THE DISTRIBUTION AND UPTAKE DYNAMICS OF MERCURY IN LEAVES OF
COMMON DECIDUOUS TREE SPECIES IN MINNESOTA, USA

A THESIS
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF THE UNIVERSITY OF MINNESOTA
BY

Aicam LAACOURI

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF SCIENCE

Edward NATER

February 2013
ACKNOWLEDGMENTS

I would like to thank my advisor Dr. Ed Nater for giving me the opportunity to be here. This work would not have been accomplished without his encouragement and scientific guidance. I would like also to thank Dr. Paul Bloom for serving in my committee and for giving me full access to his lab where the UV-Visible spectrophotometry and the FT-IR analyses were conducted. Additionally, I would like to thank Dr. Randall Kolka and Dr. Jay Bell for serving in my committee. My thanks also go to David Bell for his help with the collection and analysis of samples. Finally, I like to thank my wife Naima for being very supportive and patient with me in all aspects.
ABSTRACT

The origin of mercury in foliage has been found to be mostly atmospheric. In this study, we developed and tested an extraction technique to study mercury distribution within leaves of various temperate deciduous tree species. Knowing the exact location of mercury within the leaf structure will allow us to evaluate its potential to leach, to volatilize, or to accumulate. Additionally, we monitored mercury concentration in foliage during the entire foliage growing season to understand the intra-season variations and to compare the accumulation rates of different species. We also studied the inter-annual accumulation of Hg in foliage by comparing end of season foliage Hg concentrations of two different seasons. Mercury levels in foliage were reported in leaf area- and mass-normalized concentrations/uptake rates. Additionally, mercury levels on the surface, in the cuticle and in the inner tissues of tamarack (Larix laricina L.) needles were monitored during an entire growing season to understand the dynamics of mercury in these three compartments.

The results of this study showed that the extraction technique developed and used for separating Hg associated with the three compartments of the leaf is reliable and that leaf mercury can be separated into three fractions each associated with the surface (2%-4%), the cuticle (2%-6%), and the inner tissue of the leaf (90%-96%). While the surface and the cuticle seemed to act as transitory compartments, leaf inner tissue behaved as a permanent storage site, and Hg concentration increased steadily in this compartment. The results of the season long leaf mercury monitoring confirmed a strong and positive correlation between leaf mercury levels and the age of leaves ($r^2 > 0.81$). Tamarack
(43.13 ±1.7ng g⁻¹ – 2981.12 ±116.3 ng m⁻²) and elm (41.01 ±6.8 ng g⁻¹ – 1519.44 ±175.4ng m⁻²) showed the highest end of season foliage mercury concentrations, while gingko (19.03 ±2.8 ng g⁻¹ – 1421 ±180.5 ng m⁻²) and oak (25.17 ±5.5 ng g⁻¹ – 1144 ±250.1 ng m⁻²) showed the lowest. The difference between the coniferous species and the broadleaf species was more pronounced when surface normalized concentrations were used for the comparison. Lastly, foliage accumulation rate of mercury was found to be the highest in mid-growing season (15.76 ng m⁻² day⁻¹) when photosynthesis is known to be at its peak.
Table of Contents

ACKNOWLEDGMENTS ............................................................... i
ABSTRACT ............................................................................. ii
Table of contents ...................................................................... iv
List of Figures .......................................................................... vi
List of Tables ........................................................................... vii
List of Abbreviations ................................................................ viii
GENERAL INTRODUCTION ...................................................... 1
CHAPTER 1. LITERATURE REVIEW ........................................... 1
  1.1 Mercury as a global pollutant ............................................... 4
  1.2 Mercury’s environmental behavior and toxicity ...................... 5
  1.3 Vegetation as a sink of mercury ........................................... 6
  1.4 Pathways into vegetation .................................................... 8
    1.4.1 Leaf and root uptake .................................................. 8
    1.4.2 Gaseous and particulate deposition ............................... 9
    1.4.3 Wet and dry deposition ............................................. 9
  1.5 Deposition and transfer of Hg to the leaf surface ................. 10
  1.6 Leaf Hg accumulation and the reversibility of the uptake ....... 14
  1.7 Mercury distribution within the leaf and the extraction of the cuticle ...

REFERENCES ........................................................................... 18

CHAPTER 2. MERCURY DISTRIBUTION WITHIN LEAVES ......... 26
  2.1 Introduction ....................................................................... 26
    2.1.1 Objectives .............................................................. 28
  2.2 Materials and methods ..................................................... 30
    2.2.1 Sampling procedures ................................................. 30
    2.2.2 Dry weight/fresh weight ratio, surface area, specific leaf area and stomatal
density determination .......................................................... 33
    2.2.3 Cuticle separation .................................................... 35
    2.2.4 Sequential extraction of leaf Hg ................................. 36
    2.2.5 Hg Analysis ............................................................ 36
    2.2.6 Quality Assurance/Quality Control (QA/QC) ................ 39
    2.2.7 Evaluation of the extraction technique ......................... 39
    2.2.8 Statistical analyses .................................................. 39
  2.3 Results and discussion ..................................................... 40
    2.3.1 Cuticle Extraction technique ...................................... 40
    2.3.2 Distribution of Hg in leaves ....................................... 47
      • Hg on the leaf surface .................................................. 49
      • Cuticle-bound Hg ....................................................... 50
      • Inner tissue Hg .......................................................... 53
    2.3.3 Mercury and foliage properties .................................. 54
    2.3.4 Reversibility of uptake .............................................. 57
2.3.5 Uptake scenarios ........................................................................................................... 58
2.4 Conclusions .................................................................................................................... 62
REFERENCES ......................................................................................................................... 65

CHAPTER 3: MERCURY ACCUMULATION IN FOLIAGE OF DECIDUOUS TREE SPECIES IN MINNESOTA; INTRA-SEASONAL AND INTER-SEASONAL OBSERVATIONS ......................................................................................................................... 74
3.1 Introduction ..................................................................................................................... 74
3.2 Materials and methods .................................................................................................. 77
  3.2.1 Sampling rationale .................................................................................................... 77
  3.2.2 Sampling procedures ............................................................................................... 78
  3.2.3 Sample processing .................................................................................................... 78
  3.2.4 Hg analysis ............................................................................................................. 79
  3.2.5 Quality assurance and quality control (QA/QC)..................................................... 80
  3.2.6 Statistics ................................................................................................................ 80
3.3 Results and discussion ................................................................................................. 81
  3.3.1 Intra-seasonal foliage mercury accumulation ...................................................... 81
  3.3.2 Inter-seasonal variation in mercury accumulation ................................................. 91
  3.3.3 Intra-seasonal Accumulation of Hg in the surface, cuticular, and inner tissues of Tamarack needles................................................................................................................................. 93
3.4 Conclusions .................................................................................................................. 96
REFERENCES ......................................................................................................................... 98
GENERAL CONCLUSIONS ................................................................................................. 104
List of Figures

Figure 1.1: Diagram of a hypothetical cuticular pathway for atmospheric leaf uptake of mercury ................................................................. 11

Figure 1.2: Section through a leaf................................................................. 12

Figure 1.3: Hypothetical stomata pathway for Hg........................................ 13

Figure 1.4: Hypothetical polar and non-polar routes .................................... 14

Figure 2.1: Sampled trees in Saint Paul Campus......................................... 32

Figure 2.2a: UV reading for a cuticle extraction no chlorophyll .................... 41

Figure 2.2b: UV reading for a cuticle extraction showing a positive test ........... 42

Figure 2.3: IR Transmittance reading .......................................................... 45

Figure 2.4: Simplified polar and non-polar routes in the leaf......................... 52

Figure 2.5: Correlation between leaf surface Hg and leaf inner tissue Hg ........... 62

Figure 3.1: Intra-seasonal accumulation of Hg ........................................... 84

Figure 3.2: Intra-seasonal accumulation of Hg ........................................... 85

Figure 3.3: Temporal trend in surface, cuticle and inner tissue Hg ................. 93
List of Tables

Table 2.1: Tree locations sampled in experiment 2 and 3 ......................................................... 33
Table 2.2: Cuticle extraction time in CH$_2$Cl$_2$ for 5 deciduous species ................................. 43
Table 2.3: Total leaf Hg content for treated and untreated leaves. ........................................... 47
Table 2.4: Average dry weight/fresh weight ratios and SLA for four deciduous 48
Table 2.5: Mercury distribution and concentration in leaves of four deciduous species 49
Table 2.6: SLA and Hg levels for red maple leaves ................................................................. 52
Table 2.7: Average Hg leaf uptake rate for 4 deciduous species ............................................... 57
Table 2.8: Hg content for Norway maple .................................................................................. 59
Table 2.9: Stomata density for four deciduous species .............................................................. 60
Table 3.1: Early and End of season foliage Hg concentrations. ................................................. 81
Table 3.2: Regression fit for leaf Hg accumulation ................................................................. 82
Table 3.3: Early, Mid and late season uptake rates................................................................. 83
Table 3.4: Inter-annual comparison of foliage mercury between 2005 and 2011 ............. 91
List of Abbreviations

Hg: Mercury
Hg\(^0\): Elemental gaseous mercury
Hg\(_p\): Particle-bound mercury
RGM: Reactive gaseous mercury
MeHg: Methylmercury
PCB: Polychlorinated biphenyl
PAH: Polycyclic aromatic hydrocarbons
PCP: Pentachlorophenol
CH\(_2\)Cl\(_2\): Dichloromethane
SLA: Specific leaf area
FT-IR: Fourier transform infra-red spectroscopy
[Hg]: Mercury concentration
THg: Total mercury
GENERAL INTRODUCTION

Mercury interaction with vegetation has received a great deal of attention in the last two decades. Several studies have focused on determining the source of mercury in leaves. As a result, there is a consensus among scientists that most of the mercury in leaves is obtained from the atmosphere and little if any from the soil. This is similar to leaf uptake of many persistent organic pollutants. However, the location of mercury within the leaf structure is not known. Additionally, there is some disagreement on the variation in mercury accumulation among different tree species. A few studies have found that the species of tree had no effect on mercury accumulation (i.e. Obrist et al., 2011), while other studies found a significant interspecies effect (i.e. Bushey et al., 2008; Tabatchnick and Hammerschmidt, 2010; Juillerat and Ross, 2011). Likewise, there is also disagreement regarding the rate of accumulation of mercury throughout the leaf growth cycle, from emergence to senescence. In one study under a controlled environment, leaves were found to reach equilibrium with the atmosphere within 2-3 months (Ericksen et al., 2003). In contrast, other researchers have found that mercury levels in foliage continue to increase until the end of the leaf growing season (Bushey et al., 2008).

The aim of this study is to further our understanding of Hg accumulation and distribution within leaves of dominant deciduous tree species. Knowing the location of mercury in the leaf will help us evaluate and assess its potential to leach, to move or to accumulate. Furthermore, defining the exact location of Hg within the leaf may give us an indication of what forms of Hg are being taken up by trees. For this purpose, a sequential extraction that allows the removal of the cuticle, and therefore, the
determination of mercury fractions associated with the surface, the cuticle and the inner tissue of the leaf was developed, tested, and applied. Additionally, foliage Hg levels and leaf uptake rates were monitored and compared during the entire growing season. The comparison was made using levels normalized by dry weight and by leaf surface area.

The following questions are addressed:

- What is the distribution of mercury within leaves of boreal and temperate deciduous tree species?
- Is there any interspecies variation in the accumulation of mercury during the growing season?
- Is the rate of accumulation of mercury uniform during the growing season?
- Is there any inter-seasonal variation in the accumulation of mercury?

**Thesis outline**

In chapter 1 a literature review on mercury interaction with vegetation, and factors that influence mercury uptake are discussed. A comparison of the uptake of foliage mercury to that of persistent organic pollutants is also discussed. In chapter 2, we developed and applied a technique for the extraction of the cuticle to study Hg distribution within leaves of five deciduous tree species commonly found in north central urban areas. The results of the sequential extraction are presented as percentages of the total Hg concentration of the leaf and as concentrations normalized by leaf surface area, and by dry weight. In chapter 3, we monitored the accumulation of mercury by six temperate and boreal tree species from emergence until senescence. Mercury uptake rates
are also studied and compared between early, mid and late growing season. Additionally, we monitored and studied mercury levels in the surface, the cuticle and inner tissues of tamarack needles during the entire growing season. Lastly, we investigated the inter-seasonal accumulation of mercury in two deciduous species.
CHAPTER 1. LITERATURE REVIEW

1.1 Mercury as a global pollutant

Mercury (Hg) is a significant global pollutant due to its chemical and physical properties (Nater and Grigal 1992; Fitzgerald et al., 1998). It is easily transported by air in both gaseous and aerosol forms. It moves from emission sources to the most pristine regions, causing the contamination of aquatic ecosystems and producing adverse effects on wildlife and humans. Studies have shown that the residence time of Hg in the atmosphere can exceed one year (Lindqvist and Rodhe, 1985), making it possible for Hg to travel long distances from the source of emission to the deposition point with global implications. For example, in the state of Minnesota only 10% of the Hg atmospheric deposition originates from within the state (Edward Swain, Pers. Comm.).

Anthropogenic activities related to industrialization are blamed for the increased emission of Hg to the atmosphere (Mason et al., 1994; Hurley et al., 1998; Pacyna et al., 2006). According to Fitzgerald et al. (1998) global atmospheric Hg deposition has tripled since pre-industrial time. This conclusion was based on the results of studies using lake sediment records (Aston et al., 1973; Engstrom et al. 1994; Balogh et al., 1999), bog records (Madsen, 1981; Swain et al., 1992), and ice core records (Schuster et al. 2002). The primary anthropogenic sources of Hg are fossil fuel combustion, metal and cement production, artisanal gold mining, solid waste incineration, and chlor-alkali plants. China is thought to be responsible for over 25% of current global anthropogenic emissions (Pirrone et al., 2010). Natural sources on the other hand are dominated by surface water
evasion and emission from volcanoes and wildfires (Pirrone et al., 2010). In Minnesota, coal combustion along with wood and waste incineration contribute more than 50% of the total anthropogenic emissions (MPCA Report, 2008).

**1.2 Mercury’s environmental behavior and toxicity**

As a result of industrialization, mercury deposition fluxes have increased in the last 160 years (Swain et al., 1992; Benoit et al., 1998; Schuster et al., 2002) causing more Hg to be available in the ecosystem for transformation to methyl mercury (MeHg), a potent neurotoxin. MeHg is produced mainly in anoxic conditions by sulfate- and iron-reducing bacteria in sediments and wetlands (Compeau and Bartha, 1985, Kerin et al., 2006). Methylmercury’s hazard can be explained by 1) its chemical stability 2) its lipophilicity, or its ability to be absorbed in the gut, and 3) its slow elimination, which contributes to its tendency to biomagnify in aquatic food chains (Fitzgerald et al., 2007). As a direct result of Hg bioaccumulation and biomagnification, fish consumption advisories have routinely been issued in northern hemisphere lakes (Watanabe et al., 2003) as well as duck consumption advisories (Utah Department of Health, 2010). MeHg can also cross the placenta and the blood brain barrier causing symptoms of sensory, motor and visual disturbances (Skerfving and Copplestone, 1976). Because of these characteristics, the US Environment Protection Agency (EPA) has classified Hg as a priority pollutant and a potent neurotoxin.

The first significant incidence of mercury poisoning dates to the 18th and 19th centuries when mercuric nitrate compounds were used in the manufacture of felt hats, causing poisoning of hat makers, popularly termed “mad hatters disease”. Perhaps the
most tragic case of mercury poisoning occurred in Minamata in southern Japan (1932-1968). During this period, an acetaldehyde plant was discharging MeHg and inorganic forms of Hg in surface water, causing contamination of fish and marine life in the area. This directly caused the deaths of 1,784 people. Tens of thousands more were diagnosed with brain and nervous system damage. Another case of Hg poisoning that gained worldwide attention occurred in Iraq in (1971-1972) and was caused by the consumption of wheat coated with an organomercury fungicide intended for agricultural use. The incident was responsible for the hospital deaths of over 700 persons (Skerfving andCopplestone, 1976). Similar incidents occurred in Iraq in 1956 and 1960, in West Pakistan in 1961, and in Guatemala in 1965.

While these cases are all related to point sources, they helped raise awareness of the dangers of mercury poisoning, as well as providing the basis for the establishment of dose-response relationships for chronic and acute exposures. Global Hg pollution, however, is dominated by long range atmospheric deposition. In boreal ecosystems, vegetation provides large surface areas which could play a significant role in the cycling and movement of Hg by capturing dry deposition and/or taking up mercury vapor through their stomata.

1.3 Vegetation as a sink of mercury

It is of particular interest to us to study the role of vegetation in the uptake and cycling of Hg in these ecosystems. After being overlooked in many of the early Hg biogeochemical studies, Hg interaction with vegetation has received a great deal of attention in recent research (Kolka et al., 1999; Grigal et al., 2000; Graydon et al., 2006;
Millhollen and Gustin, 2006; Lindberg et al., 2007; Gustin et al., 2008; Stamenkovic and Gustin, 2009; Risch et al., 2011). The role of vegetation in the uptake of other air pollutants, however, has long been recognized, and plants are found to play a key role in the redistribution and movement of many air pollutants such as persistent organic pollutants (POPs) in terrestrial ecosystems; (i.e. “forest filter effect”). For instance, in the northern hemisphere, vegetated watersheds capture higher levels of air pollutants than the adjacent open fields, mainly in the form of litterfall (Barber et al., 2004). Similarly, forested watersheds have been found to capture Hg more efficiently than open fields (Kolka et al., 1999; Grigal et al., 2000; Rea et al., 2000). Furthermore, litterfall Hg levels were found to correlate with the atmospheric levels of Hg rather than soil Hg (Mosbaek et al. 1988; Bishop et al., 1998; Fleck et al., 1999; Rea et al., 2000; Ericksen et al., 2003, Erickson and Gustin, 2004; Frescholtz and Gustin, 2004).

Vascular plants have large adaxial and abaxial surface areas, which can account for more than 20 times the ground surface (Schulze, 1982), thus providing more area for the interception of atmospheric pollutants. As the leaves senesce, they integrate into the soil in the form of litterfall, acting as a vehicle for atmospheric Hg to reach the soil (Browne and Fang, 1978; Lindberg et al., 1979; Kolka et al., 1999; Rea et al., 2002; Ericksen et al., 2003). These findings have been corroborated with data from Hg mass balance studies in the last decade using gas exchange systems such as flux chambers and EcoCells (Gustin et al., 2004), micrometeorological methods including conditional sampling (Cobos et al., 2002), and the modified Bowen ratio method (Meyers et al., 1996; Fritsche et al., 2008). It is possible that both litterfall and soils act as a mercury
sink slowing the movement of Hg to nearby surface waters, which could make vegetation an essential element in the global biogeochemical cycle of mercury.

Many factors affect the uptake of air pollutants by vegetation. In the case of POPs, the kinetics of the air/plant exchange process are controlled by factors including the micrometeorology, leaf morphology and plant species characteristics such as the specific leaf area, the cuticle thickness and composition, and the stomatal density (Bakker et al., 2000).

1.4 Pathways into vegetation
1.4.1 Leaf and root uptake

According to Cousins and Mackay (2001), the uptake of chemicals by plants occurs through two principal routes; either from the air through the leaves or from the soil solution by the roots. The authors suggested that the uptake from the atmosphere is significant for chemicals with log $K_{oa}$ (Octanol-air partition coefficient)$>6$ and log $K_{ow}$ (Octanol-water partition coefficient)$<-6$, and the uptake from soil is important for chemicals that have a $K_{ow}<2.5$ and log $K_{aw}$ (Air-water partition coefficient)$<-1$. For Hg, however, most studies have suggested that uptake occurs mainly via the leaves and that the source of the majority of leaf Hg is atmospheric, with soils playing only a minor role (Beauford et al., 1977; Lindberg et al., 1979; Gaggi et al., 1991; Nater and Grigal, 1992; Cocking et al., 1995; Bishop et al., 1998; Cavallini et al., 1999; Fleck et al., 1999; Grigal et al., 2000; Ericksen et al., 2003; Schwesig and Krebs, 2003; Gregger et al., 2005).

Elemental gaseous mercury ($\text{Hg}^0$) is the dominant form in the atmosphere (Hall, 1995; Schroeder and Munthe, 1998), and its octanol-air partitioning coefficient is $K_{oa} =$
4.5 (Okouchi and Sasaki, 1985). Because it is mildly lipophilic, mercury may potentially cross the cuticle of the leaf, as well as enter through the stomata.

1.4.2 Gaseous and particulate deposition

In the atmosphere, Hg exists as elemental gaseous mercury (Hg\(_0\)), reactive gaseous mercury (RGM) and in aerosol- or particle-bound (Hg\(_p\)) forms. Studies by Hall (1995) and by Schroeder and Munthe (1998) have shown that Hg\(_0\) is the dominant form (typically >99%), with a global background concentration of 1.5 ±0.2 ng m\(^{-3}\) in the northern hemisphere, and 1.2 ±0.1 ng m\(^{-3}\) in the southern hemisphere (Lindberg et al., 2007). The three forms of atmospheric Hg have different physical and chemical properties and therefore will likely follow different routes to enter the plant. In comparison to organic pollutants, the deposition and uptake of POPs were reported to occur by partitioning equilibrium for the gaseous forms, while the particle-bound forms are captured by the leaves at varying efficiencies based on leaf morphology, and the nature and size of the atmospheric aerosol (Barber et al., 2004). For example, for polychlorinated biphenyls (PCBs), dry gaseous deposition was determined to be the major uptake pathway (Welsh-Pausch et al., 1995). Similar dynamics are likely to control Hg uptake, stipulating that the dry gaseous deposition of Hg\(_0\) is the dominant form of deposition/uptake by the leaf (Choi et al., 2007; Stamenkovic and Gustin, 2009).

1.4.3 Wet and dry deposition

Wet and dry deposition are the processes by which Hg and other atmospheric chemicals enter terrestrial ecosystems from the atmosphere. Dry deposition can occur
either by partitioning of vapor phase from the air to the leaf surface (dry gaseous deposition, dependent on $K_{oa}$) or dry particulate deposition that is controlled by many factors including particle size, wind speed, surface area, surface roughness, and other characteristics of the deposition surface. Wet deposition occurs during precipitation events, dew formation, mists and fog, and is defined by the washout of both vapor phase, more likely reactive gaseous mercury, and particle bound phase Hg from the atmosphere, a process which is dependent on the air—water partitioning coefficient, $K_{aw}$. When wet deposition is intercepted by the canopy, it becomes throughfall. This canopy interception is responsible for the cleansing of foliage and the removal of at least a portion of aerosol-associated Hg adhering to the surface of the leaves. This causes significantly higher Hg concentrations in throughfall and stemflow than occur in the initial wet deposition (Kolka et al., 1999; Choi et al., 2007). Wet deposition can also have higher Hg concentrations during the initial phase of precipitation because of the scavenging of both gaseous and particle bound Hg from the air.

Differences exist between deciduous and coniferous trees in Hg deposition fluxes. For instance, Kolka et al. (1999) demonstrated that conifers are more efficient scavengers of Hg than broadleaf species. A similar result was found by Witt et al. (2009) in a boreal forest in the Superior National Forest. However, according to Demers et al. (2007), litterfall fluxes of Hg to the soil are greater in deciduous forests while throughfall fluxes are greatest in coniferous forests.

### 1.5 Deposition and transfer of Hg to the leaf surface
For atmospheric Hg to reach the leaf, it must first be transferred from the lower troposphere, where eddy dispersion is dominant, to the leaf boundary layer surrounding the leaf and then transferred into the leaf (Figure 1.1). The transfer of air pollutants to the leaf can occur via adsorption of particulate or other atmospheric forms of Hg to the leaf surface, uptake through the stomata, or uptake through the cuticle. In the case of organic pollutants, such as PCBs, the rate of transfer has been described by diffusion coefficients, and/or conductance or deposition velocities. The cuticle has been found to play a significant role in the uptake of POPs (Schreiber and Schonherr, 1992).

**Figure 1.1:** Diagram of a hypothetical cuticular pathway for atmospheric leaf uptake of mercury

[Diagram showing the pathway from bulk air to leaf interior]
Leaves are generally covered with a thin layer of non-living lipid called the cuticle. The role of the cuticle is to protect the leaf against external aggression and also from water loss (Kerstiens, 1996). Embedded in the cuticle are the stomatal pores, which are responsible for regulating the leaf temperature and the exchange of gases between the interior of the leaf and the surrounding air (Figures 1.2 and 1.3). Research on PCBs has shown that they can penetrate the plant via the cuticle (Riederer, 1990) and also via the stomata. This is especially true for pollutants with low $K_{oa}$ (Tao and Hornbuckle, 2001). In terms of polarity (Figure 1.4), the cuticle can be divided into a polar section and a non-polar section, and therefore, each of these sections has a different affinity for xenobiotics based on the polarity. The cuticle is composed of epicuticular and intracuticular waxes,
cutin, cutan and polysaccharides (Holloway, 1982). Given the lipophilic nature of the cuticle and the solubility of Hg\(^0\) in lipids, the cuticle is likely to be involved in the uptake of gaseous Hg (Stamenkovic and Gustin, 2009; Converse et al., 2010). Diffusion is the mechanism of transport across the cuticle along a gradient of chemical potential. Studies demonstrate that there can be significant cuticle variation between species, such as thickness, composition, and morphology, which in turn may affect leaf uptake of air pollutants (Bakker et al., 2000).

**Figure 1.3:** Hypothetical stomata pathway for Hg (modified from Arizona MG manual, 1998)
1.6 Leaf Hg accumulation and the reversibility of the uptake

Research on the accumulation of Hg in foliage is inconclusive. For instance, Poissant et al. (2008) and Bushey et al. (2008) reported a season long increase in leaf Hg concentrations, with foliage Hg reaching maximum levels at the end of the season. Ericksen et al. (2003) however, reported that leaf Hg contents leveled off after 2-3 months of growth in a controlled environment. Additionally, Demers et al. (2007) reported an increase in litterfall Hg concentrations, long after the fall of the leaves, during the decomposition stage for both deciduous and conifers in the Adirondack region.
There is limited research on the reversibility of leaf Hg uptake. An early study by Gaggi et al. (1991) found that elemental Hg taken by Azalea leaves (Azalea japonica) in a controlled environment was not released back to the atmosphere. The authors found a high uptake rate of 66 µg of Hg hr⁻¹ g⁻¹ dry weight when the plant was exposed to high levels (10 µg m⁻³) of Hg. Recently, Stamenkovic and Gustin (2009) studied the foliage uptake of Hg in a controlled environment and found that once Hg was removed from the atmosphere it was not released back. Likewise, Lodenius et al. (2003) subjected leaves to intense washing and weeks of drying at 60° C without noticing any loss of leaf Hg. However, Graydon et al. (2006) reported that leaf Hg uptake is bidirectional, suggesting the existence of an atmospheric Hg compensation point that controls the direction of the flux. Nevertheless, there is agreement among scientists that foliage is a sink for atmospheric Hg, with the implication that litterfall management strategies that limit litterfall movement and increase its residence time in soil will reduce Hg loading to surface waters.

In comparison with POPs, studies on the reversibility of PCB uptake have found that more than 75% of the PCB accumulated in the leaf is not released back to the atmosphere (Bacci and Gaggi, 1987). A similar conclusion was reached by Schreiber and Schonherr (1992) that nearly 90% of the sorbed pentachlorophenol (PCP) penetrates the surface of the pine needle. Barber et al. (2003), however, conducted a depuration experiment on Skimmia plants and found that only 35% of the accumulated PCBs remained in the foliage after a 52-day depuration experiment.
1.7 Mercury distribution within the leaf and the extraction of the cuticle

Understanding the distribution and location of Hg in the leaf is important to assess its potential to accumulate or leach. However, limited research has attempted to study the distribution of Hg within leaves and the precise location of Hg in the leaf remains unknown. Leaf Hg could be located on the surface of the leaf, in the cuticle, or deep in the inner tissue of the leaf. Studies have speculated that the majority of Hg is held inside the leaf, which makes it unavailable for surface removal (Lodenius et al., 2003; Stamenkovic and Gustin, 2009). Research conducted on foliage uptake of organic pollutants involving the extraction of the cuticle has allowed scientists to identify the distribution of these pollutants within the leaf.

There are different methods for the extraction of the cuticle reported in the literature. These techniques are referred to as “dipping studies” and require careful attention so as to avoid the extraction of the contents (such as the chlorophyll) of internal cells of the leaf (Bakker et al., 2000). Other methods that can provide information about the location of air pollutants within the leaf include autoradiography of cross sections of leaves that have been spiked with radiolabelled chemicals (Amado Filho et al., 2002; Barber et al., 2004) and the use of partitioning coefficients. However, the use of $K_{OA}$ has not been found to accurately describe the partitioning of organic pollutants between air and plant cuticles for two reasons. First, octanol is a poor proxy for plant lipids (Bohme et al., 1999). Secondly, lipids exist in other areas of the leaf aside from the cuticle and can store significant amounts of these air pollutants. For these reasons, recent studies have aimed at compartmentalizing organic pollutants using chemical extraction methods.
It is important to consider two factors during the extraction of the cuticle; the polarity of the solvent used and the extraction time needed (Kylin et al., 1996). In POPs uptake studies, different organic solvents have been utilized for the extraction of leaf cuticular waxes including chloroform, methanol, petrol, and dichloromethane (CH$_2$Cl$_2$) (Bakker et al., 2000). Dichloromethane is especially useful in the extraction of hydrophobic surfaces such as the cuticle of the leaf. Most cuticle extraction studies have been conducted on pine needles because of their widespread geographic distribution, longevity and ease of age identification (Eriksson et al., 1989). However, there are only a few reports addressing the cuticle extraction of deciduous species in the context of air pollutants.
### REFERENCES


Food and Agriculture Organization of the United Nations: F.A.O, URL:


(108x708)20


In press.

- Utah Department of Health, 2010 (http://waterfowladvisories.utah.gov/).


CHAPTER 2. MERCURY DISTRIBUTION WITHIN LEAVES

2.1 Introduction

Vegetation covers nearly 80% of terrestrial surfaces allowing foliage to play a significant role in the cycling of many ubiquitous air pollutants (Simonich and Hites, 1994). This is especially true in temperate and boreal ecosystems, where trees can have large adaxial and abaxial leaf surface areas 4 to 20 times greater than the ground surface (Schulze, 1982). This high surface area allows leaves to be in contact with larger volumes of air which can translate into a significant uptake of atmospheric pollutants. An air pollutant’s location - whether it is on the surface of the leaves or inside the inner tissues - will affect its fate and potential to persist and accumulate. Xenobiotics that stay on the surface of leaves would be susceptible to loss via biotic and abiotic degradation, wind and cuticle erosion, and wash out by precipitation. On the other hand, those that penetrate the leaf, either via the stomata or the cuticle, will increase their likelihood of accumulation.

It is of particular interest to us to study the role of vegetation in the uptake of atmospheric mercury, especially because foliage Hg was documented to be mostly of atmospheric origin (Lindberg et al., 1979; Nater and Grigal, 1992; Bishop et al., 1998; Grigal, 2002). This finding was also inferred from Hg mass balance studies using controlled gas exchange systems (Gustin et al., 2004), the gradient approach (Lindberg et al., 1992), and micrometeorological methods including conditional sampling (Cobos et al., 2002), and the modified Bowen ratio (Meyers et al., 1996). Likewise, litterfall Hg
levels were found to correlate with atmospheric levels of Hg rather than soil levels of Hg (Mosbaek et al., 1988; Nater and Grigal, 1992; Bishop et al., 1998; Fleck et al., 1999; Rea et al., 2000, Ericksen et al., 2003, Erickson and Gustin, 2004, Frescholtz and Gustin, 2004), which points to the significant role of foliage in the interception of atmospheric Hg.

Previous studies showed that root uptake of Hg is negligible or even nonexistent (Beauford et al., 1977; Lindberg et al., 1979; Gaggi et al., 1991; Cocking et al., 1995; Bishop et al., 1998; Cavallini et al., 1999; Ericksen et al., 2003; Schwesig and Krebs, 2003; Greger et al., 2005).

Leaves can intercept atmospheric Hg via dry and wet deposition processes. However, recent studies speculated that the dry gaseous deposition of Hg\(^0\) is the dominant form of uptake (Choi et al., 2008; Stamenkovic and Gustin, 2009). Hg\(^0\) is also the dominant form of Hg in the atmosphere (Hall, 1995; Schroeder and Munthe, 1998). Other forms in the atmosphere are reactive gaseous mercury (RGM) and aerosol or particle-bound mercury (Hg\(_p\)) (Lindberg et al., 2007).

Despite numerous studies evaluating the interaction between atmospheric Hg and forest canopies, little information is available about the distribution of Hg in the leaf with regard to its surface, cuticle and internal tissues including epidermis, mesophyll and vascular tissue.

Extensive work has been conducted on the involvement of the cuticle in the uptake of persistent organic pollutants (POPs). Their rate of uptake has been shown to be controlled by local micrometeorology and plant species characteristics such as specific
leaf area (SLA), leaf morphology, stomatal density, and cuticle composition and thickness (Walton, 1990; Hellstrom, 2003; Barber et al., 2004; Moeckel et al., 2007). Additionally, foliage uptake research on polychlorinated biphenyls (PCBs) showed that they can penetrate leaves via the cuticle and the stomata (Riederer, 1990; Tao and Hornbuckle, 2001). Given the lipophilic nature of the cuticle and the weak lipophilicity of Hg⁰, the cuticle is likely to allow Hg⁰ to pass into the leaf interior by diffusion (Rea et al., 2000). Hg⁰ is mildly lipophilic with an octanol-water partition coefficient of $K_{ow} = 4.5$ (Okouchi and Sasaki, 1985), and octanol-air partition coefficient of $K_{oa} = 13$ (Bacci, 1994) and therefore, it could cross the leaf cuticle. However, the degree of cuticle involvement in the uptake of Hg is not as well studied as POPs.

2.1.1 Objectives

The objective of this study is to further our understanding of the location of Hg in the leaves of dominant deciduous species in temperate and boreal environments with regard to the surface, the cuticle, and the inner tissues. Determining the distribution of Hg in the leaf will help us evaluate and assess its potential to leach, to volatilize, to move or to accumulate. Furthermore, this information will also give us an indication of what forms of Hg are taken up (aerosols in surface and cuticle vs. gas in inner tissues). To achieve this objective, we propose to test the following null hypotheses:

**Null Hypothesis 1:** Leaf Hg is located on the surface of leaves (surface and cuticle), and does not penetrate the inner tissues.

$$[\text{Hg}]_{\text{tissue}} = 0$$
In other words, internal leaf tissue Hg concentration ([Hg]) is insignificant. To reject $H_0$, Hg levels in the inner tissue of leaves have to be significant for different species and different times of the growing season.

**Null Hypothesis 2:** Crown position has no effect on whole leaf Hg concentration. In other words, outside crown and inside crown foliage have similar Hg concentrations;

$$[\text{Hg}] \text{ outside crown} = [\text{Hg}] \text{ inside crown}$$

**Null Hypothesis 3:** For leaves collected near a point source of particulate Hg (a major roadside, as traffic is known to be a source of Hg$_p$ [Keeler et al., 1998]), crown position has no effect on Hg concentration in any leaf compartment (surface, cuticle, tissue);

$$[\text{Surface Hg}] \text{ outside crown} = [\text{Surface Hg}] \text{ inside crown}$$

$$[\text{Cuticle Hg}] \text{ outside crown} = [\text{Cuticle Hg}] \text{ inside crown}$$

$$[\text{Tissue Hg}] \text{ outside crown} = [\text{Tissue Hg}] \text{ inside crown}$$

**Approach**

The approach used to test the first hypothesis consists of separating the three components of the leaf using a sequential extraction technique. The technique consists of three steps, each allowing the extraction of Hg associated with the surface, the cuticle, and the inner tissues of the leaf. The hypothesis will be tested by comparing Hg concentrations in each leaf compartment in term of percentages, concentration by dry matter weight, and concentration by surface area.

For hypotheses 2 and 3, two additional experiments were conducted. These experiments were complementary studies and were based on leaves of red maple (*Acer*
rubrum L.) collected from Minnehaha Park for Experiment 2 and on leaves of Norway maple (Acer platanoides L.) collected alongside a busy interstate highway for Experiment 3.

2.2 Materials and methods
2.2.1 Sampling procedures

For Experiment 1 leaf samples from four different deciduous species gingko (Ginkgo biloba L.), American elm (Ulmus americana L.), tamarack (Larix laricina L.), and sugar maple (Acer saccharum L.) were collected during June, August and October of 2005. Trees were located on the Saint Paul Campus of the University of Minnesota, Saint Paul, MN, USA (Figure 2.1). The leaves were carefully selected to be representative of each species by choosing fully developed, undamaged leaves from several branches at heights over 2m. Samples were collected using the clean hand/dirty hand technique where only the "clean-hand" person, wearing two pairs of PVC gloves touches the foliage (Ahlers et al., 1990). A composite sample was taken for each species from two adjacent trees (except for Tamarack), double bagged, and transported to the lab for analysis. These four species were studied to determine interspecies variability. The sampled trees were all from the same general location and therefore assumed to have similar atmospheric exposure profiles. Each composite sample contained enough material to allow for a number of procedures and analyses, including: wet-to-dry weight ratio determination, determination of optimal cuticle extraction time, Hg analysis and recovery tests for the cuticle extraction technique.
In Experiment 2 the leaves of red maple (*Acer rubrum* L.) were sampled in the summer of 2005 (August 11th) using the same technique described in the previous experiment. Red maple trees were sampled from Minnehaha Park (Table 2.1) (n=6). Two composite samples were taken from each tree; one from the outside of the crown and one from the inside of the crown to determine potential differences in Hg concentrations between the two crown positions. The objective was to compare Hg concentrations between inside and outside crown leaves. Comparisons were made based on dry weight and surface area, and taking into account the SLA (specific leaf area which is defined as surface area of the leaf divided by its dry mass).

In Experiment 3 we studied Hg concentrations of maple leaves (*Acer platanoides* L.) that were exposed to traffic generated mercury. In the summer of 2005 (June 16th) leaves were sampled from Norway maple trees (n=6) growing within 30 meters of a busy interstate highway (Interstate 35W) that has a daily traffic volume of 173,000 (http://www.dot.state.mn.us) vehicles. Highway traffic is known to generate Hg (Keeler *et al.*, 1998; Hoyer *et al.*, 2004). As in Experiment 2, composite samples of leaves were collected from the inside and outside of the crowns and analyzed for Hg. The same sampling technique described in the first experiment was followed for foliage collection.
Figure 2.1: Sampled trees on the Saint Paul Campus.
Table 2.1: Tree locations sampled in experiment 2 and 3

<table>
<thead>
<tr>
<th>Trees</th>
<th>Location</th>
<th>Latitude (WGS84)</th>
<th>Longitude (WGS84)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red maple</td>
<td>Minnehaha Park</td>
<td>44.91598</td>
<td>-93.23607</td>
</tr>
<tr>
<td>Red maple</td>
<td>Minnehaha Park</td>
<td>44.91592</td>
<td>-93.23625</td>
</tr>
<tr>
<td>Red maple</td>
<td>Minnehaha Park</td>
<td>44.91598</td>
<td>-93.23599</td>
</tr>
<tr>
<td>Red maple</td>
<td>Minnehaha Park</td>
<td>44.91598</td>
<td>-93.23582</td>
</tr>
<tr>
<td>Red maple</td>
<td>Minnehaha Park</td>
<td>44.91592</td>
<td>-93.23547</td>
</tr>
<tr>
<td>Red maple</td>
<td>Minnehaha Park</td>
<td>44.91598</td>
<td>-93.23539</td>
</tr>
<tr>
<td>Norway Maple</td>
<td>Roadside</td>
<td>44.91039</td>
<td>-93.27418</td>
</tr>
<tr>
<td>Norway Maple</td>
<td>Roadside</td>
<td>44.91057</td>
<td>-93.27418</td>
</tr>
<tr>
<td>Norway Maple</td>
<td>Roadside</td>
<td>44.91075</td>
<td>-93.27418</td>
</tr>
<tr>
<td>Norway Maple</td>
<td>Roadside</td>
<td>44.91130</td>
<td>-93.27418</td>
</tr>
<tr>
<td>Norway Maple</td>
<td>Roadside</td>
<td>44.91142</td>
<td>-93.27410</td>
</tr>
<tr>
<td>Norway Maple</td>
<td>Roadside</td>
<td>44.91154</td>
<td>-93.27410</td>
</tr>
</tbody>
</table>

2.2.2 Dry weight/fresh weight ratio, surface area, specific leaf area and stomatal density determination

2.2.2.1 Dry weight/fresh weight ratio

Dry weight reporting is necessary to obtain reproducible and comparable data by eliminating the bias caused by water content differences between leaves. Techniques for the determination of the dry weight of leaves include oven drying, air drying and lyophilization. Air drying has the disadvantage of depending on the desiccation power of air, which in turn depends on the moisture and temperature of the air. As a result, air drying could yield different results in different air environments, and thus hinder comparison efforts. Likewise, lyophilization tends to leave residual water content between 1 and 4% (Goodrich and Meiske, 1971) which could negatively affect comparison between samples and studies. On the other hand, oven drying is a reliable drying technique with reproducible results. In this study, the dry weight/fresh weight
(dw/fw) ratio was determined in a subsample of each composite sample by weighing leaves before and after 24 hours of drying at an oven temperature of 60° C.

2.2.2.2 Surface area and specific leaf area

For each of the broadleaf samples, the surface area was measured using a leaf area meter after measuring the fresh weight of the sample. The leaf area meter defines the leaf area as the one-side leaf surface. The specific leaf area was calculated as the ratio of the surface area of the leaf and the corresponding dry weight.

For needles, the total surface area was calculated from geometric measurements (Sellin, 2000) of the needle length and circumference in a random sample of 40 needles. We used a cotton string to measure the average circumference (C) of each needle from ten readings. Assuming the needle has a cylindrical shape, the total needle surface area (TSA) was calculated using the formula: TSA = L \times C, where L is the needle length.

Using this method, half of the total needle surface area (equivalent to the one-side surface area measured by the leaf area meter for the broadleaf species) was used for the calculation of the SLA.

2.2.2.3 Stomatal density measurements

The stomatal density for each of the broadleaf species was measured from epidermal impressions of the abaxial (lower) surface of mature leaves. The impressions were made using clear finger nail polish (Maxiflex 2000, USA). We applied the polish to the center of the blade since it contains representative stomatal densities of the entire leaf (Willmer and Fricker, 1996). The epidermal impressions were placed in a microscope slide and mounted to a compound microscope (Leica, Germany). Stomata on Tamarack
needles were counted directly without the impression (McElwain et al., 1995). The stomatal density for all the species was calculated in a random field of view at magnification of 400 ×. Ten random readings were made per leaf/needle. Species values for the stomatal density were averages from 3 random leaves/needles for each species. The diameter of the field of view at the magnification of 400 × is 0.52 mm and the stomatal densities were reported per square millimeter (mm²).

2.2.3 Cuticle separation

We developed a technique for cuticle extraction using dichloromethane, similar to methods used for extraction of cuticle to determine foliar uptake of POPs (Eriksson et al., 1989; Kylin et al., 1996; Bakker et al., 2000). The technique consists of taking a subsample of 2 to 5 g of freshly sampled leaves from each of the composite samples, rinsing it with distilled de-ionized water (DDI), and then placing the subsample in a 125 ml Teflon bottle with 100 ml of CH₂Cl₂.

An appropriate extraction time was determined for the leaves of each tree species using the techniques described below. Differences in leaf cuticle thickness and composition may affect the time required to remove the entire cuticle without extracting other portions of the leaf, such as chlorophyll. The leaves were allowed to agitate for different periods of time. The appropriate duration was defined as the longest extraction time that did not extract chlorophyll from the leaves (chlorophyll a and b). A UV-Visible spectrophotometer was used to detect the presence of chlorophyll in the extract for each of the extraction times tested (MacKinney, 1941). A reading was conducted every two hours and the results of the UV-Visible spectrophotometer screening were recorded. A
change in color of the CH$_2$Cl$_2$ extract was an indication of chlorophyll leaking out of the leaf and was therefore considered an overexposure (confirmed by the UV-Visible spectrophotometer).

We used Fourier transform infrared spectroscopy to determine that the extract contained the cuticular material of the leaf. Using this protocol, the detection of cuticular lipids can be achieved in an appropriate screening window. Infrared spectrometric methods (IR) are able to detect functional groups present in the cuticle such as the carboxylic functional group (Machrius et al., 2009).

An additional test was conducted to confirm that the cuticle was indeed removed from the leaves after the CH$_2$Cl$_2$ extraction experiment. A subsample of leaves for each species was first subjected to the CH$_2$Cl$_2$ extraction, and then to an application of nail polish. A transparent 3M Scotch tape was attached to the surface of the leaves after the nail polish application dried. Once removed, the clear tape gave a visual confirmation for the absence of the cuticle.

2.2.4 Sequential extraction of leaf Hg

After the determination of dw/fw ratio, SLA, and the cuticle extraction time, we proceeded to determine the amount of Hg associated with the surface, the cuticle and the inner tissue of the leaves. There were three steps involved in the sequential extraction of Hg from the leaf.

2.2.4.1 Leaf surface-associated Hg

The first step in the sequential extraction aims at removing and measuring Hg associated with the surface of leaves. To do so, a sample of 2 to 5 g of freshly sampled
fully expanded leaves (ca. 2-5 months) was weighed, placed in a 125 ml Teflon bottle with 100 ml DDI water, and allowed to gently shake for 2 hours in a horizontal shaker. The rinsate was then recovered and transferred to a 125 ml Teflon bottle, where the recovered Hg was oxidized to Hg$^{2+}$ by adding 1 ml of bromine monochloride (BrCl). The sample was then heated overnight at 70° C and refrigerated at 4° C until processed. This operation was conducted for each of the samples collected.

2.2.4.2 Cuticle-associated Hg

The next step in the sequential extraction was to extract the cuticle from the leaves following the procedure explained in 2.2.3. The dichloromethane extract was placed in a 45 mL impinger vessel and covered with 20 mL of nanopure water. A stream of N$_2$ gas (45° C) was gently bubbled through the cuticle extract to evaporate it. Mercury present in the dichloromethane extract is transferred into the aqueous phase as the dichloromethane evaporates. Once the dichloromethane had evaporated completely the mercury in the aqueous phase was digested with BrCl at 70° C overnight, then stored at 4° C until Hg analysis. It is important to mention that this extraction technique was used only on fresh leaves, as frozen leaves/dried leaves suffered severe cracks that resulted in a significant leakage of the internal constituents of the cells, which may result in a biased Hg result. The potential for contamination was assessed by including analytical blanks in Hg determinations.

2.2.4.3 Inner tissue-associated Hg

To measure the amount of Hg associated with the inner tissue of the leaf, the leaf samples used in the previous treatment, now minus their cuticle, were transferred into a
Teflon digestion bomb, then digested with 40 mL of concentrated (15.8 M) nitric acid (HNO₃) overnight at a temperature of 70° C. The resulting digested liquid was left to cool and then analyzed for total Hg content. The results are reported both in ng of Hg per g of dry weight of leaves and ng of Hg per unit of surface of leaves.

2.2.5 Hg Analysis

Samples were analyzed for Hg in a clean room laboratory using cold vapor atomic fluorescence spectroscopy (CVAFS) by the double gold amalgamation method of Bloom and Crecelius (1983), similar to EPA Method 1631. With this technique, sample extracts from step 1 and step 2 of the sequential extraction were first digested overnight with BrCl at 70° C. The analysis relies on the strong oxidizing capacity of BrCl to completely oxidize mercurial compounds to the inorganic Hg²⁺ (Bloom and Crecelius, 1983). The remaining leaf tissue (step 3) was digested with concentrated nitric acid.

All three digests were pre-reduced by addition of hydroxylamine hydrochloride prior to addition of stannous chloride (SnCl₂) and CVAFS determination of Hg.

The only form of Hg that is detectable by CVAFS is the elemental form, Hg⁰ (Fitzgerald and Gill, 1979; Bloom and Fitzgerald, 1988). Therefore, water digests containing inorganic forms of Hg, predominantly Hg²⁺, are reduced to Hg⁰ by addition of 0.5mL of 10% SnCl₂ to an aliquot of the sample solution in a closed reaction flask. The volatile Hg⁰ is carried from the reaction flask in a stream of N₂ gas and captured on a sample gold trap after 21 minutes of reaction. The sample gold trap is heated to release Hg to the analytical trap, which in turn releases Hg to the CVAFS analyzer. This method has an instrumental detection limit of less than 1 pg.
2.2.6 Quality Assurance/Quality Control (QA/QC)

In addition to developing a calibration curve with a determination coefficient ($R^2$) of 0.95 or larger, rigorous QA/QC measures were undertaken to assure the validity of the results of this study. These measures include the use of two field replicates, two analytical lab replicates, two analytical lab blanks, lab blanks for each step of the extraction, one analysis of standard reference materials (SRM), four control check values, one standard addition (spike) and reagent testing for each batch of 40 samples. The acceptance criteria for replicates, control check values and standard addition was 90-110%. Only results that met these criteria were used.

2.2.7 Evaluation of the extraction technique

An additional experiment was conducted to evaluate the cuticle extraction technique. The evaluation was assessed by comparing Hg content of two sets of the leaves. The first set was immediately digested with HNO$_3$ without undergoing sequential extraction (untreated leaves), while a second set from the same leaves underwent the sequential extraction technique (treated) as described previously. The recovery efficiency of the cuticle extraction technique was thus determined by summing the Hg content of the three individual steps of the sequentially-extracted leaves to the Hg content of the whole leaf digest.

2.2.8 Statistical analyses

Statistical analyses were conducted using R (R Development Core Team, 2008). Bootstrapping was used to estimate confidence interval for means, indicated by the standard error (S.E.) with an alpha level of 0.05. Analysis of variance (ANOVA) was
conducted to test for the significant difference between means. When this difference was significant, Tukey’s honest significant difference (HSD) was used to find homogeneous groups. Means compared in Experiment 1 included specific leaf area (SLA), dry weight/fresh weight ratio (dw/fw), and Hg concentrations normalized by weight and by surface area. In Experiment 2, SLA and Hg concentrations were compared for red maple leaves by crown position. In the third experiment, Hg concentrations for maple leaves (Acer platanoides L.) collected from trees alongside interstate 35W were compared by crown position. To evaluate the extraction technique, we used One-Sample Student’s t-test to compare leaf Hg concentration for cuticle extracted leaves (treated) and unextracted leaves (untreated). Additionally, we used Pearson correlation coefficients to investigate the possible correlation between Hg concentrations and SLA, and also between Hg concentrations in the three compartments of the leaf.

2.3 Results and discussion

2.3.1 Cuticle Extraction technique

2.3.1.1 Cuticle separation

We determined the optimal time for cuticle extraction by CH₂Cl₂ by checking for the presence of chlorophyll in the extract, which was determined by UV-Visible spectrometry. Peaks of chlorophyll a were observed at about 413nm and 666nm while chlorophyll b peaked at about 454nm and 650nm. These numbers are similar to chlorophyll peaks reported by Larkum et al. (2003). The optimal extraction time was considered to be the longest extraction time that did not extract chlorophyll from the leaf tissue. Figures 2.2a shows the absorbance spectrum, for a negative reading (no peaks)
and Fig. 2.2b that for a positive reading (leakage of chlorophyll a and b). The optimal extraction times are summarized in Table 2.2.

**Figure 2.2a:** UV reading for a cuticle extraction showing a negative test with no chlorophyll.
Figure 2.2b: UV reading for a cuticle extraction showing a positive test.
We also studied leaf samples that had been preserved by freezing or by drying. Extracts from these samples turned green within minutes (Table 2.2), suggesting that the chlorophyll had leaked from the inner tissue of the leaves. Therefore, only fresh leaves that were collected within 24 hours were used to quantify the amount of Hg associated with each leaf component.

**Table 2.2:** Cuticle extraction time in CH₂Cl₂ for 5 deciduous species

<table>
<thead>
<tr>
<th>Species</th>
<th>Frozen/dried leaves</th>
<th>Fresh leaves CH₂Cl₂ reading</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15 minutes</td>
<td>16 hrs</td>
</tr>
<tr>
<td>Sugar maple</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Elm</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Gingko</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Red maple</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Maple</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Tamarack</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

A “+” sign means a detection of the chlorophyll in the extract using UV-visible spectrophotometer.

Table 2.2 indicates that different species have different cuticle extraction times. The appropriate extraction times were 16, 18, 22 and 22 hours, respectively for maple species, elm, gingko and tamarack. These durations are not to be taken as standards since they will vary, among other factors, with the age of the leaves, the thickness of the cuticle, and the polarity of the solvent (Holloway, 1974; Kylin *et al*., 1994; Bakker *et al*., 2000). Other researchers reported different extraction times for studies that focused on the uptake of organic pollutants by plants. According to Bakker *et al*., (2000), 30 seconds were sufficient for the extraction of the cuticle of lettuce (*Lactuca sativa* L.) and 15 minutes were necessary for Torpedo grass (*Panicum repens* L.) However, longer
extraction times were reported for pine needles, reaching up to 48 hours (Kylin and Sjodin, 2003).

An additional visual test consisting of a nail polish application and removal by a transparent 3m tape visually confirmed the absence of the cuticle from the CH$_2$Cl$_2$ extracted broadleaves. The same technique, when performed on untreated leaves, resulted in an impression on the transparent tape due to the removal of the cuticle.

2.3.1.2 Technique evaluation

After determination of the appropriate cuticle extraction time for each of the species in this experiment, the presence of the cuticle in the extract was confirmed using FT-IR. Figure 2.3 shows a positive FT-IR spectrophotometer reading from the cuticle extract of sugar maple leaves using the subtraction technique where the reading of the blank sample is deducted from the reading of the extracted sample. The reading confirms the presence of cuticular components in the extract as indicated by the peak of the carbonyl group (C=O) at 1710-1740 cm$^{-1}$ (Tegelaar et al., 1989), which is known to be present in the wax esters and fatty acids of the cuticle (Szafranek et al., 2008). The results obtained were highly complementary with the UV results described above and clearly demonstrated the presence of the cuticle in the extract.
Figure 2.3: IR Transmittance reading using the subtraction method for cuticular wax.
2.3.1.3 Quality Assurance/Quality Control results

The method detection limit for Hg was 0.06 ng L\(^{-1}\) in the reagent blank, which was three times the standard deviation of seven blanks. All samples were corrected by their associated blanks. Spike and SRM recovery for all samples were between 93% and 105%. Replicate analyses of field, laboratory, and analytical duplicates were all within 95-105%.

2.3.1.4 Evaluation of cuticle extraction technique

The results of the efficiency test of the sequential extraction showed no significant difference between the means of treated and untreated leaves for all the species studied (Table 2.3). In other words, the sum of the three extractions added up to the total amount of Hg present when whole fresh leaves were digested and analyzed. Thus, the sequential extraction technique did not cause any significant loss of leaf Hg. Similarly, the technique did not result in a contamination of the leaves, and therefore, it could be used to separate the three fractions of leaf Hg without affecting foliage Hg content. A similar technique has been applied in many studies examining foliage uptake of POPs (Bakker et al., 2000; Kylin and Sjodin, 2003).
### Table 2.3: Total leaf Hg content for treated and untreated leaves.

<table>
<thead>
<tr>
<th>Species</th>
<th>Total Hg Treated leaves (ng g⁻¹)</th>
<th>Total Hg Untreated leaves (ng g⁻¹)</th>
<th>One-Sample t-test (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tamarack</td>
<td>10.9 ±1.75</td>
<td>11.36</td>
<td>0.68</td>
</tr>
<tr>
<td>Norway maple</td>
<td>48.5 ±3.53</td>
<td>44.67</td>
<td>0.12</td>
</tr>
<tr>
<td>Sugar maple</td>
<td>33.9 ±1.73</td>
<td>35.00</td>
<td>0.13</td>
</tr>
<tr>
<td>Elm</td>
<td>41.7 ±1.48</td>
<td>39.22</td>
<td>0.14</td>
</tr>
<tr>
<td>Gingko</td>
<td>20.9 ±0.72</td>
<td>19.12</td>
<td>0.12</td>
</tr>
</tbody>
</table>

#### 2.3.2 Distribution of Hg in leaves

2.3.2.1 Specific leaf area and dry weight to fresh weight ratio

Means of dw/fw ratio and the SLA for the four deciduous species investigated in Experiment 1 are presented in table 2.4. Sugar maple, elm and tamarack leaves had the highest dw/fw ratio, while gingko leaves had the lowest. This finding could be interpreted as meaning sugar maple, elm and tamarack leaves have less water content than gingko, and therefore, have a higher ratio of dry mass to fresh mass. This result could also be attributed to cuticle thickness difference between the species. Indeed, the thickness of the cuticle varies between species and even between leaves of the same tree (Yoko, 1955). The cuticle plays an important role in the protection of leaf surfaces from biotic and abiotic stressors and limits the amount of water loss by transpiration. The cuticle is also involved in the regulation of gas exchange in plants (Kerstiens, 1996), and its thickness and composition is likely to vary depending on species and the environmental conditions. This may explain the difference in the dw/fw ratio among species. The dw/fw ratios were used to calculate leaf Hg concentration on a dry weight basis without actually drying the leaves.
Table 2.4: Average dry weight/fresh weight ratios and SLA for four deciduous species

<table>
<thead>
<tr>
<th>Species</th>
<th>Sampling time</th>
<th>Dry weight/fresh weight</th>
<th>Specific leaf area cm² g⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sugar maple</td>
<td>June</td>
<td>0.40a*</td>
<td>215a</td>
</tr>
<tr>
<td></td>
<td>August</td>
<td>0.42a</td>
<td>210a</td>
</tr>
<tr>
<td></td>
<td>October</td>
<td>0.38a</td>
<td>205a</td>
</tr>
<tr>
<td>Elm</td>
<td>June</td>
<td>0.40a</td>
<td>161b</td>
</tr>
<tr>
<td></td>
<td>August</td>
<td>0.40a</td>
<td>150b</td>
</tr>
<tr>
<td></td>
<td>October</td>
<td>0.40a</td>
<td>152b</td>
</tr>
<tr>
<td>Gingko</td>
<td>June</td>
<td>0.27b</td>
<td>151b</td>
</tr>
<tr>
<td></td>
<td>August</td>
<td>0.28b</td>
<td>149b</td>
</tr>
<tr>
<td></td>
<td>October</td>
<td>0.26b</td>
<td>148b</td>
</tr>
<tr>
<td>Tamarack</td>
<td>June</td>
<td>0.37a</td>
<td>152b</td>
</tr>
<tr>
<td></td>
<td>August</td>
<td>0.36a</td>
<td>140b</td>
</tr>
<tr>
<td></td>
<td>October</td>
<td>0.33a</td>
<td>142b</td>
</tr>
</tbody>
</table>

*Means with different letters in the same column are statistically different (p<0.05)

The dw/fw and SLA are known to change over time (Yoko, 1955; Venera et al., 2002). This was not seen in this study, which could be explained by our sampling time that started late in June after the leaves became well developed. Although, the SLA did not statistically decrease over time between June and October, nevertheless, it decreased numerically, and the decrease was statistically significant for a 90% confidence level. In comparison with other studies, Venera et al. (2002) found that the SLA changes over time in a study on the accumulation of polycyclic aromatic hydrocarbons (PAH) in leaves of two deciduous species. Among the species in the current study, sugar maple leaves showed the highest SLA followed by elm, gingko and tamarack.

SLA also varies among leaves within the same tree. According to Yoko (1955), exposed leaves (outside crown) tend to be thicker, tougher and thus have smaller SLA than shaded leaves. The results in table 2.6 provide more insights into the variation of
SLA within the same tree based on crown position. A significant difference was found between leaves taken from inside the crown and outside the crown, with the inside crown leaves having the highest SLA. It is clear that SLA varies not only between species as seen in experiment 1, but also between leaves of the same tree based on the crown position.

2.3.2.2 Distribution of Hg in leaves

Leaf analyses from Experiment 1 revealed the presence of Hg in the three compartments of the leaf. The first fraction is surface-associated Hg, which constitutes 2-4\% of total leaf Hg. The second fraction is cuticle Hg which also contains about 2-6\% of total leaf Hg. The third, and most significant fraction, is leaf tissue Hg which contains more than 90\% of total leaf Hg for the deciduous species investigated in Experiment 1 (Table 2.5).

### Table 2.5: Mercury distribution and concentration in leaves of four deciduous species.

<table>
<thead>
<tr>
<th>Species</th>
<th>Leaf surface Hg</th>
<th>Leaf cuticle Hg</th>
<th>Leaf internal tissues Hg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>ng g⁻¹</td>
<td>ng m⁻²</td>
</tr>
<tr>
<td>Sugar maple</td>
<td>1.6</td>
<td>0.5ᵃ</td>
<td>23ᵃ</td>
</tr>
<tr>
<td>Elm</td>
<td>2.2</td>
<td>0.8ᵃ</td>
<td>51ᵃ</td>
</tr>
<tr>
<td>Gingko</td>
<td>2.1</td>
<td>0.3ᵃ</td>
<td>27ᵃ</td>
</tr>
<tr>
<td>Tamarack</td>
<td>4.0</td>
<td>2.7ᵇ</td>
<td>190ᵇ</td>
</tr>
</tbody>
</table>

*Means with different letters in the same column are statistically different (p<0.05).

- **Hg on the leaf surface**

  The proportion of surface Hg was between 2\% and 4\% of the total leaf Hg for all the four species. The data presented in table 2.5 represents the average of nine
measurements for each species. Some variation between species was noted. For example, sugar maple had only 1.6% ±0.4 of the total leaf Hg on the surface of the leaf, while the remaining 98.4% ±5.6 of leaf Hg occurred in the cuticle and inner tissue. This percentage is higher for Tamarack where more than 4% ±2.2 of the total leaf Hg was found on the surface. In terms of concentrations by mass, Tamarack had the highest concentration of surface Hg (2.66 ±1.5 ng g⁻¹), while gingko had the lowest concentration with 0.34 ±0.1 ng g⁻¹ (Table 2.5). Similar results were obtained when using Hg concentration normalized by leaf surface area (Table 2.5). Tamarack had the highest concentration of Hg on the leaf surface (190 ±111 ng m⁻²), followed by elm (51 ±23 ng m⁻²) and sugar maple (23 ±6 ng m⁻²). In comparison to other studies, Rea et al. (2000) reported a concentration 3 times higher (75 ng m⁻²) on the surface of maple leaves in the eastern United States compared to levels found in this study.

- Cuticle-bound Hg

Similar to surface Hg, the concentrations of Hg associated with the cuticle varied among the four species (Table 2.5). Tamarack had the highest proportion of cuticular Hg (6.0% ±2.5) followed by elm (4.3%±2.6), gingko (3.2% ±1.9), and sugar maple (2.5% ±1).

In terms of concentration normalized by mass, elm had significantly higher concentration of leaf cuticle Hg (1.5 ±0.8 ng g⁻¹), while tamarack, sugar maple and gingko had lower concentrations of less than 1.0 ng g⁻¹ (Table 2.5). Similarly, elm had the highest cuticular Hg by surface area (102 ±59 ng m⁻²) followed by sugar maple (39 ±19 ng m⁻²), while tamarack had the lowest concentration (11.7 ±8 ng m⁻²) (Table 2.5).
The significant difference between elm and the three remaining species in cuticle Hg levels may possibly be explained by differences in the thickness and/or composition of the cuticle, which unfortunately could not be verified in the scope of this study. Overall, about 4% of the total leaf Hg was found in the cuticle and this observation suggests a potential role of the cuticle in the storage and/or penetration of Hg in the leaf. The cuticle structure has both polar and non-polar accumulation routes (Overbeek, 1955) and can, in theory, permit the passage of gaseous and ionic Hg (Figure 2.4). Ionic forms of Hg could reach the inside of the leaf the same way herbicides and foliar fertilizers do, in that they can follow polar routes to gain access to the inside tissue of the leaf (Schreiber, 2005). The role of the cuticle in the accumulation of Hg was an indirect subject of a study by Stamenkovic and Gustin (2009). The authors monitored, in a controlled environment, the leaf-atmosphere Hg flux, and noticed that mercury deposition occurred during darkness and elevated CO₂ levels. Thus, the authors suggested that a nonstomatal pathway plays a significant role in leaf Hg uptake. In this study, we speculate the nonstomatal pathway could involve the cuticle. To our knowledge, this is the first study to report the presence of Hg in the cuticle.
In comparison to POPs, Kuhn et al., (1998) found that the cuticle plays a significant role both as a route and as an accumulation site for PAH. In this study, the role of the cuticle as an accumulation site was ruled out after comparing cuticle Hg concentrations of Tamarack needles between early and late season (Experiment 1). There was no significant increase in the amount of Hg associated with the cuticle ($p=0.35$). In fact, needle Hg concentrations increased steadily and significantly during the growing season (Chapter 3), but when cuticle Hg was studied during the same period, fluctuations were observed instead of the steady increase expected. This could be an indication that the cuticle is more likely a route rather than a storage site of atmospheric Hg as previously discussed. However, it is possible that the cuticle was saturated with Hg early
in the season which may also explain the lack of significant increase in its Hg concentration during the growing season. In fact, Schreiber and Schonherr (1992) studied the uptake of POPs by Pine needles and found that the cuticle rapidly reaches equilibrium with the atmosphere at much faster rates than the inner leaf tissues. Given the low levels of Hg encountered in the cuticle, it is likely that the stomatal route is more dominant in atmospheric gaseous Hg uptake while the cuticle is involved in ionic Hg uptake.

- Inner tissue Hg

The results of the leaf tissue analysis revealed that the bulk of Hg in leaves is located in the internal tissue of the leaf consisting of the epidermis, mesophyll and vascular tissues (Table 2.5). Sugar maple (96% ±2) and gingko (95% ±2) have the highest proportions of leaf tissue Hg followed by elm (94% ±2.8), while tamarack had the lowest proportion (90% ±3.2).

When the concentrations were reported by mass (Table 2.5), tamarack (36.8 ±9.6 ng g⁻¹), elm (35.12 ±5.7 ng g⁻¹) and sugar maple (27.1 ±6.24 ng g⁻¹) displayed significantly higher concentrations of leaf tissue Hg than gingko (16.55 ±2.37 ng g⁻¹). However, when the concentration was normalized by surface area, tamarack (2626 ±690 ng m⁻²) and elm (2341 ±379 ng m⁻²) had significantly higher concentrations of Hg than sugar maple (1294 ±297 ng m⁻²) and gingko (1167 ±159 ng m⁻²). Despite the large variation in Hg concentration between these species, leaf tissue was the dominant reservoir of Hg for all of the species (Table 2.5).
2.3.3 Mercury and foliage properties

The result of the linear regression analysis of tamarack, elm, sugar maple, and gingko foliage Hg on surface area and dry weight suggests the importance of both parameters in Hg uptake. Leaf total Hg content (THg) for these four species combined was significantly and positively correlated with the surface area of the leaves \( y = 31.4x - 0.12 \); where \( x \) is the dry weight of the sample in g and \( y \) is the leaf sample total Hg content in ng, \( r = 0.76, n=12, p < 0.05 \). Likewise, the correlation between leaf THg and dry weight was significant and positive \( y = 0.16x + 2.63 \); where \( x \) is the surface area of the sample in cm\(^2\) and \( y \) is the leaf sample total Hg content in ng, \( r = 0.76, n=12, p < 0.05 \). However, when fresh weight was used instead of the dry weight, the correlation was insignificant \( R^2 = 0.09, p = 0.28 \). These results may suggest that leaf uptake of atmospheric Hg relies on both the surface area (its physical and morphological characteristics), and the biomass of the leaf (for storage). Therefore, reporting the leaf Hg concentration both in ng g\(^{-1}\) and in ng m\(^{-2}\) would provide complementary information that would be useful for species comparison.

A particular foliage parameter that includes both the leaf area and the dry weight is the specific leaf area. To investigate the importance of using surface area in the reporting of foliage Hg concentration we evaluated the effect of the crown position on red maple leaf total Hg (ng g\(^{-1}\) and also in ng m\(^{-2}\)) (Table 2.6). Outside and inside crown leaves had, on average, similar Hg concentrations of 53.91±6.72 ng g\(^{-1}\) and 52.46±5.5 ng g\(^{-1}\) respectively. However, when foliage Hg concentrations were normalized by surface area (Table 2.6), outside crown leaves contained 77% more Hg than inside crown leaves.
While this may seem surprising given the results obtained using leaf Hg concentration normalized by dry weight, taking into account the SLA difference between the outside crown and inside crown foliage better accounts for the apparent difference. In other words, outside crown leaves are thicker and have more mass per unit of surface, which may be used to store Hg. This observation is supported by leaf physiology studies that showed outside crown leaves have thicker palisade mesophyll layers and highly vacuolated cells (Yoko, 1955). Additionally, outside crown leaves have been found to have higher stomatal conductance (Morecroft and Roberts, 1999; Herrick et al., 2004), which supports the higher surface area based Hg concentration in the outside crown foliage, especially if the majority of uptake occurs through the stomata.

**Table 2.6:** SLA and Hg levels for red maple leaves from Minnehaha Park

<table>
<thead>
<tr>
<th>Crown position</th>
<th>SLA (cm$^2$ g$^{-1}$)</th>
<th>Leaf Hg (ng g$^{-1}$)</th>
<th>Leaf Hg (ng m$^{-2}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inside</td>
<td>261$^{a}$ ±31.1</td>
<td>52 ±5.5</td>
<td>2031$^{a}$ ±142</td>
</tr>
<tr>
<td>Outside</td>
<td>149$^{b}$ ±1.8</td>
<td>54 ±6.7</td>
<td>3602$^{b}$ ±412</td>
</tr>
</tbody>
</table>

*Means with different letters in the same column are statistically different (p<0.05).

Other authors have indirectly documented the importance of leaf surface area and leaf position in the uptake of Hg. For example, Juillerat and Ross (2011) found a negative correlation between leaf height and leaf Hg content for several hardwood species in Vermont. Similarly, Bushey et al. (2008) studied the accumulation of Hg in a northern hardwood canopy and found that understory leaves have on average 42% more Hg than overstory leaves. In both studies, SLA could possibly explain the difference reported between understory and overstory leaves on the one hand, and between lower leaves and
higher leaves on the other. Overstory foliage and higher leaves are comparable to outside crown leaves in this study (Table 2.6). Therefore it is possible that the differences in Hg levels encountered are due to differences in the SLA between the sun-exposed leaves (overstory/outside crown) and the shaded leaves (understory/inside crown). However, these differences in leaf Hg concentration between understory and overstory foliage could also be attributed to the presence of a Hg air concentration gradient decreasing upward from the soil as a result of volatilization of surface soil Hg (Grigal et al., 2000). In comparison with POPs, Simonich and Hites (1995) recommended normalizing leaf concentrations of atmospheric pollutants by surface area for comparison purposes. Moeckel et al. (2008) reached the same conclusion while studying the uptake of PCBs by vegetation. In their study, the authors hypothesized that leaf area is likely to control the ability of the vegetation to scavenge PCBs and used concentrations normalized by surface area in their results.

These results highlight the challenges associated with the quantification of foliage Hg. Such challenges arise from the variability in Hg concentration within foliage of the same tree. These challenges can be addressed by standardizing foliage sampling techniques among scientists for reliable foliage mercury measurements.

These results also underline the need for reporting leaf Hg concentrations normalized by surface area and not only by weight. Reporting the Hg content of foliage by surface area (i.e. ng m⁻²) reveals more information about the leaf surface and its ability to scavenge Hg from the atmosphere. Thus, it can be a complementary indicator to the concentration by mass (i.e. ng g⁻¹), especially for uptake comparison between species.
2.3.4 Reversibility of uptake

In Experiment 1, leaf tissue Hg (leaf interior) increased steadily between June 22nd and October 5th with leaf inner tissues behaving as a storage site for leaf Hg. There was no indication that the accumulation of Hg in foliage was reversible since total leaf Hg increased steadily between June and October. Tamarack leaves displayed the highest daily accumulation rate followed by elm and sugar maple, while gingko had the lowest (Table 2.7). Interestingly, tamarack was the only conifer in the experiment and this result highlights its efficiency in scavenging atmospheric Hg. Kolka et al. (1999) and Witt et al. (2009) also found that conifer canopies are more efficient in scavenging atmospheric Hg than broadleaf trees.

Table 2.7: Average Hg leaf uptake rate for 4 deciduous species between June and October

<table>
<thead>
<tr>
<th>Species</th>
<th>Daily accumulation rate</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ng g⁻¹</td>
<td>ng m⁻²</td>
</tr>
<tr>
<td>Sugar maple</td>
<td>0.15±0.01</td>
<td>7.83ab±0.6</td>
</tr>
<tr>
<td>Elm</td>
<td>0.18±0.04</td>
<td>12.09b±2.5</td>
</tr>
<tr>
<td>Gingko</td>
<td>0.07±0.01</td>
<td>4.36a±0.6</td>
</tr>
<tr>
<td>Tamarack</td>
<td>0.30±0.02</td>
<td>24.44c±4.4</td>
</tr>
</tbody>
</table>

*Means followed by different letters in the same column are statistically different (p<0.05)

Studies on the reversibility of the uptake of atmospheric Hg found that Hg accumulated in the leaf is not available for exchange back to the atmosphere (Gaggi et al., 1991; Lodenius et al., 2003; Stamenkovic and Gustin, 2009). Because of the irreversibility of Hg uptake, it is possible to estimate the annual quantity of Hg scavenged by vegetation using litterfall Hg concentration and leaf surface area. Poissant et al. (2008)
used a similar approach to estimate Hg loads in a Canadian forest watershed using maple leaf Hg concentration and surface area. This concept was also used in POPs studies to estimate the amount of PAH scavenged by vegetation. For example, Simonich and Hites (1994) estimated that 44% of the PAH emitted to the atmosphere from local sources in northeastern United States is removed by vegetation. This estimate was later revised to about 4% according to Wagrowski and Hites (1996), and 10% according to Tian et al. (2008). In Minnesota, anthropogenic Hg emissions are estimated to be 1193 kg per year (MPCA Report, 2008). Deciduous forest covers about 18.8% of the total area of the state with surface area of about 10.2 million acres (National Land Cover Dataset, USGS Eros). Using LAI of 2 to 4 (Pucket, 1990; Wythers et al., 2003; Zhang et al., 2005; Lee Frelich, Pers. Comm., 2011; Marvin Bauer, Pers. Comm., 2011) for the entire state of Minnesota and a Hg leaf concentration of 1.33 µg m\(^{-2}\) (this study), deciduous trees are likely to remove or displace the equivalent of 9-18% of the state's anthropogenic Hg emissions. This estimate would increase significantly if throughfall is taken into consideration.

### 2.3.5 Uptake scenarios

In order to get some insights as to what forms of Hg could be associated with leaf surfaces, we compared the Hg content of leaves collected from inside and outside the crown of maple trees growing alongside Hwy 35W from Experiment 3 (Table 2.8). Road traffic is considered point source of Hg\(_p\) (Keeler et al., 1998; Hoyer et al., 2004). The result of the comparison showed that outside crown foliage had significantly more surface Hg (mean of 232.46 ±29.6 ng m\(^{-2}\)) than leaves collected from the inside crown (mean of 114.21 ±39.8 ng m\(^{-2}\)). These observations could indicate that Hg\(_p\) is the
The dominant form of Hg on the surface of leaves (fine particle Hg). However, this fraction of Hg\textsubscript{p} is likely to be washed off frequently by precipitation, causing high levels of Hg observed in throughfall (Iverfeldt, 1991; Lindberg \textit{et al.}, 1994; Munthe \textit{et al.}, 1995, Kolka \textit{et al.}, 1999; Witt \textit{et al.}, 2009b).

\textbf{Table 2.8:} Hg content for maple (\textit{Acer platanoides} L.) trees alongside interstate 35W

<table>
<thead>
<tr>
<th>Crown position</th>
<th>SLA (cm\textsuperscript{2}g\textsuperscript{-1})</th>
<th>Surface Hg (ng m\textsuperscript{-2})</th>
<th>Cuticle Hg (ng m\textsuperscript{-2})</th>
<th>Tissue Hg (ng m\textsuperscript{-2})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inside</td>
<td>238.3\textsuperscript{a}</td>
<td>114 ±39.8\textsuperscript{a}</td>
<td>82 ±27.1</td>
<td>2096 ±422</td>
</tr>
<tr>
<td>Outside</td>
<td>205\textsuperscript{b}</td>
<td>232 ±29.6\textsuperscript{b}</td>
<td>84 ±17.2</td>
<td>2161 ±219</td>
</tr>
</tbody>
</table>

*Means followed by different letters in the same column are statistically different (p<0.05)

Despite the significant difference in surface Hg content (Hg\textsubscript{p}) between inside crown and outside crown leaves (Table 2.8), this difference did not seem to affect leaf tissue Hg (leaf interior). If Hg\textsubscript{p} was a major source of leaf tissue Hg, one would expect a significant difference between inside and outside crown leaf tissue Hg (leaf interior) in samples collected alongside interstate 35W (Experiment 3). Samples gathered from the outside crown would have more Hg since motor emissions are considered a point source of Hg\textsubscript{p} (Keeler \textit{et al.}, 1998; Hoyer \textit{et al.}, 2004; Conaway \textit{et al.}, 2005). These observations could indicate the involvement of other mechanisms in leaf Hg uptake that involve other forms of Hg such as Hg\textsubscript{0}. Traffic also generates gaseous forms of Hg (Keeler \textit{et al.}, 1998; Kelly \textit{et al.}, 2003; Conaway \textit{et al.}, 2005), which unlike Hg\textsubscript{p}, are more likely to evenly affect outside and inside crown leaves, and rely on the stomata to gain entry to the leaf. A similar conclusion was reached by Bushey \textit{et al.} (2008). In their study, the authors found that Hg\textsubscript{p} does not play a significant role in leaf Hg uptake. The SLA also did not seem to affect Hg concentrations for Norway maple leaves (\textit{Acer platanoides} L.). However the
difference between the inside and outside crown SLA was not as large as was the case for Red maple in Experiment 2 which may explain the difference between these two cases.

**Table 2.9: Stomatal density for four deciduous species**

<table>
<thead>
<tr>
<th>Species</th>
<th>Stomata Density (no mm$^{-2}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sugar maple</td>
<td>307$^c$ ± 41</td>
</tr>
<tr>
<td>Elm</td>
<td>246$^b$ ± 22</td>
</tr>
<tr>
<td>Gingko</td>
<td>50$^a$ ± 8</td>
</tr>
<tr>
<td>Tamarack</td>
<td>60$^a$ ± 6</td>
</tr>
</tbody>
</table>

*Means with different letters in the same column are statistically different (p<0.05)

We also investigated the importance of stomata ( stomates) in Hg uptake (Table 2.9). We multiplied the stomatal density for each of the four deciduous species by its corresponding surface area (area), resulting in the total number of stomata per sample ( stomates). During the calculation of the total number of stomata per sample of leaves, we took into consideration the fact that Tamarack needles have stomates in both sides of the needles unlike the broadleaves species. Regression analyses showed that stomates per sample was significantly and positively correlated with the total Hg content (THg) of the leaf interior ($y = 3.96 \times 10^{-6} x + 10.6$ where $x$ is the total number of stomates in the sample and $y$ is Hg content of the sample in ng, $n = 12$, $R^2 = 0.64$, $p < 0.001$). This correlation was better than that provided by Hg/surface area or Hg/mass ($R^2 = 0.58$, $p < 0.05$). This result further suggests that the stomata are a potential route for Hg to the interior of the leaf.

Cuticular Hg also provided insights on Hg uptake scenarios; maple foliage from interstate 35W showed no significant difference in the amount of cuticular Hg between
inside and outside crown leaves (Table 2.9). If cuticle Hg was mainly Hgₚ, we would expect outside crown leaves to have a higher cuticular Hg content as was the case for leaf surface Hg. Since this is not the case, it is likely that cuticle Hg is instead related to gaseous Hg (elemental and reactive) that is taken up by the leaf (dry deposition). It is possible that gaseous Hg is sorbed at the cuticle surface before it migrates inside the leaf. A study by Schreiber and Schonherr (1992) found that 89% of the sorbed pentachlorophenol (PCP) on the cuticle of 5 conifer species was not released back to the atmosphere. Similarly, Lodovici et al. (1998) showed that PAHs penetrate the cuticle and accumulates in the lipophilic sections of the leaf and were not removable by rinsing.

We also investigated the correlation between leaf surface Hg and leaf inner tissue Hg for the five deciduous tree species from Experiments 1 and 2. The results revealed large differences between the five deciduous species (Figure 2.5). The linear correlation was insignificant for gingko (ρ = 0.5) but was significant for the remaining species. The correlation was strong for elm (ρ = 0.95) and sugar maple (ρ = 0.86) and less strong for red maple (ρ = 0.78) and tamarack (ρ = 0.67). This could indicate that leaf surface Hg contributes a portion of leaf inner tissue Hg for Sugar maple and elm. This result, in addition to the earlier finding showing the weak correlation between Hgₚ and leaf interior Hg, seems to indicate that Hg forms other than Hgₚ are moving from the surface to the interior of the leaf. In theory, for charged Hg molecules to reach the inside tissue of the leaf, they have to follow the polar route of the cuticle in a manner similar to foliar fertilizers and herbicides (Foy, 1964; Schreiber, 2005). It is therefore possible that sugar maple and elm provide a more polar route than gingko (Figure 2.4).
2.4 Conclusions

In conclusion, our study shows that leaf Hg can be separated into three fractions: Hg associated with the surface, the cuticle, and the inner tissue of the leaf. The majority of leaf Hg (90%) is found in interior of the leaf (leaf tissue) and only about 10% is found on the surface and in the cuticle, disproving the null hypothesis. These results were
validated for four deciduous tree species and at three different times of the growing season. Both mass-based (ng g\(^{-1}\)) and surface area-based (ng m\(^{-2}\)) measures of leaf Hg content provide complementary information that can help explain leaf uptake of Hg. The correlation between leaf tissue Hg from four different deciduous species combined and the surface area was improved when the stomatal density for each species was multiplied by its corresponding surface area suggesting a significant role of the stomata in the uptake. Crown positions significantly affect leaf Hg concentration and therefore need to be taken into consideration in foliage Hg quantification. The involvement of the cuticle in the uptake of Hg was investigated but is likely limited to a route for the transit of Hg instead of storage since no accumulation was detected in a 100 day period. Based on observations in the current study, gaseous Hg is likely to be the dominant form of leaf uptake. Finally, our study demonstrates a steady and irreversible accumulation of Hg in leaves for four deciduous species. Knowing that deciduous litterfall decomposes at a faster rate than evergreens in lake water, good management of this litterfall is crucial in order to prevent it from reaching surface waters. In urban areas, it will be beneficial to have soil underneath the canopy to intercept the litterfall. This may increase mercury’s residence time in the landscape and help slow its movement to surface waters. Such movement has been chiefly linked to significant increases in stream MeHg concentration in Minnesota by Balogh et al. (2002) who found that leaf inputs to streams constitute a major Hg input and also provide sites for rapid methylation. Further research is needed to study the role of the structure of the leaf (i.e. roughness, presence of hair, etc) on the capture of atmospheric Hg. The role of the cuticle in Hg uptake warrants further
investigation by separating Hg associated with the polar cuticle and Hg in the non-polar section of the cuticle (waxes).
REFERENCES


CHAPTER 3. MERCURY ACCUMULATION IN FOLIAGE OF DECIDUOUS TREE SPECIES IN MINNESOTA

3.1 Introduction

Foliage has long been recognized as a sink for atmospheric mercury (Hg) (Lindberg et al., 1979; Grigal, 2002), and the contribution of soil solution to foliage Hg has been deemed marginal (Bishop et al., 1998). Foliage surfaces and leaf area index (LAI) can be up to 20 times the ground surfaces (Schulze, 1982), giving leaves the ability to scavenge significant amounts of atmospheric Hg via both dry and wet deposition. Foliage surfaces have unique morphological features, such as surface roughness, that allow them to be efficient scavengers of atmospheric pollutants (Barber et al., 2004). The large surface area coupled with unique surface morphology allows tree canopies to capture and contribute a significant amount of Hg to temperate and boreal ecosystems in the form of throughfall and litterfall (Kolka et al., 1999; Grigal et al., 2000). Mercury concentrations in throughfall often reach 2-5 times the concentration measured in open air precipitation (Kolka et al., 1999; Zhang et al., 2009). The majority of leaf Hg is known to be associated with dry deposition of Hg⁰ via stomatal route (Browne and Fang, 1978; Mosbaek et al., 1988; Ericksen et al., 2003; Choi et al., 2008) and non stomatal routes (Frescholt and Gustin, 2004; Stamenkovic and Gustin, 2009; Converse et al., 2010). The role of dry deposition is supported by a recent study by Risch et al. (2011) that found no correlation between Hg levels in wet deposition and foliage.
Watershed Hg budget studies have reported that conifer cover types contribute higher Hg loadings in litterfall than broadleaf cover types (Kolka et al., 1999; Grigal et al., 2000; Witt et al., 2009a), but a more recent study reported no significant difference in Hg contribution between the two types (Obrist et al., 2011).

There are many large scale studies that have evaluated the significant role of vegetation and particularly deciduous forests in the uptake and cycling of atmospheric Hg (i.e. Lindberg et al., 1991; Kolka et al., 1999; Rea et al., 2000; Demers et al., 2007; Obrist et al., 2011; Risch et al., 2011). However, there is limited research on interspecies variation in the accumulation of Hg at the leaf scale. Additionally, there is limited research on the intra-seasonal variation in the accumulation of Hg in leaves from emergence of buds until senescence, and such research sometimes reported contradictory results. Bushey et al. (2008) reported that leaf Hg concentrations increase throughout the growing season to reach a 10 fold increase by the end of the season. On the other hand, laboratory studies showed that leaf Hg concentrations leveled off after 2-3 months of leaf growth (Ericksen et al., 2003). Likewise, data on interspecies variation with regards to Hg uptake are unsettled. Some researchers found that oak leaves accumulated significantly less Hg than other broadleaf species (Bushey et al., 2008; Juillera and Ross, 2011). However, Obrist et al. (2011) reported no significant interspecies variation in the accumulation of Hg in senescing foliage of 17 different tree species from fourteen forest sites in the United States.
In this study, we attempt to expand our current understanding of bud-to-litterfall accumulation of Hg in deciduous species with emphasis on interspecies comparison, including a comparison between a conifer species and five broadleaf species. We also address the inter-seasonal dynamic of leaf Hg accumulation. Additionally, we will investigate the involvement of the cuticle in needle Hg accumulation by closely monitoring the intra-seasonal Hg accumulation in the surface, cuticle and inner tissues of Tamarack needles. To achieve these goals, we test the following hypotheses:

**Null Hypothesis 1:** There is no interspecies variation in foliage Hg accumulation, and therefore, foliage of different deciduous tree species will reach similar end of season Hg concentrations ([Hg]):

\[
[Hg]_{Maple} = [Hg]_{Elm} = [Hg]_{Oak} = [Hg]_{Gingko} = [Hg]_{Horse Chestnut} = [Hg]_{Tamarack}
\]

**Null Hypothesis 2:** There is no inter-annual variation in foliage Hg accumulation. In other words, the end of year Hg concentration in leaves is relatively constant in any given year;

\[
[Hg]_{2005} = [Hg]_{2011}
\]

**Null Hypothesis 3:** The rate (ng/g/d) of Hg accumulation in foliage is constant throughout the growing season. In other words, foliage Hg accumulation rate in early, mid-and late-season is a constant;

\[
\text{ACCUMULATION RATE}_{\text{Early season}} = \text{ACCUMULATION RATE}_{\text{Mid-season}} = \text{ACCUMULATION RATE}_{\text{Late season}}
\]
Null Hypothesis 4: The cuticle of Tamarack needles does not accumulate Hg during the growing season. In other words, Hg accumulation occurs mainly in the inner tissue of the needles and therefore there is no difference between early and late season needles cuticle Hg concentration ([Cuticle Hg]);

\[ [\text{Cuticle Hg}] \text{ Early season} = [\text{Cuticle Hg}] \text{ Late season} \]

3.2 Materials and methods

3.2.1 Sampling rationale

We monitored six deciduous species to study changes in foliage Hg concentrations throughout the growing season and to evaluate the interspecies variation in leaf Hg accumulation. These species are the deciduous conifer, tamarack (\textit{Larix laricina} (Du Roi) K. Koch) and five broadleaf species; gingko (\textit{Ginkgo biloba} L.), northern red oak (\textit{Quercus rubrum} L.), horse chestnut (\textit{Aesculus hippocastanum} L.), sugar maple (\textit{Acer saccharum} L.) and American elm (\textit{Ulmus Americana} L.). Additionally, we measured Hg concentrations in senescing foliage of sugar maple and elm in 2011 and compared those concentrations to Hg concentrations of leaves collected from the same trees in 2005 to study the inter-seasonal variability in senescing foliage Hg concentrations.

During the 2004 growing season, we collected foliage of gingko, oak, horse chestnut and sugar maple at regular intervals and analyzed their total Hg concentrations. Likewise, during the 2005 growing season, we monitored and studied Hg accumulation in tamarack, sugar maple and elm. Additionally, at the end of the 2011 growing season, we
measured senescing foliage Hg concentrations for the same sugar maple and elm trees that were sampled in 2005 for inter-annual accumulation comparison.

For Tamarack needles studied during the 2005 growing season, we monitored Hg accumulation in the surface, cuticle and inner tissues of the needle from emergence to senescence. We reported foliage Hg content by concentration normalized by dry weight or by surface area.

3.2.2 Sampling procedures

We sampled foliage as described in Chapter 1. Briefly, in the 2004 growing season, composite samples of sugar maple, oak, horse chestnut, and gingko leaves were collected between May and October, at 1-4 week periods depending on weather conditions. The same procedure was followed to collect Tamarack needles in 2005. The first set of leaves was sampled at the emergence of the buds and the last one during the senescence of leaves but while foliage was still on the tree. Additionally, in 2005, the same sugar maple and gingko trees sampled in the previous year were resampled. An American elm tree was also sampled in May, June, August and October of 2005. The same sampling protocol was used in 2011 for the collection of senescing foliage of sugar maple and elm. After collection, the samples were refrigerated at 4°C overnight for processing the following day.

3.2.3 Sample processing

Broadleaf foliage was oven dried at 60°C for 24 hours before total Hg analysis. A preliminary study showed no significant loss of Hg using this drying method (data not shown). Lodenius et al. (2003) reached a similar conclusion and found no significant
effect of drying at temperature of 60° C on leaf Hg over a period of four weeks. For needles, a subsample was used to determine the dry weight to fresh weight ratio (dw/fw), which was used to infer Hg concentration by dry weight. This procedure was performed to avoid drying the needles used for the sequential extraction analysis, since drying causes leakage of the internal constituents of needles. Fresh needles were subjected to the cuticle extraction technique described in Chapter 1. Subsequently, Hg concentrations in the surface, cuticle and inner tissue of the leaf were measured at each sample collection date. We measured the leaf surface area of the broadleaf species using a leaf area meter, which measures the one-side leaf surface area. Specific leaf area (SLA) was determined as the ratio of the leaf surface area to leaf dry weight. For needles, the total surface area was calculated from geometric measurements (Sellin, 2000) of the needle length and circumference in a random sample of 40 needles. We used a cotton string to measure the average circumference (C) of each needle from ten readings. Assuming the needle has a cylindrical shape, the total needle surface area (TSA) was calculated using the formula: TSA = L × C, where L is the needle length.

Using this method, half of the total needle surface area (as was the case for the broadleaf species) was used for the calculation of the SLA. The SLA of each species was used to report Hg concentrations normalized by surface area.

3.2.4 Hg analysis

Samples were analyzed for Hg in a clean room laboratory using cold vapor atomic fluorescence spectroscopy (CVAFS) by the double gold amalgamation method of Bloom and Crecelius (1983), similar to EPA Method 1631. Details of the method are provided in
Chapter 1. For broadleaf species, leaves were prepared for Hg determination by acid digestion. Details of the acid digestion are given in Chapter 1. The sequential Hg analysis for needles was also conducted following the procedures explained in Chapter 1. Total needle Hg was determined by the sum of the three Hg fractions associated with the surface, the cuticle and the inner tissue of the needles.

3.2.5 Quality assurance and quality control (QA/QC)

Acid blanks were analyzed at the beginning of each analysis, in addition to analytical blanks after every 20 samples. Two analytical duplicates, digestion blanks and matrix spikes were also conducted for every 40 samples. Control check standards were analyzed every 10 samples in addition to a certified plant reference material, NIST SRM #1515 (Apple leaves). Results of the analysis showed that acid blanks were low (lower than 2 ng L\(^{-1}\)). The percentage of recovery from duplicates, control standards, spikes and SRM was in the range of 95-105%.

3.2.6 Statistics

Statistical analyses were conducted using the R software (R Development Core Team, 2008). Bootstrapping was performed to calculate confidence intervals for the means indicated by the standard error (S.E.) with an alpha level of 0.05. The significance of the accumulation was assessed by Analysis of Variance (ANOVA) by comparing the initial foliage Hg concentration to the senescing foliage Hg concentration for all of the species. ANOVA was also used to compare end of season foliage Hg concentrations between the species. Single linear regression was used to fit prediction models for the
temporal trends in foliage Hg accumulation. These trends were compared using the significance test for the regression slope with confidence intervals.

3.3 Results and discussion

3.3.1 Intra-seasonal foliage mercury accumulation

3.3.1.1 Foliar Hg concentrations and magnitude of change

Foliation mercury concentrations were measured early and late in the growing season. The results, reported on a dry weight basis, are provided in Table 3.1.

**Table 3.1: Early and End of season foliage Hg concentrations**

<table>
<thead>
<tr>
<th>Species</th>
<th>Early season leaf Hg</th>
<th>End of season leaf Hg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ng g⁻¹</td>
<td>ng m⁻²</td>
</tr>
<tr>
<td>Gingko</td>
<td>1.73 ±0.6ᵃ</td>
<td>167.8 ±37.8</td>
</tr>
<tr>
<td>Horse chestnut</td>
<td>1.51 ±0.8ᵃ</td>
<td>60.47 ±29.3</td>
</tr>
<tr>
<td>Oak</td>
<td>2.10 ±0.7ᵃ</td>
<td>93.90 ±29.4</td>
</tr>
<tr>
<td>Sugar maple</td>
<td>2.70 ±2.1ᵃ</td>
<td>113.6 ±77.1</td>
</tr>
<tr>
<td>Elm</td>
<td>3.00 ±1.7ᵃ</td>
<td>133.6 ±27.2</td>
</tr>
<tr>
<td>Tamarack</td>
<td>2.85 ±1.3ᵃ</td>
<td>338.4 ±50.5</td>
</tr>
</tbody>
</table>

*Means followed by different lower case letters in the same row are statistically different (p<0.05). Means followed by different upper case letters in the same column are statistically different. The uncertainty represents the 95% C.I.

All species accumulated Hg (p<0.05) with varying efficiencies during a 162-day growing season. Early and late growing season foliage mass based Hg concentrations were 2.7 ±2.1 and 30.59 ±4.8 ng g⁻¹ for sugar maple, 3 ±1.7 and 41.01 ±6.8 ng g⁻¹ for elm, 2.85 ±1.3 and 43.13 ±1.7 ng g⁻¹ for tamarack, 1.73 ±0.6 and 19.05 ±2.8 ng g⁻¹ for gingko, 2.1 ±0.7 and 25.17 ±5.5 ng g⁻¹ for oak and 1.51 ±0.8 and 29.93 ±3.4 ng g⁻¹ for horse chestnut (Figure 3.2). These results are roughly comparable to results reported in other studies; Siwik et al. (2009) measured 23.6 ng g⁻¹ for sugar maple and 20.6 ng g⁻¹ for
oak at the end of the 2004 growing season in Ontario, Canada. In that same season, Bushey et al. (2008) reported sugar maple leaf Hg concentrations of 32.3±9.4 ng g\(^{-1}\) in an upland forest in NY. In Vermont, Juillerat and Ross (2011) reported fall foliage Hg concentrations of 33.5±5.6 ng g\(^{-1}\) for sugar maple and 29.4±7.4 ng g\(^{-1}\) for red oak.

The results of these analyses are also reported on leaf area basis. Mercury concentration in sugar maple leaves increased from 113.8 ±77.1 ng Hg m\(^{-2}\) leaf surface area at emergence to 1461.3 ±123 ng m\(^{-2}\) at senescence, displaying a 13 fold increase over the course of the growing season. A similarly significant increase was observed in horse chestnut with a 19 fold increase in leaf Hg, reaching 1199 ±134 ng m\(^{-2}\) in autumn. Oak foliage Hg concentrations increased by 12 fold to reach 1144 ±250 ng m\(^{-2}\) by mid-October. Likewise, elm increased 11 fold to reach 1519.44 ±175 ng m\(^{-2}\), while gingko increased 8 fold and reached 1421 ±180 ng m\(^{-2}\). Tamarack, the only conifer in the study, had a 9 fold increase in needle Hg concentration, but reached significantly higher Hg concentrations than the broadleaf species (2981.12 ±116 ng m\(^{-2}\)). Stupple (2010) reported similar Hg concentrations at the end of the growing season for sugar maple foliage in Canada ranging from 1090 ng m\(^{-2}\) to 1630 ng m\(^{-2}\). Siwik et al. (2009) however, reported a higher concentration (1900 ng m\(^{-2}\)) for five temperate deciduous species in Canada compared to our average of 1326.45 ng m\(^{-2}\) for the five broadleaf species (Figure 3.1).

The increase in foliar Hg observed in this study (8 to 19 fold increase) is comparable to or higher than that reported in other studies (Bushey et al., 2008; Poissant et al., 2008; Siwik et al., 2009). However, due to a lack of control of the start of Hg
monitoring and potential differences in the length of the growing season among these studies, comparisons among them have little quantitative value.

Leaf Hg concentrations among the broadleaf species showed no significant inter-species difference when end of season leaf Hg concentrations were normalized by surface area (Table 3.1). A similar result was found by Siwik et al. (2009), who reported a lack of significant difference between various broadleaf species when they reported leaf Hg concentration by surface area rather than by mass. When the different species were compared on a mass normalized basis, however, significant differences were observed among species (Table 3.1). Elm and sugar maple reached higher Hg concentrations than horse chestnut, and ginkgo had the lowest Hg concentrations. Null hypothesis 1 was accepted based on similar Hg area-based concentrations.

Interestingly, tamarack, the only conifer in this study, had the highest concentrations of Hg by surface area and by dry mass (p<0.001) compared to broadleaf species, although the distinction was less obvious when leaf Hg concentration was reported by dry mass (Table 3.1). Conifers are known to have greater surface roughness, more leaf hairs, and a structure that slows air flow (Rea et al., 2002), and in the case of Tamarack, the needles have stomata in both the abaxial and adaxial surfaces, unlike the broadleaf species. These unique properties may allow needles to intercept larger amounts of atmospheric Hg if Hg uptake is diffusion limited. Additionally, needles are known to have more lipids than broadleaves (Franzaring et al., 2000; Jien et al., 2011), which could be a factor in Hg uptake and/or accumulation given the hydrophobicity of Hg. The higher scavenging ability of Hg by conifers was also observed in watershed studies that
evaluated mass-based Hg concentrations and uptake by deciduous and coniferous species (Kolka et al., 1999; Grigal et al., 2000; Demers et al., 2007; Witt et al., 2009a).

Figure 3.1: Intra-seasonal accumulation of Hg in foliage (ng m$^{-2}$) of 6 deciduous species in Minnesota

At the end of the growing season, oak, like gingko, had the lowest leaf Hg concentration per surface area among the six deciduous species (Figure 3.1). Interestingly, Siwik et al. (2009) reported a similar finding when they compared oak Hg uptake to other deciduous species in Canada and found that oak had the lowest leaf Hg concentration at different times and sites. Similarly, Juillerat and Ross (2011) measured leaf Hg for various deciduous species and also found oak to have the lowest Hg concentrations. However, Lindberg (1996) has reported exceptionally high oak foliage
concentrations in a contaminated site in the Walker Branch Watershed exceeding 100 ng g$^{-1}$.

Figure 3.2: Intra-seasonal accumulation of Hg in foliage (ng g$^{-1}$) of six deciduous species in Minnesota.

3.3.1.2 Trends and regression analysis

We used linear regression analysis to compare the growing season leaf Hg accumulation rates for the six deciduous species in this study (Table 3.2). The slope of the regression provides the average growing season foliage Hg accumulation rate. Maple, gingko, oak and horse chestnut showed similar seasonal accumulation rates with similar slopes (Table 3.2) with no statistical difference. Tamarack on the other hand, had the
The steepest slope of 16.09 ng of Hg per m² leaf area per day, and was significantly higher than the group of the four broadleaf species mentioned above. Among the broadleaf species, oak had the lowest Hg accumulation rate of 4.71 ng m⁻² day⁻¹ corroborating results of recent studies where oak foliage Hg concentrations were found to be the lowest (Siwik et al., 2009; Juillerat and Ross, 2011). Elm foliage had an accumulation rate of 8.78 ng of Hg m⁻² day⁻¹ but with a large standard error, and therefore, belonged to both groups. Overall, leaf Hg concentration correlated significantly and positively with leaf age for all of the species. Similar results regarding the correlation between leaf age and Hg content were found in laboratory studies (Ericksen et al., 2003) and field studies (Millhollen et al., 2006; Rea et al., 2002; Poissant et al., 2008). However, Siwik et al. (2009) reported steeper slopes for oak (17) and maple (9) while studying leaf uptake of Hg in Ontario, Canada using a similar regression model.

The accumulation of Hg in foliage continued throughout the entire growing season and did not level off after two months as was found in laboratory studies (Ericksen et al., 2003). Despite a decline in Hg uptake rate by foliage later in the season, foliage Hg concentrations continued to increase until the last weeks of the growing season (Figure 3.1).
Table 3.2: Regression fit for seasonal leaf Hg accumulation in six deciduous trees

<table>
<thead>
<tr>
<th>Species</th>
<th>Regression equation</th>
<th>Adjusted R²</th>
<th>p-value</th>
<th>95% C.I. (slope)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maple</td>
<td>$y^* = 7.72 x^* + 78.12$</td>
<td>0.97</td>
<td>&lt;0.05</td>
<td>5.39-10.58</td>
</tr>
<tr>
<td>Gingko</td>
<td>$y = 7.37x + 99$</td>
<td>0.92</td>
<td>&lt;0.05</td>
<td>5.60-8.44</td>
</tr>
<tr>
<td>Oak</td>
<td>$y = 4.71x + 100$</td>
<td>0.82</td>
<td>&lt;0.05</td>
<td>3.23-6.33</td>
</tr>
<tr>
<td>Horse chestnut</td>
<td>$y = 7.50 x + 11.16$</td>
<td>0.91</td>
<td>&lt;0.05</td>
<td>5.86-9.10</td>
</tr>
<tr>
<td>Tamarack</td>
<td>$y = 16.09 x + 265.19$</td>
<td>0.93</td>
<td>&lt;0.05</td>
<td>12.57-19.61</td>
</tr>
<tr>
<td>Elm</td>
<td>$y = 8.78 x + 190.49$</td>
<td>0.95</td>
<td>&lt;0.05</td>
<td>4.43-13.13</td>
</tr>
</tbody>
</table>

y is mercury concentration in ng m⁻² and x is leaf age in days; n=3.

Broadleaf litterfall Hg concentrations reported in this study averaged 1.33 µg m⁻².

Using a maximum LAI of 7 (Urban average LAI is higher than the State average; Marv Bauer, pers. comm., 2011) calculated from assessment of maple stands in the Great Lakes region (Burton et al., 1991) and assuming that maple foliage LAI is representative of all deciduous species in the area, this would result in a maximum litterfall deposition flux of 9.3 µg m⁻² year⁻¹ under foliage canopies in the Twin Cities. This urban flux is slightly lower than the measured litterfall inputs of 12.5 µg m⁻² year⁻¹ in a remote watershed in Minnesota (Grigal et al., 2000). This is possibly due to the significant influence of conifers in the watershed which have showed higher Hg concentrations relative to broadleaves in this study. However, our estimated flux is higher than the 7.8µg m⁻² year⁻¹ litterfall dry deposition flux reported for an urban area (NADP MN 98) in Minnesota (Risch et al., 2011) which could be explained by uncertainties in LAI estimation and interspecies leaf Hg variation. Additionally, the measurement of dry deposition is exceedingly difficult as it depends not only on the concentration of the contaminant in the atmosphere but also on the nature of the surface area, its orientation, stickiness, hairiness,
etc. The combination of all these factors may explain the difference between our estimate and that of other studies.

3.3.1.3 Intra-seasonal variation in Hg accumulation rate

To get a better insight into the variation in the rate of foliage Hg accumulation during the growing season, we divided the season into three periods of two months each; early season (May-June), mid-season (July-August) and late season (September-October). We determined the rate of Hg accumulation in foliage in each of these three periods using foliage Hg concentrations from three sampling events in each window (Table 3.3).

Table 3.3 suggests that the bulk of Hg accumulation occurred in July-August when leaves reached maturity and photosynthetic activity was at its peak (Bassow and Bazzaz, 1998; Royer et al., 2005). The highest Hg uptake rate (13.67 ng m$^{-2}$ day$^{-1}$) was observed in the mid-growing season, similar to the rate calculated by Bushey et al. (2008), who found that sugar maple from the Huntington Wildlife Forest in NY accumulated a daily average of 14.40 ng m$^{-2}$ in the 2005 growing season. Similarly, using litterfall for calculation of the uptake rate, Poissant et al. (2008) reported an accumulation rate of 13.20 ng m$^{-2}$ day$^{-1}$ for maple foliage in a Canadian forest.
Table 3.3: Variation in foliage Hg accumulation rate for six deciduous species (ng m$^{-2}$ day$^{-1}$)

<table>
<thead>
<tr>
<th>Growing season</th>
<th>Sugar maple</th>
<th>Gingko</th>
<th>Tamarack</th>
<th>Oak</th>
<th>Elm</th>
<th>Horse chestnut</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>May-June</td>
<td>7.24ab</td>
<td>2.30Aa</td>
<td>9.80Aab</td>
<td>1.94Aa</td>
<td>13.42Bb</td>
<td>7.39ab</td>
<td>7.08A</td>
</tr>
<tr>
<td>July-August</td>
<td>10.30a</td>
<td>10.53Ba</td>
<td>40.43Bb</td>
<td>5.42Ba</td>
<td>5.36Aa</td>
<td>9.95a</td>
<td>13.67B</td>
</tr>
</tbody>
</table>

For comparison between species, means with different lower case letters in the same row are significantly different. For comparison between periods of the growing season, means with different upper case letters in the same column are significantly different ($p<0.05$).

A decline in foliage Hg uptake rate was observed later in the growing season for most species (Table 3.3). Similar declines in leaf Hg uptake rates were also observed in controlled environment studies (Ericksen et al., 2003) and in natural settings (Poissant et al., 2008). These authors studied Hg uptake in different maple forest canopies in Canada and found a decrease in the rate of Hg$^0$ uptake starting in September. This could be related to depressed photosynthetic activity at the end of summer in deciduous leaves (Koike et al., 2004), especially since leaf Hg uptake has been related to the stomatal route (Mosbaek et al., 1988; Lindberg, 1992; Fleck et al., 1999). The decline in Hg uptake later in the season could also be explained by leaves reaching a saturation point with regards to Hg assimilation. However, this scenario is less likely since foliage Hg concentration increased again just before the senescence of the leaves (Figure 3.1). Moreover, higher leaf Hg concentrations have been reported for the same species in other studies (ie. Lindberg, 1996; Juillerat and Ross, 2011) which would contradict the saturation point theory.
While most species experienced a decline in Hg uptake later in the season, gingko and oak seem to show an increase in uptake rate near the end of the season with rates reaching nearly 13.55 and 11.95 ng m$^{-2}$ day$^{-1}$ respectively (Table 3.3). In comparison with other studies, Siwik et al. (2009) also reported a higher uptake rate for oak leaves reaching 10.15 ng m$^{-2}$ day$^{-1}$ in October. These observations could be explained by the prolonged photosynthetic activity of oak which does not start to decline until later in the season (Koike, 2004). Heichel and Turner (1983) found that for oak, CO$_2$ assimilation does not start to decline until mid-September, while that for maple starts declining in mid-August in the northeastern United States.

Even when Hg uptake rates declined, foliage Hg concentrations did not show any sign of decrease during the entire growing season. Therefore, if a compensation point existed (Hanson et al., 1995), it did not seem to have an effect on the accumulation of leaf Hg, supporting the findings of Lodenius et al. (2003) on the irreversibility of foliage Hg uptake. Therefore, it is possible that boreal and temperate vegetation emissions of Hg observed in other studies (i.e. Leonard et al., 1998) are a result of surface Hg (Hg$^{2+}$) being reduced to Hg$^0$ by biotic and/or abiotic mechanisms (Zhang and Lindberg, 1999) and emitted back to the atmosphere. These circumstances could falsely indicate plant emission of Hg. Additionally, the continued increase of foliage Hg concentrations at the end of the growing season could indicate that leaf Hg is not involved in nutrient translocation from leaves before abscission unlike nitrogen (Grigal et al., 1976), starch (Hoch et al., 2003) and microelements. However, it is possible that nutrient translocation is behind the surge in leaf Hg concentration shortly before foliage senescence, especially
for elm, sugar maple and oak (Figure 3.1), and long after the slowdown of photosynthetic activity (Bassow and Bazzaz, 1998).

3.3.2 Inter-annual variation in seasonal mercury accumulation

The results displayed in table 3.4 show that the foliage of sugar maple and elm accumulated significantly higher concentrations of Hg during the 2005 growing season than in the 2011 growing season (p<0.05). In 2005 the average end of season foliage Hg concentration was 33.6 ±2.6 ng g⁻¹ and 40.6 ±3.3 ng g⁻¹ respectively for sugar maple and elm. At the end of the 2011 growing season, foliage of the same trees reached only 21.6 ±1.5 ng g⁻¹ and 30.6 ±2.2 ng g⁻¹ respectively for sugar maple and elm. Consequently, we rejected the null hypothesis 2 based on these results which show a significant difference in the inter-seasonal accumulation of mercury for sugar maple and elm trees between 2005 and 2011.

Table 3.4: Inter-seasonal comparison of foliage mercury between 2005 and 2011

<table>
<thead>
<tr>
<th>Species</th>
<th>2005 End of season foliage Hg (ng g⁻¹)</th>
<th>2011 End of season foliage Hg (ng g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sugar maple</td>
<td>33.6 ±2.6ᵇ, n=5</td>
<td>21.63 ±1.5ᵃ, n=6</td>
</tr>
<tr>
<td>Elm</td>
<td>40.6 ±3.3ᵇ, n=5</td>
<td>30.60 ±2.2ᵃ, n=4</td>
</tr>
</tbody>
</table>

*Means followed by different letters in the same row are statistically different (p<0.05). The uncertainty represents the 95% C.I.

In comparison with other studies, De Temmerman et al. (2009) studied the inter-seasonal variability in plant Hg uptake in Belgium as a function of air Hg concentrations and reported that the inter-seasonal variation is only significant when plants were grown in urban environments with high atmospheric Hg concentrations.
The decrease in senescing foliage Hg concentration observed between 2005 and 2011 in sugar maple and elm foliage could be sign of a decrease in background atmospheric mercury concentrations. Such a decrease is likely a result of declines in local mercury emissions (MPCA, 2005) as well as regional emissions (Engstrom and Swain, 1997). This result is also in agreement with the research of Nater and Grigal (1992) which has chiefly linked litterfall mercury concentrations to regional anthropogenic activities. This finding is also supported by a recent analysis of global trends in Hg concentrations by Slemr et al. (2011). These authors showed a 20 to 38% decline in global atmospheric Hg concentrations between 1996 and 2009.

Other factors may also be responsible for our observed decrease in senescing foliage mercury. Many environmental parameters have been documented to affect plant Hg uptake including temperature (Lindberg et al., 1992), CO₂ levels (Mosbaek 1988; Millhollen et al., 2006), solar radiation (Graydon et al., 2004), and light exposure (Frescholtz and Gustin, 2004). Additionally, foliage uptake is likely to be affected by tree health status which affects the photosynthetic activity of plants. One would also suspect the water balance to affect Hg uptake since it partially controls the opening of the stomates. This panoply of factors and their complex interactions are likely to change year to year which could also explain the inter-seasonal variability in Hg accumulation observed in sugar maple and elm.
3.3.3 Intra-seasonal Accumulation of Hg in the surface, cuticular, and inner tissues of Tamarack needles

3.3.3.1 Needle surface Hg

Surface Hg concentrations varied throughout the growing season and were between 9.03 and 263.16 ng m\(^{-2}\). Low values were recorded early in the season but were also seen in mid growing season with no clear accumulation trend. The contribution of surface Hg to total needle Hg varied between 1 and 14%. Needle surface Hg (Figure 3.3)
showed periods of accumulation (high surface Hg concentrations) followed by depletion (low surface Hg concentrations), likely indicating rainfall events and surface Hg removal via throughfall (Grigal et al., 2000). This observation is supported by recent research by Witt et al. (2009b) who observed a significant increase in two coniferous species’ throughfall Hg concentrations following a forest fire in a boreal forest in northern Minnesota. The authors attributed the enhanced increase in throughfall Hg to the ability of the needles to scavenge smoke from the atmosphere. Depletion of surface Hg could also be caused by biotic and abiotic reduction processes (Zhang and Lindberg, 1999) causing re-emission of Hg from the surface of needles. Alternatively, depletions can be caused by penetration of surface Hg into the cuticle which could render it unavailable at the surface.

3.3.3.2 Cuticular Hg

Cuticular Hg behaved similarly to surface Hg such that it showed periods of accumulation followed by depletion (Figure 3.3). Cuticle Hg concentration varied from 9 to 446 ng m\(^{-2}\) contributing 3 to 24% of total needle Hg. However, no significant accumulation occurred over the growing season when early season and late season needle cuticular Hg concentrations were compared (p=0.35). Therefore, there was not enough evidence against the null hypothesis 4 and thus it was not rejected. There was a period between day 80 and 160 of the growing season where cuticle Hg concentration steadily increased (Figure 3.3). It is possible that this temporary increase in cuticle Hg concentration is related to an increase in the cuticle thickness (Bernstein and Carroll,
1977; Petrini and Carroll, 1981) and thus its storage capacity. Conversely, the observed decrease in Hg concentration in the cuticle may be due to erosion of the cuticle by microbial activity. This observation corroborates recent suggestions on the involvement of the cuticle in Hg uptake (St Louis et al., 2001; Stamenkovic and Gustin, 2009; Converse et al., 2010). The lack of a steady increase in cuticle Hg throughout the growing season could possibly suggest a role of the cuticle in the penetration of Hg inside the needles rather than a storage site as is the case of POPs (Schreiber and Schonherr, 1992).

3.3.3.3 Tissue Hg

Needle tissue Hg increased significantly between emergence and senescence of needles (Figure 3.3). Periods of high and low uptake rates were observed. Tissue Hg contribution to total needle Hg varied from 75% to 94%. Tissue Hg concentrations increased continuously and reached an average concentration of 2422 ±664 ng m\(^{-2}\) at the end of the growing season with an average Hg accumulation rate of 14.4 ±4 ng m\(^{-2}\) day\(^{-1}\). Additionally, there was a surge in the uptake rate in the last two weeks, which could be due to many factors including increase in atmospheric mercury concentration, increase in the photosynthetic activity of the needle and nutrient translocation.
3.4 Conclusion

In this study, we investigated the rate of accumulation of Hg in foliage of six deciduous species. Total areal leaf Hg concentrations (ng Hg m\(^{-2}\) leaf area) increased 8 to 19 fold from the initial measurement. Tamarack, the only conifer evaluated in this study, had significantly higher Hg concentrations at the end of the growing season than the broadleaf species. Foliage Hg concentrations correlated positively and significantly with foliage age for all the species, but the linear regression slope, and therefore the uptake rate, was steeper for the conifer species.

Total foliar Hg concentrations increased throughout the growing season and showed no sign of loss. Hence, it was hard to support the theory of a compensation point in the absence of a temporary decrease in foliar Hg concentrations.

End of season foliage Hg concentration differed between the species with the coniferous tamarack scavenging more Hg than the broadleaf species. Additionally, senescing sugar maple and elm foliage Hg concentrations declined between 2005 and 2011 by nearly 30% possibly indicating changes in Hg exposure. Mercury distribution in tamarack needles was dominated by the inner tissue compartment which contained 75-94% of total needle Hg.

This study highlights the importance of leaf age in estimating litterfall Hg and dry deposition. Sampling should be conducted up to leaf senescence to avoid underestimating foliage Hg. The underestimation can be caused by rapid Hg accumulation rates that can take place even late in the season.
An increased understanding of interspecies and intra-seasonal foliage Hg changes could be useful for urban and forest managers to adequately assess the impact of foliage and litterfall on the environment, particularly aquatic ecosystems.
REFERENCES


• Jien S., Chen T. and Chiu C. Effects of afforestation on soil organic matter characteristics under subtropical forests with low elevation. J. For. Res. 16: 275–283


• Stupple G. 2010. Air mercury speciation, foliar uptake, and wash-off along an urban-rural gradient. A thesis submitted for the degree of Masters of Science, University of Toronto.


GENERAL CONCLUSIONS

Mercury in foliage of temperate deciduous trees can be separated into three fractions, each associated with the surface, the cuticle, and the inner tissue of the leaf. The majority of leaf mercury (90%) is held inside the leaf and less than 10% remains on the surface of the leaf (surface and cuticle mercury). This study showed the involvement of the cuticle in the uptake of mercury for the first time, although its role is likely limited to a pathway. The specific leaf area was found to correlate better with leaf mercury in comparison to surface area or dry mass. It is also shown that concentration by mass (i.e. ng g⁻¹) and by surface area (i.e. ng cm⁻²) provide complimentary information, and therefore, could be used side by side to explain and compare leaf mercury levels and uptake rates between species. We hypothesized that gaseous mercury is likely the dominant form of uptake based on the dynamics of mercury associated with the three fractions of leaves collected from roadside trees.

Foliage mercury concentrations increased 8-19 fold during the growing season. Tamarack accumulated significantly more mercury at the end of the growing season than the broadleaf species. Mercury concentrations correlated positively and significantly with foliage age for all the species, but the linear regression slope was steeper for tamarack. Inter-season leaf mercury variation was species-specific with sugar maple foliage displaying a significant difference in mercury accumulation between two successive growing seasons.

A good understanding of interspecies and intra-seasonal foliage mercury changes could be useful for urban and forest managers to adequately assess foliage mercury and
the impact of foliage and litterfall on the environment, particularly aquatic ecosystems. Sampling must take place within a short period of time, in the last week of the growing season, to avoid underestimating foliage Hg.

Further research is needed to study the role of the structure of the leaf (i.e. roughness, presence of hair, etc.) on the capture of atmospheric mercury. The role of the cuticle in mercury uptake also warrants further investigation by separating Hg associated with the polar cuticle and Hg in the non-polar section of the cuticle (waxes).