identified. Tree response to above- and belowground conditions in urban landscapes can provide valuable insight into the physiobiochemical response patterns at the leaf level that control whole tree growth response (Gregg et al. 2003; Rossini et al. 2006; Norton et al., 2015; Sonti et al. 2019).

Trees in cities experience a wide range of environmental pressures that create both growth stimulating and stress inducing conditions (Table 2.1). For example, the urban heat island can stimulate plant growth (Livesley et al. 2016; Founda and Santamouris, 2017; Sonti et al. 2019), or alternatively lead to soil and tree moisture stress (Cregg and Dix, 2001; Breda et al. 2006). In addition, elevated atmospheric reactive nitrogen (N) deposition can stimulate plant growth or induce nutrient deficiencies depending upon the initial N status (land use history) of a site (Craine et al. 2002; Silva et al. 2015; Zhang et al. 2017). Interestingly, Vallano and Sparks (2013) have reported significant impacts of direct foliar uptake of gaseous reactive N on foliar δ^{15} N in red maple and other tree species. Similarly, storm run-off, wet and dry deposition, and environmental legacies can exacerbate soil nutrient and toxin levels that can either negatively or positively impact plant growth (Pouyat and McDonnell, 1991; McDonnell et al. 1997; Bignal et al. 2007).

Within individual tree species and genotypes, leaf-pigment and leafbiochemical traits may be a response to above- and below-ground conditions due to current environmental tolerances (Minocha et al. 1997; Kolb and Evans, 2002; Sharma and Diez, 2006; Lichtenthaler et al. 2007). The metabolic response of an individual tree is controlled primarily by the magnitude of environmental pressure intensity at the tree-soil scale that ultimately determines the level of productivity, oxidative stress signaling and stress mitigation, and physiological acclimation in the plant (Table 2.1).

Since above- and below-ground conditions of urban environments simultaneously impact the physiology and biochemistry of trees in urban forests, there might be a tight coupling of soil-to-leaf-pigment and soil-to-leaf-biochemical patterns. This integrated response to soil biogeochemistry and air chemistry that can be reflected in the leaf chemistry and metabolic trait responses is largely dependent on the local magnitude of the urbanization intensity.

Bioindicator	Growth indicator	Stress indicator	Acclimation trait
Cell chlorophyll (Chl)	Increase chl: greater light capture and higher photosynthesis rate	Low Chl:Car: Greater antioxidant ability	Elevated Chl and Car contents
Cell carotenoids (Car)	High Chl:Car Increase photosynthesis	Low Chl:Car: Increase antioxidation	Elevated Car content
Nutrients (in soil and plant cell)	Increase macro: Ca, P, S (enzyme and ATP synthesis); K for extrachloroplastic K ⁺ , Mg (chlorophyll biosynthesis), adequate quantity of N (amino acids and protein synthesis). Threshold level micro: Zn, Mn, Cu, Ni, Se, Fe	Lower than threshold quantities of Ca, K, N; Very high Zn, Mn, Cu; low Ca/Mg; high K:Ca; low Ca/Al; low Mg/Mn; high Zn/Fe; high P/Al; Mn/Ca	Elevated foliar K, P and Zn, Mn and Cu, Ca, and Mg and N

Table 2.1.Foliar metrics as bioindicators for growth or stress acclimation responses
to the urban conditions.

Table 2.1 -Continued

Bioindicator	Growth indicator	Stress indicator	Acclimation trait
Stress-inducible metals (in soil and plant cell)	Increase in Zn, Ni and Cu but within the tolerance range of tissues: increase in RuBisCO enzyme activity and increase in CO ₂ assimilation	Very high intolerable foliar Al, Cr, Cd, Co, Pb, Ni, Cu, Zn, Mn: increase in cellular reactive oxygen species	Elevated Zn, metal phyto-extraction/ accumulation
Polyamines: putrescine, spermidine, spermine (plant cell)	Putrescine, spermidine and spermine concentrations in normal range for these tissues: cell division and cell growth (biomass) with one exception of excess N	Very high Put, Spd and Spm: osmoprotection, heat, drought, temperature, salinity, acidity stress. All three polyamines high: antioxidation, signaling, excess N sequestration	Elevated putrescine, spermidine, and spermine contents
Amino acids: Arg, Thr, GABA, Pro, Gly, Orn, Gln (plant cell)	Amino acids concentrations within normal range for these tissues. No large accumulations	Very high Arg, Thr, Pro, GABA, Gly, Gln: Excess N sequestration, antioxidation, stress signaling, metal chelation	Elevated amino acids (Big five) contents: Arg, Thr, GABA, Pro, Gly

Elevated leaf pigments (e.g., chlorophyll) and accumulation of nitrogenmetabolites such as free amino acids and free polyamines may also represent accompanying shifts in the allocation of nonstructural carbohydrates (NSCs), pathways and mechanisms for maintenance of basal metabolism, stress mitigation, and acclimation for plant productivity and survival (Wargo et al. 2002). Plant species that grow in adverse environmental conditions can exhibit morphological and physiological damage brought on by abiotic stressors (Näsholm et al. 1994; Minocha et al. 2014; Tubby and Weber, 2010; Fernando et al. 2016; Molnár et al. 2018). The physiological relationship between abiotic stress and nitrogen-utilizing amino acids and polyamines, as well as some exchangeable ions in plants has been explored and proposed as a suite of potential physio-biochemical indicators of persistent environmental stresses (Hoffman and Samish, 1971; Moschou et al. 2012; Sharma and Dietz, 2006; Winter et al. 2015; Majumdar et al. 2016). Observed increases in tolerance to abiotic stress when cellular polyamines, amino acids, and nutrients are elevated is indicative of a protective role (e.g., antioxidation, osmoprotection, signaling and metal-chelation) to cellular organelles. This results in the establishment of new cellular reactive oxygen/nitrogen species (ROS, RNS) homeostasis in trees in which phenotypic symptoms of stress are not already obvious (Moschou et al. 2002; Sharma et al. 2012; Shi et al. 2013; Minocha et al. 2014; Majumdar et al. 2016). Thus, leaf pigments, amino acids, polyamines, and nutrient ions can act as a suite of combined physiological biomarkers to altered biogeochemistry and atmospheric conditions in cities.

Many studies have focused on drivers of plant physiologic and metabolic traits (e.g., net photosynthesis rate, chlorophyll content, C/N ratio, polyamine content, nutrients and amino acids (e.g. Gregg et al. 2006; Groffman et al. 2006; Zhang et al. 2017) in contrasting environments and experimental forests. However, there is difficulty in conducting long-term manipulative experiments in situ that mimic global change impacts (e.g., urbanization) on the physio-biochemical plant traits (Calfapietra et al. 2015). Cities are experimentally underutilized and can serve as a surrogate to investigate different magnitude of urbanization intensities that impact *in situ* forest tree physiology and free metabolites leading to stress mitigating or acclimating responses to multiple stresses. Urbanization creates different magnitudes of urban intensity that vary with city size and are unique to the city. Thus, differing above- and below-ground controls related to city size (e.g., urban heat island intensity) can influence plant physiology in different pathways, and trees in urban forests can be biomonitors of these varying environment conditions and impacts. Research that exploits this understanding and an interdisciplinary approach will provide baseline scientific data on 'urban tree physiology' and develop the field. Previous work on tree response to urban conditions utilized a gradient approach of urbanization intensity from one large city (McDonnell et al. 1990; 1997; Gregg et al. 2006; Kaye et al. 2006; Ziska et al. 2004; Niinemets, 2010; Vallano and Sparks, 2013), yet the majority of cities in the U.S. have low-intensity land cover and more open-area developed spaces. Thus, to build collective understanding of how multiple environmental changes influence tree physiology, we need research that focuses on tree response in multiple size cities with differing magnitude of urban intensities (metro-scale impacts). Furthermore, a comparison of tree physiology and biochemistry in cities with different

sizes can help isolate the controls (e.g., metro-scale versus site-scale) on tree response to urban environmental conditions.

To understand the impacts of different magnitude of urbanization intensity on the physiology and stress mitigating-acclimating response (i.e., effects on pigments and free metabolites) in cities, we investigated mature native red maples in small deciduous forests embedded in two cities (i.e., Newark, DE and Philadelphia, PA). These cities differ in total population, developed land area, and impervious surface cover, and provide a natural experiment for discerning urban impacts on tree physiology and biochemistry response to urbanization. This research utilized a longterm urban forest ecology network, the FRAME (FoRests Among Managed Ecosystems), to study how red maples respond to urbanization in a small city (Newark, DE) and a large city (Philadelphia, PA). We measured foliar pigments (chlorophyll and carotenoids), foliar nitrogen (%N and δ^{15} N isotope), nutrient elements (e.g., Ca, Mg, P, K, S, Fe, Mn), stress-inducible elements (e.g., Ni, Zn, Al, Na, Cd, Cu, Co, Cr, Se, Pb), stress mitigating and signaling free polyamines (putrescine, spermidine and spermine), and free amino acids (e.g., Arg, Thr, Pro, GABA, Gly, Gln, Glu) in red maple trees (Table 2.1). We also measured the subcanopy soil nutrient elements and stress-inducible elements, soil bulk density, soil organic matter, and soil pH. We asked the following questions: 1) Do red maple foliar chlorophyll, carotenoids, chlorophyll/carotenoid ratio, nitrogen, nutrient, and stress metal concentrations in urban forests differ with city size? And 2) do observed differences in foliar and soil chemistry between cities indicate differing metabolic responses? We hypothesized that red maples in Philadelphia forests compared to Newark forests will have higher foliar pigments, nutrients, and stress-inducible

elements, and more enriched δ^{15} N isotope due to greater nutrient and metal inputs, urban heat island, and elevated atmospheric CO₂ that create stronger above- and below-ground controls with respect to city size leading to stress acclimation. We also hypothesized higher foliar polyamines and amino acids, and nutrients (K, P) in trees in Philadelphia forests relative to Newark forests would indicate elevated stress mitigating tolerance to the cumulative impacts of a large city tolerance. The results from this study provide valuable insight into how above- and below-ground conditions in cities drive differing patterns in tree response based on the magnitude of the (city size as a proxy for) urbanization intensity.

2.3 Materials and Methods

2.3.1 Study Species and Geographic Region

In the present study, we focused on mature native red maple (*Acer rubrum* L.) trees in ten forests in a long-term urban forest ecology study, the FRAME (FoRest Among Managed Ecosystems). We studied red maple trees in five forests in Newark, DE and five forests in Philadelphia, PA, U.S (Fig. 2.1). In 2009, researchers at the USDA Forest Service and University of Delaware selected forest sites located on public lands across the Coastal Plain and Piedmont region of the Mid-Atlantic for long-term establishment and research. The FRAME traverses an urban gradient extending across Newark, DE and Philadelphia, PA into nearby rural areas. Newark, DE has a population of 31,454 and a mean density of 1,403 people km⁻² and Philadelphia, PA has a population of 1,526,006 people and density of 4,405 people km⁻² (US Census Bureau, 2010). The forest canopy is dominated by native tree species in both cities: red maple (*Acer rubrum*), American beech (*Fagus grandifolia*), black

gum (*Nyssa sylvatica*), sweetgum (*Liquidambar styraciflua*), tulip poplar (*Liriodendron tulipifera*), red oak (*Quercus rubra*). The forest understory is dominated by non-native and native species: multiflora rose (*Rosa multiflora*), spicebush (*Lindera benzoin*), greenbrier (*Smilax rotundifolia*), Japanese honeysuckle (*Lonicera japonica*), southern arrowwood (*Viburnum dentatum*), autumn olive (*Elaegnus umbellata*), and sweet pepperbush (*Clethra alnifolia*) (Trammell et al., *in revision*).



Figure 2.1. Map of the USA east coast showing the location of Newark, DE and Philadelphia, PA where ten forests and seventy-nine red maple trees were selected and sampled in July 2016 and August 2017. Forests in Newark, DE: Folk (FO), Rittenhouse (RH), Chrysler Woods (CW), Ecology Woods (EW) and Webb Farm (WF). Forests in Philadelphia, PA: Smith Memorial (SM), Chamounix (CX), Park Line Dr (PL), Pennypack Dr (PP) and Walton's Run (WR).

2.3.2 Experimental Design, Tree Selection, and Sample Collection

For this study, we selected five small forests experiencing high levels of urbanization in Newark, DE (forest size: 4.0 - 16.3 ha) and in Philadelphia, PA (forest size: 3.5 - 7.0 ha); therefore, focusing this study on the impact of large-scale urbanization pressures associated with city size on tree physiology and metabolism. We randomly selected five or 10 red maple trees in five forests in Newark (n = 39) and Philadelphia (n = 40) on availability of red maples and proximity to each other. Seventy-nine adult, native red maple trees (diameter breast height [dbh]: 7.6 - 23 cm) were randomly sampled at low-canopy in late July 2016 and combined mid- and upper-canopy in August 2017 from our forests. Red maple trees in Newark (dbh: 13.7 ± 0.5 cm; p = 0.06) and Philadelphia (dbh: 12.2 ± 0.5 cm) forests did not differ significantly in mean dbh. The trees were located at least 10 meters from the forest edge, and many trees were near a creek and/or close to a trail. The trees sampled experienced sub-canopy shade as they were not tall enough to reach the upper forest canopy.

Leaf sampling was conducted at mid-upper-canopy of red maple trees (n = 79) using a pole pruner, a slingshot, or by climbing the tree in August 2017. Light-exposed leaf samples were stored at 4°C during collection in the field prior to transport to the laboratory. Leaf samples (n = 79) were also stored at -20°C for pigment analysis, and oven dried at 55°C for nutrient, stress-inducible metal, and %N and natural abundance δ^{15} N isotope analyses. Leaf disks from three to five fresh, clean leaves (n = 79) were collected with a paper puncher to obtain ~0.5 cm³ (~100 g) of tissue and stored in 5% perchloric acid (PCA) [PCA, 1 mL; stops biochemical reactions instantly] at 4°C prior to transport to laboratory and stored at -20°C until the analyses for polyamine, amino acid, and exchangeable ion.

Under each red maple tree, a composite of four soil cores (0-10 cm) were collected at the mid-point between the tree trunk and canopy edge for nutrient and stress-inducing metal analyses. One intact soil core (diameter = 5 cm, depth = 15 cm; n = 79) was also collected under each tree for soil bulk density (BD), soil organic matter (SOM) and soil pH measurements. Soil samples were oven dried at 105°C for dry soil BD measurement.

2.3.3 Foliar %N and natural abundance δ^{15} N isotope measurement

All leaves were oven-dried at 55°C for 48 h and subsamples (~ 3500 µg) were ground to a fine powder using a Retsch Ball Mixer Mill (MM200, Haan, Germany). Nitrogen concentrations (%) and δ^{15} N values (‰) were measured with the Costech EA (California) connected to a Thermo Delta V spectrometer (Thermo, Bremen, Germany) interfaced with an elemental analyzer. Two USGS standards (external) and one secondary standard (acetanilide, internal) were used, with precision less than 0.02‰ (Ladin et al. 2015). The natural abundance stable isotope values were expressed relative to the international standard (atmospheric N₂) in the conventional δ notation:

$$\delta^{15}N = [({}^{15}N_{sample}/{}^{14}N_{sample})/({}^{15}N_{standard}/{}^{14}N_{standard}) - 1] * 1000\%$$

2.3.4 Foliar chlorophyll and carotenoid concentration

Freshly frozen leaf samples were brought to room temperature and a plunger used to remove three discs (~ 8.5 - 11.5 mg). Dimethylformamide (DMF, 1 mL) was added to the leaf discs and shaken for 24 h in the dark for extraction, then transferred to a refrigerator at 4°C for 48 h for complete pigment extraction. The pigment extracts
were filtered and further diluted with DMF to attain absorbance values below 1 absorbance to obey Beer's Law. The absorbance was measured with a spectrophotometer (Thermo Evolution 60S UV-Visible, Waltham, MA, USA) at wavelengths 647 nm, 664 nm, and 480 nm, respectively, in triplicate. The total chlorophyll (a + b) and carotenoid concentrations were calculated according to equations by Porra et al. (1989) and Minocha et al. (2009):

[Chlorophyll *a*] equation = (12 x A664) - (3.11 x A647) x dilution factor [Chlorophyll *b*] equation = (20.78 x A647) - (4.88 x A664) x dilution factor [Total Chlorophyll (a + b)] equations = [Chlorophyll a] + [chlorophyll b] [Total carotenoids] equation = (1000A480 - 1.12Ca - 34.07Cb)/ 245 x dilution factor

2.3.5 Foliar Polyamine, Amino Acid, and Exchangeable Ion Measurement

In order to extract 5% PCA soluble polyamines and amino acids, previously frozen leaf discs were thawed and frozen (-20° C) three times and then centrifuged at 13 500 × g for 10 min according to Minocha et al. (1994). The supernatant was used for quantitation of both polyamines and amino acids according to the procedure described in Minocha and Long (2004). A fluorescent tag (dansyl group) was attached to polyamines and amino acids using dansyl chloride and the derivatives were quantified by HPLC (Perkin Elmer Inc., Waltham, MA, USA) according to Minocha and Long (2004). The reaction was terminated using L-asparagine (Asn) (50 µL of 20 mg mL⁻¹ in water) rather than with alanine as described in the original method. Following this protocol, the separation of Arginine (Arg) and Threonine (Thr) was not always complete. For their quantitation, the concentrations of these two amino acids

were added together for each standard to formulate a combined calibration curve; at equal concentrations arginine (Arg) contributed most of the combined peak area. For the simultaneous separation of dansyl amino acids and dansyl polyamines, high performance liquid chromatography (HPLC) measurement was employed for analysis with the excitation and emission wavelengths set at 340 nm and 510 nm, respectively. A TotalChrom HPLC software package (Perkin-Elmer, version 6.2.1) was used for data interpretation. Exchangeable inorganic ions (soluble in 5% PCA) were quantified using a simultaneous axial inductively coupled plasma emission spectrophotometer (Vista CCD, Varian Inc., Palo Alto, CA, USA) and Vista Pro software (Version 4.0), following appropriate dilutions of the PCA extracts with deionized water (Minocha et al. 2010; 2013).

2.3.6 Foliar and Soil Nutrient and Heavy/Toxic Metal Concentration

Leaf samples (~ 0.5 g) were oven-dried at 55°C for 48 h and ground to fine powder prior to digestion with a CEM MARs5 microwave digestion system (CEM, Matthews, NC) using concentrated nitric acid and 30% hydrogen peroxide. Nutrient elements (B, Ca, Mg, K, P, S, Mn and Fe) were analyzed by inductively coupled plasma optical emission spectroscopy (ICAP; 7600 Duo view Inductively Coupled Plasma – Optical Emission Spectrometer [ICP-OES], Thermo Elemental, Madison, WI) and heavy/toxic or stress-inducible elements (Na, Al, Cu, Cr, Co, Ni, Zn, Cd, As, Se and Pb) by Inductively Coupled Plasma Mass Spectrophotometer (ICP-MS; Agilent 7500cx, Wilmington, DE). Helium gas was introduced into the octopole reaction cell for the analysis of Cr, Co, Ni, As and Se (Miller, 1998).

Soil samples collected at 0-10 cm depth were dried at 105°C for 48 h, sieved with a 2 mm pore sieve, and ground to fine powder (Retsch MM200, Haan, Germany).

Soils (0.5 g) were extracted using the Mehlich 3 soil test extractant protocol. The supernatants were analyzed for plant available elements (B, P, K, Ca, Mg, Mn, Fe, and S) and heavy/toxic elements (Na, Al, Zn, Cr, Co, Cu, Ni, As, Se, Cd and Pb) using the same protocol described for foliar samples above. (Wolf and Beegle, 2011)

2.3.7 Soil Bulk Density, Organic Matter and pH Measurement

Soil samples were oven-dried at 105°C prior to analysis. The intact soil core dried mass to total volume ratios were calculated to determine the soil BD. SOM content was analyzed by the combustion method, loss on ignition (LOI; Schulte 1995), and carried out at 350°C. Soil pH (1:1; Eckert 1995) was also determined on sub-samples.

2.3.8 Statistical Analysis

All data analyses were conducted in R (Version 3.3.3 and x64 3.6.1; R Core Team, 2016). Statistical significance is reported at the $\alpha = 0.05$ critical value. Analysis of differences in foliar chlorophyll, carotenoid, % N, and δ^{15} N between the cities were analyzed using a one-way analysis of variance (ANOVA) for data that met the assumptions of normality and homoscedasticity, followed by post-hoc Tukey HSD tests. Additionally, the Kruskal-wallis non-parametric analysis of variance was performed when the assumptions of normality and homoscedasticity were not met. Principal component analysis (PCA) was used to determine whether patterns in foliar chemistry and metabolism, and soil chemistry were discernible among trees (Version 3.3.3; R Core Team, 2016). PCAs were conducted separately for foliar metabolites and soil chemistry to explore potential differences in tree pigments, elements, and metabolites in response to soil chemistry.

2.4 Results

2.4.1 Foliar Chlorophyll, Carotenoid, N, and $\delta^{15}N$

The leaf total chlorophyll concentration was significantly greater in red maples in Philadelphia forests than in Newark forests (p = 0.004; Table 2.2). Similarly, mean total chlorophyll-a (p = 0.002) and chlorophyll/carotenoid ratio (p = 0.044) were also significantly higher in red maples in Philadelphia forests compared to red maples in Newark forests (Table 2.2). There were no significant differences in total carotenoid concentration in red maples between Philadelphia and Newark forests (p = 0.08). The foliar mean %N was significantly higher in Philadelphia forests compared to Newark forests (p < 0.001; Table 2.2). Similarly, foliar mean natural abundance δ^{15} N was significantly more enriched in red maples in Philadelphia forests compared to Newark forests (p < 0.02; Table 2.2).

Forest	Chlorophyll	Chlorophyll-a	Carotenoids	Chl/Car	N (%)	δ ¹⁵ N (‰)
Newark						
FO	3.40 ± 0.18	2.40 ± 0.13	1.22 ± 0.05	2.79 ± 0.04	1.47 ± 0.07	-2.44 ± 0.4
EW	3.12 ± 0.18	2.20 ± 0.06	1.16 ± 0.06	2.69 ± 0.03	1.58 ± 0.05	-10.60 ± 1.2
CW	3.10 ± 0.15	2.16 ± 0.11	1.10 ± 0.05	2.88 ± 0.10	1.53 ± 0.03	-11.51 ± 0.7
RH	3.23 ± 0.20	2.03 ± 0.16	1.21 ± 0.06	2.65 ± 0.08	1.61 ± 0.11	$\textbf{-8.79} \pm \textbf{0.8}$
WF	3.12 ± 0.14	2.20 ± 0.11	1.17 ± 0.51	2.66 ± 0.03	1.68 ± 0.05	-11.62 ± 0.3
Average	3.17 ± 0.08^{a}	2.25 ± 0.07^{a}	$1.16\pm0.03^{n.s.}$	2.74 ± 0.04^{a}	1.57 ± 0.03^{a}	-9.6 ± 0.6^{a}
n= 39						
Philadelphia						
PL	3.60 ± 0.40	2.57 ± 0.30	1.30 ± 0.16	2.79 ± 0.05	1.71 ± 0.06	-11.68 ± 0.4
SM	3.60 ± 0.22	2.58 ± 0.20	1.24 ± 0.09	2.97 ± 0.18	1.79 ± 0.05	$\textbf{-6.87} \pm 0.7$
WR	3.16 ± 0.12	2.30 ± 0.10	1.15 ± 0.33	2.74 ± 0.04	1.58 ± 0.04	-8.59 ± 0.5
PP	3.71 ± 0.22	2.70 ± 0.15	1.35 ± 0.08	2.76 ± 0.02	1.52 ± 0.05	-7.24 ± 1.5
CHX	3.72 ± 0.14	2.69 ± 0.10	1.24 ± 0.06	3.03 ± 0.11	1.93 ± 0.05	-9.87 ± 0.3
Average	$3.53 \pm \mathbf{0.09^{b}}$	2.54 ± 0.07^{b}	$1.24 \pm 0.03^{\text{n.s.}}$	2.88 ± 0.06^{b}	1.72 ± 0.03^{b}	-8.43 ± 0.4^{b}
n=38						

Table 2.2. Foliar total chlorophyll, chlorophyll-*a*, carotenoids, Chl:Car ratio, N content, and natural abundance δ^{15} N for red maple trees in ten forests in Newark, DE and Philadelphia, PA.

Forest: FO, Folk; EW, Ecology Woods; CW, Chrysler Woods; RH, Rittenhouse; WF, Webb Farm; PL, Park Line Dr; SM, Smith Memorial; WR, Walton's Run; PP, Pennypack Dr; CHX, Chamounix

Letters 'a' and 'b' represent significant differences between means when $\alpha = 0.05$ ^{n.s.} denotes 'not significant'

Concentration of pigments are in mg g-1 FW

2.4.2 Foliar Nutrients, Stress-inducible Metals, and Exchangeable Ions

The foliar nutrient concentrations of total P, K, and S were significantly higher, and co-linked Mg and Mn were significantly lower in red maples in Philadelphia

forests compared to red maples in Newark forests (p < 0.05; Table 2.3). Red maples in

Newark forests had significantly greater foliar concentrations of total Ni (p = 0.012),

Cr (p = 0.02), and Co (p = 0.006), whereas red maples in Philadelphia forests had

significantly greater foliar concentrations total Pb (p = 0.03) and Cd (p = 0.04; Table

2.3). Interestingly, similar to total Mg and Mn concentrations, foliar concentrations of

exchangeable Mg and Mn were significantly higher in red maples in Newark forests compared to Philadelphia forests (p < 0.05; Table 2.4). Additionally, foliar concentrations of exchangeable P and K (p < 0.05), and Zn (p = 0.003), and stressinducible metal ion Al (p = 0.04) were significantly higher in red maples in Philadelphia forests compared to Newark forests (Table 2.4), though foliar concentrations of total Al and Zn in red maples were not significantly different between cities.

Foliar element	Newark, DE	Philadelphia, PA
	forests (n=5)	(n=5)
Ca§	$8330 \pm 349^{n.s.}$	$7907 \pm 332.6^{\text{n.s.}}$
K§	6610 ± 211.5^a	8234 ± 305^{b}
Mg [§]	$2355\pm120^{\rm a}$	2089 ± 102^{b}
$\mathbf{P}^{\mathbf{\bar{\$}}}$	1198 ± 45^{a}	1369 ± 63^{b}
$\mathbf{S}^{\$}$	1019 ± 18^{a}	1099 ± 18^{b}
Fe [§]	$64.5 \pm 2^{n.s.}$	$67.8 \pm 1.7^{\text{n.s.}}$
Mn [§]	$714 \pm 45^{\mathrm{a}}$	425 ± 33^{b}
Zn [§]	$38\pm0.12^{n.s.}$	$41 \pm 0.12^{\text{n.s.}}$
Cu [§]	$8.5\pm0.4^{n.s.}$	$8.7 \pm 0.5^{n.s.}$
Na [§]	$14 \pm 3^{\text{n.s.}}$	$13 \pm 1^{n.s.}$
Al§	$32 \pm 1^{\text{n.s.}}$	$30.9 \pm 1^{\text{n.s.}}$
Cr¶	$14 \pm 1.2^{\mathrm{a}}$	9.2 ± 0.8^{b}
Co¶	3.6 ± 1.1^{a}	0.6 ± 0.08^{b}
Ni¶	$25 \pm 4.4^{\mathrm{a}}$	14.6 ± 1.2^{b}
Cd^{\P}	$2.3\pm0.3^{\rm a}$	3.3 ± 0.4^{b}
As¶	$0.55 \pm 0.04^{n.s.}$	$0.27 \pm 0.03^{n.s.}$
Se¶	$2.9\pm0.18^{\text{n.s.}}$	$2.8\pm0.14^{n.s.}$
Pb¶	4.9 ± 0.5^{a}	8.2 ± 1^{b}

Foliar total nutrient and stress-inducible element concentrations of red Table 2.3. maples in ten forests in Newark, DE (n=39) and Philadelphia, PA (n=38).

Element: Ca= calcium; K= potassium; Mg= magnesium; P= phosphorus;

S= sulfur; Fe= iron; Mn= manganese; Zn= zinc; Cu= copper; Na= sodium; Al= aluminum; Cr= chromium; Co= cobalt; Ni= nickel; Cd= cadmium; As= arsenic; selenium= Se; Pb= lead

Letters 'a' and 'b' represent significance at $\alpha = 0.05$

Bold values represent significantly greater concentrations

[§] denotes units are mg kg⁻¹ DW

[¶] denotes units are $\mu g kg^{-1} DW$

^{n.s.} denotes 'not significant'

Exchangeable ion	Newark, DE	Philadelphia, PA
K^{\ddagger}	35.9 ± 1.4^{a}	48.5 ± 3^{b}
Mg^\ddagger	$26.8 \pm 1.9^{\rm a}$	23.1 ± 1.4^{b}
\mathbf{P}^{\ddagger}	$7.7\pm0.3^{\mathrm{a}}$	9.3 ± 0.7^{b}
Mn^{\ddagger}	$2.7 \pm 0.3^{\mathrm{a}}$	1.6 ± 0.2^{b}
Al‡	0.1 ± 0.01^{a}	0.2 ± 0.02^{b}
Zn^{\ddagger}	0.2 ± 0.01^{a}	$0.3\pm0.04^{\rm b}$
Ca‡	$44.7 \pm 2.3^{n.s.}$	$46.4 \pm 3.7^{n.s.}$
Fe [‡]	$0.16 \pm 0.013^{n.s.}$	$0.2 \pm 0.017^{n.s.}$
S‡	$1.6 \pm 0.07^{n.s.}$	$1.7 \pm 0.1^{\text{n.s.}}$
Cu [‡]	$0.1 \pm 0.024^{\text{n.s.}}$	$0.1 \pm 0.033^{n.s.}$
Ni [‡]	$0.01 \pm 0.002^{\text{n.s.}}$	$0.01 \pm 0.003^{n.s.}$
Se^{\ddagger}	$0.003 \pm 0.001^{\text{n.s.}}$	$0.003 \pm 0.001^{\text{n.s.}}$
Co^{\ddagger}	$0.004 \pm 0.0005^{n.s.}$	$0.003 \pm 0.0004^{\text{n.s.}}$
As^\ddagger	$0.006 \pm 0.0001^{\text{n.s.}}$	$0.01 \pm 0.0001^{\text{n.s.}}$
Cd‡	$0.025 \pm 0.01^{\text{n.s.}}$	$0.03 \pm 0.02^{\text{n.s.}}$
Pb [‡]	$0.003 \pm 0.001^{\text{n.s.}}$	$0.002 \pm 0.001^{\text{n.s.}}$

Table 2.4.Foliar exchangeable nutrient and stress-inducible metal ion concentrations
of red maples in ten forests in Newark, DE and Philadelphia, PA.

Letters 'a' and 'b' represents significant differences between means when $\alpha = 0.05$ Bold values represent significantly greater concentrations [‡]denotes units are µmol g⁻¹ FW

^{n.s.} denotes 'not significant'

Principle component analysis (PCA) was used to characterize the relationship of foliar nutrients and stress-inducible elements in red maples in Newark and Philadelphia forests. The first two principle components for the foliar chemistry described 36.8% of the variation among the elements (Fig. 2.2) which was not very high likely due to lots of variation and/or little separation. PCA1 and PCA2 variables explained 19.1% and 17.7%, respectively, of the variation in correlation in the foliar elements of the red maple trees in both cities (Fig. 2.2). The variables having the strongest correlation with PCA1 were Co and Ni (negative), and Zn (positive) in the order of strongest to weakest. The variables most significantly correlated on PCA2 were Ca and Mg (in the order of strongest to weakest) and all had strong, positive loadings. The patterns in foliar chemistry of red maple trees in Newark and Philadelphia forests formed two circles of ellipses that represent the group mean for each city (Fig. 2.2A).

To determine relationships between foliar exchangeable ions (i.e., nutrient and stress-inducible) in red maple trees across Newark and Philadelphia forests, we conducted principle component analyses. The PCA (PCA1, 43.8% and PCA2, 19.8%) explained 63.5% of the variation in foliar exchangeable ions across the cities (Fig. 2.2B). The foliar exchangeable ions having the strongest positive correlation with PCA1 were Al, P, K and Zn (Fig. 2.2B). The foliar exchangeable ions with strongest loading on PCA2 was Mn (positive; Fig. 2.2B). The patterns in foliar exchangeable ions of red maple trees in Newark and Philadelphia forests formed two circles of ellipses that represent the group mean (Newark and Philadelphia forests; Fig. 2.2B).



Figure 2.2. Principal component analysis of seventy-seven mature native red maple trees leaf total (A) and exchangeable (B) elemental composition in ten urban deciduous forests in Newark, DE and Philadelphia, PA. PCA1 (19.1%) and PCA2 (17.7%) variables explained 36.8% of the variation in the leaf chemistry of red maples (legend A). PCA1 (43.7%) and PCA2 (19.8%) variables explained 63.5% of the variation in correlation (legend B). Leaf nutrient and stress-inducible elements that most strongly loaded on each axis are listed next to their respective axis in order of strongest to weakest. (+) represents values that indicate variables are positive and strongly loaded on the axis; (-) represents values that indicate variables are ellipses that represent the group mean.

2.4.3 Soil Bulk Density, Organic Matter and pH

Sub-canopy soil bulk density (BD) showed very little variation among forests within Newark and Philadelphia forests. The mean soil BD was significantly higher at the sub-canopy of red maples in Newark forests $(1.14 \pm 0.02 \text{ g cm}^{-3})$ compared to Philadelphia forests $(1.01 \pm 0.04 \text{ g cm}^{-3}; p = 0.0027)$. In addition, the percent soil organic matter (SOM) at the sub-canopy of red maples was significantly higher in Philadelphia forests $(13.8 \pm 2.4\%)$ compared to Newark forests $(7.7\% \pm 0.6\%; p = 0.016)$. Alternatively, sub-canopy aqueous soil pH (1:1) was not significantly different between Philadelphia forests (4.7 ± 0.1) and Newark forests $(4.5 \pm 0.1; p = 0.11)$.

2.4.4 Soil Nutrients and Stress-inducible Metals

Red maple sub-canopy soil macronutrients were 1.5 to 2.5-fold higher in Philadelphia forests compared to forests in Newark. The mean soil concentrations of Ca, K, Mg, P, S were significantly higher at the sub-canopies of red maples in Philadelphia forests compared to Newark forests (p < 0.001, Table 2.5). The subcanopy soil stress-inducible Cr, Co, Ni, and Se concentrations were significantly greater at the canopy of red maple trees in Newark forests than in Philadelphia forests (p < 0.001; Table 2.5). In contrast, red maple sub-canopy soil concentrations of Al, Zn, Cu, Pb, and Cd were significantly greater in Philadelphia forests compared to Newark forests (p < 0.05; Table 2.5).

The soil element ratios for element availability and uptake, along with soil pH and SOM measured, indicated altered soil biogeochemistry of element pools in Newark and Philadelphia forests. In Philadelphia forests compared to Newark forests, higher soil element ratios of K/Ca (1.77 vs 1.14), Ca/Al (0.08 vs 0.06), and P/Al (0.03 vs 0.02) with the exception of lower Ca/Mg ratio (0.45 vs 0.7) and Mn/Ca (0.23 vs

0.32) were measured at the sub-canopy of red maples, which paralleled higher foliar exchangeable ratios of K/Ca (1.06 vs 0.8), Ca/Mg (2.01 vs 1.66) and Fe/Mn (0.11 vs 0.06) with the exception of lower Mn/Ca (0.03 vs 0.06), Ca/Al (232.3 vs 259.3) and P/Al (46.06 vs 51.8).

Total soil element	Newark, DE	Philadelphia, PA
Ca [§]	1153 ± 125^{a}	1821 ± 132^{b}
K [§]	1312 ± 61^{a}	3216 ± 236.5^{b}
Mg [§]	1647 ± 94^{a}	4073 ± 364.2^{b}
$\mathbf{P}^{\$}$	448 ± 35^{a}	691 ± 33^{b}
S§	420 ± 49^{a}	638 ± 77^{b}
Fe [§]	18799 ± 1634^{a}	26606 ± 1577^{b}
Mn§	$370 \pm 54^{\text{n.s.}}$	$416 \pm 39^{\text{n.s.}}$
Zn [§]	$58\pm 6^{\mathrm{a}}$	92 ± 5^{b}
Cu [§]	19 ± 2^{a}	36 ± 2^{b}
Al§	20619 ± 1159^a	24006 ± 1388^{b}
Na [§]	167 ± 7^{a}	184 ± 9^{b}
Pb¶	973 ± 0.6^{a}	1007 ± 91^{b}
Cd¶	1.0 ± 0.1^{a}	2 ± 0.1^{b}
Cr^{\P}	1165 ± 15^{a}	396 ± 0.4^{b}
Co¶	146 ± 23^{a}	46 ± 0.2^{b}
Ni¶	$351 \pm 74^{\mathrm{a}}$	120 ± 15^{b}
As¶	39 ± 1.5^{a}	35 ± 2^{b}
Se¶	82 ± 4^{a}	36 ± 2^b

Table 2.5. Sub-canopy soil total element concentrations for the upper 0-10 cm soil horizon of seventy-seven red maples in ten forests in Newark, DE and Philadelphia, PA (Mehlich 3 extraction).

Letters 'a' and 'b' represent significant differences when $\alpha < 0.05$ Bold values represent significantly greater concentrations

 $\ensuremath{^\$}$ denotes units are mg kg^-1 DW

 ¶ denotes units are $\mu g \; kg^{\text{-1}} \; DW$

Urban conditions can homogenize soil elements unique to city pressures, and PCA can reveal relationships among soil elements across the two cities. Principle component analysis was used to characterize the variability in soil elements across Newark and Philadelphia forests. The first two axes in the PCA explained 54.9% of the variation in sub-canopy elements (nutrient and stress-inducible) in the sub-canopies of red maples in Newark and Philadelphia forests. The variables with the strongest positive (Zn, K, Cd, P, Cu, Ca) loadings on the first principle component (PCA1) explained 35.0% of the variation. The variables with the strongest positive (Co, Fe, Ni, Mn) loadings on PCA2 explained 19.9% of the variation in the soil chemistry (Fig. 2.3). These strongly correlated sub-canopy soil variables of PCA1 and PCA2 (Fig. 2.3a) formed two circles are ellipses that represent the group mean of Newark and Philadelphia soil chemistry.



Figure 2.3. Principal component analysis for seventy-seven mature, native red maple trees sub-canopy elements and foliar metabolites in ten urban deciduous forests in Newark, DE and Philadelphia, PA. PCA1 (35.0%) and PCA2 (19.9%) variables explained 54.9% of the variation in the soil chemistry (Legend a). PCA1 (54.7%) and PCA2 (11.9%) variables explained 66.7% of the variation in the leaf metabolites correlation (Legend b). Soil elements and foliar metabolite that most strongly loaded on each axis are listed next to their respective axis in order of strongest to weakest. (+/-) represents values that indicate variables are positive/negative and strongly loaded on the axis. The circles are ellipses that represent the group mean.

Lower total leaf/soil element concentration ratios for the macronutrients (Ca, Mg, P, K, S), micronutrients (Zn, Cu, Mn) and stress-inducible elements (Cd, Co, As) were measured (except for Cr, Ni, Se, and Pb) in Philadelphia forests compared to Newark forests, largely reflecting the higher soil element pools (except for Co and As), nutrients imbalance, within-species variability, pH, SOM, and BD. Therefore, linear regressions of the leaf/soil element concentration ratios showed tighter relationships between trees and soil (environment) in red maples in Philadelphia forests for Co ($r^2 = 0.4$, p < 0.001), As ($r^2 = 0.34$, p < 0.001), Pb ($r^2 = 0.13$, p = 0.025), Mn ($r^2 = 0.13$, p = 0.03), Ni ($r^2 = 0.12$, p = 0.033) compared to Mn ($r^2 = 0.24$, p = 0.002), P ($r^2 = 0.21$, p = 0.003), Ni ($r^2 = 0.18$, p = 0.007) and Cr ($r^2 = 0.11$, p = 0.034) in Newark forests.

2.4.5 Amino Acid and Polyamine Accumulation

Free foliar metabolite accumulation may explain tree response to nutrients (i.e., K and N) and stress-inducible metals (i.e., traffic-related-Zn versus geology-related-Mn versus legacy-related-Cr) in red maples in Newark and Philadelphia forests. Greater concentrations of foliar free amino acids and free polyamines were measured in red maples in Philadelphia forests compared to Newark forests. Foliar serine, arginine-threonine (unseparated), proline, glutamic acid, glutamine, ornithine, and glycine were significantly higher in red maple trees in Philadelphia forests compared to Newark forests (p < 0.05; Table 2.6). In addition, concentrations of methionine and *gamma* amino butyric acid were marginally significantly higher in Philadelphia forests (p < 0.1). Foliar polyamine concentrations (and the Spd/Put ratio) were not different in red maple trees between Newark and Philadelphia forests (p > 0.05). Spermine was

the only polyamine that showed marginal significantly higher concentration in red maples in Philadelphia forests compared to Newark forests (p = 0.08).

Free metabolites	Newark, DE	Philadelphia, PA	p-value
Gluγ	127.6 ± 6.1^{a}	151.92 ± 8.2^{b}	P = 0.02
Gln ^γ	134.2 ± 12.7^{a}	185.28 ± 9.7^{b}	P = 0.002
Arg-Thr ^γ	46.8 ± 2^{a}	122.4 ± 5.5^{b}	P < 0.001
Glyγ	$28.9 \pm 1.5^{\rm a}$	35.4 ± 1.9^{b}	P = 0.008
Pro ^γ	37.3 ± 2.39^{a}	45.86 ± 3.5^{b}	P = 0.046
\mathbf{GABA}^{γ}	339.8 ± 15.4	405.21 ± 31	P = 0.059
Orn ^γ	0.3 ± 0.33^{a}	6.95 ± 0.6^{b}	P < 0.001
Ser ^γ	112.8 ± 7.3^{a}	204.6 ± 12^{b}	P < 0.001
Met ^γ	147 ± 30	214.7 ± 38.6	P = 0.09
Put ^γ	$24.5 \pm 22^{n.s.}$	$30.52 \pm 3.7^{n.s.}$	P > 0.05
\mathbf{Spd}^{γ}	$25.3 \pm 1.76^{\text{n.s.}}$	$29.73 \pm 2.6^{\text{n.s.}}$	P > 0.05
Spd/put ^γ	$1.2\pm0.1^{n.s.}$	$1.42 \pm 0.2^{\text{n.s.}}$	P > 0.05
Spm ^γ	16.2 ± 0.86	19 ± 1.4	P = 0.08

Table 2-6.Foliar amino acids and polyamines concentration of seventy-six redmaples in ten forests in Newark, DE and Philadelphia, PA.

Amino acid: Glu, Glutamic acid; Gln, Glutamine; Arg, Arginine; Thr, Threonine; Gly, Glycine; Pro, Proline; GABA, *gamma* Amino Butyric Acid; Orn, Ornithine; Ser, Serine; Met, Methionine; Put, Putrescine; Spd, Spermidine; Spm, Spermine. Letters 'a' and 'b' represent significant difference in means when α = 0.05. Bold values represent significantly greater concentrations ⁷ denotes nmol g⁻¹ FW

^{n.s.} denotes 'not significant'

PCA was used to characterize the relationship of foliar metabolites (free polyamines and amino acids) in red maple trees between Newark and Philadelphia forests (Fig. 2.3b). The first two principle components explained 66.7% of the variation in amino acids and polyamines. PCA1 and PCA2 explained 54.7% and 11.9% of the variation in foliar metabolites in red maples in the two cities. The

metabolites with the strongest correlation on PCA1 were Ser, GABA, Pro, Leu, Ala, Ile, and Arg-Thr (in order of strongest), and all of the variables had significant positive loadings on PCA1 (Fig. 2.3b). The variable with the strongest correlation on PCA2 was Spd/Put ratio, which had a significant positive loading on PCA2. These free amino acids and polyamines of PCA1 and PCA2 formed two circles of ellipses that represent the group means of red maple metabolites in Newark and Philadelphia forests (Fig. 2.3b).

2.5 Discussion

2.5.1 Foliar Physio-Biochemistry of Red Maple Trees in Philadelphia Forests Significantly Differs from Newark Forests

Between Philadelphia and Newark forests, we found differences in mature native red maple chlorophyll, chlorophyll-*a*, chlorophyll/carotenoid ratio, N (%), ¹⁵N (‰), nutrients, stress-inducible metals, free amino acids, and polyamines (Tables 2.4 and 2.6). In addition, we found differences in the sub-canopy soil nutrients and stressinducible metals (Table 2.5), and soil physical properties between Newark and Philadelphia forests. More specifically, forests in the large city showed the highest concentrations of foliar total chlorophyll, chlorophyll/carotenoid ratio, %N, ¹⁵N‰, nutrients (i.e., K, P, S, Zn), stress inducible metals (i.e., Al, Mn, Zn, Cd, Pb), free amino acids (i.e., Arg, Orn, Pro, Glu, Gln, Gly, Ser) and marginal higher spermine in red maple trees that were growing in nutrient-rich (i.e., Ca, Mg, K, P, S, Fe, Cu) soils that were also contaminated with traffic-related (i.e., Na, Zn, Cd, Cu, Pb; Pouyat et al. 2007; Trammell et al. 2012) and site-linked stress-inducible metals (Al, Mn). Forests in the small city showed higher concentrations of foliar Mg, stress-inducible Mn, Cr, Co, and Ni in red maple trees that were growing in surface geology-related (e.g., Mn, Cr, Co, Ni) contaminated soils disturbed by previous land use changes (i.e., agricultural practices). Increased foliar chlorophyll concentration (and chlorophyll-*a*) in red maples in Philadelphia indicates a net adaptive effect on plastids (chloroplasts, mitochondria) in response to simultaneous increase in soil nutrient availability (Ca, Mg, K, P, S, Zn, Cu; positive effects) and elevated stress-inducible metals (Na, Al, Mn, Cd, Pb; negative effects) (Manta et al. 2002; Kramer 2010; Silva 2012; Wang et al. 2013; Minocha et al. 2014; Nahar et al. 2016; Lambrecht et al. 2016; Razaq et al. 2017; Moser et al. 2017; Pretzsch et al. 2017).

According to previously published literature, more enriched foliar $\delta^{15}N$ and elevated foliar %N in red maple trees in Philadelphia suggest differences in N deposition and N sources, differences in soil N cycling rates and associated ¹⁴N losses. and/or within plant N allocation differences in forests within the larger city relative to the small city (Craine et al. 2009; Redling et al. 2013; Silva et al. 2015; Moser et al. 2017; Kanwan et al. 2019). Moreover, direct foliar reactive N uptake could contribute to enriched foliar δ^{15} N in red maple (Vallano and Sparks, 2013) because of a metroscale N deposition gradient. Importantly, elevated chlorophyll and marginal higher carotenoids observed in red maple trees in nutrient-rich soils in Philadelphia forests relative to Newark forests were strongly correlated with an accumulation of free amino acids (Pro, Gln, Arg, Gly), spermine, and stress regulating ions (Ca, K, P, Zn), even though the foliar stress-inducible (except for $Mn: > 285 \text{ mg kg}^{-1}$) metals were not at or above the foliar threshold levels of toxicity for land plants (Markert 1991; Kramer 2010). Lahr et al. (2018) reported that wildtype red maples had higher WUE, stomatal conductance and overall photosynthesis rate as air temperature increase in urban settings likely attributed to their genetic background and local adaptation. These

findings suggest varying magnitudes of urban intensity impact chlorophyll, amino acids, polyamines, macro- and micro-nutrients, stress-inducible elements, elements interaction, and soil physical properties (e.g., SOM, pH and BD) differently in Newark and Philadelphia area forests. The accumulation of amino acids, spermine, carotenoids and nutrients were likely responsible for ROS detoxification, ion homeostasis, and osmotic adjustment that afford physiological acclimation in red maples in Philadelphia forests (Sharma and Dietz, 2006; Shi et al. 2013; Minocha et al. 2014; 2019; Winter et al. 2015; Nahar et al. 2016; Gupta et al. 2016).

Several studies have reported similar adaptive stress response traits in other plant species (Breda et al. 2006; Lambrecht et al. 2016; Dahlhausen et al. 2017; Jia et al. 2018) similar to what was observed in red maple trees in Philadelphia forests and to a greater degree than red maples in Newark forests. Previous reports have revealed an accumulation in K with salt stress (Wang et al. 2013), Zn with altered Na⁺ and K⁺ homeostasis (Zhang et al. 2016), amino acids with heavy metals exposure (Sharma and Dietz, 2006; Thangavel et al., 2007, Fernando et al. 2016), and spermine under drought and temperature stress (Takahashi and Kakehi, 2010; Nahar et al. 2016; Fu et al. 2014; Li et al. 2015). In the present study, red maple trees growing in urban environments are likely to respond to combined salt and heavy metal (oxidative or osmotic) stresses by triggering the accumulation of N-signaling arginine, ornithine, and proline (Gzik, 1996; Shi et al. 2013; Winter et al. 2015; Nahar et al. 2016; Anwar et al. 2018), and multiple cumulative stresses (e.g., low pH, high N, drought) by accumulating polyamines and amino acids (Moschou et al 2012; Minocha et al. 1997; 2014). Furthermore, red maple trees may have exploited novel epigenetic strategies (new genotypes) that regulate cell physiology (Shi et al. 2013; Winter et al. 2015;

Gupta et al. 2016; Gömöry et al. 2017; Lahr et al. 2018). In response to the differing environmental gradients, gene up-regulation can result in biosynthesis and accumulation of soluble proteins and sugars, increased ions, and increased enzymes activity (RuBisCO, nitrate reductase, arginase, ornithine aminotransferase [OAT]), leading to osmotic adjustment using a network of N-C interactions to achieve ROS/RNS homeostasis (Pérez-Clemente et al. 2012; Villar-Salvador et al. 2015; Minocha et al. 2014; 1997; Zhang et al. 2016; Majumdar et al. 2016).

The accumulation of foliar chlorophyll, amino acids, polyamines, and nutrients in red maples in Philadelphia forests (Tables 2.2, 2.3 and 2.6) is evidence of a possible shift in the N-C-metabolism pathways and interactions in response to elevated nitrogen (N-containing Arg-Orn-Gln-Glu), stress-inducible metals (Pro, Ser and Gly signaling), and temperature and salt (spermine, K^+ , Zn^{2+}). The overall cumulative combined metro-scale (elevated UHI, N deposition and CO₂) impacts and the resulting coordinated physio-metabolic shifts (robust genetic background) suggest enhanced productivity and stress physiological acclimation in Philadelphia red maples, which was observed to a lesser extent in Newark (Table 2.6; Fig. 2.3). Here, we have demonstrated that in the larger (e.g., higher population density), warmer (UHI and dryer soils), browner (higher N-S deposition inputs), and nutrient-rich (higher Mg-Ca-P-K inputs) city (metro-scale impacts), the urban conditions appear to be stimulating chlorophyll accumulation and inducing stress mitigating-acclimating responses in red maples. Our research provides evidence that nutrient rich urban environments (Lovett et al. 2000; Wang et al. 2013; Zhang et al. 2016; Lahr et al. 2018) may compensate for environmental perturbations on forest trees growing in a large city.

2.5.2 Urban Soil-Tree Interactions Indicate Differing Above- and Below-Ground Impacts in Newark and Philadelphia Forests

Forest trees in cities are subjected to multiple simultaneous above- and belowground pressures that influence the physiology, biochemistry, and stress mitigationacclimation patterns unique to soil type, local environmental conditions, and metroscale impacts (e.g., Gregg et al. 2003; 2006; Lahr et al. 2018). Soil-tree interactions possibly initiate a network of molecular cross-talks with other signaling compounds within the cells (Sharma and Dietz, 2006; Mujumdar et al. 2016; Gupta et al. 2016) to allow plants to avoid/resist stressful conditions (Gregg et al. 2006; Minocha et al. 2014; Stein et al. 2017) and effectively respond to growth-stimulating conditions (Lovett et al. 2000). In the present study, the leaf-soil chemistry patterns (linear regression) depicted strong relationships between leaf element composition and the sub-canopy soil element pools, particularly the stress-inducible metals in Philadelphia (As, Co, Mn, Ni, Pb) and in Newark (Mn, Cr, Ni) forests. Sub-canopy soil and foliar elements showed evidence of traffic-related sources (e.g., Zn, Cu, Pb; Trammell et al. 2011) in Philadelphia forests, and land use/land cover legacy-associated sources (e.g., Cr, Co, Se; Pouyat et al. 1991;1997; Fraterrigo et al. 2005; Fernando et al. 2016; Richardson 2017) in Newark forests, and geology-related origin (e.g., Al, Mn; Pouyat et al. 2007) in both Newark and Philadelphia forests.

In Philadelphia forests relative to Newark forests, foliar total Ca, Mg, and Mn did not reflect the elevated sub-canopy soil nutrient composition due to altered nutrient biogeochemistry of soil elements (i.e., lower Ca/Mg, higher K/Ca, higher Mn/Ca) in low pH soils (pH = 4.7) that negatively impact the accumulation of Mg and Mn (Table 2.3 and 2.4; Viet, 1944; Jacobson et al. 1961; Maas et al. 1968; DeHayes et al. 1999; Kogelmann and Sharpe, 2006; Richardson 2017). However, higher soil K, P,

K/Ca, and P/Al correlate with increased foliar total and exchangeable P, K, and Al (Tables 2.3 and 2.4) in Philadelphia, similar to what was reported previously in soils (Twyman, 1950; Jacobson et al. 1961; Scharenbroch et al. 2005). Elevated soil Al increases soil acidity and plant toxicity (Maas et al. 1968; Abrahamsen, 1983; Minocha et al. 1997), and competes with P in plant root hair channels for uptake in acid soils with low P, Ca, and SOM (Viet, 1944; Twyman, 1950; Alva et al. 1986). However, Philadelphia forests had significantly greater soil P, Ca, (Table 2.5) and SOM, and higher foliar P than Newark forests (Table 2.3 and 2.4) suggesting Al (e.g., as AIPO₄) may not be detrimental to red maple trees in Philadelphia forests (Minocha et al. 1996). The observed greater SOM in Philadelphia forests may be due to more recalcitrant organic matter litter from higher levels of stress acclimating compounds (e.g., spermine and several amino acids) observed in our trees (Table 2.6, Fig. 2.4) and/or these systems may be achieving steady-states (i.e., SOM decay rates similar to litter production rates). Higher amounts of N in soil have been reported to increase litter accumulation (Li et al., 2015, Krishna and Mohan, 2017). Alternatively, lower SOM in Newark forests may be due to previous land use practices such as intense agriculture that has long-term negative impacts on the SOC, N and BD (Murty et al. 2002). Lower SOM and higher soil BD found in Newark forests versus Philadelphia, PA suggest the urban landscapes are altered differently over time and soils in Philadelphia forests are likely moving toward steady-state (Scharenbroch et al. 2005).

Elevated base elements in the sub-canopy soils in Philadelphia forests marginally higher soil pH (4.7) compared to Newark forests (soil pH = 4.5). However, the soil pH in these urban forests are (reflective of northeastern temperate forests that suffer from many decades of acid rain) lower than the average natural soil pH of 5.6

for growing healthy plants (DeHayes et al. 1999; Kogelmann and Sharpe, 2006). The soil acidity is exacerbated under elevated N deposition inputs (N saturation), Al solubility, and increased leached-base elements (Twyman, 1950; Jacobson et al. 1961; Shortle and Smith, 1988; DeHayes et al. 1999). In addition, plants are challenged with nutrient imbalances and metal toxicity (Mn, Al) in low pH soils making them more vulnerable abiotic and biotic stresses (Maas et al. 1968; DeHayes et al. 1999; Kogelmann and Sharpe, 2006; Minocha et al. 2010; Fernando et al. 2016). Quantification of Mn enrichment (using [Mn/Ca]) in urban forests is novel and this could be used to determine spatio-temporal, and above- and belowground impacts of Mn concentration on biotic and abiotic characteristics and nutrient relationships (Richardson 2017).

The soil-tree element interactions to the altered edaphic properties in urban environments may likely dictate urban tree physiology and stress mitigating strategies for element uptake at the city scale. Foliar Co, As, Pb, Mn and Ni were the strongly correlated leaf/soil element concentration ratios in Philadelphia forests suggesting that the uptake and transport (or lack thereof) of these elements may impact acclimation against stress-inducible elements (Fernando et al. 2016; Stein et al. 2017). In Newark forests, stress-inducible elements Mn, Ni, and Cr along with P were the strongest correlated leaf/soil element ratios suggesting that these elements were likely taken up during the absorption of P in red maple trees (Stein et al. 2017). Furthermore, trees in Newark forests likely suffered greater levels Mn toxicity (relative to red maples in Philadelphia forests) as high concentrations of Mn above threshold levels (> 285 mg/kg DW; Altland 2006; Kramer 2010) were found in the leaves in flood-prone, waterlogged soils (i.e. Ecology Woods and Chrysler Woods), which favors decreasing

redox potential and increasing oxygen demand (Smiley et al. 1986; Pezeshki and DeLaune, 2012; Lambers et al. 2015). Red maple leaves sampled in Newark forests may be exposed to lower temperature, and acidic, water-logged soils (summer rainfall; Li et al. 2019) that increase bioavailable Mn²⁺ (Smiley et al. 1986; St. Clair and Lynch, 2004; Kogelmann and Sharpe, 2006). Moreover, leaf P and soil P had the strongest correlation among nutrient elements suggesting red maple trees in Newark forests may possibly experiencing P imbalances, and micronutrient (Ni, Cr) deficiencies (Markert 1991; Kramer 2010), which subsequently increase the concentration and uptake of Mn to toxic levels (Lambers et al. 2015).

Elevated foliar Mn was the strongest correlated stress-inducible element with soil Mn in Newark forests (Tables 2.3 and 2.4) suggesting possible plant upregulating strategies for P uptake (carboxylate-releasing P-acquisition) may have created mimicking strategies for Mn uptake and accumulation to toxic levels (Marker 1991; Kramer 2010). Cellular sequestration of Mn and Mg in red maple suggests Mn and Mg were possibly co-linked during accumulation (St. Clair, Lynch, 2004; Fernando et al. 2016), and a similar Mn-Mg accumulation pattern was observed in Newark forests relative to Philadelphia forests. Conversely, Co was the strongest correlated stress-inducible element between red maple foliage and soil in Philadelphia forests suggesting likely up-regulation mechanisms at the cellular level for more Co uptake with subsequent physiological effects on chlorophyll and osmotic adjustment (Palit et al. 2004) since observed foliar Co concentration was critically low (Markert 1991). Furthermore, the release of root organic acids for micronutrient acquisition may have resulted in accumulation of Mn to toxic levels (Markert 1991; Altland 2006; Kramer 2010; Lambers et al. 2015) in trees in Philadelphia forests.

The findings suggest that red maple trees were subjected to similar anthropogenically-inherited (Zn, Cu, Pb; Trammell, et al. 2011) and surface geologyrelated stress-inducible metals (Al, Mn; Pouyat et al. 2007) in Philadelphia and Newark forests. However, the magnitude of the urban intensity influenced the soil biogeochemistry differently between cities (Pouyat et al. 2007), and thus leaf-soil interactions (Stein et al. 2017). The differences in absolute values of the foliar and soil total elements, foliar exchangeable ions, metabolites, soil element ratios, leaf/soil element relationships, SOM, and BD between Newark and Philadelphia forests suggest very complex but dominant metro-scale and site-scale impacts on soil-tree interactions. These findings help discern potential drivers of tree physiology and acclimation and may be useful in understanding the importance of balanced nutrient supply for plant productivity and oxidative stress acclimation for growth and survival of urban trees.

2.5.3 Red Maple Trees in Philadelphia Forests Demonstrate Physiological Acclimation to Urban Conditions

Co-occurring metro-scale and site-scale impacts influenced soil factors and plant traits between Newark and Philadelphia forests that resulted in different physiology and acclimation of red maple trees between the two cities. Similar to other previously published reports on other plant species, red maple foliar free polyamines and amino acids, and specific nutrients were important players in stress-signaling, stress-mitigating, and nitrogen storage compounds in plants experiencing high soil N, heavy/toxic metals, salt, drought, acidity and heat stresses (Kogelmann and Sharpe, 2006; Takahashi and Kakehi, 2010; Wang et al. 2013; Minocha et al. 2014; Winter et al. 2015; Zhang et al. 2016; Gupta et al. 2016; Nahar et al. 2016; Seifi and Shelp, 2019). Higher levels of metal-chelating amino acids (proline, glycine), N-storing arginine and glutamine, and intermediate ornithine (for proline and arginine anabolism) were likely accumulated in response to greater levels of cellular ROS and RNS caused by elevated heavy/toxic metals, salt ions, and nitrogen (Gzik, 1996; Minocha et al. 1997; 2014; Sharma and Dietz, 2006; Kalamaki et al. 2009). In addition, spermine accumulated (Table 2.6) with only a marginal increase in putrescine and spermidine in response to broad spectrum stresses such as salt, heat, N, and drought (Fu et al. 2014; Gzik, 1996; Minocha et al. 2014; Gupta et al. 2016; Nahar et al. 2016; Seifi and Shelp, 2019). Therefore, the greater metro-scale impacts of elevated sub-canopy soil stress-inducible metals (e.g. Mn, Zn, Al), UHI, elevated N deposition inputs and their combined effect in the large city (Philadelphia) possibly result in tightly coupled root-leaf communication that allows amino acids and polyamines to accumulate for signaling, ROS homeostasis, heavy metal chelation and storing excess N for osmotic adjustment and physiological acclimation relative to trees in Newark forests that mainly encounter Mn toxicity.

The dynamic shift in cellular metabolites and nutrients as heavy/toxic metals accumulated in red maple leaves in Philadelphia forests suggests a unique shift in the N-C-metabolism pathways under stressful conditions that was partially or fully controlled by changes in nutrient concentrations (Minocha et al. 2014; Zhang et al. 2016; Majumdar et al. 2016). Such shifts in C-N reallocation and remobilization pathways may utilize ornithine aminotransferase (OAT) for the biosynthesis of more arginine and subsequent more proline and spermine from arginine metabolism using arginase (Shi et al. 2012; Winter et al. 2015; Anwar et al. 2018). Higher foliar free amino acids and spermidine further suggests tightly physiologically regulated

ROS/RNS homeostasis that may be responsible for higher level of physiological acclimation (Wang et al. 2013; Zhang et al. 2016; Lahr et al. 2018; Seifi and Shelp, 2019) in trees in Philadelphia forests experiencing multiple cumulative abiotic/biotic stress complexities, such as excess N relative to Newark forests.

The present study demonstrates evidence of physiological acclimation in red maple trees in Philadelphia forests, which experience a greater magnitude of urban intensity. Accumulated chlorophyll, free amino acids and spermine, along with higher foliar nutrients and stress-inducible metals in red maple trees suggest higher levels of stress tolerance and higher cellular physiological acclimation in Philadelphia forests relative to Mn-stressed red maple trees in Newark forests. Urban intensity can be a metro-scale phenomenon that appears to override above- and below-ground conditions in these forests as reflected by the altered soil biogeochemistry of elements, soil physical properties, tree/soil element interactions, and tree physio-biochemistry patterns in Philadelphia and Newark forests. The multiple combined stress impacts were more pronounced in the belowground processes in forests in both cities. However, metro-scale impacts seem to dominate the combined above- and belowground impacts such that red maple trees possibly acclimate in Philadelphia forests whereas they become more vulnerable in Newark forests.

2.6 Conclusions

Our study shows that greater magnitudes of urban intensity affected leaf physiology, biochemistry and elemental composition of red maples in urban forests in the large city, which may lead to acclimation and prolonged tree health and productivity. In Philadelphia, higher pigments, accumulated N-containing metabolites, and increased nutrient ions demonstrate an acclimation response to increased nutrient

loads, toxic metals, UHI, increased atmospheric CO₂, and higher N deposition inputs. While red maples in urban forests in a small city shows a lower tendency to acclimate to toxic metal inputs due to nutrient imbalance and a less robust antioxidant system. These results support that city size can be a proxy for evaluating the differing magnitude of urban intensities (metro-scale impacts) on the physiology and acclimation of trees in urban forests. The observed acclimation of red maple trees to altered air and soil pollution (Abrams 1998), while maintaining higher productivity (Sonti et al. 2019), indicates the suitability of this tree species for biomonitoring urban forest health and urban conditions.

Chapter 3

ABOVE- AND BELOWGROUND URBANIZATION IMPACTS ON RED MAPLE (Acer rubrum L.) PHYSIOLOGY AND MORPHOLOGY: A MANIPULATIVE FIELD EXPERIMENT

3.1 Abstract

Metro-scale and site-scale impacts have high potential to influence the physiomorphology and acclimation of plants in urban environments. My goal was to isolate metro-scale and above- and below-ground impacts on the physiology and morphology of red maple saplings in forests in a small (Newark, DE) and a large (Philadelphia, PA) city. The study was conducted in the FRAME network exploiting a manipulative field experiment in small forests in Newark and Philadelphia (metro-scale). Red maple (Acer rubrum) saplings (n = 54) can serve as a biomonitor for aboveground (AGB), belowground (BGB), and field control (FC) conditions for both cities. I hypothesized that aboveground conditions will positively influence physiology (e.g., chlorophyll), site-linked belowground conditions will positively influence tree morphological plasticity (e.g., leaf shed, root metal tolerance) and field conditions will positively influence physio-morphology plasticity (overall plant survival and adaptation) in red maple saplings. I measured foliar chlorophyll, carotenoids, % carbon and nitrogen, δ^{13} C and $\delta 15N$, nutrient elements, stress-inducible metals, leaf shed, bud survival, and plant survival. My results revealed higher foliar chlorophyll in Philadelphia and higher foliar Ca and greater occurrence of leaf shed in Newark early in the growing season. Additionally, above- and combined above/below-ground conditions increased foliar

Cu uptake, while the interaction of metro-scale impacts and above/belowground conditions increase foliar % C and toxic levels of Mn as well as greater occurrence of leaf shed and bud mortality in Newark saplings early in the growing season. Later in the growing season, saplings had higher foliar Mg and greater occurrence of re-bud and plant survival in Newark and in response to above-/below-ground conditions. In addition, saplings had higher foliar % C, depleted δ^{13} C, toxic levels of Mn and greater occurrence of re-bud and plant survival in forest soils lacking forest canopy (BGB biomonitors). Combined in situ above/belowground conditions increase foliar Fe, Na, Zn, Al, Cr, and Ni near and above toxic levels, and decreased sapling survival (FC biomonitors). Moreover, aboveground conditions increase foliar Zn (toxic levels) and Na and decrease occurrence of re-bud survival late in the growing season. The results suggest differences in metro-scale intensity is an important driver of the physiology and morphology of red maples early in the growing season. Long-term responses suggest saplings acclimate to metro-scale and site-scale impacts exploiting plastic responses to avoid and cope with stress-inducible metals, and summer heat waves and intense rainfalls that result in altered microclimates late in the growing season. My findings highlight the transient role of metro-scale impacts to positively influence physiology and site conditions to drive local acclimation to prolonged stress complexity in urban forests. The physio-morphology plasticity responses to urbanization and above/belowground conditions make red maple trees a good biomonitor of urban forests and urban conditions.

3.2 Introduction

Urbanization impacts have high potential to influence the physio-morphology and adaptation of plants in urban environments (Grimm et al. 2008; Lahr et al. 2018;

Steele and Wolz, 2019). Plants respond physiologically and morphologically to altered urban environmental conditions, yet our knowledge of urban plant physiology and adaptation research is nascent and lingers behind research on plants in other ecosystems (Gregg et al. 2003; Lambrecht et al 2016). Depending on the magnitude of urban intensity at the city level (metro-scale impacts), and alteration in above (AG) and below-ground (BG) conditions at the site scale, plants may exploit physiological (e.g., chlorophyll shifts) and morphological (e.g., leaf shed) differences to environmental variation over different spatial scales (Macel et al. 2007; Raabová et al. 2011; Liu et al. 2012). At larger metro-scales, altered precipitation and elevated temperatures may be dominant factors and important drivers of plant physiology, and at site scales soil conditions and element biogeochemistry may be potential drivers of plant morphology and species local adaptation (Macel et al. 2007; Lambrecht et al 2016; Wright et al. 2017). In addition, both metro location and site conditions (above and below-ground) can induce synergistic and opposing effects on the physiology, morphology and growth of plants (Gregg et al. 2006; Falxa-Raymond et al. 2014; Sonti et al. 2019).

Plants in cities experience a wide range of environmental complexities because of heterogeneity in land use/land cover and morphology and age of cities (Fraterrigo et al. 2005; Steele and Wolz, 2019) that create growth stimulating and/or stress inducing conditions in plants (Gregg et al. 2003; Niinemets 2010; Trammell et al. 2011; Sonti et al. 2019). Cities are characterized by altered above- and below-ground impacts with respect to metro-scale urban intensities and site conditions that influence urban ecosystem processes. For example, at the city level, the urban heat island (UHI) and elevated atmospheric CO_2 can promote plant growth because of enhanced soil and

plant processes (Livesley et al. 2016; Founda and Santamouris, 2017; Sonti et al. 2019), or alternatively lead to soil and plant water stress because of elevated evapotranspiration (Cregg and Ellen, 2001; Breda et al. 2006). Similarly, land-use legacies (e.g., agricultural/commercial activities) at the site-scale can exacerbate soil nutrient and heavy/toxic metal levels that can impact plant growth and survival (McDonnell et al. 1997; Bignal et al. 2007). At the metro-scale, elevated atmospheric reactive nitrogen (N) deposition and CO₂ can promote plant growth or restrict nutrient availability depending upon the initial N and water status of a site (Craine et al. 2002; Silva et al. 2015; Zhang et al. 2017).

From site- to metro-scales, summer ground-level ozone (O₃) formed from nitrogen oxide (NO) and biogenic volatile organic compounds (BVOCs), can negatively influence stomatal response, plant growth, chlorophyll, photosynthesis, and plant survival in cities (Gregg et al. 2006). However, the underlying mechanism governing O₃ damage needs further study (Gao et al. 2016). Increasing atmospheric temperatures and decreasing humidity can positively impact nutrient uptake, physiology, and morphology of plants (Islam and McDonald, 2009; Kukk et al. 2015) at the metro-scale and site-scale during the growing season. Moreover, metro-scale impacts and simultaneous above- and below-ground conditions may influence the physiology and morphology differently within individual plant species and genotypes (Lichtenthaler et al. 2007; Liu et al. 2012; Lambrecht et al. 2016).

Plant response to metro-scale, and above- and below-ground conditions in urban landscapes can provide valuable insight into the physio-morphological response patterns at the leaf level in saplings that control whole plant growth response (Scharenbroch et al. 2005; Gregg et al. 2006; Raabová et al. 2011; Redling et al.

2013). Above-ground factors in urban environments, such as increased temperature and atmospheric CO_2 , create a warm, CO_2 rich environment that positively influences plant stomatal conductance, photosynthesis and transpiration rates, chlorophyll concentrations, and delayed flowering in urban forests (Lambrecht et al 2016; McDermot *in Chapter 2*). In addition, large plants with greater number of leaves, higher leaf area, higher leaf nitrogen concentration, larger biomass, greater plant survival, and faster growth in urban environments suggests metro-scale conditions are favorable for plant growth (Gregg et al. 2006; Lambrecht et al 2016; Lahr et al. 2018; Sonti et al. 2019). On the other hand, site-specific conditions (i.e., soil properties and soil chemistry) due to land use legacies and current land cover can have very strong impacts on soil health and plant-soil element interactions (McDermot in Chapter 2). For example, nutrient and heavy/toxic metal (herein referred to as stress-inducible elements) uptake can result in varied morphology responses such as leaf senescence, leaf shed/drop, death of vertical and lateral buds, or whole plant mortality (Lambrecht et al 2016). Additionally, plants may respond to low soil organic matter (SOM) content and low soil pH that create nutrient deficiency/imbalance, such as Ca or P, by accumulating stress-inducible metals (e.g., Mn, Al, Cr) that trigger chlorophyll degradation and leaf necrotic symptoms (Christensen et al. 1950; Smiley et al. 1986; Silva 2012). Previous land use changes and current urban-rural phenomena that effect nutrient availability and stress-inducible metal toxicity/deficiency (Pouyat et al. 1991; Fraterrigo et al. 2005; Alves et al. 2016) can be indicated by symptoms of nutrients imbalance, chlorosis, reduced chlorophyll or photosynthesis, or early leaf senescence and plant mortality (Kogelmann et al. 2006; Lichtenthaler et al. 2007; Lambers et al. 2015; Lambrecht et al. 2016; Fernando et al. 2016). Plant responses to multiple above-

and belowground factors in urban environments can stimulate or dampen plant growth, and foliar characteristics can mirror such impact, but these biotic-abiotic responses are often complex in nature. Foliar characteristics such as concentrations of chlorophyll, δ^{13} C, δ^{15} N, and nutrients are good bioindicators of plant responses to these altered environmental conditions. Therefore, we need to measure plant variables that can help us to understand these complex relationships in urban forests.

The chloroplast exhibits evolutionary conservative characteristics unique to species, and foliar chlorophyll concentration can be a good indication of plant photosynthesis capacity (Lichtenthaler et al. 2007; Du et al. 2016; Lahr et al. 2018; Molnár et al. 2018; McDermot in Chapter 2). Co-measured carotenoids can aid in light energy capture for photosynthesis and indicate photooxidative stress in the chloroplast (St. Clair and Lynch, 2004; Sun et al. 2018). Under favorable conditions and in plants exhibiting plasticity, elevated CO₂ concentrations and higher temperatures (aboveground conditions) directly increase the net photosynthesis and stomatal conductance rates of the species (Lahr et al. 2018). Altered air temperatures in cities experiencing the urban heat island and frequent summer heat waves can influence soil moisture and nutrient availability (belowground conditions) that control stomatal conductance. The natural abundance of δ^{13} C in plants can indicate stomatal closure under altered soil moisture conditions (i.e., drought or flood) since the CO₂ diffusion rate into the leaf determines ¹³C discrimination during CO₂ assimilation (Farquhar et al. 1989). Furthermore, plant δ^{13} C can be a useful tracer of atmospheric CO₂ sources if the sources are isotopically distinct, such as fossil fuel-derived CO₂ (depleted: -24 to -44‰; Tans 1981, Andres et al. 2000) relative to background atmospheric CO₂ (-8.7‰; NOAA 2019). Elevated concentrations of fossil fuel-derived

CO₂ in cities (Idso et al. 2001; 2002) has the potential to deplete plant δ^{13} C (e.g., Lichtfouse et al. 2003). Similarly, the natural abundance of δ^{15} N in plants can integrate the influence of N sources and N cycling rates on plant N acquisition and availability (Högberg 1997, Evans 2001). Enhanced soil N cycling rates enrich soil δ^{15} N pools due to ¹⁵N discrimination during most soil N processes (e.g., nitrification; Dawson et al. 2002). While foliar δ^{15} N is a useful indicator of soil N cycling rates, plant δ^{15} N can also act as a tracer for N sources, if the δ^{15} N in N sources are isotopically distinct (Robinson 2001). Pollution-derived N sources are typically enriched in ¹⁵N (e.g., Redling et al. 2013). Measurements of foliar chlorophyll, carotenoids, C, and N (i.e., physiology) can give an indication of metro-scale and aboveground impacts (i.e., UHI and elevated N deposition) on plant response to these altered air and soil conditions.

Foliar nutrient (i.e., P, K, Ca, Mg) imbalances can be a result of altered soil pH, soil moisture, soil N, organic matter, and element biogeochemistry (Pouyat et al. 1991; Gregg et al. 2003; Momen et al. 2015; Silva et al. 2015; Lambers et al. 2015; Fernando et al. 2016). Foliar concentrations of phosphorus and potassium (macronutrients), calcium and magnesium (secondary nutrients), and manganese, iron and zinc (micronutrients) are an indication of plant cellular health and nutrition due to enhanced/reduced nutrient inputs (belowground conditions). Plants will respond to excess nutrients by enhancing growth, chlorophyll, and photosynthesis (Maclachlan et al. 1963; Rossini et al. 2006; Momen et al. 2015; Zhang et al. 2017). Additionally, excess micronutrients (e.g., Mn and Zn) can acutely or chronically hyperaccumulate in plant leaves causing cellular oxidative stress due to soil nutrient imbalances, increased available soil moisture and low pH conditions (Kogelmann and Sharpe, 2006; Altland 2006; Lambers et al. 2015; Fernando et al. 2016; McDermot *in Chapter 2*). Similarly,

concentrations of stress-inducible metals (e.g., Al, Na, Co, Cr, Ni, Pb) are an indication of cellular oxidative stress due to altered edaphic conditions (Manta et al. 2002; Sharma and Dietz, 2006; Minocha et al. 2014; McDermot *in Chapter 2*). Plants can respond to acute nutrient toxicity/deficiency (e.g., Mn, Ni) or chronic stress-inducible metal toxicity (e.g., Al, Cr, Pb), drought, and anoxic conditions by displaying strategies such as increased leaf senescence, due to cellular necrosis followed by leaf shedding, and increased bud and plant mortality. Foliar nutrient and stress-inducible metal concentrations, leaf shed, and bud and plant survival measurements (i.e., morphology) can indicate plant response to altered belowground conditions due to urban intensity and site level impacts.

Populations of native red maple (*Acer rubrum* L.) are found predominantly in forests, roadsides, residential yards and parks, and span a wide range of ecological biomes from Newfoundland, Canada to southern Florida (Little 1971; Townsend et al. 1979; Abrams 1998; Wiersma et al. 2007). Red maple trees have been shown to be variable genetically in many traits associated with adaptability (Wiersma et al. 2007; Townsend et al. 1979). Thus, red maple trees provide opportunities to evaluate how trees respond to above- and below-ground impacts in habitats influenced by urbanization (i.e., city size as a proxy for urban intensity; McDermot *in Chapter 2*). For example, red maple trees exhibit variation in foliar chlorophyll, photosynthesis, nutrients (i.e., P, K, Ca), heavy/toxic metals, and morphology responses to elevated atmospheric CO₂, temperature, and reactive N deposition inputs (Stitt and Krapp, 1999; Vallano and Sparks, 2013; Silva et al. 2015; Momen et al. 2015; Zhang et al. 2017). Red maple foliar manganese sensitivity can result in foliar Mn toxicity (Mn accumulation) when soil P, pH, oxygen, and SOM are low, and foliar chlorosis can
result when soil is deficient in Mn and Mg (Smiley et al. 1986; Minocha et al. 1997; St. Clair et al. 2004; Fernando et al. 2016). Red maple response to metro-scale and site-specific impacts can reveal very important traits of plasticity that will make red maple trees ideal biomonitors to altered above- and belowground conditions (Ziska et al. 2003; Calfapietra et al. 2015; McDermot *in Chapter 2*).

To understand above-and below-ground impacts of different magnitudes of urban intensity (i.e., large versus small city) on tree physiology (i.e., pigments, C and N isotopes, and nutrient and metal elements) and morphology (i.e., leaf shed bud survival, plant re-bud and plant survival), I investigated red maple saplings in a manipulative field experiment in small forests embedded in two cities (i.e., Newark, DE and Philadelphia, PA). These cities differ in total population, developed land area, and impervious surface cover, and provide a natural experiment for discerning urban impacts on tree physiology and morphology response to above- and below-ground conditions. This research utilized a long-term urban forest ecology network, the FRAME (FoRests Among Managed Ecosystems; McDermot *in Chapter 2*), to study how red maple saplings respond to above- and below-ground impacts in a small city (Newark, DE) and a large city (Philadelphia, PA). I measured foliar pigments (chlorophyll and carotenoids), carbon (% C and δ^{13} C), nitrogen (% N and δ^{15} N), nutrient elements (e.g., Ca, Mg, P, K, Mn, Fe, Cu), stress-inducible elements (e.g., Mn, Ni, Zn, Al, Na, Cu, Cr), and leaf shed, bud survival, and whole plant survival in red maple saplings. I asked the following research questions: 1) Do red maple saplings exhibit differing physio-morphology response patterns to metro-scale (i.e., Newark and Philadelphia) and site-scale (i.e., AG, BG and FC) impacts?, 2) Are there differences in physiology to transient (i.e., early in the growing season) and prolonged

(i.e., late in the growing season) exposure to the metro and site-scale impacts?, and 3) Are there morphology patterns that indicate acclimation of saplings to metro and sitescale impacts earlier and/or later in the growing season? I hypothesized that greater magnitude of metro-scale impacts on aboveground site-scale conditions will stimulate physiology and foliar nutrients early in the growing season, whereas site-level impacts will suppress the physiology and foliar nutrients later in the growing season. Furthermore, site-scale conditions will drive plasticity to nutrient deficiency and stress-inducible metal accumulation.

3.3 Materials and Methods

3.3.1 Study Area

In the present study, I focused on four-year old red maple (*Acer rubrum* L.) saplings in six forests in a long-term urban forest ecology study, the FRAME (Forest Among Managed Ecosystems) network. I studied fifty-four red maple saplings at the University of Delaware Botanical Garden (n = 18), three forests in Newark, DE (n = 18), and three forests in Philadelphia, PA (n = 18), U.S. In 2009, researchers at the USDA Forest Service and University of Delaware selected forest sites located on public lands across the Coastal Plain and Piedmont region of the Mid-Atlantic for long-term establishment and research. The FRAME traverses an urban gradient extending across Newark, DE and Philadelphia, PA into nearby rural areas. Newark, DE has a population of 31,454 and a mean density of 1,403 people km⁻² and Philadelphia, PA has a population of 1,526,006 people and density of 4,405 people km⁻² (US Census Bureau, 2010). The forest canopy is dominated by native tree species in both cities, and the most common tree across all forests is red maple (*Acer rubrum*).

The forest understory is dominated by non-native and native species (Trammell et al., *in review*). The manipulative field experiment was conducted in the six selected urban forests of the FRAME, which represented forests in a large city (Philadelphia, PA) and a small city (Newark, DE) experiencing different magnitudes of urbanization intensities (a proxy for city size effect and referred to as metro-scale impacts) (Fig. 2.1).

3.3.2 Growth and Establishment of Red Maple Saplings

Sixty-five four-year old red maple saplings were obtained from Octoraro Native Plant Nursery (Kirkwood, PA, U.S.), transplanted in 10-gallon pots containing NX6 potting media (Frey's Brothers, Quarryville, PA, U.S.), and grown for six weeks (April – May 2018) in the greenhouse. The saplings were transferred from the greenhouse to the University of Delaware Botanical Gardens (UDBG, Newark, DE, U.S.) and allowed to acclimate to outdoor conditions for another four weeks (June – July) in 2018. Red maple saplings were watered once daily and fertilized (N-P-K: 21-5-20; 200 ppm N with chelated Fe/gallon) weekly over the ten-week period. Plants were sprayed once with insecticide, cyantraniprole, to treat aphids while inside the greenhouse.

3.3.3 Experimental Design

The red maple saplings (n = 54; root collar diameter (RCD): 0.13 - 0.26 cm; height: 100 - 196 cm) were used in a manipulative field experiment between July and September 2018 in six forests in Newark, DE and Philadelphia, PA (Fig. 3.1). The saplings served as biomonitors of above- (n = 18) and below-ground (n = 18) conditions, and field controls (n = 18) for forests in Newark, DE (small city) and

Philadelphia, PA (large city). Saplings as 'biomonitors' were placed under the subcanopy of *in situ* mature red maple trees (n = 36, Fig. 3.1A) in six urban forests for which data on foliar pigment, nitrogen, element, and sub-canopy soil element were collected in 2017.

<u>Belowground biomonitors (BGB)</u>: Red maple saplings were transplanted to a 10-gallon pot containing *in situ* forest soil (layers maintained as much as possible) obtained from three forests (n = 9) in Newark, DE and in three forests (n = 9) in Philadelphia, PA. The BGB saplings were grown at the University of Delaware Botanical Gardens (UDBG) after sapling transplantation on July 2, 2018. The biomonitors were only watered for establishment and survival initially then left to experience natural precipitation and environmental conditions outside the UDBG (Fig. 3.1B).

Aboveground biomonitors (AGB): Red maple saplings grown in potting mix were transferred to forests and placed under the canopy of three *in situ* adult red maple tree (<10 meters apart) in three forests (n = 9) in Newark, DE and in three forests (n = 9) in Philadelphia, PA (Fig. 3.1C). The AGB saplings were placed where the soil was excavated for the BGB to represent field conditions similar to *in situ* trees (e.g., soil temperatures), and AGB saplings were placed on a block to avoid water pooling around the pot. The AGB saplings were watered initially for establishment and survival, and two 500 mL water bottles filled with water were placed upside down in the 10-gallon pot to drip slowly. The AGB saplings were biweekly fertilized (N-P-K: 21-5-20; 200 ppm N with chelated Fe/gallon) and at times weekly fertilization (N-P-K: 25-5-20; 250 ppm N with chelated Fe/gallon) was done to avoid soil nutrient limitation due to frequent summer rains and hot moments in July and August 2018. The AGB saplings grew under natural precipitation and environmental conditions in the forests. A fence was installed around each AGB sapling for deer protection.

<u>Field control (FC)</u>: Red maple saplings were transplanted into the forest floor under the *in situ* adult red maple trees and next to the above-ground biomonitor as the 'field control' for the BGB and AGB saplings in three forests (n = 9) in Newark, DE and in three forests (n = 9) in Philadelphia, PA (Fig. 3.1D). The field control saplings were watered initially for establishment and survival. The FC grew under natural precipitation and environmental conditions. A fence was installed around each FC sapling for deer protection.



A: *In* situ mature red maple tree

B: Belowground biomonitor (BGB)

C: Aboveground biomonitor (AGB)

D: Field control (FC)

Figure 3.1. Manipulative field experimental design showing *in situ* mature red maple tree as the field active point of experimental set-up for belowground (BGB), aboveground (AGB), and field control (FC) saplings of red maple in six forests in Newark and Philadelphia from July to September 2018.

3.3.4 Foliar Collection

Red maple saplings were grown in the forests and UDBG during summer 2018, and leaf samples were collected after fourteen (14) days and 90 days after transplant. Leaf samples (n = 54) were collected at mid-upper-canopy and wrapped in wet paper towels and stored in zip-lock bag on ice (4°C) during transportation from the forest to laboratory. Leaf samples were stored at -20°C for pigment analysis, and oven dried at 55°C for nutrient and stress-indicating elements, carbon and nitrogen concentration, and natural abundance δ^{13} C and δ^{15} N isotope analyses.

3.3.5 Foliar %C, %N and Natural Abundance δ^{13} C and δ^{15} N Isotopes

Leaf subsamples (~ 3500 µg) were ground to powder in a ball mill using a Retsch Ball Mixer Mill (MM200, Haan, Germany). Carbon and nitrogen

concentrations (%) and δ^{13} C and δ^{15} N values (‰) were measured with the Carlo Erba NC2500 elemental analyzer (California) interfaced with a Thermo Delta V+ isotope ratio mass spectrometer (IRMS; Thermo, Bremen, Germany). Two external known reference materials (USGS 40 and USGS 41) and an in-house standard (spinach leaves), that were previously calibrated against internationally known reference materials, were used. The precision is 0.12‰ for δ^{13} C and 0.11‰ for δ^{15} N. The natural abundance stable isotope values were expressed relative to the international standard for δ^{13} C (Vienna PeeDee Belemnite) and δ^{15} N (atmospheric N₂) in the conventional δ -notation:

$$\delta^{13}C = [({}^{13}C_{sample}/{}^{12}C_{sample})/({}^{13}C_{standard}/{}^{12}C_{standard}) - 1] * 1000\%$$

$$\delta^{15}N = [({}^{15}N_{sample}/{}^{14}N_{sample})/({}^{15}N_{standard}/{}^{14}N_{standard}) - 1] * 1000\%$$

3.3.6 Foliar Chlorophyll and Carotenoid Concentration

Freshly frozen leaf samples were brought to room temperature and a plunger used to remove three discs (~ 8.5 - 11.5 mg). Dimethylformamide (DMF, 1 mL) was added to the leaf discs and shaken for 24 h in the dark for complete extraction, then transferred to a refrigerator at 4°C for 48 h for complete pigment extraction. The pigment extracts were filtered and further diluted with DMF to attain absorbance values below 1. The absorbance was measured with a spectrophotometer (Thermo Evolution 60S UV-Visible, Waltham, MA, USA) at wavelengths 647 nm, 664 nm, and 480 nm in triplicate. The total chlorophyll (a + b) and carotenoid concentrations were calculated according to equations by Porra et al. (1989) and Minocha et al. (2009):

[Chlorophyll *a*] equation = $(12 \times A664) - (3.11 \times A647) \times dilution factor;$

[Chlorophyll *b*] equation = (20.78 x A647) - (4.88 x A664) x dilution factor; [Total Chlorophyll (a + b)] equations = [Chlorophyll a] + [chlorophyll b], and [Total carotenoids] equation = (1000A480 - 1.12Ca - 34.07Cb)/ 245 x dilution factor.

3.3.7 Foliar Nutrient and Stress-indicating Metal Concentration

Leaf subsamples (~ 0.5 g) ground to fine powder prior to digestion with a CEM MARs5 microwave digestion system (CEM, Matthews, NC) using concentrated nitric acid and 30% hydrogen peroxide. Nutrient elements (B, Ca, Mg, K, P, S, Mn and Fe) were analyzed by inductively coupled plasma optical emission spectroscopy (ICAP; 7600 Duo view Inductively Coupled Plasma – Optical Emission Spectrometer [ICP-OES], Thermo Elemental, Madison, WI) and heavy/toxic or stress-indicating elements (Na, Al, Cu, Cr, Co, Ni, Zn, Cd, As, Se and Pb) by Inductively Coupled Plasma Mass Spectrophotometer (ICP-MS; Agilent 7500cx, Wilmington, DE). Helium gas was introduced into the octopole reaction cell for the analysis of Cr, Co, Ni, As and Se (Miller, 1998).

3.3.8 Sapling Morphology Measurement

Red maple sapling with < 50% canopy leaves at day 28 of transplant was considered an empty canopy plant (n = 9 saplings for each treatment in Newark and Philadelphia forests). The number of saplings having greater than 50% leaf shed was counted and the percentage leaf shed was calculated for each treatment (n = 9) in Newark and Philadelphia forests. Similarly, individual sapling possessing more than 50% viable buds after defoliation (28 days after field transplant) and re-foliation (56 days after transplant) was considered as a live bud sapling, thus initial bud and re-bud survival were counted and the percentage survival was calculated for each treatment in Newark and Philadelphia forests. Whole plant survival (visible green leaves and viable buds) was counted at the end of the experiment (day 90 of field transplant) and the percentage whole plant survival was calculated for each treatment (n = 9) in Newark and Philadelphia forests.

3.3.9 Statistical Analyses

All data analyses were conducted in R (Version 3.3.3 and x64 3.6.1; R Core Team, 2016). Statistical significance is reported at the $\alpha = 0.05$ critical value. A two factor ANOVA (balanced and unbalanced design) was performed to analyze differences in foliar chlorophyll, carotenoid, δ^{13} C, % C, % N, δ^{15} N, and elements between the cities (Newark and Philadelphia) and treatments (AGB, BGB, and FC) for data that met the assumptions of normality (Shapiro test) and homoscedasticity (Levene test), followed by post-hoc Tukey HSD tests for differences between means. Kruskal-Wallis test was used to determine significance when data did not meet the assumptions of normality and equal variance.

3.4 Results

3.4.1 Early Growing Season Response to Above- and Below-ground Urban Conditions

Foliar total chlorophyll concentrations were significantly greater in red maple saplings in Philadelphia forests (11.95 \pm 0.71 mg g⁻¹ FW) than in Newark forests (10.15 \pm 0.56 mg g⁻¹ FW; p = 0.05; Fig. 3.2); however, there were no significant differences in foliar chlorophyll between the treatments, or the interaction between

cities and treatments. I found significant differences in foliar C (%) between treatments (p = 0.03) and the interaction (p = 0.01) between treatments and cities. Foliar C was significantly lower in the FC saplings in Newark (49.50 ± 0.23 % C) compared to both Newark (50.74 ± 0.18 % C) and Philadelphia BGB saplings (50.78 ± 0.12 % C; p = 0.03) and Philadelphia FC sapling (50.6 ± 0.5 % C; p = 0.05; Fig. 3.2). There were no significant differences in the mean concentrations of the total carotenoids, δ^{13} C, % N, and δ^{15} N in red maple saplings between Philadelphia and Newark forests, treatments, or the interactions between cities and treatments (p >0.05).



Figure 3.2. Foliar total chlorophyll concentration and % C of belowground (BGB), aboveground (AGB) and field control (FC) saplings of red maple in three Newark forests and three Philadelphia forests 14 days after field transplant (early growing season) in July 2018. Upper case letters (A, B) represent differences between treatment means, lower case (a, b) letters represent interaction differences between treatment and city (treat: city), and asterisks represent differences between city means.

Regardless of the treatments, foliar total Ca was significantly higher in saplings in Newark forests (11456 \pm 541 mg Ca kg⁻¹ DW) compared to saplings in Philadelphia forests (9611 \pm 631 mg Ca kg⁻¹ DW, p = 0.03; Fig. 3.3). Additionally, I found differences in the interaction between cities and treatments as foliar Ca was significantly lower in Philadelphia BGB saplings ($8078 \pm 821 \text{ mg Ca kg}^{-1} \text{ DW}$) compared to Newark FC saplings (12258 \pm 1004 mg Ca kg⁻¹ DW; p = 0.05; Fig. 3.3). I found significant differences across treatments for mean concentration of foliar Mn and Cu. The AGB saplings (266.5 \pm 41 mg Mn kg⁻¹ DW) had significantly greater foliar Mn than BGB (148 \pm 18 mg Mn kg⁻¹ DW) and FC saplings (188 \pm 28 mg Mn kg⁻¹ DW, p = 0.02; Fig. 3.3). I also found differences in the interaction between cities and treatments. Foliar Mn was significantly greater in Newark AGB saplings (356 \pm 67 mg Mn kg⁻¹ DW) compared to all the other saplings (Fig. 3.3). Foliar Cu was significantly lower in BGB saplings (6.35 ± 0.7 mg Cu kg⁻¹ DW) compared to AGB $(8.9 \pm 0.8 \text{ mg Cu kg}^{-1} \text{ DW})$ and FC saplings $(9.1 \pm 0.75 \text{ mg Cu kg}^{-1} \text{ DW}; p = 0.04;$ Fig. 3.3). There were no significant differences in foliar Mg, K, P, S, Fe, or stressinducible elements across cities, treatments, or the interaction between cities and treatments.



Figure 3.3. Foliar concentrations of total Ca, Mn and Cu of belowground (BGB), aboveground (AGB) and field control (FC) saplings of red maple in three Newark forests and three Philadelphia forests 14 days after field transplant in the early growing season (July 2018). Upper case letters (A, B) represent differences between treatment means, lower case (a, b) letters represent interaction differences between treatment and city (treat: city), and asterisks represent differences between city means.

Canopy leaf shed occurred in more red maple saplings in Newark forests (82%) than in saplings in Philadelphia forests (63%; Table 3.1). Furthermore, across treatments BGB saplings (78%) showed the greatest occurrence of leaf shed compared to FC (73%) and AGB saplings (67%). Initial bud survival occurred the least in BGB (23%) saplings in Newark forests compared to the highest occurrence in BGB (56%) saplings in Philadelphia forests. Across treatments, AGB saplings (55%) showed the greatest occurrence of bud survival compared to BGB (40%) and FC saplings (33%; Table 3.1).

Table 3-1.Leaf shed and bud survival of belowground (BGB), aboveground (AGB),
and field control (FC) saplings of red maple in three Newark forests and
three Philadelphia forests 28 days after field transplant in the early
growing season (July 2018).

Urban forests	Biomonitor	Leaf shed (%)	Bud survival (%)
Newark, DE (small city)	BGB	89	23
	AGB	78	66
	FC	78	33
Philadelphia, PA (large city)	BGB	67	56
	AGB	56	44
	FC	67	33

3.4.2 Late Growing Season Response to Above- and Below-ground Urban Conditions

I found no significant differences in foliar total chlorophyll and carotenoid concentrations, % N, or δ^{15} N in red maple saplings between cities or treatments in the

late growing season (p > 0.05). However, I found significant differences in foliar δ^{13} C and % C across treatments, but no significant differences between the cities in the late growing season. Foliar δ^{13} C was significantly depleted in BGB saplings (-29.5 ± 0.3 ‰) compared to AGB (-27.9 ± 0.3 ‰) and FC saplings (-27.3 ± 0.5 ‰, p < 0.01; Fig. 3.4), and foliar %C was significantly higher in BGB (48.7 ± 0.2 %) and AGB saplings (48.9 ± 0.5 %) compared to FC saplings (47.1 ± 0.5 %, p < 0.001; Fig. 3.4).



Figure 3.4. Foliar concentrations of δ^{13} C and % C of belowground (BGB), aboveground (AGB) and field control (FC) saplings of red maple in three Newark forests and three Philadelphia forests 90 days after field transplant in the late growing season (September 2018). Upper case letters (A, B) represent differences between treatment means.

In the late growing season, I found significant differences in three foliar nutrients (i.e., Mg, Fe, and Mn) between cities and across the treatments. At the city level, foliar Mg was significantly higher in Newark forests saplings ($4524.6 \pm 271 \text{ mg}$ Mg kg⁻¹ DW) compared to Philadelphia forests saplings ($3502 \pm 241 \text{ mg}$ Mg kg⁻¹ DW, p < 0.02; Fig. 3.5). Across treatments, foliar Mg was significantly higher in AGB ($4635.5 \pm 387 \text{ mg}$ Mg kg⁻¹ DW) and FC ($4396.5 \pm 343 \text{ mg}$ Mg kg⁻¹ DW) saplings compared to the BGB saplings ($3334 \pm 263 \text{ mg}$ Mg kg⁻¹ DW, p = 0.015; Fig. 3.5). Foliar Fe was significantly higher in FC saplings ($394 \pm 82 \text{ mg}$ Fe kg⁻¹ DW) compared to BGB ($102 \pm 5 \text{ mg}$ Fe kg⁻¹ DW) and AGB saplings ($207 \pm 44 \text{ mg}$ Fe kg⁻¹ DW, p =0.0001; Fig. 3.5). Alternatively, foliar Mn was significantly greater in the BGB ($547 \pm$ 109 mg Mn kg⁻¹ DW) compared to the AGB ($148.5 \pm 29 \text{ mg}$ Mn kg⁻¹ DW) and FC ($84 \pm 11 \text{ mg}$ Mn kg⁻¹ DW) saplings (p < 0.001; Fig. 3.5). I found no significant differences in foliar Ca, K, P, or S across cities, treatments, or their interaction in the late growing season.



Figure 3.5. Foliar nutrient concentrations of Mg, Fe, and Mn of belowground (BGB), aboveground (AGB) and field control (FC) saplings of red maple in three Newark forests and three Philadelphia forests 90 days after field transplant (September 2018). Upper case letters (A, B) represent differences between treatment means and asterisks represent differences between city means. In the stress-inducible metals, I found consistent differences across the treatments for Na, Zn, Al, Cr and Ni. The BGB saplings have significantly lower foliar Na (25.6 ± 4.2 mg Na kg⁻¹ DW) and Zn (48 ± 4 mg Zn kg⁻¹ DW) than the AGB (Na: 70 ± 12.6 mg kg⁻¹ DW; Zn: 156.4 ± 38 mg kg⁻¹ DW) and FC (Na: 67 ± 10.6 mg kg⁻¹ DW; Zn: 119 ± 22 mg kg⁻¹ DW) saplings (p < 0.01; Fig. 3.6). Foliar concentrations of Al were significantly higher in FC saplings (122.8 ± 25.6 mg Al kg⁻¹ DW) than in BGB (43 ± 5 mg Al kg⁻¹ DW) and AGB (65.7 ± 7.7 mg Al kg⁻¹ DW) saplings (p < 0.001; Fig. 3.7). Similarly, foliar concentrations of Cr and Ni were significantly higher in FC (Cr: 36 ± 9 mg kg⁻¹ DW; Ni: 16 ± 4.8 mg kg⁻¹ DW) than in BGB (Cr: 4 ± 0.8 mg kg⁻¹ DW, p < 0.01; Ni: 3.3 ± 0.5 mg kg⁻¹ DW, p = 0.05) saplings (Fig. 3.7). There were no significant differences in Cu, Co, Cd, As, Se and Pb stress-inducible metals across treatments, cities, and their interaction in the late growing season.



Figure 3.6. Foliar stress-inducible metal concentrations of Na and Zn of belowground (BGB), aboveground (AGB) and field control (FC) saplings of red maple in three Newark forests and three Philadelphia forests 90 days after field transplant (September 2018). Upper case letters (A, B) represent differences between treatment means.



Figure 3.7. Foliar stress-inducible metal concentrations of Al, Cr, and Ni of belowground (BGB), aboveground (AGB) and field control (FC) saplings of red maple in three Newark forests and three Philadelphia forests 90 days after field transplant (September 2018). Upper case letters (A, B) represent differences between treatment means. Red maple sapling re-bud or bud break was greater in Newark forests (71%) than in Philadelphia forests (37%), and AGB saplings had the lowest re-bud when compared to BGB and FC saplings (Table 3.2). Whole plant survival was highest in BGB (100%) and lowest in FC (77%) saplings (Table 3.2).

Table 3.2. Sapling re-bud and sapling survival 56 and 90 days, respectively of belowground (BGB), aboveground (AGB) and field control (FC) red maples in three Newark forests and three Philadelphia forests after field transplant late in the growing season (August/September 2018).

Urban forest	Biomonitor	Re-bud (%)	Plant survival (%)
Newark, DE (small city)	BGB-soil	89	100
	AGB-air	56	100
	FC	67	77
Philadelphia, PA	BGB-soil	78	100
(large city)	AGB-air	11	77
	FC	22	77

3.5 Discussion

Red maple saplings demonstrate differing responses to above- and belowground conditions across the growing season indicating acclimation through plastic response to altered urban environmental conditions in urban forests. Early in the growing season, differences in foliar characteristics suggest red maple saplings were less stressed after day 14 in Philadelphia forests and soils relative to saplings in Newark forests and soils. I found greater foliar chlorophyll concentrations in saplings growing in Philadelphia forests, yet greater foliar Ca concentrations, greater occurrence of leaf shed, and lowest bud survival in saplings growing in Newark forests. Late in the growing season, I found few differences (except foliar Mg, re-bud and plant survival) in saplings growing in Newark versus Philadelphia forests or soils. However, there were significant differences in foliar characteristics of saplings exposed to above- and/or below-ground conditions. In general, foliar nutrients and stress-inducible metals were lowest in the red maple saplings growing in the soils from the Newark and Philadelphia forests compared to saplings exposed to the aboveground conditions) in Newark and Philadelphia forests. This suggests the presence of isolated belowground conditions reduce the uptake of stress-inducible metals, whereas saplings growing in the forests had greater nutrient and metal uptake. My study indicates above- and below-ground conditions in red maple saplings.

3.5.1 Early Growing Season Physio-morphology of Red Maple Saplings

Early in the growing season, increased concentrations of foliar chlorophyll in red maple saplings in Philadelphia forests suggests a positive effect of greater urban intensity on chloroplasts (i.e., chlorophyll biosynthesis) for photosynthesis and growth (Gregg et al. 2006; Lichtenthaler et al. 2007; Momen et al. 2015; Lambrecht et al. 2016; Zhang et al. 2017; Lahr et al. 2018; Sonti et al. 2019). Since urban centers have elevated temperature (UHI), N deposition inputs, and CO₂ concentrations (aboveground impacts; Founda D, Santamouris, 2012; Watson-Lazowski et al. 2016; Livesley et al. 2016; Moser et al. 2017), I expected red maple saplings in the larger city (Philadelphia) to have a greater response to aboveground conditions relative to red maple saplings in the smaller city (Newark). The increased chlorophyll in all saplings experiencing aboveground conditions in Philadelphia supports this expectation. Alternatively, higher concentration of foliar Ca in saplings in Newark forests early in the growing season suggests a positive effect of less urban intensity on soil Ca availability (i.e., favorable Ca/Mg ratio) (Maas et al. 1968; Abrahamsen, 1983; Pouyat et al. 2007; Macel et al. 2007; McDermot, *in Chapter 2*). Peak growing season (July) rainfall was 5.5 cm above 30-yr normal close to the Newark forests, whereas Philadelphia experienced less than average rainfall (-3.3 cm) relative 30-yr normal in July 2018 ([1981- 2010]; NOAA 2019). Therefore, root uptake of Ca may respond to lower temperature (~1°C), heat waves (Kornhuber et al. 2018; Vogel et al. 2019), lower N deposition inputs, lower CO₂, and higher soil moisture (Momen et al. 2015; Silva et al. 2015; NOAA 2019; McDermot *in Chapter 2*) in this small city relative to red maple saplings in Philadelphia forests.

Similarly, I found higher foliar % C in red maple saplings growing in Philadelphia and Newark forest soils compared to the FC saplings in Newark. This suggests positive effects of belowground conditions with the absence of the combined above- and belowground (i.e., forest canopy and increase soil water) impacts on foliar % C (i.e., plant nutrition) in Newark (Garten and Taylor, 1992; Falxa-Raymond et al. 2014). Elevated foliar chlorophyll in Philadelphia saplings and increased foliar Ca in Newark saplings growing in belowground conditions (intense rainfall on Ca availability) indicate positive responses to metro-scale impacts earlier in the growing season (Kornhuber et al. 2018), whereas lower foliar % C in Newark FC saplings indicates above- and below-ground conditions combined (e.g., intense rainfall and intact forests canopy) reduce C assimilation in Newark.

The metro-scale influence on site-specific soil conditions from Newark and Philadelphia forests can induce contrasting plant nutrient or stress inducible metal responses. Higher foliar micronutrients in AGB saplings suggests a positive response via above ground conditions for Mn plant uptake (i.e., plant nutrition or increased light; Smiley et al. 1986; St. Clair and Lynch, 2004; Altland, 2006: Fernando et al. 2016) and establishment early in the growing season. This pattern is driven by greater foliar Mn in saplings growing in pots in Newark forests (i.e., AGB; Fig. 3.3). Since urbanization can impact forest humidity, humid forest conditions (aboveground impact) are expected to have slower rates of evaporation of water from pots in Newark (AGB saplings). In combination with the high rainfall and heat waves (Vogel et al. 2019; NOAA 2019) in Newark early in the growing season, it is possible that greater soil water availability due to over-watering (rainfall and pot watering) and root action (e.g., root acid release) create unfavorable conditions in pots (AGB-Newark forests) that alter root and stomatal response likely accumulated Mn (and less mobile Cu) for root uptake, especially when conditions are anoxic, acidic and nutrient limited (altered leaching rate, nutrient imbalances, altered transpiration). Similarly, more available Cu in AGB media and FC soils (belowground impact) should be more available for root uptake in AGB and FC saplings when other metals (e.g., Zn, Cd, Mn, Al) are already taken up or leached from the pot or soil when moisture conditions are great (e.g., very wet July month in 2018; Kornhuber et at. 2018; NOAA 2019) and soil pH is low in soils (McDermot in Chapter 2). I found an increase in foliar Cu in AGB and FC saplings experiencing aboveground and belowground conditions that are similar, which supports this possibility that wetter soils, heat waves, greater transpiration rates, and more root exudates facilitated plant Cu uptake.

The foliar accumulation of Ca, Mn, and Cu in red maple saplings provides insight on the potential controls on leaf shed and post-defoliation bud survival patterns across cities and at site scale. Forests in the small city (Newark) showed the highest occurrence of leaf shed/drop (e.g., BGB saplings) and the lowest occurrence of bud survival (e.g., BGB) relative to saplings in Philadelphia forests. This suggests that metro-scale/site-level interaction resulted in foliar Mn toxicity (> 285 mg kg⁻¹ DW; Altland, 2006; Kramer 2010; Lambrecht et al 2016; McDermot in Chapter 2) in AGB saplings in Newark forests, and site-scale impacts resulted in increased foliar Cu (> 5mg kg⁻¹ DW; Altland, 2006; Kramer 2010) in AGB and FC saplings growing in Philadelphia and Newark forests early in a wet year (NOAA 2019). Saplings in wetter Newark forest soils may have responded to Mn toxicity via symptoms of leaf chlorosis and subsequent leaf shed/drop (stress avoidance strategy) to reallocate resources from leaf to stem and root (root tolerance) until favorable conditions return for re-growth. AGB (and FC) saplings with higher foliar Cu in Philadelphia forests may have exploited the positive impacts of metro-scale (e.g., UHI, atmospheric CO₂) conditions and played a positive role in greater chlorophyll production.

The combined implications of greater foliar chlorophyll, lower leaf shed, and greater bud survival in red maple saplings growing in Philadelphia forests suggests metro-scale impacts potentially promote (AGB media and FC soil conditions) and override site-specific belowground conditions to stimulate saplings 14 days after field transplant in Philadelphia forests. Alternatively, the combined effects of greater foliar Ca (i.e., Newark) and accumulated foliar Mn (AGB in Newark) and Cu (AGB and FC in both cities) coupled with greater leaf shed and lower bud survival in red maple saplings growing in Newark forests suggest metro-scale impacts and site-specific

belowground conditions likely acted positively to influence foliar Cu uptake. However, microclimate conditions (excess water in pot) likely induced Mn toxicity and suppressed saplings early in growing season in Newark forests. Red maple sapling response to soil stress-inducible metals and nutrient imbalances after 28 days field transplant highlights red maple's ability to acclimate and exploit plastic strategies to resist stress-inducible conditions and prolong sapling survival in Newark and Philadelphia forests for late growing season plant responses.

3.5.2 Late Growing Season Physio-morphology of Red Maple Saplings

The metro-scale differences I observed in the late growing season were higher foliar Mg, greater occurrence of re-bud and plant survival in saplings growing in Newark forests and soils. This suggests above- and below-ground differences between the cities drive Mg availability for plant uptake. Late growing season rainfalls were 11 cm (close to the Newark forests) and 9.6 cm (close to Philadelphia forests) higher than the 30-year normal in September 2018 ([1981- 2010; NOAA 2019). In Newark, root uptake of Mg may respond to lower temperatures, increase soil moisture (intense rainfalls), and nutrient imbalance/deficiency relative to red maple saplings in Philadelphia forests. Elevated foliar Mg in Newark saplings suggests a possible nutrient competition (e.g., increased Ca observed during early growing season) or nutrient imbalance/deficiency in soils after high precipitation inputs that lead to increase Mg (and Fe) availability for uptake later in growing season in 2018 (Fraterrigo et al. 2005; NOAA 2019). Additionally, soil Mg as reflected in saplings in Newark forests was the nutrient differentiating metro-scale impacts and site level conditions, likely driven by agriculture legacies (dolomite-Ca/Mg fertilizers or geology related-Mg) that has enduring effect on soil Mg (Fraterrigo et al. 2005; Pouyat et al. 2007).

The saplings growing in Newark and Philadelphia forest soils (BGB saplings) likely had greater nutrient deficiencies/imbalances and high Mn (lack of an intact forest canopy) since the foliar concentrations of Mn (Fig. 3.5) were two times higher than the threshold value of 285 mg kg⁻¹ DW (Altland, 2006; Kramer 2010; Fernando et al. 2016; McDermot *in Chapter 2*). Throughout summer and early fall 2018, both Newark and Philadelphia experienced intense rainfalls, yet early fall rainfalls were on average 10 cm above the 30-yr normal (NOAA 2019) in both cities. This has the potential to saturate soils causing high nutrient leaching, nutrient imbalances, altered soil biogeochemistry, and altered soil pH and, thus, create reducing conditions for Mn²⁺ formation (Christensen et al. 1950; Fraterrigo et al. 2005; Kogelmann et al. 2006). Observed toxic levels of foliar Mn (> 400 mg kg⁻¹ DW; Altland 2006; Kramer 2010) in BGB saplings in soils from both Newark and Philadelphia forests exposed to greater light (higher stomatal conductance and transpiration xylem pull), high soil moisture (slowly accumulate plant usable Mn^{2+}), and low soil nutrients suggest that light and soil moisture conditions, low pH, and nutrient deficiencies/imbalances increase foliar Mn uptake to toxic levels (St. Clair and Lynch, 2004; Lambers et al. 2015; Fernando et al. 2016; McDermot in Chapter 2).

In the late growing season, I found red maple saplings growing in Newark and Philadelphia soils had δ^{13} C depleted foliage. In 2018, intense rainfall and temperature (September: 72.3 – 72.5°C compared to 67.8 – 69.1°C 30-yr normal) were almost twice as high as the 30-yr normal in Newark and Philadelphia regional areas (Vogel et al. 2019; NOAA 2019). This suggests potentially greater stomatal opening being

outside of the urban forests (lacked canopy) due to CO₂ source differences, which can lead to ¹³C depletion while the city impacts resulted in ¹³C enrichment (Garten and Taylor, 1992; Dawson et al. 2002). It is possible the potted soils at the greenhouse (BGB) experienced higher temperatures due to the absence of an intact forest canopy and increased light conditions, which can lead to greater stomatal activity (Drake et al. 2013; Lawson and Blatt, 2014; Urban et al. 2017). Contrasting foliar δ^{13} C responses to potential differences in soil water availability, soil stress-inducible metal contamination, and altered light and temperatures provide evidence that red maple sapling exhibits a stomatal plastic response with differing above- and below-ground conditions (Liu et al. 2013; Drake et al. 2013; McDermot *in Chapter 4*). Furthermore, the observed variations also suggest differences in CO₂ sources on foliar δ^{13} C (BGB versus AGB and FC sources) between the treatments (Lichtfouse and Eglington, 1995; Lichtfouse et al. 2003). Instead higher temperatures combined with intense rainfall in potted samplings at the greenhouse (belowground impacts) potentially influence stomatal plastic responses and foliar δ^{13} C.

In general, I observed plant required threshold levels of foliar Fe, Zn, and Ni (micronutrients; Markert 1991; Kramer 2010) and lower levels of stress-inducible metals such as Na, Cr and Al in the BGB saplings compared to the AGB and/or FC saplings (Markert 1991; Kramer 2010). The intense early and late summer rainfalls (and root exudate production to nutrients deficiency/imbalance) may have concentrated stress-inducible elements (e.g., Fe, Zn, Al, Cr, Ni) to toxic levels in the FC saplings (Markert 1991; Kramer 2010) in these low pH forest soils (McDermot *in Chapter 2*). Furthermore, without access to nutrient uptake from deeper soil layers or higher and more frequent nutrient recharge (e.g., fertilization) in the AGB saplings,

concentrated stress-inducible metals (e.g., Na, Zn, Cr, Ni) may have been taken up by the action of root exudates and accumulated in leaves to toxic levels (e.g., Zn; Christensen et al. 1950; Kramer 2010; McDermot *in Chapter 2*). Nutrient deficiencies observed in BGB (and AGB and FC) saplings may be influenced by high elemental leaching and/or a loss of mycorrhizal association responsible for nutrient uptake in the root rhizospheres (Phillips et al. 2013) due to soil disturbance followed by intense rainfall in 2018 or absence of mycorrhizae in potting media as pots hot and cool.

Across treatments, higher foliar Mg and Zn in AGB saplings, higher foliar Mn in BGB saplings, and greater foliar Mg, Fe, Al, Cr, Ni, and Zn in FC saplings suggest above- and belowground conditions are important drivers of stress-inducible metal uptake that can lead to plant stress (Markert 1991; Fraterrigo et al. 2005; Kramer 2010; Li et al. 2019; McDermot Chapter 2). There is a possibility that nutrient imbalances/deficiencies may have been caused in potted media (AGB), due to soil water saturation and/or field soil metals reaching pots (contamination in forests) as a result of intense rainfalls and even flooding within the forests during summer 2018 (Li et al. 2019). In situ forest soils (FC) may have experience similar impacts as AGB potted media, and summer rainfall in the forests most likely increased a variety of stress-inducible metals that accumulated in FC saplings. Toxic levels of foliar Fe, Zn, Al, Cr and Ni, and nutrient Mg in the saplings growing in the forests (FC saplings) suggest in situ forest soils possibly experience surface geology-related site-linked (Mg, Fe, Al, Cr, Ni) and traffic-linked (e.g., Zn) heavy/toxic metal contamination in soils (Fraterrigo et al. 2005; Pouyat et al. 1997; Trammell et al. 2011; McDermot in Chapter 2). During intense rainfall, forest conditions may result in continuous recontamination by the soil mineral pools in FC saplings compared to potted plants

(AGB and BGB) that possibly experienced greater nutrient leaching. Furthermore, greater foliar stress-inducible metals in FC saplings suggest these metals outcompete available nutrients in the root rhizospheres in response to nutrient imbalances caused by high soil moisture (Islam and McDonald, 2009), root hypoxia (Dreyer 1994), low soil pH (Altland, 2006; McDermot *in Chapter 2*), altered redox potential or a combination of the above factors (Christensen et al. 1950). Differences in foliar stress-inducible metal accumulation across treatments, yet no differences across cities (except Mg) late in the growing season suggest stronger edaphic (Guerrero et al. 2018) and microclimate controls on soil stress-inducible metal availability for plant uptake and adaptability than metro-scale impacts.

Combined metro-scale and site-level impacts such as decreasing atmospheric temperature and increasing humidity late in the growing season following intense rainfalls and UHI impacts can negatively affect re-bud frequency and whole plant morphology (Oke 1973; Dreyer 1994; Islam and McDonald, 2009; Kukk et al. 2015; Li et a. 2019). Red maple sapling re-bud (bud break) after defoliation and whole plant survival may reveal plant vulnerability or resistance to impacts of intense rainfalls and heavy metal accumulation. Intense rainfall in Newark and Philadelphia forests in 2018 combined with UHI and frequent heat waves (Vogel et al. 2019) likely resulted in altered humidity that influence bud break and plant survival at the metro-scale (Philadelphia versus Newark) and across treatments (FC vs. BGB saplings) late in the growing season. I observed the lowest proportion of re-bud and whole plant survival in saplings exposed to greater urban intensity (Philadelphia – ABG and FC saplings) and combined above/belowground field conditions (FC saplings) in both cities. The greatest re-bud and sapling survival occurred in the BGB saplings, which had the

lowest concentrations of stress-inducible metals (except Mn). Greater re-bud (after low occurrence of bud survival) and higher occurrence of whole plant survival in Newark forests relative to Philadelphia forests suggest that sapling root sensitivity to soil conditions potentially exploits root/shoot stress signaling during early/late growing season to avoid (i.e., leaf shed) and cope (i.e., root and stem dormancy) with altered conditions.

3.6 Conclusions

The data presented in this study provide a novel approach to assess metroscale, and above- and belowground drivers of foliar nutrient, stress-inducible metal concentrations and plant survival across two cities differing in size and urbanization intensity. To best of my knowledge, this is the first study to attempt to isolate aboveand belowground impacts on foliar pigment, C and N concentration, natural abundances of C and N isotopes, nutrients, stress-inducible metals and plant survival in urban forests embedded in a small (Newark, DE) and large (Philadelphia, PA) city. The findings from this manipulative experiment suggests early in the growing season metro-scale, above- and below-ground conditions, and their interactions impact chlorophyll, % C, Ca, Mn, Cu, and leaf shed and bud survival patterns after hot and wet conditions. Later in the growing season after frequent hot conditions (Vogel et al. 2019) and intense rainfall, differences in above- and below-ground conditions influenced patterns in foliar ¹³C and % C, secondary nutrients (i.e., Mg and Fe), stressinducible metals, and re-bud and plant survival patterns. Saplings growing in urban forest soils with the absence of aboveground impacts appear to accumulate foliar Mn, yet no other stress-inducible metals. Saplings growing in the absence of belowground impacts accumulate Mg and Zn, while saplings growing in combined above- and

below-ground impacts accumulate foliar Fe, Al, Cr, Ni, and Zn. The combined influence of metro-scale and site-specific aboveground and belowground impacts on plant physiology and morphology, and survival in urban forests suggests red maple trees demonstrate acclimation exploiting inherent physio-morphological plastic responses to complex urban environmental conditions throughout the hot and wet growing season. My findings also suggest that red maple can adjust in urban forests to improve the capacity of the ecosystem to respond positively to intense rainfall. While the above normal rainfall and humidity experienced during our manipulative experiment in 2018 may represent unusual conditions, climate change predictions for the northeast United States are for warmer, wetter conditions. Therefore, the results from my study may provide important information for potential responses of red maple saplings to metro-scale, above- and below-ground conditions, and their interactions in the future.

Chapter 4

URBANIZATION IMPACTS ON LEAF MORPHO-ANATOMY TRAITS IN RED MAPLE TREES IN TEMPERATE DECIDUOUS FORESTS

4.1 Abstract

We investigated metro-scale impacts of urban heat island, reactive N deposition inputs, and elevated CO₂ on leaf anatomy and morphology of trees growing in urban forests. Our goal was to compare metro-scale impacts on leaf morphoanatomy traits in red maple (Acer rubrum) trees in deciduous forests in a small (Newark, DE) and a large (Philadelphia, PA) city. The study was conducted in six 'urban' forests in the FRAME network on eighteen mature red maple trees in Newark and Philadelphia forests. We hypothesized that red maples in Philadelphia forests compared to Newark forests will have greater fresh leaf weight, dry leaf weight, leaf dry matter content, leaf thickness, upper epidermal thickness, spongy palisade length, stomatal density, stomatal length, and stomatal width. In addition, lower leaf water content, specific leaf area and spongy mesophyll length are expected in trees from Philadelphia forests compared to Newark forests. Our results indicate higher stomatal length and width and lower relative leaf water content in leaves of red maples in Philadelphia forests compared to Newark forests (p < 0.10). The increased stomate size and decreased leaf water content in these red maple trees suggest potential altered water-use efficiency and photosynthesis in response to greater metro-scale impacts in Philadelphia forests. This supports previous findings of possible physiological and

biochemical acclimation of red maples to urban conditions. Furthermore, the findings from this study suggest red maple trees may be a good biomonitor of the 'metro-scale' impacts in urban environments. Metro-scale drivers likely override site-scale controls of plant acclimation and adaptation in urban forests.

4.2 Introduction

Leaf morpho-anatomy responses in trees are a good indicator of local site conditions and regional scale climate impacts (Abrams 1998). Urban environments are characterized by elevated temperatures (urban heat island, UHI; Oke 1973) and atmospheric CO₂ concentrations (Idso et al. 2001; 2002), increased reactive N deposition inputs (Lovett et al. 2000), and nutrient-rich soils (Pouyat et al. 2007; McDermot *in Chapter 2*) compared to their suburban and rural counterparts. These altered environmental conditions in cities can impact leaf morpho-anatomical characteristics of in situ trees in urban forests. Leaf morpho-anatomy can change with pollution (Royer et al. 2008; Miller-Rushing et al. 2009; Pourkhabbaz et al. 2010), yet little is known about how multiple global change factors, such as climate and pollution, will influence leaf morpho-anatomy. In addition, long-term exposure of tree leaves in urbanized settings to the altered above- and below-ground conditions may result in more robust morpho-anatomy traits that allow plants to better mitigate urban conditions. Therefore, leaf structural traits of plants growing in highly urbanized areas can be an ideal study system for understanding how multiple environmental changes influence plant anatomy and morphology, which is an important determinant of plant growth, health, and function.

Urban forests and trees have the potential to improve air, soil, and water quality in urban environments through blockage, uptake, and immobilization of

pollutants (Nowak and Dwyer 2007; Livesley et al. 2016), and plant leaves have the ability to trap, sequester, and compartmentalize pollutants (e.g., Yadav 2010; Chaturvedi et al. 2013). Therefore, urban trees may provide important benefits via pollution regulation of altered urban conditions; however, this is debatable due to biogenic volatile organic compound (BVOC) production by urban trees (Eisenman et al. 2019). In fact, tropospheric-ozone has been reported to induce visible injuries, deregulate stomatal conductance, and reduce photosynthesis and leaf area at the expense of BVOC production (Jahan and Iqbal, 1992; Gregg et al. 2006). For some pollutants, like particulates, the aerodynamic effect of tree canopies was reported to increase particulates residence time and recirculate and concentrate airborne particulates in urban environments (Vos et al. 2013, Tong et al. 2015). These complex interactions with urban tree canopies and atmospheric pollutants may stress trees leading to shorter life-span even with enhanced growth rates (Sonti et al. 2019) or increased tree species colonization tendencies with prolonged life-span (Abrams 1998). Thus, the ability of urban trees to mitigate urban conditions and improve environment quality and sustainability of cities may be dampened by their response to altered urban environmental conditions (Setälä et al. 2012; Vos et al. 2013; Phillips et al. 2019). Moreover, leaves of trees growing in urban environments can serve as a biomonitor of altered urban conditions (Redling et al. 2013; Mohasseli et al. 2016), but much of this data are theoretical in approach (Nowak 2005).

Leaf morphology and anatomy characteristics can serve as bioindicators of plant response to altered environmental conditions and land-use change, specifically air (Pal et al. 2002; Chaturvedi et al. 2013) and soil (Abrams 1998; Tomasevic et al. 2004; Sharma and Dietz, 2006) pollution. Tree species exposed to auto-exhaust

pollution exhibited leaf structural changes (e.g., increased stomatal frequency, trichome length, collapsed epidermal cell), yet no differences in leaf phenology (Pal et al. 2002). London plane trees had reduced the leaf size and stomate density in a highly urbanized environment compared to a rural area; however, area of palisade parenchyma cells and spongy parenchyma cells showed no change with urbanization (Pourkhabbaz et al. 2010). Furthermore, Royer et al. (2008) reported toothier and more highly dissected red maple leaves in response to cold versus hot climates. Interestingly, leaf structural characteristics of red maple trees do not particularly support evidence for its wide range adaptability of this species, yet the tree persists in hot, dry, and nutrient-poor forest stands across northeastern United States (Abrams 1998).

The impacts of altered air and edaphic conditions on plant characteristics are largely a role of geo-location (Abrams 1998; *in Chapter 1*), motor vehicle traffic emission (>70% total emission; WHO 2006), distance from urban center (Trammell et al. 2011), land-use change (Abrams 1998), and local site-scale conditions (Clark and Kjelgren, 1990; Egilla et al. 2005; Macel et al. 2007). In many disturbed ecosystems and experimental studies, reports of dryer or wetter soils (Whitlow and Bassuk, 1988; Abrams 1998), soil contaminant enrichment (de Silva et al. 2012), and altered soil biogeochemistry (Zhang et al. 2017) seem to result in contrasting plant responses (e.g., leaf size and shape, stomata density, spongy and palisade mesophyll cells, leaf senescence, and epidermal thickness; Jahan and Iqbal, 1992; Pal et al. 2002; Gregg et al. 2006; Royer et al. 2008; Pourkhabbaz et al. 2010; Lambrecht et al. 2016). Tree species that display morpho-anatomical acclimation and tolerance to contrasting
environmental impacts can provide more insights into multiple cumulative impacts on urban trees.

Since the turn of the late twentieth century, red maple (*Acer rubrum*) trees have dominated a wide range of ecological biomes, particularly forest stands in the northeastern to southeastern United States (Little 1971; Townsend et al. 1979; Abrams 1998). The morphology and growth characteristics of red maple in forest stands in the eastern U.S. have resulted in wide scale cultivation because of its aesthetics, marketability, and adaptability in many urban landscapes (Little 1971; Townsend et al. 1979; Wiersma et al. 2007). Importantly, red maple trees are a dominant tree in urban forests (Trammell et al. *in revision*), but very little is known about urban impacts on the leaf morphology and anatomy of red maple trees.

The purpose of this study was to determine the impacts of urbanization on red maple leaf morphology and anatomy as an indicator of tree response to multiple environmental changes, such as increased atmospheric CO₂, elevated temperatures, increased ground-level O₃, and greater nutrient availability. To understand the combined impacts of different magnitudes of urban intensity (i.e., large versus small city) on leaf anatomy and leaf morphology of trees, we investigated red maple trees in small forests embedded in two cities (i.e., Newark, DE and Philadelphia, PA). These cities differ in total population, developed land area, and impervious surface cover. This research utilized a long-term urban forest ecology network, the FRAME, to study how red maple leaves respond to combined metro-scale and site-level impacts in a small city (Newark, DE) and a large city (Philadelphia, PA). We measured fresh leaf weight, dry leaf weight, specific leaf area, leaf thickness, leaf dry matter content, leaf water content, upper epidermal thickness, palisade mesophyll thickness, spongy

mesophyll thickness, stomata density, and stomate length and width. I asked the following questions: 1) Do red maple trees exhibit differing leaf morphology in response to combined metro-scale impacts? 2) Are there differences in leaf anatomy in response to differing magnitudes of urbanization? and 3) Are there morpho-anatomy patterns that indicate plasticity of trees to the combined metro-scale impacts? I hypothesized that red maples in Philadelphia forests will have greater fresh leaf weight, dry leaf weight, leaf dry matter content, leaf thickness, upper epidermal thickness, palisade mesophyll length, stomatal density, stomatal length, and stomatal width compared to red maples in Newark forests. In addition, lower leaf water content, specific leaf area, and spongy mesophyll length are expected in trees from Philadelphia forests compared to Newark forests. A greater magnitude of UHI, atmospheric CO₂, anthropogenic N deposition inputs, and nutrient inputs on red maple trees in urban forests in Philadelphia compared to Newark will result in altered leaf morpho-anatomy and plasticity that support the physiology and acclimation of red maple trees to urban conditions.

4.3 Materials and Methods

4.3.1 Study Species and Geographic Region

We studied leaf morphology and anatomy of mature red maple (Acer rubrum L.) trees in six forests in the FRAME (Forest Among Managed Ecosystems) network. The FRAME traverses an urban gradient extending across Newark, DE (small city) and Philadelphia, PA (large city). Philadelphia has a population mean density three times higher and a population nearly fifty times larger than that of Newark, yet there is a similar impervious surface cover gradient extending across both Newark and Philadelphia (Trammell et al. *in revision*). The magnitude of urban intensity at the city scale is referred to as metro-scale impacts (proxy for urban intensity) that can stimulate growth and/or induce stress in plants in urban forests. The forest canopy of the FRAME is dominated by native tree species in both cities (Trammell et al. *in revision*): red maple (*Acer rubrum*), American beech (*Fagus grandifolia*), black gum (*Nyssa sylvatica*), sweetgum (Liquidambar styraciflua), tulip poplar (*Liriodendron tulipifera*), and red oak (*Quercus rubra*). The forest understory is dominated by non-native and native species: multiflora rose (*Rosa multiflora*), spicebush (*Lindera benzoin*), greenbrier (*Smilax rotundifolia*), Japanese honeysuckle (*Lonicera japonica*), southern arrowwood (*Viburnum dentatum*), autumn olive (*Elaegnus umbellata*), and sweet pepperbush (*Clethra alnifolia*).

4.3.2 Experimental Design and Tree Selection

I selected three forests experiencing high levels of urbanization in Newark, DE (Ecology Woods, Chrysler Woods and Webb Farm) and in Philadelphia, PA (Chamounix, Parkline Drive and Smith Memorial). Therefore, this study focused on the impacts of metro-scale urbanization intensity on leaf morphology and anatomy traits in urban trees. I randomly selected three red maple trees in three forests in Newark (n = 9) and Philadelphia (n = 9). Red maple tree selection details are described in McDermot et al. (in prep). Leaf sampling was conducted at mid-upper-canopy of red maple trees (n = 18) using a pole pruner or by climbing the tree in August 2018. Leaf samples were stored at 4° C during collection in the field prior to transport to the laboratory for leaf morphology and anatomical characterization.

4.3.3 Leaf Morphology Trait Measurements

On fully grown leaves (n = 36) collected from mature red maples in Newark and Philadelphia forests, I performed the following measurements: fresh leaf weight (FLW), dry leaf weight (DLW), dry leaf area (DLA), specific leaf area (SLA), leaf water content (LWC), and leaf dry matter content (LDMC). Fresh leaf samples (n = 180) were allowed to equilibrate to room temperature and FLW was recorded using a laboratory balance. Leaf samples (n = 180) were oven-dried at 55°C for 48 h for DLW and followed by measurements of DLA using a scanner (name, brand, state and U.S) I performed the following calculations for leaf morphology:

SLA = DLA/DLW

LDMC = DLW/FLW

LWC = ((FLW-DLW)/FLW) * 100)

4.3.4 Leaf Anatomy Trait Measurements

Two fully healthily grown and similar size leaves from each red maple tree were punched once (n = 36) with a 1 mm2 disc in areas (left or right of mid vein) that were visibly as similar as possible. Each leaf disc was added to a tray well and covered with paraformaldehyde (0.4%, 1 mL) and triton (0.005%) for fixing cells. A polystyrene sphere was placed into each well to hold the leaf disc in place. Trays containing leaf discs were placed into a bell jar and vacuumed (30 Hg pulse, X3) and stored at 4°C under vacuum overnight. Fixative was aspirated from leaf discs and washed three times with phosphate buffered saline (1xPBS, 1.5 mL) for sectioning and staining prior to microscopic analysis. Each individual leaf disc was added to a cassette and immersed in a beaker containing 1 x PBS (50 mL). Each leaf disc was processed (Leica ASP 300S tissue processor) and embedded into a paraffin. The sections were cut at on a Microm HM 335E paraffin microtome. Sectioned leaf discs (n = 36; 5 µm; 40X objective) were stained with safranin O and fast green then placed between a cover glass and imaged in brightfield. Whole slide imaging was performed on the vertical section of the leaf tissue using an Aperio AT2 brightfield scanner (Leica Biosystems), and the Aperio eSlide Manager software toolkit used to perform anatomical trait measurements of the upper epidermal thickness (UET), stomatal length (SL), stomatal width (SW), spongy mesophyll length (SML), spongy palisade length (SPL), and microscopic leaf thickness (LT_m) of the leaf tissue.

For the leaf abaxial surface imaging, each leaf disc (n = 36) was covered with 1xPBS (2 mL) solution followed by the addition of calcofluor white (1:100, 1%, 1mL) and propidium. A leaf disc was removed and placed between two pieces of cover glass. Leaf discs were imaged on the Echo Revolve microscope in the upright configuration. All images were obtained with the CFP filter. Using the 2X objective and starting at the bottom right corner of the leaf disc, samples were moved 1 field of view to the left and 1 field of view up to enable unbiased analysis of stomatal density. One image was taken at the 2X magnification to highlight the field of view. The objective was switched to 4X and another image was obtained. The leaf disc between cover glass was inverted and the process was repeated for the other side of the leaf. Quantification of stomatal density was obtained from calculations of 4X images of the leaf disc abaxial surface. All quantifications were performed using the Revolve software. Three regions of interest from each image were chosen for analysis.

4.3.5 Statistical Analysis

All data analyses were conducted in R (Version R x64 3.6.1; R Core Team, 2019). Statistical significance is reported at the $\alpha = 0.10$ critical value, and in a few cases the $\alpha = 0.1$ critical values are reported as marginally significant to identify potential trends. Analysis of differences in foliar fresh weight (FLW), dry weight (DLW), dry leaf area (DLA), specific leaf area (SLA), leaf water content (LWC), stomatal length (SL), stomatal width (SW), leaf thickness (LT_m), and upper epidermal thickness (UET) between the cities were analyzed using a one-way analysis of variance (ANOVA) for data that met the assumptions of normality and homoscedasticity. Additionally, the Kruskal-Wallis non-parametric analysis of variance was performed when the assumptions of normality and homoscedasticity were not met.

4.4 Results

4.4.1 Leaf Morphology Traits

In general, leaf morphology traits of red maple trees were similar between Newark and Philadelphia forests. We found no significant differences in fresh leaf weight, dry leaf weight, dry leaf area, specific leaf area, or leaf dry matter content of red maple trees in Newark and Philadelphia forests (Table 4.1). However, we found a significant trend in red maple leaf water content (LWC) with slightly higher LWC (%) in red maples in Newark forests compared to red maples in Philadelphia forests (p = 0.06; Table 4.1).

Morphology trait	Newark, DE	Philadelphia, PA	Unit
FLW	$4.93\pm0.4^{n.s.}$	5.3 ± 0.45 ^{n.s.}	g, FW
DLW	$2.24 \pm 0.2^{\text{ n.s.}}$	$2.6 \pm 0.2^{\text{ n.s.}}$	g, DW
LA	$46.55 \pm 4.1^{\text{n.s.}}$	$52.8\pm3.1^{n.s.}$	cm ² , DW
SLA	$21 \pm 0.9^{\text{ n.s.}}$	$20.5\pm0.8^{\text{ n.s.}}$	$cm^2 g^{-1} DW$
LDMC	$0.45 \pm 0.02^{\text{ n.s.}}$	$0.5 \pm 0.01^{n.s.}$	-
LWC	54.6 ± 1.8*	50.1 ± 1.2	%

Table 4.1.Leaf morphology traits for red maple trees in six forests in Newark, DE
and Philadelphia, PA

Values are morphology measurements of leaves (n = 10) per tree for 18 trees (mean \pm SE).

FLW: fresh leaf weight; DLW: dry leaf weight; DLA: dry leaf area; SLA: specific leaf area; LWC: leaf water content in percentage (%); LDMC: leaf dry matter content. * represents significant differences between means when $\alpha \le 0.10$ and ^{n.s.} represents no significant differences between means.

4.4.2 Leaf Anatomy Traits

Similar to leaf morphology traits, leaf anatomy traits in red maple trees were similar when growing in Newark and Philadelphia forests (Figure 4.1). There were no significant differences in leaf thickness, upper epidermal thickness, spongy mesophyll length, spongy palisade length and stomatal density in red maple trees growing in Philadelphia and Newark forests (p > 0.10; Figure 4.2, 4.3). However, we found differences in leaf stomate anatomy between the two cities. The leaf stomate length and width were significantly greater in red maple trees in Philadelphia forests (SL: 9.6 $\pm 0.2 \ \mu m$; p = 0.08; SW: 15.8 $\pm 0.3 \ \mu m$; p = 0.06) compared to red maple trees in Newark forests (SL: 8.9 $\pm 0.4 \ \mu m$; SW: 14.8 $\pm 0.8 \ \mu m$; p > 0.10; Figure 4.2).



Figure 4.1. Cross sectional view (90 µm) of internal leaf structures of red maple leaf in Newark (a) and Philadelphia (b) forests. UE: Upper epidermis; S: Stomate; SM: Spongy mesophyll; SP: Spongy palisade; LE: Lower epidermis.



Figure 4.2. Leaf anatomy traits of red maple trees in three forests (n =9 in Newark and three forests in Philadelphia (n = 9) forests. Two leaves per tree were used to determine the anatomical traits. Letters represent significant differences between means when $\alpha \le 0.10$.



Figure 4.3. Stomata density of the leaf abaxial surface of red maple trees in Newark (a) and Philadelphia (b) forests. Letters represent significant differences between means when $\alpha \le 0.10$.

4.5 Discussion

This study demonstrates the robust nature of red maple tree morphology and anatomy in altered urbanized settings since morpho-anatomy was similar in red maple trees growing in Philadelphia and Newark forests. However, I did find that red maple trees in Philadelphia forests had greater stomate length and width and lower leaf water content compared to Newark forests. The increase in stomate length and width (i.e., larger guard cells) of red maple trees growing in Philadelphia forests suggests the stomates potentially have lower stomatal conductance rates and/or slower response times (i.e., stomatal kinetics) to urban conditions, yet these are findings that may oppose previous studies (Abrams et al. 1998; Drake et al. 2013; Lawson and Blatt, 2014). Drake et al. (2013) and Lawson and Blatt (2014) reported smaller stomates, higher stomatal kinetics, and higher stomatal density in leaves experiencing altered environmental conditions. Furthermore, the trend for lower leaf water content in red maple trees in Philadelphia forests compared to in Newark forests suggests possible acclimation to water limited systems and/or greater transpiration (i.e., more water loss) as a result of slower stomatal response time with larger stomata (Drake et al. 2013).

In conjunction with stomatal size and leaf water content, foliar nutrient concentrations can indicate potential stomatal conductance and/or kinetics. Wang et al. (2013) and Egilla et al. (2005) have found that lower leaf water content was linked to lower potassium levels, which directly regulate stomatal conductance and root depth (along with N and P). However, I found lower leaf water content in red maple trees that had higher foliar K⁺ (McDermot *in Chapter 2*), which is likely due to acclimation to drier urban conditions. Higher foliar K⁺ ions may play a role in signaling accumulation of polyamines, amino acids, and solutes that may aid in osmotic adjustment and increase the intrinsic water-use efficiency (iWUE) of the trees.

Therefore, larger leaf stomates (i.e. guard cells), lower leaf water content and increased cellular free signaling ions along with reported elevated free metabolites (McDermot *in Chapter 2*) in red maple trees in Philadelphia forests are likely main players regulating intrinsic water-use efficiency and osmotic adjustment for lower leaf water content when K⁺ ions are preferentially taken up and accumulate in cells.

Environmental conditions, such as elevated atmospheric CO₂ and warmer temperatures have the potential to drive changes in stomatal conductance and stomatal kinetics (Gay and Hurd, 1975; Gray et al., 2000; Urban et al. 2017; Purcell et al. 2018; Lahr et al. 2018). Miller-Rushing et al. (2009) have found increased guard cell length and decreased stomate density in response to higher temperatures $(1.8^{\circ}C)$ independent of global CO₂ rise (> 86 ppm) over a 100-year period while intrinsic water use efficiency (iWUE) remained unchanged. While we did not observe differences in stomatal density in red maple trees in forests of differing city sizes, the trend for increased stomate size (i.e., guard cell length and width) in the large city (Philadelphia, PA) suggests some level of stomate plasticity as a result of acclimation to elevated CO_2 and temperature (Drake et al. 2013; Lawson and Blatt, 2014) in response to urban intensity. Furthermore, red maple trees in urban forests appear to respond to elevated temperature via regulatory changes in stomate size and possibly iWUE rather than increased stomatal density (Miller-Rushing et al. 2009; Drake et al. 2013; Lawson and Blatt, 2014). These findings of increased stomata length and width (and unchanged stomatal density), and many unaffected morpho-anatomical traits further indicate a complex physio-anatomy plasticity of red maple trees to greater magnitudes of urban intensity.

Leaf water content changes within tree species are linked to iWUE and may be a complex sub-cellular communication response to stomatal conductance and stomatal kinetics that signal osmoregulatory processes to bring about cellular osmotic adjustment to altered environmental cues (Lawson and Blatt, 2014). Many studies have reported the positive effects of cytosolic free K⁺, Ca²⁺, Zn²⁺ and N-containing compounds on low leaf water content and cellular osmoregulation through mechanisms that control reactive oxygen species (ROS) levels, improve membrane integrity, and regulate cellular osmotic adjustment and stomatal responsiveness, thus improving plant tolerance to multiple abiotic (and biotic) stresses (Egilla et al. 2005; Flexas et al. 2006; Drake et al. 2013; Wang et al. 2013; Hu et al. 2013).

McDermot (*in Chapter 2*) found increased foliar N, K, P, Zn, chlorophyll, proline, glutamine, arginine, and spermine in red maple trees growing in high nutrient and heavy metal soils in forests in a large city. Interestingly, K and Zn were tightly correlated to free amino acids in trees experiencing greater urban intensity. This suggests these nutrients likely communicate with N-containing metabolites to play a role in cellular osmotic adjustment response and ROS detoxification to oxidative stress induced by heavy/toxic metals (e.g., Mn, Al, Zn) and/or salinity stress in these red maple trees. Furthermore, altered cellular K⁺/Na⁺ homeostasis can induce cellular desiccation (i.e. lower cell water content) and signal an osmotic stress response that trigger the regulation of water and nutrient balance, regulation of iWUE, and cellular osmotic adjustment (Sharma and Dietz, 2006; Hu et al. 2013; Winter et al. 2015; Zhang et al. 2016). Lahr et al. (2018) reported that wildtype red maples had higher WUE, stomatal conductance and overall photosynthesis rate as air temperature increase in urban settings likely attributed to their genetic background and local

adaptation. Interestingly, McDermot (*in Chapter 2*) found increased foliar Mg, very toxic levels of Mn, and lower chlorophyll and proline in red maple trees growing in low soil pH, low soil organic matter forests in a small city experiencing lesser magnitude of urban intensity compared to the large city forests. These findings suggest faster stomatal kinetics in response to more disturbed, nutrient-limited (e.g., P, K) soils and foliar Mn-induced oxidative stress (chlorophyll degradation in chloroplasts) under excess soil moisture conditions (e.g., Ecology Woods, Crysler Woods, Webb Farm).

Red maple trees in Newark may have a quick stomatal response (guard cell kinetics) that requires adequate Ca^{2+} ions to aid the movement of lower concentrations of K⁺ ions across guard cells membrane for opening the stomate aperture to allow xylem transpirational pull for adequate nutrients uptake and removal of excess leaf water via transpiration (Drake et al. 2013; Lawson and Blatt, 2014) in wetter forests. Such robust ROS scavenging system of accumulated K^+ and Zn^{2+} , increased free proline and other amino acids, and polyamines reported in red maples in response accumulated heavy/toxic metals (e.g., Mn, Zn, Al) and other combined abiotic stresses (e.g., likely drier soils) Philadelphia forests were possibly compromised (Minocha et al. 1997; Moschou et al. 2002, Sharma and Dietz, 2006; Yadav 2010) in Newark forests due to limited stress regulating ions compared to (McDermot in Chapter 2). Therefore, cellular osmotic adjustment did not dominate and possibly result in lower iWUE and higher leaf water content. The findings of this study suggest lower leaf water content in red maple trees in Philadelphia forests may be better physiologically regulated by N-containing compounds and nutrient ions in response to cellular oxidative stress induced by complex abiotic stresses in nutrient-rich, contaminated soils that likely experience periodic water stress.

Larger leaf stomatal size (i.e., slower stomatal response time) and lower leaf water content observed in this study, along with greater foliar K^+ and Ca^{2+} , and accumulated free amino acids (e.g., Pro, Gln, Glu, Arg) and polyamine (e.g., spermine; McDermot *in Chapter 2*) suggests that red maple trees have acclimated to elevated temperature, CO₂, and N deposition inputs, increased water deficits, and accumulated heavy/toxic metals in Philadelphia forests. This acclimation was in part possible through plastic responses such as larger stomate size that alters the stomatal aperture CO₂ concentration and transpiration gradients. In addition, larger stomate and unaffected specific leaf area suggest a greater stomatal surface area-to-volume ratio for CO₂ uptake in red maple trees growing in Philadelphia forests. Red maple leaves characterized by larger stomates may be associated with slower stomatal opening regulated by an influx of K⁺ ions, possibly accumulated in the guard cells (influx of Ca^{2+} ions regulate stomatal closing) for cell turgor, resulting in slower stomatal conductance rates (Drake et al. 2013; Lawson and Blatt, 2014). Therefore, stomates are possibly partially closed most of the time to conserve adequate leaf water content (and improved iWUE) in red maple trees in Philadelphia forests, possibly experiencing periodic drought stress to greater urban intensity.

4.6 Conclusions

In summary, the study shows that greater magnitudes of urban intensity affected leaf morpho-anatomical response to urbanization impacts indicate plasticity of red maple trees to complex urban conditions. However, plant fitness is a cumulative response of many physiological, morphological, and biochemical adjustments (Minocha et al. 1997; Abrams 1998; Miller-Rushing et al. 2009; Drake et al. 2013; Hu et al. 2015). McDermot (*in chapter 2*) has reported increased foliar chlorophyll,

free amino acids, free spermidine, N, P, K, and Zn in red maple trees in forests across Philadelphia, PA compared to red maple trees in forests across Newark, DE. Therefore, a trend for increased stomate length and width, along with lower leaf water content, in red maple trees growing in Philadelphia forests suggest positive adaptive effects of urbanization impacts on morphology and anatomy traits that may enhance survival and acclimation to harsh urban conditions. In conclusion, leaf morphoanatomy patterns and plasticity strategies indicate red maple trees can serve as a biomonitor of urban forest health and urban environmental conditions.

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