Urbanization impacts on epiphytic nitrogen and metal cycling in *Acer macrophyllum* stands in Western Washington, USA

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Bryophytes have been used extensively in Europe to assess the impacts of air pollution and atmospheric deposition; however there have been no studies conducted in Washington State to examine how pollution associated with urbanization is impacting canopy and forest floor bryophyte communities. This study investigated in N\textsubscript{2} fixation rates and metal concentrations in bryophytes across *Acer macrophyllum* Pursh stands in western Washington. We used an intensive, vertically stratified sampling approach in *Acer macrophyllum* canopies in the Hoh Rainforest on the Olympic Peninsula, WA and in Seattle, WA to collect 214 samples of *Isothecium stoloniferum* (Brid.). An extensive, ground-based sampling approach was used across an urban-to-wildland gradient to collect 59 *Kindbergia praelonga* (Hedw.) Ochyra samples. Intensive samples were collected four times (April, July, and October of 2016; and in January
and extensive samples three times (April, July, and October 2016) to assess seasonal differences in N₂ fixation rates and metal concentrations across sampling locations.

Nitrogen fixation rates in canopy *I. stoloniferum* samples was found to be dependent on sampling season and location. Average N₂ fixation rates reached peak levels in spring 2016 for both peninsula and urban samples (588.34 and 179.02 µmol m⁻² day⁻¹, respectively) and were lowest in fall 2016 (18.47 and 5.29 µmol m⁻² day⁻¹). In total, *I. stoloniferum* in canopies of *A. macrophyllum* was found to yield 1.13 kg N ha⁻¹ yr⁻¹ in the Hoh Rainforest and only 0.009 kg N ha⁻¹ yr⁻¹ in the Seattle urban area.

For the heavy metal analysis, elevated concentrations of Cd, Cr, Cu, Fe, K, Mg, Mn, Ni, Pb, Sr, Ti, and Zn were found in both intensive and extensive bryophyte samples collected in Seattle across all collection seasons. The elevated concentrations of heavy metals observed in *I. stoloniferum* samples collected from Seattle may be related to the low the N₂ fixation rates observed in the same samples.

The results from this study highlight that canopy and ground floor bryophytes has the potential to be used as a low-cost screening tool to evaluate N and heavy metal pollution in urban and rural areas.
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Chapter 1

Introduction

The Pacific Northwest (PNW) is considered to be one of the cleanest air quality regions in the United States (NADP/NTN, 2015), owing primarily to a small industrial base, an extensive hydroelectric system, and a region-wide lack of coal-fired power plants. However, despite this history and continued reductions in point source and per capita vehicular emissions, recent population increases and associated transportation growth is threatening to reverse the region’s history of high air quality (Geiser & Neitlich, 2007). In the Puget Sound Region, the population is expected to grow by roughly 1.5 million people over the next 25 years, which will in turn increase demand for travel throughout the region by 40% (Puget-Sound-Regional-Council, 2014). Seattle traffic, increasingly known for rampant transportation gridlock, was recently ranked as the 10th most congested city in North America (INRIX, 2016).

This region-wide expansion in the transportation sector has introduced a new set of atmospheric pollutants to forests of the PNW. Vehicle emissions associated with exhaust, lubricants, and tire and brake attrition represent a major form of pollution in busy urban centers in the PNW. Increasing urban vehicle operation and increasing traffic on major transportation corridors throughout the region (e.g., I-5 and I-90) can adversely affect forest ecosystem health, and there is growing evidence that traffic-related emissions, such as through tire and brake attrition, can impact ecosystems (Maltby et al., 1995; Pearson et al., 2000; Bermejo-Orguna, 2014). As personal and commercial vehicle traffic continue to rise, the region will experience an increase in atmospheric deposition associated with nitrogen oxide (NOx) emissions from gasoline and diesel vehicles. Further, transportation activities can greatly increase heavy metal
emissions and subsequent deposition in heavily used transportation corridors (Maltby et al., 1995; Pearson et al., 2000; Bakirdere & Yaman, 2008). Due to the limited use of coal throughout the region, metal deposition has generally been of little concern; however zinc (Zn), copper (Cu) and titanium (Ti) deposition has been increasing primarily due to tire and brake attrition (Thorpe & Harrison, 2008; Apeagyei et al., 2011).

These transportation pollutants have the potential to negatively affect forest ecosystem health by altering canopy epiphytes and their associated foodwebs (Creamer et al., 2008, Zvereva & Kozlov, 2011). Despite the iconic image of PNW moss-covered trees, the impact of transportation-related pollutants on PNW canopy epiphytes, and their impacts on ecosystem function, has been poorly examined.

Canopy dwelling epiphytes (i.e. lichens, bryophytes, algae) play important roles in biogeochemical cycles worldwide (Cornelissen et al., 2007) and are of particular importance in both deciduous and coniferous forests of the PNW (Binkley & Graham, 1981; McCune et al., 1997; Peterson & McCune, 2003; Antoine & McCune, 2004; Pypker et al., 2006). In the PNW, canopy epiphytes represent a substantial component of the forest ecosystem biomass, with standing epiphyte loads of 900-6,870 kg ha⁻¹ on some regional tree species (Pike et al., 1977 and Nadkarni, 1984, respectively). Tree canopies are complex biogeochemical components of an ecosystem, serving as key sites for the accumulation and processing of nutrients and other chemical elements (Klopatek et al., 2006).

Despite their fundamental role in ecosystem function and nutrient cycling, epiphytes are exceptionally sensitive to both direct and indirect effects of anthropogenic stress. Bryophytes
are widely used as biomonitors to assess the impacts of air pollution and atmospheric deposition (Ruhling & Tyler, 1973; Steinnes, 1989; Berg et al., 1995; Pott & Turpin, 1996; Reimann et al., 2006; Zechmeister et al., 2005; Harmens et al., 2008; Aničić et al., 2009; Ares et al., 2010; Donovan et al., 2016). As bryophytes lack roots, they absorb nutrients and water from atmospheric deposition (Bates, 1992) rather than from the soil. Bryophyte leaves are only one cell-layer thick and do not have a protective epidermis. As a result, they demonstrate ion exchange properties, all of which allows for the accumulation of water, organic compounds and inorganic ions that are deposited on their surfaces (Gjengedal & Steinnes, 1990; Tyler, 1990; Aboal et al., 2011; Gonzalez & Pokrovsky, 2013).

Exposure to nitrogen (N) based pollutants is known to negatively influence bryophyte growth, impacting epiphyte community structure (Davies et al., 2007; Bermejo-Orduna, 2014). Moss shoot growth has been found to be is sensitive to NO₂ emissions from vehicles (Bell, 1992). Cyanobacteria, facultative autotrophs, associated with bryophytes have the ability to fix substantial quantities of N in environments that experience little N deposition (DeLuca et al., 2002). Multiples studies have demonstrated the sensitivity of forest floor N₂ fixing bryophytes to N deposition (DeLuca et al., 2008; Zackrisson et al., 2009; Gundale et al., 2011) and N₂ fixation has been shown to cease in areas with increasing N deposition (DeLuca et al., 2008, Gundale et al., 2011).

Heavy metal deposition associated with industry and urbanization are also known to negatively influence bryophyte communities (Pearson et al. 2000; Aničić et al., 2009; Zvereya & Kozloy, 2011). As bryophytes exhibit high cation exchange capacity and can generate acidity through proton release from exchange sites (Spearing, 1972), the bioaccumulation of heavy metals in bryophyte tissue may inhibit microbial populations and thus limited N₂ fixation
capacity (Tyler, 1990; Pearson et al., 2000; Basiliko & Yavitt 2001). Bengtsson et al. (1982) found a reduction in the photosynthetic tissue responsible for growth in *Hylocomium splendens* samples collected from sites with elevated Cu and Zn concentrations.

To date, no comprehensive studies have examined N\textsubscript{2} fixation in canopy bryophytes in forests of western Washington or how increasing N and metal deposition in PNW forest ecosystems may influence canopy bryophyte communities and N\textsubscript{2} fixation rates.

**Objectives**

While there has been scattered interest in epiphyte ecology and its connection to forest health (McCune, 2000; Lindo & Gonzalez, 2010), there have been no recent studies that assess the influence of transportation sector pollutants on forest canopy bryophytes in the PNW. The overarching goal of the proposed research is to evaluate whether N and metal deposition associated with urbanization is creating a serious threat to canopy epiphyte and forest floor bryophyte communities throughout Western Washington. This thesis is broken down into three primary objectives that are presented in two data chapters:

1. Evaluate N\textsubscript{2} fixation rates in canopy bryophytes in *A. macrophyllum* stands in the Hoh Rainforest on the Olympic Peninsula and in the urban center of Seattle, WA (Chapter II)

2. Assess how urbanization affects canopy bryophyte N\textsubscript{2} fixation rates in this region (Chapter II).

3. Assess whether transportation sourced metals contribute to shifts in canopy epiphyte and forest floor bryophyte biodiversity and metal retention across an urban-to-wildland gradient in Western WA (Chapter III)
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Chapter 2: Epiphytic nitrogen fixation in *Acer macrophyllum* canopies in Western Washington, U.S.A.

Abstract

Old-growth forests in the Pacific Northwest are unique in their capacity to host a variety of epiphytes on their branches and trunk. Given the high epiphytic biomass associated with *Acer macrophyllum* trees, we evaluated how seasonal weather changes and urbanization, as proxied through metal deposition, affect canopy epiphytic N\textsubscript{2} fixation in the Hoh Rainforest of the Olympic Peninsula and in urban parks and forests in Seattle. We used an intensive sampling approach to collect *Isothecium stoloniferum* (Brid.) samples from both the Hoh Rainforest and Seattle during four seasons from April 2016 through January 2017. Levels of N\textsubscript{2} fixation were found to be the highest during the April 2016 sampling period. Permutational multivariate analysis of variance (perMANOVA) revealed a significant effect of sampling location and season. Elevated levels of heavy metals were observed in *I. stoloniferum* samples collected in the urban boundaries of Seattle, suggesting that N\textsubscript{2} fixation is sensitive to the bioaccumulation of heavy metals. In total, *I. stoloniferum* in canopies of *A. macrophyllum* was found to yield 1.13 kg N ha\textsuperscript{-1} yr\textsuperscript{-1} in the Hoh Rainforest and only 0.009 kg N ha\textsuperscript{-1} yr\textsuperscript{-1} in the Seattle urban area. Our results suggest that epiphytic N\textsubscript{2} fixation in *A. macrophyllum* stands on the Olympic Peninsula may contribute nitrogen in secondary successional maple forests.

**Keywords:** nitrogen fixation; bryophytes; heavy metals; Pacific Northwest
Introduction

Canopy dwelling epiphytes play an important role in biogeochemical cycles in deciduous and coniferous forests of the Pacific Northwest (PNW) (Pike et al., 1977; Binkley & Graham, 1981; Pypker et al., 2006; Cornelissen et al., 2007; Haristoy et al., 2014); however, few studies have evaluated these epiphytic mosses for their capacity to fix N. Dense, extensive epiphytic moss mats establish on the trunk and branches of big leaf maple (*Acer macrophyllum* Pursh) trees as a result of high rainfall rates in the region, the calcium rich bark, and neutral pH of maple throughfall (Kenkel & Bradfield, 1986). On the Olympic Peninsula of Western Washington state (USA), *A. macrophyllum* canopies generally have an estimated standing epiphytic load of 6,870 kg ha\(^{-1}\) (Nadkarni, 1984). These epiphytic mats, which contain a variety of lichen and bryophyte species, contribute to a number of important ecosystem functions including the processing of nutrients and water (Proctor, 2001; Klopatek et al., 2006; Pypker et al., 2006).

Unlike vascular plants, epiphytes lack roots and obtain their nutrients from the atmosphere (Bates, 1992). Bryophytes leaves demonstrate ion exchange properties, which facilitate the absorption of organic compounds and inorganic ions that are deposited on their surfaces (Gjengedal & Steinnes, 1990; Tyler et al., 1990; Gonzalez & Pokrovsky, 2013). Symbiotic associations between cyanobacteria and forest floor bryophytes can contribute significant input of Nitrogen (N) in late successional boreal forests (DeLuca et al. 2002, Zackrisson et al., 2004). Lindo and Whiteley (2011) confirmed the presence of cyanobacteria in canopy bryophyte species associated with old growth Sitka spruce (*Picea sitchensis* (Bong) Carr.) trees in British Columbia, Canada and reported significant rates of *N\(_2\)* fixation in these mats. Cyanobacteria associated with canopy bryophytes have the potential to function as an important N source in N-limited PNW forests, which supports forest productivity by supplying stand-level N in mid to late succession.
Despite their fundamental role in ecosystem function and nutrient cycling, bryophytes are extremely sensitive to both direct and indirect effects of anthropogenic stress. Bryophytes have been widely used in Europe to assess the impacts of air pollution and atmospheric deposition (Berg et al., 1995; Berg & Steinnes, 1997; Reimann et al., 2000; Harmens et al., 2008). Heavy metal deposition and exposure to N based pollutants are known to negatively influence bryophyte growth (Bengtsson et al., 1982; Pearson et al., 2000). Previous studies have highlighted the sensitivity of forest floor N\textsubscript{2} fixing bryophytes to N deposition (DeLuca et al., 2008; Ackermann et al., 2012), and rates of N\textsubscript{2} fixation has been shown to decrease or even cease in areas with increasing N deposition (Gundale et al., 2011).

To date, no comprehensive studies have examined epiphytic N\textsubscript{2} fixation in bigleaf maple, \textit{Acer macrophyllum}, or assessed how changes in N and metal deposition associated with the increasing population and transportation growth in Western Washington could influence N\textsubscript{2} fixation in \textit{A. macrophyllum} bryophyte communities. \textit{Acer macrophyllum} is a prominent component of the rural and urban forest in Western Washington, which lies at the heart of the native range of \textit{A. macrophyllum}. In our observations, \textit{A. macrophyllum} is perhaps the only woody plant species that consistently supports epiphytes in heavily urbanized areas in Seattle, even though epiphytes are an iconic feature of rural western Washington forests that are found on a number of woody different plant species, including \textit{A. macrophyllum}. Thus, we chose \textit{A. macrophyllum} due to both its wide distribution across Western WA (urban and rural) and its support of bryophytes. We hypothesized that 1) canopy bryophytes harbor N\textsubscript{2} fixing bacteria; 2) N\textsubscript{2} fixation will be higher in samples collected from the somewhat pristine Olympic Peninsula compared to urban sites in the Puget Sound region; 3) N\textsubscript{2} fixation will vary by season across
sampling locations; and 4) urban areas will be exposed to higher concentrations of heavy metals in canopy bryophyte tissue.

**Materials & Methods**

2.1 Study Area

We collected moss samples using an intensive, vertically stratified sampling approach in *A. macrophyllum* canopies in the Hoh Rainforest on the Olympic Peninsula, WA and in the urban center of Seattle, WA. We collected samples from one stand located on Hoh River Trust land in the Hoh Rainforest (47.82°N, 124.20°W) and from three stands located in Seattle City Parks: Seward Park (47.55°N, 122.25°W), Interlaken Park (47.63°N, 122.31°W), and Ravenna Park (47.67°N, 122.30°W).

In the Hoh Rainforest stand, understory vegetation was dominated by sword fern (*Polystichum munitum* (Kaulf.) C. Presl), redwood sorrel (*Oxalis oregano* Nutt.) and vine maple (*Acer circinatum* Pursh). In the Seattle stands, understory vegetation was dominated by sword fern, salah (*Gaultheria shallon Pursh*), Oregon grape (*Mahonia aquifolium* (Pursh) Nutt.), and stinging nettle (*Urtica dioica* L.). The dominant epiphytic moss species in *A. macrophyllum* canopies in the Hoh Rainforest stand were *Isothecium stoloniferum* (Brid.), *Antitrichia curtipendula* (Hedw.), and *Hylocomium splendens* (Hedw.). The dominant epiphytic moss species in *A. macrophyllum* canopies in Seattle were *I. stoloniferum* (Brid.), *Homalothecium lutescens* (Hedw.), and *Orthotrichum lyelli* Hook. & Taylor. We chose *I. stoloniferum* as the epiphyte species in our study as it could readily be found across all stands, and because it has been used previously in biomonitoring studies from the Pacific Northwest region (Pott & Turpin, 1998; Raymond & Pott, 2003; Raymond et al., 2010).
2.2 Sample Collection

We collected replicate *I. stoloniferum* core samples every three months beginning in April 2016 to January 2017. Samples from extracted from a total of 18 *A. macrophyllum* trees: nine from the Hoh Rainforest, WA and nine from city parks in Seattle, WA, USA (three per park). We selected individual trees at stands based on safety criteria and sampling considerations. For example, selected trees had to have a least three accessible branches below a solid anchor point to allow us to obtain samples from three stratified heights throughout the crown: low (6-10 m), mid (12-15 m) and upper (15-18 m). We used the Single Rope Technique (SRT) (Coffey & Anderson, 2012) in which a rope wrench and a friction hitch was used to gain access to the canopy. We deployed throughfall collectors in the form of ionic resin (UNIBEST Ag Manager, Walla Walla, WA) lysimeters (c.f., DeLuca et al. 2008) in each tree and at each sampling height. Lysimeters were deployed in January 2016, and removed in January 2017. The lysimeters were used to evaluate wet NO$_3^-$-N and NH$_4^+$-N deposition throughfall rates (Susfalk & Johnson, 2002; Kopatek et al., 2006; DeLuca et al., 2008). We also deployed HOBO data loggers (Onset Computer Corps., Bourne, MA, USA) to record hourly temperature at the three sampling heights throughout the canopy in a total of six trees (three from the Hoh Rainforest and in one tree per city park in Seattle).

In the nine trees from the Hoh Rainforest, we also collected replicate *A. curtipendula* and *H. splendidens* cores every three months beginning in April 2016 to January 2017. These cores allowed for the comparison of difference in fixation rates across dominant canopy moss species from the Hoh Rainforest.
A black parachute cord was tied to the climbing line to facilitate sampling in the same tree during seasonal sampling. Installing the parachute cord saved time and prevented any potential damage to the tree that might occur through repeating the rigging process.

2.3 Laboratory Analysis:

i. Nitrogen Fixation Analysis

$N_2$ fixation rates were estimated using the acetylene reduction (AR) method (Schöllhorn and Burris, 1967). Three replicate moss samples were collected per sample position within each tree and placed into 20 mL glass vials and then sealed with a rubber septum. Ten-percent of the headspace was removed using a syringe and replaced with reagent grade acetylene. The tubes were allowed to incubate at 18°C for 24 hours. Ethylene generated by bacterial reduction in the headspace was analyzed using a Thermo Scientific Trace Gas Chromatograph equipped with a flame ionization detector and Porapak Q column. Controls consisted of moss without acetylene gas, and blank samples contained acetylene gas without moss. Data are presented as the average amount of acetylene reduced to ethylene in $\mu$mol m$^{-2}$ d$^{-1}$ across the three replicates per sample.

To determine the amount of $N_2$ fixed per unit area, 20 replicate samples of the three moss species for were taken with a 20.27cm$^2$ PVC tube core. For *I. stoloniferum*, 20 cores were collected from the Hoh Rainforest and 20 from Seattle *A. macrophyllum* canopies. Hoh Rainforest and Seattle *I. stoloniferum* were found to have an average of 0.42 shoots cm$^{-2}$ and 0.88 shoots cm$^{-2}$, respectively. The other two species of
interest in the Hoh Rainforest, *A. curtipendula* and *H. splendens*, were found to have an average of 0.16 shoots cm\(^{-2}\) and 0.07 shoots cm\(^{-2}\), respectively.

To quantify the amount dry-weight moss biomass per unit area for *I. stoloniferum* and *A. curtipendula*, 10cm\(^2\) core were collected along with average moss depth per sampling branch. Hoh Rainforest and Seattle *I. stoloniferum* were found to have an average of 0.43 g cm\(^{-2}\) and 0.05 g cm\(^{-2}\), respectively. *Antitrichia curtipendula* samples from the Hoh Rainforest were found to have an average of 0.80 g cm\(^{-2}\).

ii. Trace Metal Concentrations:

After samples had been analyzed for N\(_2\) fixation, they were decapped and allowed to vent for 24 hours. Next, they were dried for 24 hours at 40 °C and ground to a fine powder with the facilitation of liquid N. The HNO\(_3\) + H\(_2\)O\(_2\) digestion method was used to prepare the dried and ground samples for analysis. Four mL of concentrated reagent-grade HNO\(_3\) were added to samples in 50 mL borosilicate tubes. The tubes were covered with plastic watch glasses and sat overnight at ambient temperature in a fume hood (approximately 18°C) to allow some initial oxidation of the samples by the HNO\(_3\). Samples were digested at 95 °C for 90 minutes in borosilicate tubes heated evenly in a 36-tube graphite block digester. Samples were allowed to cool, after which 4 mL of reagent-grade 30% H\(_2\)O\(_2\) were added to each tube followed by a 30-minute digestion at 95 °C and time to cool. Next, an additional 4 mL of H\(_2\)O\(_2\) were added to each sample and heated again at 95 °C. After cooling, deionized water was added to each tube to the 20-mL mark. To remove any undigested particulates not
dissolved in the HNO₃ and H₂O₂, samples were filtered through 0.45-µm membrane syringe filters.

Digests were analyzed for a suite of metals (Cd, Cr, Cu, Fe, K, Mg, Mn, Ni, Pb, Sr, Ti, and Zn) using inductively coupled plasma optical emission spectrometry (ICP-OES). Instrument performance tests were carried out daily to ensure proper calibration. After calibration, quality control samples consisting of blanks, low and high concentration check standards were analyzed.

iii. Resin Analyses for NO₃⁻-N and NH₄⁺-N:

The ionic resins from canopy lysimeters were collected in January 2017. Resin capsules were extracted by placing the ionic resin capsules in three successive 10 mL aliquots of 0.5M HCl. Extractable NO₃⁻-N and NH₄⁺-N were analyzed using microplate colorimetric technique using the salicylate-nitroprusside method for NH₄⁺ (Mulvaney, 1996) and the vanadium method for NO₃⁻ (Miranda et al., 2001).

2.4 Statistical Analysis:

Data on N₂ fixation, represented as µmol m⁻² d⁻¹, were transformed using a log₁₀(y + 1) to normalize the distribution. A two-way permutational multivariate analysis of variance (perMANOVA) was carried out to test if N₂ fixation rates and moss metal concentrations were significantly different across location types and sampling seasons for I. stoloniferum samples. The subsequent test of multivariate homogeneity of groups (DISPER) was used to calculate significant multivariate dispersions in variables among the location types for I. stoloniferum. Euclidean distance was chose for the two-way perMANOVA, and significance of the two-way
perMANOVA R-value was determined via 1000 random permutations. For this study, effects with probabilities of $p < 0.05$ were assumed to be significant.

Principle component analysis (PCA) was selected as the appropriate ordination method where dissimilarity was calculated as Euclidean distance (Legendre & Legendre 1998). PCA was performed on the data matrix ‘location type x chemical parameters’ for $I.\ stoloniferum$ for urban and peninsula sampling sites. Thirteen parameters ($\mu$mol m$^{-2}$ d$^{-1}$, Cd, Cr, Cu, Fe, K, Mg, Mn, Ni, Pb, Sr, Ti, and Zn) were introduced as the analysis variables. PCA was performed to assess the dominant patterns in moss acetylene reduction rates and metal concentrations across locations. The loadings of variables to each PC was examined by converting eigenvector coefficients to structure correlations.

We used an Analysis of variance (ANOVA) to test the main effects of species (e.g., $I.\ stoloniferum$, $A.\ curtipendula$, and $H.\ splendens$), sampling time, and canopy height on $N_2$ fixation rates. All statistical analyses were conducted in the R Statistical Environment (R Core Team, 2013).

**Results**

Nitrogenase activity, measured by using AR, was highest in $I.\ stoloniferum$ samples collected in spring for both peninsula and urban sampling sites (Figure 1). Of the in $I.\ stoloniferum$ samples collected in spring, five peninsula samples and one urban sample nitrogenase activity exceeded 1,000 $\mu$mol m$^{-2}$ day$^{-1}$. The shoot density and biomass estimates per unit area were used to scale up the acetylene reduction rates to landscape levels $N_2$ fixation. By using a season-weighted average, we estimate a canopy $N_2$ fixation rate (given a molar ratio
of 3:1 acetylene reduced to N₂) of 1.13 kg N ha⁻¹ yr⁻¹ for I. stoloniferum and 0.63 kg N ha⁻¹ yr⁻¹ A. curtipendula in the Hoh Rainforest. N₂ fixation rates for Seattle canopy I. stoloniferum are estimated at 0.009 kg N ha⁻¹ yr⁻¹.

Canopy collection height did not have a significant effect on N₂ fixation observed across the three species (I. stoloniferum, A. curtipendula, and H. splendens) collected from A. macrophyllum canopies on the Olympic Peninsula (p=0.11). The ANOVA results across the three moss species found significant differences across species (F₂,208 = 56.70, p<0.001) and across sampling seasons(F₃,208 = 84.98, p<0.001). A significant interaction between species and sampling season was observed (F₆,208 = 4.72, p<0.001).

In the Hoh Rainforest, the highest average nitrogenase activity measured was observed in H. splendens (2,388.18 µmol m⁻² day⁻¹); followed by I. stoloniferum (588.33 µmol m⁻² day⁻¹) and A. curtipendula (309.21 µmol m⁻² day⁻¹) in April 2016 (Figure 2). N₂ fixation rates in dropped in July with the highest average rates observed in H. splendens (373.01 µmol m⁻² day⁻¹); followed by A. curtipendula (121.44 µmol m⁻² day⁻¹) and by I. stoloniferum (96.71 µmol m⁻² day⁻¹). The lowest average nitrogenase activity rates were found in October 2016 for A. curtipendula and I. stoloniferum (49.36 and 18.47 µmol m⁻² day⁻¹, respectively) and January 2017 for H. splendens (135.27 µmol m⁻² day⁻¹).

For the canopy I. stoloniferum samples, N₂ fixation and metal concentrations were significantly different across peninsula and urban locations (F₁,206 = 256.34, p<0.001) and across sampling seasons (F₃,206 = 29.69, p<0.001) (Table 3a). There was a significant interaction found between location type and sampling season (F₃,206 = 4.00, p<0.001). This would suggest that N₂ fixation rates and metal concentration amount found in the moss tissue is dependent on the
season it was collected. Considering the location type term, the DISPER results show no significant differences in between-site variation ($F_{1,212}=1.00$, $p=0.324$) and pairwise comparison also showed no significant differences among location types.

Significant proportions of the variability were explained by their first two PCs (Figure 3). Structure correlation coefficients between each variable and the first two PCs for urban *I. stoloniferum* canopy samples are presented in Table 1. The total amount of variance explained by the first two PCs were (86.27%): PC1 (60.57%) and PC2 (25.07%). Cr, Cu, Fe, K, Mg, Mn, Ni, Pb, Sr, Ti, and Zn moss tissue concentrations are highly correlated with PC1; Cd concentrations and µmol m$^{-2}$ day$^{-1}$ are highly correlated with PC2. Vector arrows for the metal concentrations point towards the urban sampling points, suggesting that the metal concentration tends to be higher in urban *I. stoloniferum* canopy samples compared to peninsula *I. stoloniferum* canopy samples. The µmol m$^{-2}$ day$^{-1}$ arrow points towards the peninsula sampling points, suggesting that biological N$_2$ fixation rates are higher in peninsula *I. stoloniferum* canopy samples compared to urban *I. stoloniferum* canopy samples.

Deposition of NO$_3^-$-N and NH$_4^+$-N in peninsula and urban A. macrophyllum canopies as estimated using resin-based throughfall collectors are reported in Figure 4. Canopy height did not have a significant influence on resin-sorbed NO$_3^-$-N and NH$_4^+$-N ($p=0.11$ and $p=0.51$, respectively), therefore an average deposition rate was used per location. Higher rates of wet NH$_4^+$-N deposition were observed in the Hoh Rainforest canopy ($p<0.1$) compared to urban canopies in Seattle, WA. No significant differences were observed across peninsula and urban canopies for annual wet NO$_3^-$-N deposition rates.
Discussion

Measurable rates of N\textsubscript{2} fixation were recorded in moss communities of A. macrophyllum canopies across the sampling area in Western WA. Rates of N\textsubscript{2} fixation in canopy mosses were found to be dependent on sampling season and location. Average N\textsubscript{2} fixation rates for I. stoloniferum reached peak levels in spring 2016 for both peninsula and urban samples, 588.34 and 179.02 µmol m\textsuperscript{-2} day\textsuperscript{-1}, respectively. Rates dropped off during summer 2016 (96.71 and 53.99 µmol m\textsuperscript{-2} day\textsuperscript{-1}) and reached the lowest average levels in fall 2016 (18.47 and 5.29 µmol m\textsuperscript{-2} day\textsuperscript{-1}) for peninsula and urban samples, respectively. There was a slight increase in N\textsubscript{2} fixation rates in winter 2017 in both sampling locations (28.18 and 20.72 µmol m\textsuperscript{-2} day\textsuperscript{-1}).

Abiotic factors such as temperature and light have been studied extensively and are known to influence N\textsubscript{2} fixation rates in ground floor mosses (Alexander et al., 1973; Basilier et al., 1978; Davey, 1983; Meeks, 1998; Zielke et al., 2002; Gentili et al., 2005). Several studies have found that temperature is a stronger driver for N\textsubscript{2} fixation than light (Chapin, 1991; Lennihan et al., 1994; Liengen & Olsen, 1997; Solheim et al., 2002; Gundale et al., 2009). Gentili et al. (2005) found that N\textsubscript{2} fixation in feather mosses reached a peak between 13°C - 22 °C. Temperature data was recorded at three heights within the canopy of sample trees, but no significant difference in temperature across canopy height was observed (p=0.75). Figure 5 shows seasonal changes in canopy temperature across the Hoh Rainforest and Seattle sampling sites. An average daily temperature on the date of collection was compared to N\textsubscript{2} fixation rates across all four sampling seasons and locations (Figure 6). The highest average N\textsubscript{2} fixation rates were observed under temperatures greater than 17°C, which occurred in April 2016. The lowest N\textsubscript{2} fixation rates, observed during October 2016, did not correspond to the lowest average daily temperature. It is possible that total precipitation during the month of October, in addition to
cooler daily temperatures, influenced N₂ fixation rates in canopy l. stoloniferum samples. There was a sharp increase in total precipitation (cm) in October (Figure 7) for both the Hoh Rainforest and Seattle locations (50.05cm and 25.53cm, respectively). Several studies have observed a positive relationship between rainfall frequency and N₂ fixation rates in arctic and boreal ecosystems (Zielke et al., 2002; Gundale et al., 2009; Jackson et al., 2011; Gundale et al., 2012; Rousk et al., 2014). It is possible that the hydrological status in the samples collected in October 2016 exceeded the optimum moisture requirement to facilitate N₂ fixation.

Lindo and Whiteley (2011) provided the first study to confirm the presence of N₂ fixation in canopy moss communities in the temperate rainforest on Vancouver Island, British Columbia, Canada. Canopy fixation rates associated old-growth Sitka spruce (Picea sitchensis (Bong) Carr) stands are estimated at 0.5 kg N ha⁻¹ yr⁻¹. Annual kg N ha⁻¹ yr⁻¹ associated with canopy mosses in this study is intermediate compared to forest floor levels reported in late successional forests in the boreal (2.1 kg N ha⁻¹ yr⁻¹) (DeLuca et al., 2002). The lower annual N₂ fixation rates in Seattle is likely a result of the limited biomass and higher heavy metal concentrations in urban A. macrophyllum stands compared to the Hoh Rainforest. However, the values reported in this study may be a conservative estimate of stand level N-inputs as it does not take not take branch density on each tree into consideration.

This study did not identify all potential cyanobacteria genera that may be living epiphytically on canopy bryophytes in A. macrophyllum trees. In the boreal, four cyanobacteria species (Nostoc, Stigonema, Calothrix, and Cylindrospermum) are known colonizers on forest floor P. schreberi (DeLuca et al., 2007; Ininbergs et al., 2011). Lindo and Whiteley (2011) found a linear relationship between density of Scytonema cells on moss and N₂ fixation in the canopy of Sitka spruce (Picea sitchensis (Bong) Carr.) trees. As Scytonema are predominately aerially
dispersed (Komarek et al., 2003), it is possible *Scytonema* would be the dominant genera in *A. macrophyllum* canopies. Future research is needed to identify the diversity of cyanobacteria living epiphytically on canopy bryophytes in old growth *A. macrophyllum* canopies.

In boreal regions, biological N$_2$ fixation associated with *Pleurozium schreberi* (Brid.) Mitt. is a primary source of ecosystem N at 1.7 kg N ha$^{-1}$ yr$^{-1}$ (DeLuca et al., 2002). However it remains unclear how N$_2$ fixed through cyanobacteria associations enters the soil for plant uptake, but it appears to be a tortuous pathway (DeLuca et al., unpublished data). As mosses are efficient in retaining nutrients (Oechel and Van Cleve, 1986; Aldous, 2002), the rate at which fixed N is transferred to the soil and plants is expected to be slow (Rousk et al., 2014). In *A. macrophyllum* canopies, fixed N may enter the environment by accumulating in canopy soil. Studies conducted in *A. macrophyllum* canopies along the Queets River, located on the Olympic Peninsula, found a C/N ratio of 22 for canopy soils and NH$_4^+$ to be the dominant form of extractable N (Haristoy et al., 2014). This would suggest that *A. macrophyllum* canopy soils have a higher potential for mineralization and thus increased N availability for plants, host tree adventitious roots, and microorganisms (Nadkarni, 1994; Brady and Weil 2000; Lindo & Winchester, 2007; Berg & McClaugherty, 2008). Another potential pathway for fixed N from canopy bryophytes is being deposited to the forest floor as epiphytic litterfall. As epiphytic litterfall decomposes on the forest floor it can be available for terrestrially root plants (Nadkarni & Matelson, 1992). Tejo et al. (2015) found a total of 495 kg ha$^{-1}$ yr$^{-1}$ input from bryophyte litterfall in the Queets River Watershed. It is possible that the input of nutrients from bryophyte litterfall is providing of a much-needed source of N in N-limited PNW forests.

Nitrogen deposition is known to negativity influence bryophyte growth and N$_2$ fixation rates (Davies et al, 2007; DeLuca et al., 2008; Gundale et al., 2011). Despite being considered
one of the cleanest air quality regions in the U.S., the PNW has experienced an increase in total wet inorganic-N deposition since 1985 (NADP/NTN, 2015). Several studies have observed shifts in lichen community composition as a result of the adverse effects of increasing N pollution throughout the region (Fenn et al., 2003; Geiser & Neitlich, 2007). Canopy ionic resin lysimeters captured wet inorganic-N across the four studies sites in 2016 and were compared to bulk wet inorganic-N levels measured at the four NADP/NTN monitoring stations across Western WA (Table2). There was a significant increase in NH$_4^+$-N deposition measured in 2016 compared to 2015 at the NADP WA-14 Hoh Rainforest monitoring site (Figure 8). Elevated rates of NH$_4^+$-N deposition were also measured in the canopy lysimeters. The elevated rates of NH$_4^+$-N deposition in 2016 may be attributed to higher rainfall inputs in spring as well as the influence of wildfire ash inputs on the Western side of the Olympic peninsula in the summer of 2015. NH$_4^+$ deposition has been shown to be more impactful than NO$_3^-$ at decreasing biodiversity of sensitive species (Erisman et al., 2007), which is of particular concern for canopy epiphytes. The transition of the dominant N species from NO$_3^-$ to NH$_4^+$ deposition in the PNW (Li et al., 2016) fit current projections for ammonia (NH$_3^+$) emission growth and increasing influences from wildfire in the western U.S. (Ellis et al., 2013). The increase in emissions throughout the region would suggest NH$_4^+$ deposition will continue to rise and have the potential to negatively impact canopy epiphyte ecosystem interactions.

As bryophytes lack roots, they primarily absorb dissolved nutrients and particulate pollutants from the atmosphere (Aboal et al., 2011) although some nutrients are thought to be collected via fungal associates (Carleton & Read, 1991) and capillary movement of nutrients upward from the humus layer (Ayres et al., 2006). They exhibit high cation exchange capacity and can generate acidity through proton release from exchange sites (Spearing, 1972), which may inhibit microbial populations and thus limited N$_2$ fixation capacity (Tyler, 1990; Pearson et
al., 2000; Basiliko & Yavitt, 2001). In this study, *I. stoloniferum* metal tissue concentrations were found to be higher in the Seattle canopies (Table 3) and N$_2$ fixation rates were significantly higher across all seasons in the Hoh Rainforest canopies in spite of slightly higher rates of N deposition in this area. Nitrogen deposition rates are low enough across this region to not directly inhibit moss N$_2$ fixation (Gundale et al., 2011). This would suggest that N$_2$ fixation is not only a function of the seasonal weather influences discussed above, but perhaps strongly influenced by metal concentrations in the moss tissue. The expected lower level of metal deposition in the Hoh Rainforest samples is likely due to lower population density and lower daily traffic counts. As the Puget Sound Region is expected to experience significant population and transportation growth by 2040 (Puget Sound Regional Council, 2014), it is likely urban canopies will continue to experience higher rates of metal deposition and decreasing rates of biological N$_2$ fixation.

**Conclusion**

The forest canopies of the world play an important role in the regulation and cycling of nutrients. Increasing atmospheric pollution associated population increases and transportation growth in the PNW threatens to uncouple the intricate ecosystem services canopy epiphytes provide. Our study is the first to assess the effects of urbanization on seasonal N$_2$ fixation associated with canopy bryophytes. The results suggest that N$_2$ fixation associated with canopy bryophytes are sensitive to seasonal changes in temperature and precipitation, and the bioaccumulation of anthropogenic heavy metals in bryophyte tissue. Nitrogen deposition did not appear to have a noted impact on N$_2$ fixation across location types. Changes in temperature and precipitation patterns associated with climate change will likely affect ecosystem N$_2$ fixation inputs through the bryophyte-cyanobacteria pathway. In order to better understand the
ecological impacts of climate change on canopy bryophytes an increase in the number of sampling sites across Western WA and an increase sampling frequency across seasons would be appropriate.
Tables:

Table 1: Structure correlation coefficients between measured parameters and the first two principal components (PC1 and PC2) for I. stoloniferum for average N\textsubscript{2} fixation rates and metal concentrations across urban and peninsula A. macrophyllum canopies.

<table>
<thead>
<tr>
<th>Principle Component</th>
<th>PC1</th>
<th>PC2</th>
</tr>
</thead>
<tbody>
<tr>
<td>(% Variance explained, significance value)</td>
<td>60.57%, p&lt;0.001</td>
<td>25.07%, p&lt;0.05</td>
</tr>
<tr>
<td>umol m\textsuperscript{-2} d\textsuperscript{-1}</td>
<td>0.899</td>
<td></td>
</tr>
<tr>
<td>Cd</td>
<td>0.978</td>
<td></td>
</tr>
<tr>
<td>Cr</td>
<td>-0.894</td>
<td></td>
</tr>
<tr>
<td>Cu</td>
<td>-0.967</td>
<td></td>
</tr>
<tr>
<td>Fe</td>
<td>-0.918</td>
<td></td>
</tr>
<tr>
<td>K</td>
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<tr>
<td>Mg</td>
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<tr>
<td>Mn</td>
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<td>Ni</td>
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<td>Pb</td>
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<td>Sr</td>
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<tr>
<td>Ti</td>
<td>-0.839</td>
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<tr>
<td>Zn</td>
<td>-0.983</td>
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</table>
Table 2: Comparison of wet inorganic-N deposition across four sampling sites instrumented with canopy resin lysimeters and four National Atmospheric Deposition Program National Trend Network sites in Washington State, USA.

<table>
<thead>
<tr>
<th>Location</th>
<th>Type</th>
<th>NO$_3^-$-N</th>
<th>NH$_4^+$-N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hoh Rainforest</td>
<td>Resin Lysimeter</td>
<td>1.92</td>
<td>2.61</td>
</tr>
<tr>
<td>Interlaken Park</td>
<td>Resin Lysimeter</td>
<td>2.28</td>
<td>1.03</td>
</tr>
<tr>
<td>Ravenna Park</td>
<td>Resin Lysimeter</td>
<td>1.87</td>
<td>0.57</td>
</tr>
<tr>
<td>Seward Park</td>
<td>Resin Lysimeter</td>
<td>2.83</td>
<td>1.14</td>
</tr>
<tr>
<td>NADP WA-14 Station (Olympic NP)</td>
<td>Rainfall Collector</td>
<td>2.97</td>
<td>3.24</td>
</tr>
<tr>
<td>NADP WA-19 Station (North Cascades NP)</td>
<td>Rainfall Collector</td>
<td>3.36</td>
<td>1.2</td>
</tr>
<tr>
<td>NADP WA-99 Station (Mt. Rainier NP)</td>
<td>Rainfall Collector</td>
<td>2.81</td>
<td>1.47</td>
</tr>
<tr>
<td>NADP WA-98 Station (Columbia River Gorge)</td>
<td>Rainfall Collector</td>
<td>2.55</td>
<td>1.37</td>
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</table>
Table 3: Element descriptive statistics in mg kg\(^{-1}\) for (a) Hoh Rainforest metal concentrations; and (b) Seattle metal concentrations from canopy I. stoloniferum samples.

(a)

<table>
<thead>
<tr>
<th>Element</th>
<th>Minimum</th>
<th>Maximum</th>
<th>Mean</th>
<th>Median</th>
<th>Standard Deviation</th>
<th>Fisher-Pearson Skewness Coefficient</th>
<th># Samples below detection limit</th>
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</thead>
<tbody>
<tr>
<td>Cd</td>
<td>BD</td>
<td>3.44</td>
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<td>0.49</td>
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<td>3.52</td>
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<td>44.47</td>
<td>38.45</td>
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(b)

<table>
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<tr>
<th>Element</th>
<th>Minimum</th>
<th>Maximum</th>
<th>Mean</th>
<th>Median</th>
<th>Standard Deviation</th>
<th>Fisher-Pearson Skewness Coefficient</th>
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<tr>
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<td>64.06</td>
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<tr>
<td>Zn</td>
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<td>191.82</td>
<td>79.46</td>
<td>76.12</td>
<td>30.58</td>
<td>0.33</td>
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Figures:

Figure 1: Nitrogen fixation rates as measured by acetylene reduction for *I. stoloniferum* samples collected across Olympic peninsula and Seattle urban *A. macropylllum* canopies in western WA.
Figure 2: Seasonal $N_2$ fixation rates across one A. macrophyllum stand on the Olympic Peninsula for (a) A. curtipendula, (b) H. splendens, and (c) I. stoloniferum.
Figure 3: Principle component analysis (PCA) ordination of seasonal *I. stoloniferum* samples collected from the Olympic Peninsula and Seattle urban and rural forests in western WA.
Figure 4: Average NO$_3^-$-N and NH$_4^+$-N wet deposition rates collected using resin lysimeters instrumented for 12-months in A. macrophyllum canopies in the Hoh Rainforest, Olympic Peninsula and three city parks in Seattle, WA.

Figure 5: Average daily temperature data gathered using HOBO monitors installed in A. macrophyllum canopies in the Hoh Rainforest and Seattle, WA for the 2016-2017 sampling period.
Figure 6: Relationship between the seasonal mean (+/- SE) acetylene reduction rates across Hoh Rainforest and Seattle *I. stoloniferum* samples and temperature.
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Chapter 3:
Bioaccumulation of trace metal deposition in mosses in Western Washington, USA

Abstract

Heavy metal pollution across Western Washington, USA was assessed in 2016 and 2017 using Isothecium stoloniferum (Brid.) and Kindberga praelonga (Hedw.) Ochyra. We used an intensive, vertically stratified sampling approach in Acer macrophyllum canopies in the Hoh Rainforest on the Olympic Peninsula, WA and in Seattle, WA to collect 214 samples of I. stoloniferum. An extensive, ground-based sampling approach was used across an urban-to-wildland gradient to collect 59 K. praelonga samples. Intensive samples were collected four times (April, July, and October of 2016 and in January 2017) and extensive samples three times (April, July, and October 2016) to assess seasonal differences in metal concentrations across sampling locations. A total of 273 moss samples were analyzed for Cd, Cr, Cu, Fe, K, Mg, Mn, Ni, Pb, Sr, Ti, and Zn. Elevated concentrations of all of these elements were found in both intensive and extensive samples collected in Seattle across all collection seasons. Permutational multivariate analysis of variance (perMANOVA) revealed a significant effect of sampling location and seasonal for both intensive and extensive samples. Metal deposition in and around Seattle appears to be primarily derived from the transportation sector.

Keywords: air quality; biomonitoring; bryophytes; heavy metals; traffic pollution
Introduction

Elevated metal deposition within urban areas of the Pacific Northwest have been recorded in Portland and Seattle; however, until recently, few studies have used mosses in this region as indicators of metal pollution. There is a long history of using moss (Ruhling & Tyler, 1968; Pott & Turpin, 1996; Reimann et al., 2001; Aboal et al., 2010; Ćujić et al, 2014; Donovan et al., 2016) and lichen (McCune, 2000; Geiser & Neitlich, 2007) as bioindicators of air quality and atmospheric pollution. Unlike vascular plants, moss lack roots and absorb most nutrients from the atmospheric (Bates, 1992). Moss leaves are only one cell-layer thick, lack a protective epidermis and demonstrate ion exchange properties, all of which allows for the absorption of water, organic compounds and inorganic ions that are deposited on their surfaces (Gjengedal & Steinnes, 1990; Tyler et al., 1990; Aboal et al., 2011; Gonzalez & Pokrovsky, 2013).

*Hylocomium splendens* and *Pleurozium schrbeberi* are two moss species that have been successfully used as bioindicators of pollution deposition in northern Europe (Berg et al, 1995; Berg & Steinnes, 1997; Reimann et al., 2000). These studies show a highly significant positive correlation between moss tissue metal concentrations and wet deposition values. The growth rate for *H. splendens* is highly constant allowing the moss to be used to assess changes in metal concentrations over time (Berg et al., 1995). In most other bioindicator species, the time period represented for pollutant accumulation is unknown, whereas for mosses it consistently ranges from several months to a maximum of three years. Generally, the top two-thirds of the shoot are used in analyses of bioaccumulation, which approximately represents up to three years of growth (Bargagli, et al., 2002; Schintu et al., 2005; Gatziolis et al., 2016). Because there is little recycling of metals from senescent tissue (Brown & Bates, 1990), the concentration of metals in
the upper two-thirds of the moss shoots can be used to infer metal deposition over a three year or less time period.

The Pacific Northwest (PNW) is considered to have high air quality among the United States (NADP/NTN, 2015), in part due to a lack of coal-fired power plants and a small industrial base. However, rapid increases in total population and increased transportation growth threatens to decrease air quality within the region (Geiser & Neitlich, 2007). In the Puget Sound Region (PSR) alone, the population is expected to grow by roughly one million people from 2010 to 2040, which will in turn increase demand for travel throughout the region by 25% (Puget Sound Regional Council, 2014). As evidence of this increase, Seattle highways and roads were recently ranked the 10th most congested urban areas in the United States (INRIX, 2016).

This region-wide expansion in the transportation sector has introduced a new set of atmospheric pollutants to western Washington. Brake and tire attrition, as well as lubricant degradation produce high rates of metal deposition in highly congested areas (Garg et al., 2000; Apeagyei et al., 2011; Hulskotte et al., 2014). Brake bands and tires contain heavy metals, including chromium (Cr), copper (Cu), iron (Fe), nickel (Ni), lead (Pb), strontium (Sr), titanium (Ti), and zinc (Zn), that are released with disintegration and can lead to adverse human health and wildlife effects (U.S. Government, 2001; Perrenoud et al., 2010; Denier van der Gon et al., 2013). These transportation pollutants as well as long-range atmospheric transports originating from Asia (Jaffe et al., 1999) have the potential to negatively impact ecosystem health by altering bryophyte communities (Davies et al., 2007; Aničić et al., 2009; Zvereva and Kozlov, 2011).
To date there have been no extensive or intensive assessment of metal deposition using moss as a bioaccumulator of metals in Washington State. We sought to quantify the extent and intensity of metal accumulation in urban and rural areas in western Washington using mosses as bioaccumulator of atmospheric heavy metal deposition. We hypothesized that: 1) moss tissue metal concentrations will be higher in samples collected from urban sites compared to suburban and rural peninsula sites; and 2) urban sites will experience higher annual average wet deposition rates of heavy metals.

Materials & Methods

2.1 Study Area & Design

We collected moss samples for metal analyses by using two approaches. One involved the use of an intensive, quantitative, vertically stratified sampling in A. macrophyllum canopies in the Hoh Rainforest on the Olympic Peninsula, WA, USA and in the urban center of Seattle, WA. The second involved an extensive, broad ranging sampling across an urban-to-wildland gradient in Western WA. We chose A. macrophyllum because it is widely distributed across the urban-to-wildland gradient and hosts abundant growth of a variety of moss species on its branches and trunk. The two sampling approaches are detailed below:

i. Intensive Approach:

We collected samples from one stand located on Hoh River Trust land in the Hoh Rainforest (47.82°N, 124.20°W) and from three stands located in Seattle City Parks: Seward Park (47.55°N, 122.25°W), Interlaken Park (47.63°N, 122.31°W), and Ravenna Park (47.67°N, 122.30°W). Replicate moss samples were collected from a
total of 18 *A. macrophyllum* trees: nine from the Hoh Rainforest, Olympic Peninsula, WA, USA, and nine from three city parks in Seattle, WA, USA over the course of four seasons (April, July and October 2016, and January 2017). Trees were selected based on several safety criteria and sampling needs. The samples trees had to have at least three accessible branches below a solid anchor point in order that researchers could obtain samples from three different heights throughout the crown. Trees were ascended using the Single Rope Technique (SRT) (Coffey & Anderson, 2012) employing the Singing Tree Rope Wrench and a friction hitch to gain access to the canopy. The 18 trees were instrumented with ionic resin lysimeters (UNIBEST Ag Manager, Walla Walla, WA) and installed over the 12-month sampling period (January 2016 – January 2017) at the three sampling heights within the canopy to evaluate wet deposition throughfall rates (Susfalk and Johnson 2002; Kopatek et al. 2006; DeLuca et al. 2008). As moss metal concentrations are not reliable integrators of metal deposition rates (Aboal et al. 2010), the lysimeters provide an estimate of annual wet deposition levels.

The dominant epiphytic moss species in *A. macrophyllum* canopies in the Hoh Rainforest includes: *Isothecium stoloniferum* (Brid.), *Antitrichia curtipendula* (Hedw.), and *Hylocomium splendens* (Hedw.). The dominant epiphytic moss species in *A. macrophyllum* canopies in Seattle includes: *I. stoloniferum* (Brid.), *Homalotheicum lutescens* (Hedw.), and *Orthotrichum lycelli* Hook & Taylor. *Isothecium stoloniferum* was chosen as the target species for the intensive design as it was the only species we found available to sample across both locations and has been used previously for biomonitoring studies conducted in the Georgia Basin,
British Columbia, Canada (Pott & Turpin, 1998; Raymond & Pott, 2003; Raymond et al., 2010).

As samples were collected once each season (April, July, and October 2016; and January 2017), a black parachute cord was tied to the climbing line to replace it when the line was pulled out of the tree. Installing the parachute cord saved time and prevented any potential damage to the tree that might occur through repeating the rigging process.

ii. Extensive Approach:

To extend the spatial coverage of the intensive sampling approach, we also sampled epiphytes using ground-based sampling techniques across an urban-to-wildland gradient in western Washington. Replicate field samples were collected from a total of twenty primarily *A. macrophyllum* stands across western Washington: five sites from the western side of the Olympic Peninsula, five suburban to rural sites in the Cascades along Interstate 90 heading towards Snoqualmie Pass, and 10 urban sites in Seattle, WA (Table 1). Samples of *Kindbergia praelonga* (Hedw.) Ochyra, a common feather moss found throughout western Washington, were collected from the trunk of *A. macrophyllum* approximately 1m above the ground. Extensive samples were collected within five days of the intensive sample collections for April, July and October 2016.

2.2 Laboratory Analysis:

iv. Trace Metal Concentrations:
Prior to analysis, all debris and necrotic tissue were removed with sterilized plastic forceps from the base of moss samples keeping only the upper two-thirds of the shoots (Gatziolis et al., 2016). We did not wash the moss samples prior to drying because previous studies have shown that is ineffective in removing particulates (Aboal et al., 2011) and that some elements such as Cd and Zn may increase in concentration after washing (Fernández et al., 2010).

Moss samples were dried for 24 hours at 40 °C and ground to a fine powder while immersed in liquid nitrogen. The HNO$_3$ + H$_2$O$_2$ digestion method was used to prepare the dried and ground samples for analysis. Four mL of concentrated reagent-grade HNO$_3$ were added to 0.2-0.3 g subsamples in 50 mL graduated glass digestion tubes. The tubes were covered with plastic watch glasses and left overnight at ambient temperature in a fume hood (approximately 18°C) to allow some initial oxidation of the samples by the HNO$_3$. Samples were digested at 95°C for 90-minute in borosilicate tubes heated evenly in a 36-tube graphite block digester. Samples were allowed to cool, following which 4 mL of reagent-grade 30% H$_2$O$_2$ were added to each tube followed by a 30-minute digestion at 95 °C and allowed to cool. Next, an additional 4 mL of H$_2$O$_2$ were added to each sample and heated again at 95°C. After cooling, deionized water was added to each tube to the 20-mL mark. To remove any undigested particulates not dissolved in the HNO$_3$ and H$_2$O$_2$, the samples were filtered through 0.45-µm membrane syringe filters. Digests were analyzed for a suite of metals (Cd, Cr, Cu, Fe, K, Mg, Mn, Ni, Pb, Sr, Ti, and Zn) using inductively coupled plasma optical emission spectrometry (ICP-OES). Instrument performance tests were carried out daily to ensure proper calibration.
After calibration, quality control samples consisting of blanks, low and high concentration check standards were analyzed.

v. Trace Metal Wet Deposition:

The ionic resin from the canopy lysimeters was collected in January 2017. Elements adsorbed in the resin capsules were extracted with 30 ml of 0.5M HCl and analyzed for Cd, Cr, Cu, Fe, Ni, Pb, Sr, Ti, and Zn by ICP-OES.

vi. Lead and Strontium Isotopic Analysis:

Utilizing the HNO₃ + H₂O₂ digestion method described above, we analyzed 10 moss samples collected in January 2017 from the intensive sites for Pb and Sr isotopes. Five samples were collected from the Hoh Rainforest site on the Olympic Peninsula, WA, five samples from Seattle, WA (Ravenna, Interlaken, and Seward Parks) and run alongside aliquots of an internal laboratory standard (UW BCR-1). All reagents used for isotopic analysis were purified by a double sub-boiling distillation in Teflon.

a. Lead separation and analysis:

Immediately following the HNO₃ + H₂O₂ digestion method, samples were dried down at 93-107 C on a hot plate overnight and redissolved in 2.5mL of 6N HCl. Samples were dried down once again and dissolved in 1N HBr. Pb separation was achieved using 0.3mL anion-exchange resin (BioRad AG 1-X8, 100-200 mesh) that were rinsed with 6N HCl, DI water, and 1N HBr. Next, the 1N HBr samples were loaded onto the column and the liquid sample collected in a 30mL Teflon beaker was saved for Sr isotopic analyses. To elute Pb off the resin, 6N HCl was added to each column. Immediately following this step, the samples were placed on a hotplate to dry down
completely for 2-3 hours at 190°C. The columns were cleaned a second time and the cooled samples, dissolved in 1N HBr, were run through the resin columns to achieve complete Pb purification.

The samples were dried down once more, fluxed in 2mL 2% HNO₃ and spiked with thallium with a known $^{203}\text{Tl}/^{205}\text{Tl}$ ratio prior to being analyzed for Pb isotopes ($^{208}\text{Pb}/^{204}\text{Pb}, ^{207}\text{Pb}/^{204}\text{Pb}, ^{206}\text{Pb}/^{204}\text{Pb}$) on the Multicollector Inductively Coupled Plasma Mass Spectrometer (MC-ICP-MS). Prior to isotopic analyses, a small aliquot of the sample solution was measured for concentration in order to adjust sample intensity to within 10% of the intensity of the Pb isotope standard (SRM 981) (Kayzar et al., 2014). Tl spiked aliquots of SRM 981 provided the mass fractionation relationship between Tl and Pb isotopes used for correction of the sample unknowns. The Pb isotope standard was run after every three samples to ensure proper calibration.

b. Strontium separation and analysis:

Strontium separation was achieved using 0.3 mL of a Sr-specific crown ether resin (Eichrom Sr-SPEC resin). The Sr-bearing elutions from the previous Pb column procedure were collected and dried down at 107°C and then redissolved in 0.4 mL of 4N HNO₃. This solution was loaded onto the Eichrom resin column and rinsed with 3.2 mL of 4N HNO₃ to elute Ca, Na, Rb, and Ba. Strontium was eluted with 3 mL of 0.05N HNO₃. Samples were dried and redissolved in 3 mL of 2% HNO₃. Concentrations were adjusted to
within 10% of the bracketing NIST-987 Sr standard. The procedure for MC-ICP-MS Sr isotope analysis follows that of Brach-Papa et al. (2009). The IAPSO seawater standard was run after every three samples to verify analytical accuracy.

2.3 Statistical Analysis:

Descriptive statistics (minimum, maximum, mean, median, standard deviation, and Fisher-Pearson Skewness coefficient) were calculated for each element across intensive and extensive moss samples. A Pearson product-moment correlation test was used to determine the relationship between moss metal concentrations and average daily traffic counts across the intensive sampling locations. Daily traffic counts were gathered from WSDOT traffic counters near the four sampling locations.

Data on metal concentrations, represented as mg kg$^{-1}$, were transformed using a $\log_{10}(y + 1)$ to normalize the distribution. We used a two-way permutational multivariate analysis of variance (perMANOVA) to test if moss metal concentrations were significantly different across location types and sampling seasons for both intensive and extensive sites. The subsequent test of multivariate homogeneity of groups (DISPER) was used to calculate significant multivariate dispersions in variables among the location types for intensive and extensive moss samples. Euclidean distance was chosen for the two-way perMANOVA, and significance of the two-way perMANOVA R-value was determined via 1000 random permutations. For this study, effects with probabilities of $p < 0.05$ were assumed to be significant.
Principle component analysis (PCA) was selected as the appropriate ordination method where dissimilarity was calculated as Euclidean distance (Legendre & Legendre 1998). PCA was performed on the data matrix ‘location type x metal concentration parameters’ for both intensive (*I. stoloniferum* for urban and peninsula) and extensive (*K. praelonga* for urban, suburban, and peninsula) sampling sites. Twelve metal concentration parameters (Cd, Cr, Cu, Fe, K, Mg, Mn, Ni, Pb, Sr, Ti, and Zn) were introduced as the analysis variables. PCA was performed to assess the dominant patterns in moss metal concentrations across locations. The loadings of variables to each PC was examined by converting eigenvector coefficients to structure correlations.

Linear regression models were used to determine the relationship between metal concentrations in *I. stoloniferum* tissue with average annual wet deposition measured using resin lysimeters across intensive sampling sites. All statistical analyses were conducted in the R Statistical Environment (R Core Team, 2013).

**Results**

For intensive (*I. stoloniferum* at peninsula and urban sites) and extensive (*K. praelonga* at peninsula, suburban, and urban sites) moss samples, descriptive statistics are shown in table 2. Fisher-Pearson skewness coefficient was calculated to identify elements with extremely high concentrations (Shepard, 1968). Within the intensive sites, urban *I. stoloniferum* samples exhibited higher metal concentrations compared to peninsula *I. stoloniferum* samples. For the extensive sites, *K. praelonga* metal concentrations were generally highest in the urban sites and lowest in the peninsula sites with suburban metal concentrations falling in the middle range as hypothesized. Both intensive and extensive samples were relatively free of exceptionally high
concentrations, resulting in smaller skewness coefficients. The Pearson correlation was used to reveal any potential correlations between elemental concentrations and daily traffic counts across the intensive sites. Correlation coefficient values greater than 0.22 indicate significant correlation at $P \leq 0.001$ (Table 3). Cadmium concentrations showed no correlation either with other elements or with daily traffic rates.

For the intensive canopy samples, metal concentrations were significantly different in comparing between peninsula and urban *I. stoloniferum* samples ($F_{1,206} = 326.41$, $p<0.001$) and across sampling seasons ($F_{3,206} = 5.25$, $p<0.01$). Canopy height is not correlated with metal concentrations ($p = 0.67$) and therefore was not included in the perMANOVA. There was a significant interaction between location type and sampling season ($F_{3,206} = 2.67$, $p \leq 0.05$). This would suggest that metal concentration in the moss tissue is dependent on the season it was collected. Considering the location type term, the DISPER results show no significant differences in between-site variation ($F_{1,212}=1.35$, $p=0.245$) and pairwise comparison also showed no significant differences among location types.

For the extensive moss samples, metal concentrations were significantly different across the three location types ($F_{2,50}= 33.33$, $p<0.001$) and across sampling seasons ($F_{2,50} = 12.5$, $p<0.001$). There was a significant interaction between location type and season ($F_{4,50} = 2.7$, $p<0.01$), which suggests a seasonal effect on metal concentrations across the sampling locations. Considering the location type term, the DISPER results showed no significant differences in between-site variation ($F_{2,56} =1.14$, $p=0.326$) and pairwise comparison also showed no significant differences among location types.
Significant proportions of the variability in the ‘location type x metal concentration parameters’ for both intensive and extensive samples were explained by their first two PCs. Structure correlation coefficients between each variable and the first two PCs for intensive and extensive samples are presented in Table 4. The total amount of variance explained by the first two PCs in PCA 1 were (85.26%): PC1 (78.2%) and PC2 (7.06%) (Figure 1). In PCA 1, representing the intensive sampling data, Cr, Cu, Fe, K, Mg, Ni, Pb, and Ti moss tissue concentrations are highly correlated with PC1; Cd, Mn, Sr, and Zn concentrations are highly correlated with PC2. Vector arrows point towards the urban sampling points, suggesting that the metal concentration tends to be higher in urban *I. stoloniferum* canopy samples compared to peninsula *I. stoloniferum* canopy samples (Figure 1).

The total amount of variance explained by the first two PCs in PCA 2 were (80.57%): PC1 (70.29%) and PC2 (10.26%) (Figure 2). In PCA 2, representing the extensive sampling data, Cr, Cu, Fe, Pb, Ti, and Zn were negatively and heavily loaded to PC1; Cd, K, Mg, Mn, Ni, and Sr were positively and heavily loaded to PC2. As hypothesized, PCA 2 revealed that the three location types (peninsula, suburban, and urban) are environmentally distinct from each other. Vector arrows point towards the urban sampling points, suggesting metal concentration tends to be greater in urban *K. praelonga* samples than in suburban or peninsula *K. praelonga* samples (Figure 2).

No significant differences in wet deposition values collected in the resin lysimeters were observed across the canopy heights, resulting in the use of an average annual precipitation-weighted wet deposition value per tree (Chance et al., 2015; Wetherbee et al., 2010). The moss values used are the arithmetic mean of metal concentrations per tree from the January 2017
collection. Linear regression analyses were only carried out on moss samples collected from the January sampling round as they were exposed to the full 12-months of wet deposition captured by the canopy lysimeters. Plots of metal concentrations in the intensive *I. stoloniferum* moss vs. wet atmospheric deposition of the same metal are shown in Figure 3. Of the nine metals, Cr, Cu, Fe, Ni, Pb, Ti, and Zn showed significant correlation (p<0.05) between moss tissue concentrations and annual average wet deposition rates. No correlation was observed for Cd and Sr between annual average wet deposition rates and moss tissue concentrations (\(r^2 = 3.8 \times 10^{-5}\), \(p=0.983\); \(r^2 = 0.085\), \(p=0.238\) respectively).

Table 5 lists the trace element concentrations and isotopic composition for the ten *I. stoloniferum* samples analyzed in this study. In general, elemental concentrations for Cu, Pb, Sr, and Zn were elevated in the Seattle samples compared to those from the Hoh Rainforest. Trace metal concentrations are also expressed in term of metal/metal ratios for Cu/Pb and Zn/Pb (Figure 4). Samples from Seattle contain lower ratios of Cu/Pb and Zn/Pb ratios and less radiogenic Pb isotope values (\(^{206}\text{Pb}/^{207}\text{Pb} = 1.1751-1.1859\)). Samples from the Hoh Rainforest are characterized by higher ratios of Cu/Pb and Zn/Pb ratios and more radiogenic Pb isotope values (\(^{206}\text{Pb}/^{207}\text{Pb}\) generally >1.1849). Measured Pb isotope compositions (Figure 5) show that natural geologic samples (Chan et al., 2012) are more radiogenic than Seattle or West Coast industrial sources (Jones et al., 2000; Bollhöfer & Rosman, 2001). Samples from the Hoh Rainforest contain higher \(^{86}\text{Sr}/^{87}\text{Sr} (0.7051 – 0.7067)\) and lower Sr concentrations compared to Seattle samples (0.7047- 0.7052) (Figure 6).
Discussion

Metal deposition and accumulation in *I. stoloniferum* and *K. praelonga* were higher in urban sites in Seattle than in suburban and remote sites. For the intensive sites, the metal concentrations are significantly correlated with heavier daily traffic rates, with the exception of Cd (Table 3). The traffic density is highest in Seattle with a citywide average daily traffic volume of 1,010,000 vehicles (WSDOT, 2015) in areas where metal concentrations are among the highest recorded. The expected lower metal concentrations on the western side of the Olympic Peninsula are correlated with lower daily traffic counts of 1,300 (WSDOT, 2015) on few nearby highways. As the PSR is expected to experience significant population growth by 2040, increasing pressure from construction and the transportation sector is likely lead to higher levels of heavy metal pollutants in the region.

Apeagyei et al. (2011) sampled 115 different tires and found that the most abundant element is Zn (17,720 ppm), followed Ca>W>K>Fe>Ti>Cr>Mo>Cu>V>Sr>Zr>Pb. In our study we observed a significant correlation (p<0.001) between higher daily traffic rates with Cr, Cu, Fe, K, Pb, Sr, Ti, and Zn suggesting a common pollution source (Table 2). Fugitive dust from tire attrition is a potentially substantial source of Zn entering the environment (Councell et al., 2004). The higher levels of Zn found in the intensive and extensive urban sites may be attributed to tire attrition from major roadways such as Interstate 5 and WA State Route 99.

Although Cu is a plant-essential micronutrient, it is of particular concern in the PNW as it can be hazardous to wildlife in high concentrations in the environment. This is a particular problem in road runoff where Cu concentrations ranging from 1-30 µg/L have been shown to produce sublethal neurotoxicity in salmon (Linbo et al., 2006; Sandahl et al., 2004; Sandahl et al.
In an attempt to regulate sources of Cu entering waterways by surface runoff and dust deposition, Washington State passed the Better Brakes Law (Chapter 173-901 WAC) in 2010. The law will be fully implemented in 2025, but until then brake pads will still be a source of Cu pollution in Washington State. We observe a significant correlation between Cu concentrations in intensive *I. stoloniferum* samples with daily traffic rates (*p*<0.001), as well as a significant (*p*<0.01) relationship between annual average Cu deposition rates and moss metal concentrations. These correlations suggest that Cu released as fugitive dust from brake pads along major roadways, such as Interstate 5 and Washington State Route 99, may account for the higher levels of Cu we found in the intensive and extensive urban moss samples.

Lead is a more obvious trace metal of environmental concern given that it is not an essential nutrient and it is toxic to mammals in relatively low concentrations. A multitude of studies have examined the impact of Pb pollution on human and wildlife health (Lanphear et al. 2005; Nevin, 2000; Needleman & Gatsonis, 1990; Holcombe et al., 1976; Spehar et al., 1978). It is clear that *I. stoloniferum* collected from Seattle have slightly less radiogenic isotopic composition, which is consistent with the anthropogenic Pb composition of other West Coast cities (Figure 5). In this study, Pb was strongly correlated with average daily traffic counts (*p*<0.001) for the intensive moss samples (Table 3). This would suggest that the source of Pb most likely originates from brake pad and tire abrasion (Apeagyei et al., 2010). Hallock (2010) found that Pb is likely to be transported as particle-bound fugitive dust rather than in the dissolved phase as Pb has a high affinity for particulate matter. Existing studies suggest a significant relationship between bulk deposition levels and the concentrations of Pb in moss (Ross, 1990; Berg et al., 1995; Thoni et al., 1996; Berg & Steinnes, 1997; Schintu et al., 2005; Fowler et al., 2006), which is supported by the findings in our study (*p*<0.05). Emissions from Asia reach the western U.S. within 10 days (Jaffe et al., 1999) and Chinese aerosol is enriched in
\(^{208}\)Pb (Bollhöfer & Rosman, 2001; Ewing et al., 2010). The Pb isotopic compositions for Hoh Rainforest samples indicate potential trans-Pacific contamination by Asian emissions.

Approximately 1.0 Mg yr\(^{-1}\) of Cd is released from anthropogenic sources in Puget Sound, which includes leaching from roofing materials, release from agricultural fertilizer, air emissions for industrial facilities, and road-related sources such as tire and brake pad wear (Ecology & King County, 2011). As all of the sampling sites in this study are located in either in the Hoh Rainforest or in city parks, they are unlikely to experience Cd leachate from roofing materials. Spectrum Glass was the only industrial facility in the Puget Sound region reporting fugitive air releases of cadmium in the last 10 years, but they ceased production in July 2016. We did not observe significantly high levels of Cd in either intensive or extensive samples; therefore we do not believe emissions from Spectrum Glass were a source of Cd in the urban moss samples. As Cd did not show significant correlation with daily traffic rates (Table 3), it is unlikely that tire and brake wear are the source of cadmium detected in \(I.\ stoloniferum\) and \(K.\ praelonga\) samples across the sampling locations.

Elevated levels of Sr were observed in urban areas in \(I.\ stoloniferum\) and \(K.\ praelonga\) samples (122.89 mg kg\(^{-1}\) and 101.48 mg kg\(^{-1}\), respectively) compared to rural samples (51.19 mg kg\(^{-1}\) and 50.40 mg kg\(^{-1}\), respectively). Strontium is an alkaline earth element with an average concentration in the Earth’s crust of 370ppm and 240ppm in soil minerals (Sposito, 1989). The \(^{87}\)Sr/\(^{86}\)Sr isotope ratio has been used extensively to measure atmospheric inputs of Sr to soils and vegetation (Graustein & Armstrong, 1983; Graustein, 1989; Miller et al., 1993; Capo and Chadwick, 1999). The relationship in Figure 7 between \(^{86}\)Sr/\(^{87}\)Sr and Sr (mg kg\(^{-1}\)) indicates Hoh Rainforest samples may be influenced by seawater input given the proximity to the Pacific.
Ocean. However, it is not clear what is causing the higher levels of Sr associated with urban samples is. One possible source for the elevated Sr, given its high correlation with daily traffic rates (Table 3), may be from diesel soot associated with traffic along major corridors in Seattle.

A comparison of mean element concentrations in mosses obtained from this study with recent moss monitoring studies in the PNW region is presented in Table 6. The Portland study used Orthotrichum lyellii to monitor pollution patterns, whereas this study and that conducted in British Columbia (B.C.) used I. stoloniferum (Gatziolis et al., 2016; Potts & Turpin, 1998). While interspecies variation may affect the comparison of metals levels to some extent, it is unlikely to affect the general trend of higher metal levels in urban vs. rural environments. Mean metal concentrations in the urban I. stoloniferum samples gathered in this study were higher than values previously reported in the PNW, with the exception of Cd in the Portland study, and Cd and Pb in the B.C. study. The higher levels of Cd and Pb found in the Canadian samples collected in 1993 may be attributed to legislative policies that did not remove leaded gasoline from the Canadian market until December 1990. Several studies that have attempted to address the time period represented by metals in moss tissue (Boquete et al., 2013; Fernadez et al., 2013), none of which have used I. stoloniferum, K. praelonga, or O. lyellii. The metal range captured in these three studies likely represents a maximum of a three-year exposure period (Gatziolis et al., 2016) as only the top two-thirds of the moss shoot was analyzed. The sources of the Cd levels reported in the Portland study were identified as industrial atmospheric deposition originating from two stained-glass manufacturers (Donovan et al., 2016). With the exception of Cd, both Ni and Pb levels found in the urban I. stoloniferum from this study were 1.42 and 1.74 higher, respectively, than the O. lyellii levels reported in Portland. It is possible that the combustion of diesel fuels is a main source of Ni pollution in the canopy environment (National Research
Council, 1975). This study would support that suggestion due to the significant correlation between daily traffic rates and Ni concentrations from the intensive sampling (Table 3).

**Conclusion**

In the PNW, lichen has been widely used as biomonitors to assess the impacts of air quality and regional atmospheric deposition (Berg et al., 1995; McCune, 2000; Geiser & Neitlich, 2007). Results from this study suggest moss has the potential to be used as a low-cost biomonitor to evaluate heavy metal pollution in urban and rural areas. The values found in this study cannot be treated as an absolute measure of metal deposition, but rather used as a comparative index of metal deposition and a screening tool to assess areas of metal pollution.

In the PSR, the public is increasingly concerned with the effect of the growing rate of urbanization on regional air quality. The moss metal concentration data presented in this paper helps elucidate the impacts of the transportation sector on ecosystem health throughout the PSR. It seems appropriate to expand the existing lichen monitoring network to include moss sampling and better establish regional distribution patterns of transportation-based pollutants. As the transport and fate of heavy metals in the environment is complex, particularly in urban environments where natural biogeochemical pathways have been highly altered, calibration of moss values with instrument measurements for both wet and dry deposition is needed.
Tables:

Table 1: GPS coordinate locations for Extensive sampling sites.

<table>
<thead>
<tr>
<th>Site Name</th>
<th>Coordinates</th>
<th>Location Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coppermine Bottom</td>
<td>47.65544, -124.19863</td>
<td>Peninsula</td>
</tr>
<tr>
<td>Upper Clearwater</td>
<td>47.67858, -124.11877</td>
<td>Peninsula</td>
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<td>Peninsula</td>
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<tr>
<td>Hard Rain</td>
<td>47.81609, -124.13964</td>
<td>Peninsula</td>
</tr>
<tr>
<td>South Fork Snoqualmie River</td>
<td>47.43216, -121.63053</td>
<td>Suburban</td>
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Table 2: Element descriptive statistics (a) Peninsula intensive metal concentrations I. stoloniferum; (b) Urban intensive metal concentrations I. stoloniferum; (c) Peninsula extensive metal concentrations in K. praelonga; (d) Suburban extensive metal concentrations in K. praelonga; and (e) Urban extensive metal concentrations in K. praelonga.

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Table 3: Pearson’s correlation coefficients between annual average daily traffic counts and metal concentrations in I. stoloniferum samples collected the intensive sampling sites. * = p<0.05, ** = p<0.01, *** = p<0.001.
Table 4: Structure correlation coefficients between measured parameters and the first two principal components (PC1 and PC2) for (a) intensive metal concentrations I. stoloniferum and (b) extensive metal concentrations in K. praelonga.

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(b)

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<td>Sample</td>
<td>Elemental Concentration (mg kg⁻¹)</td>
<td>Isotopic Composition</td>
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<tr>
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<tr>
<td></td>
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<td>Pb</td>
</tr>
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Table 6: Comparison of mean element concentrations (mg/kg) in mosses obtained in this study with those collected in British Columbia, Canada and Portland, OR, USA. The Hoh Rainforest and Bridal Veil Falls represent rural sites, whereas Seattle, Vancouver, and Portland represent urban sites. a= I. stoloniferum, b= O. lyelli

<table>
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<tr>
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<th>Washington, USA&lt;sup&gt;a&lt;/sup&gt;</th>
<th>British Columbia, Canada&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Oregon, USA&lt;sup&gt;b&lt;/sup&gt;</th>
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<td></td>
<td>Cd  0.17</td>
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<td>Zn  44.47</td>
<td>Zn  79.46</td>
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Figures:

Figure 1: Principle component analysis (PCA) ordination of Intensive I. stoloniferum samples
Figure 2: Principle component analysis (PCA) ordination of Intensive *K. praelonga* samples.
Figure 3: Relationship between trace element concentrations in *I. stoloniferum* tissue (mg kg\(^{-1}\)) and annual atmospheric wet deposition (mg kg\(^{-1}\)).

**Cr**

\[ y = 1.345x - 0.3161 \]
\[ r^2 = 0.847 \]
\[ p < 0.001 \]

**Cu**

\[ y = 1.549x + 4.264 \]
\[ r^2 = 0.359 \]
\[ p = 0.01 \]

**Fe**

\[ y = 1.2314x - 142.42 \]
\[ r^2 = 0.806 \]
\[ p < 0.001 \]
Figure 4: Illustration of the variation between Zn/Pb versus Cu/Pb ratios from samples analyzed for isotopic composition.
Figure 5: Plot of $^{208}\text{Pb}/^{206}\text{Pb}$ vs. $^{206}\text{Pb}/^{207}\text{Pb}$ for samples collected from the Hoh Rainforest and Seattle. Also shown are the compositions for samples representative of cities in Western U.S., Western Canada, and Asia (Bollhöfer & Rosman, 2001), Chinese loess (Jones et al., 2000), Asian industries (Tan et al., 2006), and geologic influences from Washington State, USA (Chan et al., 2012). The red line represents the North American array and the blue line represents the Asian array of isotopic ratios.
Figure 6: Plot of $^{87}\text{Sr}/^{86}\text{Sr}$ vs. Sr concentrations (mg kg$^{-1}$) for samples collected from the Hoh Rainforest and Seattle. Also shown are the compositions for samples representative of saltwater influences (IAPSO certified standard) and geologic influences from Washington State, USA (Chan et al., 2012).
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