NRC Research Press

ARTICLE

Differences in foliar endophyte communities of red alder (*Alnus rubra*) exposed to varying air pollutant levels

Emily R. Wolfe, Stefanie Kautz, Sebastian L. Singleton, and Daniel J. Ballhorn

Botany Downloaded from www.nrcresearchpress.com by 73.11.45.213 on 03/25/19
For personal use only.

Abstract: In the Pacific Northwest, *Alnus rubra* Bong. (red alder) is a common deciduous tree species especially prevalent in riparian corridors and disturbed sites, including metropolitan areas undergoing land use changes and development. Importantly, red alder is also considered a bioindicator for ozone pollution and, like all plants, harbors a diverse endophyte community that may interact with aerial pollutants. In this study, we surveyed foliar fungal endophyte communities (microfungi) in red alder leaves from the metropolitan area of Portland, Oregon, USA, using culture-based techniques, and found that communities differed significantly by site. Our results suggest that the fungal endophyte community composition in red alder leaves may be influenced in part by local air pollution sources, likely in conjunction with other site characteristics. As urban areas expand, more studies should focus on how the urban environment affects plant–microbe community ecology and endophyte–host interactions, as well as on the long-term consequences for other ecosystem processes such as leaf litter decomposition.

Key words: microbial community, diversity, air pollution, microfungi, urban ecosystems.

Résumé : Dans le Nord-Ouest Pacifique, *Alnus rubra* Bong. (aulne rouge) est une espèce fréquente d'arbre décidu, spécialement répandue dans les corridors riverains et les sites perturbés, y compris les agglomérations soumises à des changements ou à des développements en matière d'utilisation des sols. Fait important, l'aulne rouge est aussi considéré comme bio-indicateur de la pollution par l'ozone et, comme tous les végétaux, héberge une communauté d'endophytes divers qui peut interagir avec les polluants de l'air. Dans cette étude, les auteurs ont examiné les communautés d'endophytes fongiques foliaires (microchampignon) d'aulnes rouges de l'agglomération de Portland, Oregon, États-Unis, à l'aide de méthodes basées sur la culture, et trouvé que les communautés différaient significativement d'un site à l'autre. Leurs résultats suggèrent que la composition de la communauté d'endophytes fongiques de l'aulne rouge peut être influencée en partie par les sources de pollution atmosphérique locales, probablement conjuguées à d'autres caractéristiques du site. Au fur et à mesure de l'expansion des zones urbaines, un plus grand nombre d'études devraient se concentrer sur la manière par laquelle l'environnement urbain affecte l'écologie de la communauté plante–microbe et les interactions endophyte–hôte, de même que sur les conséquences à long terme d'autres processus écosystémiques comme la décomposition de la litière de feuille. [Traduit par la Rédaction]

Mots-clés : communauté microbienne, diversité, pollution de l'air, microchampignon, écosystèmes urbains.

Introduction

Plant and microbial communities are sensitive to local environmental conditions, especially those resulting from anthropogenic sources. An increase in the levels of atmospheric CO_2 has been shown to interact with nitrogen availability and plant community species richness to influence community biomass (He et al. 2002), while warming has been shown to negatively affect plant productivity in a grassland community (De Boeck et al. 2007). The microbial community composition in the soil of grassland plots exhibited similar responses to warming (Frey et al. 2008), and was further affected by drought conditions (Sheik et al. 2011). Likewise, soil pollution has been found to negatively affect plant symbionts such as mycorrhizae (Cairney and Meharg 1999). The plant–microbe holobiont, which is the sum of a plant and all of its microbial symbionts, has been shown to critically determine the performance and resistance of plants to a variety of stresses, including environmental pollutants (Li et al. 2012), yet still little information exists on the interactions between pollutants and plant and microbial communities.

Received 24 April 2018. Accepted 10 July 2018.

E.R. Wolfe, S. Kautz, S.L. Singleton, and D.J. Ballhorn. Department of Biology, Portland State University, P.O. Box 751, Portland, OR 97207-0751, USA.

Corresponding author: Emily R. Wolfe (email: emwolfe@pdx.edu).

Copyright remains with the author(s) or their institution(s). Permission for reuse (free in most cases) can be obtained from RightsLink.

Published at www.nrcresearchpress.com/cjb on 22 August 2018.

Botany Downloaded from www.nrcresearchpress.com by 73.11.45.213 on 03/25/19
For personal use only.

Plant responses to air pollution can vary with species, exposure duration, pollutant concentration, or a combination of factors, but may include the production of reactive oxygen species (Schützendübel and Polle 2002; Wohlgemuth et al. 2002) and phytoalexins (Rakwal et al. 2003). Further, beyond affecting the plants themselves, pollutants may have effects on the microbial communities associated with every plant, which can further reduce plant growth and fitness (Porter and Sheridan 1981). Well-studied symbionts determining plant performance include nitrogen-fixing bacteria and mycorrhizal fungi that inhabit plant roots. However, microbial colonization can occur in any part of the plant, and communities may vary temporally and by plant tissue type (Ek-Ramos et al. 2013; Younginger and Ballhorn 2017). A particularly diverse group of plant-associated microbes are foliar endophytic fungi.

Fungal endophytes are ubiquitous, asymptomatic plant symbionts (Stone et al. 2004; Rodriguez et al. 2009) that can affect host-plant chemistry (Arnold 2007; Suryanarayanan 2013), as well as competition and survival (Rudgers and Clay 2007; Aschehoug et al. 2012; McCulley et al. 2014), and as pioneer colonizers directly contribute to leaf litter decomposition (Lemons et al. 2005; LeRoy et al. 2011; Grimmett et al. 2012). Importantly, fungal endophytes have been shown to increase drought tolerance (Hesse et al. 2003; Khan et al. 2016), pathogen resistance (Waller et al. 2005; Lin et al. 2015; Busby et al. 2016), and metal tolerance in a variety of host plants (Ren et al. 2011; Li et al. 2012). Although endophytes may colonize any plant tissue, we focus here on foliar endophytes because of their eventual contribution to ecosystem processes like litter decomposition and nutrient cycling.

In this study we examined the taxonomic diversity and frequency of endophytic fungi in red alder (*Alnus rubra* Bong.) in urban and rural sites that have previously been identified as differentially exposed to a variety of air pollutants, including sulfur, lead, iron, and nickel (Gatziolis et al. 2016). Red alder is a common deciduous tree species occurring throughout the Pacific Northwest in riparian areas and moist forests mostly within 200 km of the Pacific coast. The species is of significant economic importance in the timber industry, exceeding the annual value of the dominant softwood species Douglas-fir (*Pseudotsuga menziesii*) (Warren 2009). Ecologically, red alder is a key species in the Pacific Northwest. As an early pioneer tree, red alder colonizes disturbed sites and stabilizes eroded soils as well as river banks. Like other members of its genus, red alder is especially important in riparian corridors and disturbed sites (Harrington et al. 1994) because of its association with N_2-fixing *Frankia* bacteria. Red alder in particular also serves as a bioindicator of ozone damage (Campbell et al. 2000; Bennett and Tkacz 2008), which may be useful in impacted urban areas, while other members of the genus have been shown to tolerate metal-polluted soils at other sites (Lee et al. 2009; Printz et al. 2013).

Here, we surveyed fungal endophyte communities in red alder leaves from the metropolitan area of Portland, Oregon, USA, using culture-based techniques, and add to the findings of another culture-based study of red alder foliar endophytes by Sieber et al. (1991). The Portland-Metro area has poor air quality that has recently garnered media attention due to a variety of industrial point sources of pollutants (Department of Environmental Quality 2017). The region's air pollution has previously been found to contribute to reduced diversity in lichen communities (Geiser and Neitlich 2007), and a recent study by Gatziolis et al. (2016) revealed possible pollution hotspots in several areas by measuring the concentrations of 22 elements in mosses sampled from across Portland. In addition to our culture-based survey, we used several publicly-available datasets to characterize the environments surrounding our metropolitan sample sites, including data provided by Gatziolis et al. (2016). By sequencing culturable endophytes in red alder exposed to varying influences of air pollutants, we draw connections between the hosts' growing environment and foliar fungal community composition. We hypothesized that the sampling sites closer to these pollution hotspots would have lower biodiversity, and that fungal communities would differ among sites based on the local point sources of pollutants.

Materials and methods

Red alder (*Alnus rubra*) leaves were sampled 9–15 November 2016 from three sites around the metropolitan area of Portland, Oregon (see Fig. 1), and one site located in the Tillamook State Forest, approximately 65 km west of downtown Portland (see Table 1). The metropolitan sites included Errol Heights Park (referred to as "Flavel"), the Delta Park/Vanport Park & Ride (referred to as "Raceway"), and the Overlook neighborhood ("Overlook"), and were chosen according to their relative proximity to possible sources of air pollution suggested by several recent studies (Geiser and Neitlich 2007; United States Environmental Protection Agency 2011; Gatziolis et al. 2016; Donovan et al. 2016). The Tillamook State Forest site ("Tillamook") served as a rural site. For Overlook, we used the Portland Tree Inventory Project (Parks & Recreation 2017) to locate street trees, and then averaged the GPS locations of the trees for the overall site coordinates to maintain the privacy of the property owners.

At each site, five trees were selected and three leaves were collected from each using clean gloves doused with 95% ethanol, and stored in paper envelopes. We randomly selected leaves without visible lesions or other defects when possible on branches between 1.0 and 2.5 m from the ground. Leaves were no more than 15 cm long from apex to petiole and all of the trees had a DBH < 17.5 cm. Samples were returned to the lab and surface-sterilized in a

Fig. 1. Map of the four study sites in the metropolitan area of Portland and part of the Oregon Coast Range; service layer credits: Esri, HERE, Garmin, USGS, Intermap, INCREMENT P, NRCan, Esri Japan, METI, Esri China (Hong Kong), Esri (Thailand), NGCC, OpenStreetMap contributors. [Colour online.]

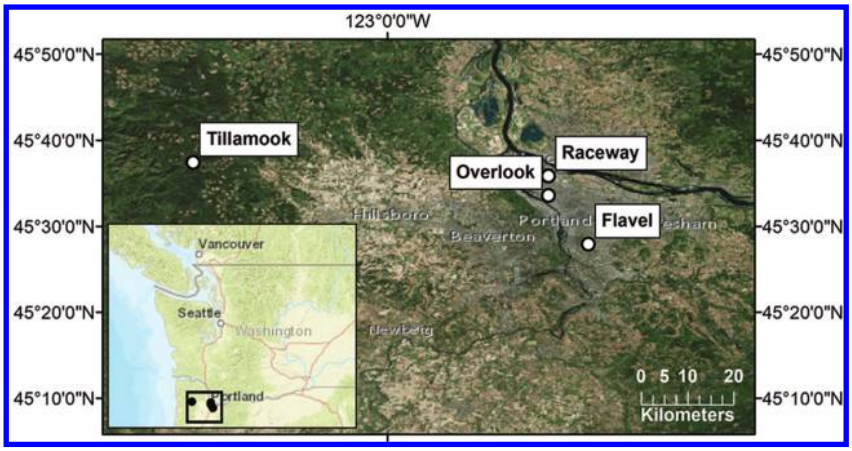


Table 1. GPS coordinates and descriptions of study sites across the metropolitan area of Portland.

Site	Coordinates	Elevation (m a.s.l.)	Description
Flavel	45°27′55.1″N, 122°36′36.0″W	59	Errol Heights Park is a small, wooded preserve between an industrial plant near SE Flavel Street and a busy intersection (near SE Johnson Creek Boulevard)
Raceway	45°35′49.6″N, 122°41′13.6″W	5	The Delta Park/Vanport Park & Ride in North Portland is located at the entrance to the Portland International Raceway, and adjacent to Interstate 5. This site is within a few kilometers of the Columbia River Crossing and sees heavy traffic volumes daily
Overlook	45°33′33.8″N, 122°41′14.2″W	63	Overlook is a North Portland neighborhood bordered by the Willamette River, and busy portions of Interstate 5 and Interstate 405. The neighborhood is adjacent to shipyards and industrial areas along the river
Tillamook	45°37′25.4″N, 123°22′35.2″W	483	The Tillamook State Forest is part of the Coast Range and is surrounded by rural communities

laminar-flow cabinet using the following procedure: dip in 0.1% Tween 20, rinse for 30 s in deionized (DI) water, soak for 10 s in 95% ethanol, soak for 2 min in 0.5% sodium hypochlorite (8.25%), and then soak for 2 min in 70% ethanol, followed by air-drying in the laminar-flow cabinet (Younginger and Ballhorn 2017). Leaves were then sectioned into pieces approximately 5 mm in diameter with flame-sterilized scalpels; four pieces were plated on malt extract agar (MEA) and sealed with Parafilm, and the remaining pieces were placed into Eppendorf tubes and stored at –80 °C until further analysis by direct sequencing of the ITS region. All of the samples were plated or processed for storage less than 24 h after collection.

Once leaves were plated, they were checked every few days for new fungal growth. These isolates were transferred to new MEA plates until they were axenic (i.e., no new morphotypes appeared), at which point DNA was extracted using the Sigma REDExtract-N-Amp Tissue Kit (St. Louis, Missouri, USA). DNA was amplified using the primer pair ITS1F and ITS4, and 34 cycles of 94, 50, and 72 °C for 1 min each, followed by a final extension at 72 °C for 10 min. PCR products were sent to Functional Biosciences (Milwaukee, Wisconsin, USA) for Sanger sequencing, after which the sequencing data were cleaned and analyzed using Geneious 10.2.3. Cleaned sequences were checked against both NCBI GenBank and UNITE to determine identity at the lowest possible taxon, using 97% similarity as the assignment threshold. If GenBank accession numbers differed between NCBI and UNITE, the UNITE accession and taxon were used.

Gatziolis et al. (2016) overlaid a grid system on the metropolitan area of Portland, sampled mosses from within these sites, and measured the concentrations of 22 elements that had accumulated in the moss biomass. We used coordinates from those sampling locations to first determine the 10 closest locations to each of our metropolitan alder-sampling sites, averaged the concentrations for each of the 22 elements measured in moss biomass collected from those locations, and then examined between-site differences in elemental concentra-

Botany Downloaded from www.nrcresearchpress.com by 73.11.45.213 on 03/25/19
For personal use only.

Botany Downloaded from www.nrcresearchpress.com by 73.11.45.213 on 03/25/19
For personal use only.

tions previously measured in moss in the context of our alder sites. Pollution point-sources identified by Donovan et al. (2016) in a similar study of epiphytic mosses were also considered during site selection. We attempted to use air quality data provided by the Environmental Protection Agency (EPA) to find monitoring stations adjacent to our sampling sites, although comparable data between those three stations (EPA sites 0080, 2001, and 0246 in Multnomah County, OR) were limited (United States Environmental Protection Agency 2017). Traffic volume tables produced by the Oregon Department of Transportation provided average daily traffic on each of the major roadways neighboring our sites (Transportation Data Section 2016). Comparisons of elemental concentrations previously measured in moss and traffic volume were made to further interpret fungal community data against the background of pollution and to identify possible trends for future studies.

Statistics

All statistical analyses were performed using R version 3.4.1. Community data were analyzed with the "vegan" and "indicspecies" packages (Cáceres and Legendre 2009; Oksanen et al. 2017). All analyses were performed after operational taxonomic units (OTUs) were combined by common species assignments (e.g., all *Alternaria alternata* OTUs with similarity ≥97% were combined into a single *Alternaria alternata* column within the community matrix, while OTUs with similarity <97% were combined as *Alternaria* sp.). Two extreme outliers (i.e., two leaf samples), both from the Overlook site, were removed because they were singletons that prevented examination of beta diversity. Non-metric multidimensional scaling (NMDS) with Bray–Curtis distances was used to model community composition overlap between sites, and permutational multivariate analyses of variance (PERMANOVA) were used to examine statistical differences, also with Bray–Curtis distances and using 999 permutations. A Bray–Curtis dissimilarity matrix represents the differences in community composition among sites (0 = identical species composition, and 1 = no shared species). PERMANOVA then assumes that random permutation of the values in the matrix (e.g., 999 times) results in the sites having the same calculated centroids, meaning that community composition does not differ significantly among sites. To determine pairwise differences, PERMANOVAs were run for each site pair and the *P*-values adjusted with Bonferroni correction. Shannon diversity indices were also calculated by site after determining relative abundance with the "phyloseq" package (McMurdie and Holmes 2013). Indicator species analysis was performed to determine whether particular taxa were significantly associated with specific sites or site combinations (Dufrêne and Legendre 1997; De Cáceres et al. 2010). Analysis of variance (ANOVA) and Tukey HSD post-hoc tests were used to assess differences in the concentrations of 22 elements among our study sites, which was done by using measurements from the 10 closest moss locations to our alder study sites (i.e., each of our Portland sites were composed of $n = 10$ locations from Gatziolis et al. (2016). If data did not meet the assumptions for ANOVA, even after transformation, nonparametric Kruskal–Wallis tests and Dunn post-hoc tests were used.

Maps and Venn diagrams

Figure 1 was generated with ArcGIS Desktop 10.5, and Fig. 6 was generated in R with the ggmap package (Kahle and Wickham 2013). Figure 4 was generated in R with the VennDiagram package (Chen and Boutros 2011).

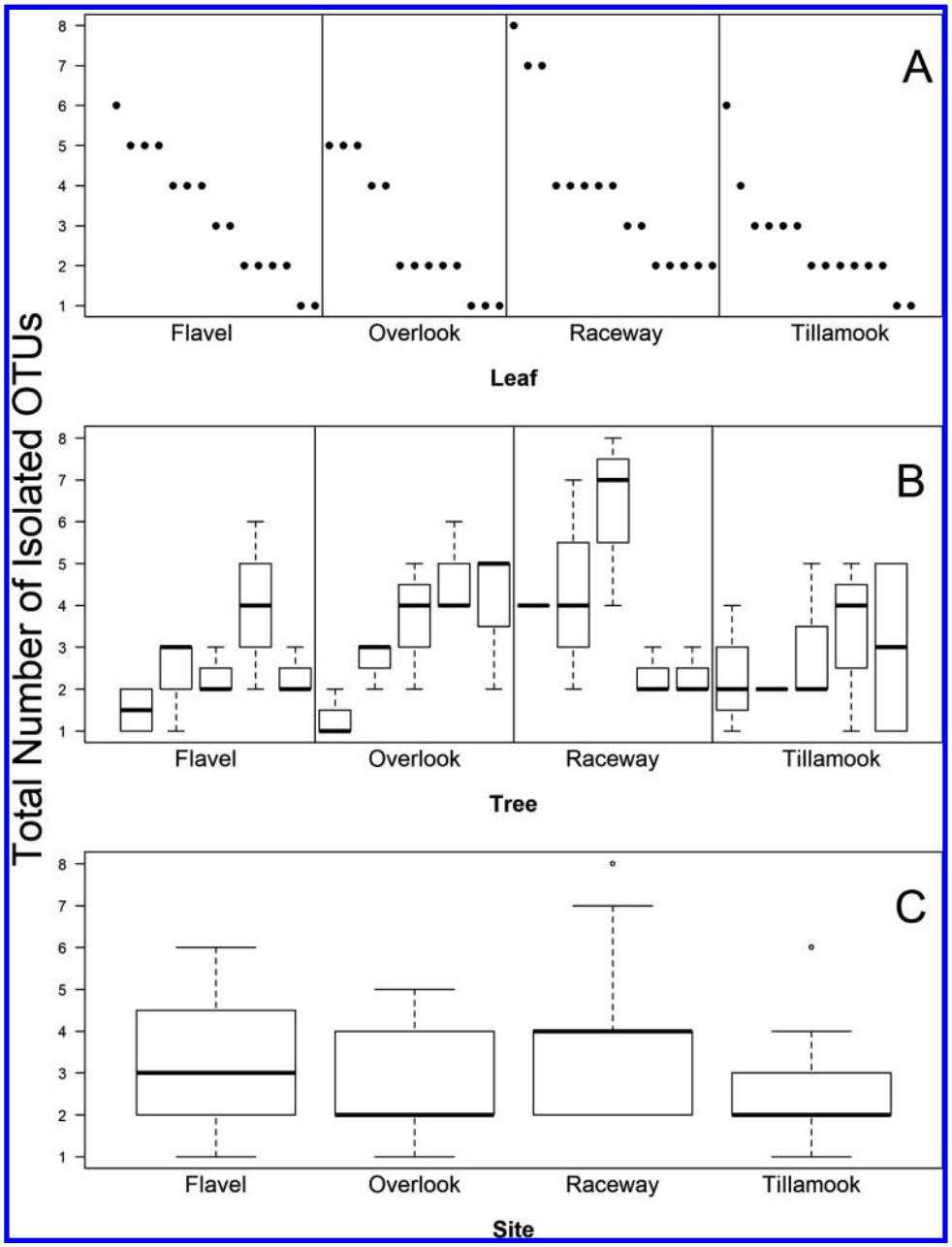
Results

Fungal endophyte communities isolated from leaves of *Alnus rubra* differed significantly across sites in the metropolitan area of Portland during November 2016. A total of 267 OTUs representing 56 assigned species and 36 assigned genera were isolated (Supplementary data, Table S1[1]), averaging 3.14 ± 1.67 species per leaf, with varying relative abundances by site (Supplementary data, Fig. S1). The number of different assigned species isolated from each tree varied from one (Tillamook; Fig. 2) to as many as eight (Raceway) while the average frequency of isolation for all assigned species across all sites was 5.6% ± 5.4%. *Epicoccum nigrum* (15 leaves), *Asteroma alneum* (12 leaves), and *Cladosporium allicinum* (10 leaves) were the three most frequently isolated assigned species, but they occurred unevenly among sites. *Epicoccum nigrum* occurred mostly in leaves from Raceway (7/15 leaves or 46.7% of all occurrences), *A. alneum* occurred frequently in leaves from Tillamook (41.7%) and also Flavel (33.3%), and *C. allicinum* was found in leaves from Raceway (40.0%) and Overlook (60.0%). The species accumulation curve did not level off, which suggests that sampling more leaves, trees, and (or) sites would reveal new species (Fig. 3).

Shannon diversity indices were only marginally different among sampling sites (Kruskal–Wallis, $\chi^2 = 7.35$, df = 3, $P = 0.06$); 19, 27, 28, and 23 species were isolated from leaves from Tillamook, Flavel, Raceway, and Overlook, respectively. Of these species, four were isolated exclusively from Tillamook samples: *Diaporthe cotoneastri*, *Cladosporium macrocarpum*, *Colletotrichum gloeosporioides*, and *Trichoderma harzianum*; seven exclusively from Overlook samples: *Chaetomium* sp., *Boeremia exigua*, *Cryptostroma corticale*, *Lophiostoma* sp., *Penicillium glabrum*, and *Podospora curvicolla*; nine exclusively from Raceway samples: *Sordaria humana*, *Colletotrichum salicis*, *Colletotrichum* sp., *Pyrenophora erythrospila* (as *Drechslera erythrospila*), *Cryptodiaporthe pulchella*, *Curvularia inaequalis*, *Ophiognomonia ibarakiensis*, *Ophiognomonia*

[1]Supplementary data are available with the article through the journal Web site at http://nrcresearchpress.com/doi/suppl/10.1139/cjb-2018-0085.

Fig. 2. Numbers of assigned species (OTUs) cultured at the leaf (A), tree (B), and site (C) level demonstrate variability between sampling units.



Botany Downloaded from www.nrcresearchpress.com by 73.11.45.213 on 03/25/19
For personal use only.

sp., and *Preussia* sp.; and 11 exclusively from Flavel samples: *Diaporthe viticola*, *Diaporthe nobilis*, *Phoma* sp., *Botryosphaeria stevensii* (as *Diplodia mutila*), *Glomerella acutata* (as *Colletotrichum acutatum*), *Gnomoniopsis idaeicola*, *Leptosphaeria* sp., *Nigrospora oryzae*, *Phaeosphaeriaceae* sp., *Pseudopithomyces chartarum* (as *Pithomyces chartarum*), and *Ramularia archangelicae* (Fig. 4).

Fungal endophyte community composition differed significantly by site (PERMANOVA, $F_{[3,56]} = 2.86$, $P = 0.001$), illustrated in part by NMDS ordination that demonstrates the high level of variation within samples and some overlap among sites (Fig. 5). All pairwise site comparisons of endophyte communities were significantly different from one another, except between Tillamook and Flavel, which were marginally different ($P = 0.06$; Table 2). Tillamook and Flavel also shared indicator species within the genus *Diaporthe* (Table 3) and Raceway and Overlook shared *C. allicinum* as an indicator species, whereas two species of *Alternaria* were positively correlated with Raceway only. Several species that approached significance as indicators for Overlook, Flavel, and Raceway are included in Table 3.

After clustering sites from the Gatziolis et al. (2016) dataset to coincide with our three metropolitan sampling sites, we found differences in several previously measured concentrations of elements in moss biomass among our study sites. All 22 elemental concentrations

Fig. 3. Species (OTUs) accumulation curve showing that a larger sample of leaves would have yielded additional fungal taxa.

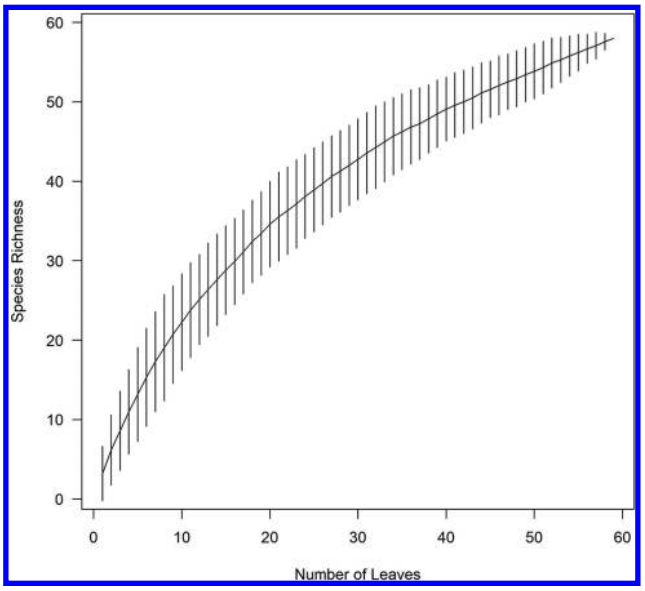
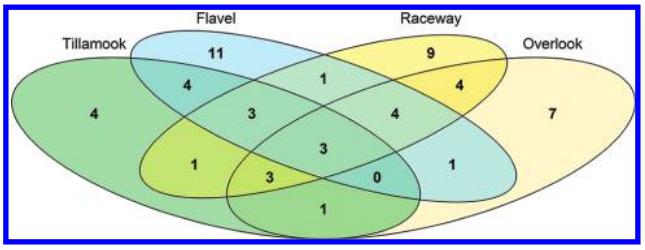


Fig. 4. Venn diagram of number of taxa shared among and between our four study sites. [Colour online.]



were tested, but only significant results are shown (Table 4; P and K are included in Fig. 6 as well). In general, mosses from areas clustered around Raceway and Overlook had accumulated insignificantly different amounts of S (%), Mn (mg/kg), Ni (mg/kg), and Zn (mg/kg) (Tukey HSD, $P > 0.5$). Mosses from areas clustered around Flavel had insignificantly different amounts of S and Mn content compared with Overlook (Tukey HSD, $P > 0.06$), significantly lower amounts of S and Mn content compared with mosses from areas clustered around Raceway (Tukey HSD, $P < 0.05$), and significantly higher Ni content than either mosses from areas clustered around Overlook or Raceway (Tukey HSD, $P < 0.0001$). Mosses from areas around Overlook had significantly higher levels of Zn compared with mosses from around Flavel. Both Pb and Cu concentrations in mosses were significantly lower in areas clustered around Flavel compared with mosses clustered around Overlook (Dunn's test, $P < 0.01$). Areas clustered around Raceway had significantly higher concentrations of Cu in mosses compared with mosses clustered around either Overlook or Flavel (Dunn's test, $P < 0.01$). Areas clustered around Flavel had significantly lower concentrations of Fe in mosses than areas clustered around either Raceway or Overlook (Tukey HSD, $P < 0.01$), which had insignificantly different concentrations.

Discussion

In this study, we determined that foliar fungal endophyte communities may be structured in part by proximity to specific point sources of air pollution in sites across a metropolitan area. We did observe high variability in isolation frequency among both trees and sampling sites, underscoring the dynamic nature of endophyte communities, which can vary temporally and spatially within host plant tissues (Ek-Ramos et al. 2013; Younginger and Ballhorn 2017). While overall community composition varied by site, the most abundant species were recovered from multiple sites and represent a considerable population of generalist endophytes (Stone et al. 2004), including several species in *Alternaria* and *Cladosporium* as well as *Diaporthe*, another common endophyte genus that contains many pathogenic species. Our findings corroborate many of the fungi identified in the earlier survey of red alder by Sieber et al. (1991), but also suggest that a large component of the foliar fungal endophyte community widely occurs among hosts and also within different host tissues, lending support to the concept of a "core microbiome" (Shade and Handelsman 2012) at least within plants although next-generation sequencing analyses will better identify core OTUs. A majority of the fungal genera, if not species, isolated in this study were also found in stem tissues of Spanish olive trees (Fisher et al. 1992), in twigs from ash, oak, and beech (Griffith and Boddy 1990), in various tissues of *Arabidopsis thaliana* (Junker et al. 2012), and in nine different tree species in urban and rural sites in Japan (Matsumura and Fukuda 2013). Shared genera include *Aureobasidium*, *Botrytis*, *Cytospora*, *Epicoccum*, *Phoma*, *Phomopsis*, and *Sordaria*. Additionally, this study may be the first to report the presence of *Pyrenophora erythrospila* in *Alnus*.

Contrary to earlier studies of urban versus rural endophyte communities (Jumpponen and Jones 2009, 2010; Matsumura and Fukuda 2013), we only found a marginally significant difference between fungal community diversity between our study sites, which included three metropolitan locations and one forested location. Previous work anticipates lower fungal endophyte diversity in plants growing in polluted soils at former industrial sites (Lappalainen et al. 1999; Renker et al. 2005; Likar and Regvar 2009). However, while this effect may be due to small sample size, the trends in the number of species isolated by site appear to be the opposite of our expectations, given that about 30% more species were cultured from the Raceway and Flavel sites compared with the Tillamook site. Given that Wężowicz et al. (2014) also reported higher diversity in more polluted sites, these trends may be driven in part by local pollution sources or dispersal limitation; additionally, priority effects may be

Botany Downloaded from www.nrcresearchpress.com by 73.11.45.213 on 03/25/19
For personal use only.

Botany Downloaded from www.nrcresearchpress.com by 73.11.45.213 on 03/25/19
For personal use only.

Fig. 5. Non-metric multidimensional scaling ordination of fungal endophyte communities of *Alnus rubra* across the metropolitan area of Portland; ellipses represent 95% confidence intervals based on the standard error.

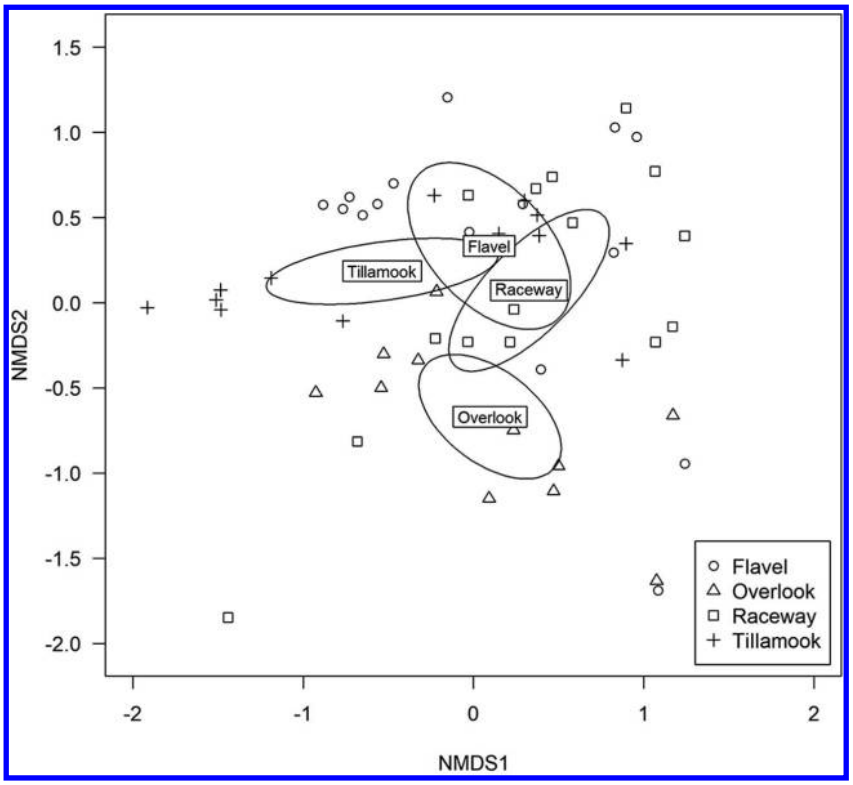


Table 2. perMANOVA results of pairwise site comparisons of fungal endophyte communities, following Bonferroni adjustment.

Site Pair	*F*	R^2	*P*
Tillamook–Flavel	2.41	0.082	0.060
Tillamook–Raceway	3.43	0.113	0.006
Tillamook–Overlook	3.59	0.126	0.006
Flavel–Raceway	2.46	0.081	0.024
Flavel–Overlook	2.81	0.098	0.012
Overlook–Raceway	2.54	0.089	0.030

Table 3. Results of indicator species analysis by study site, showing the dominant members of site and site-combination communities.

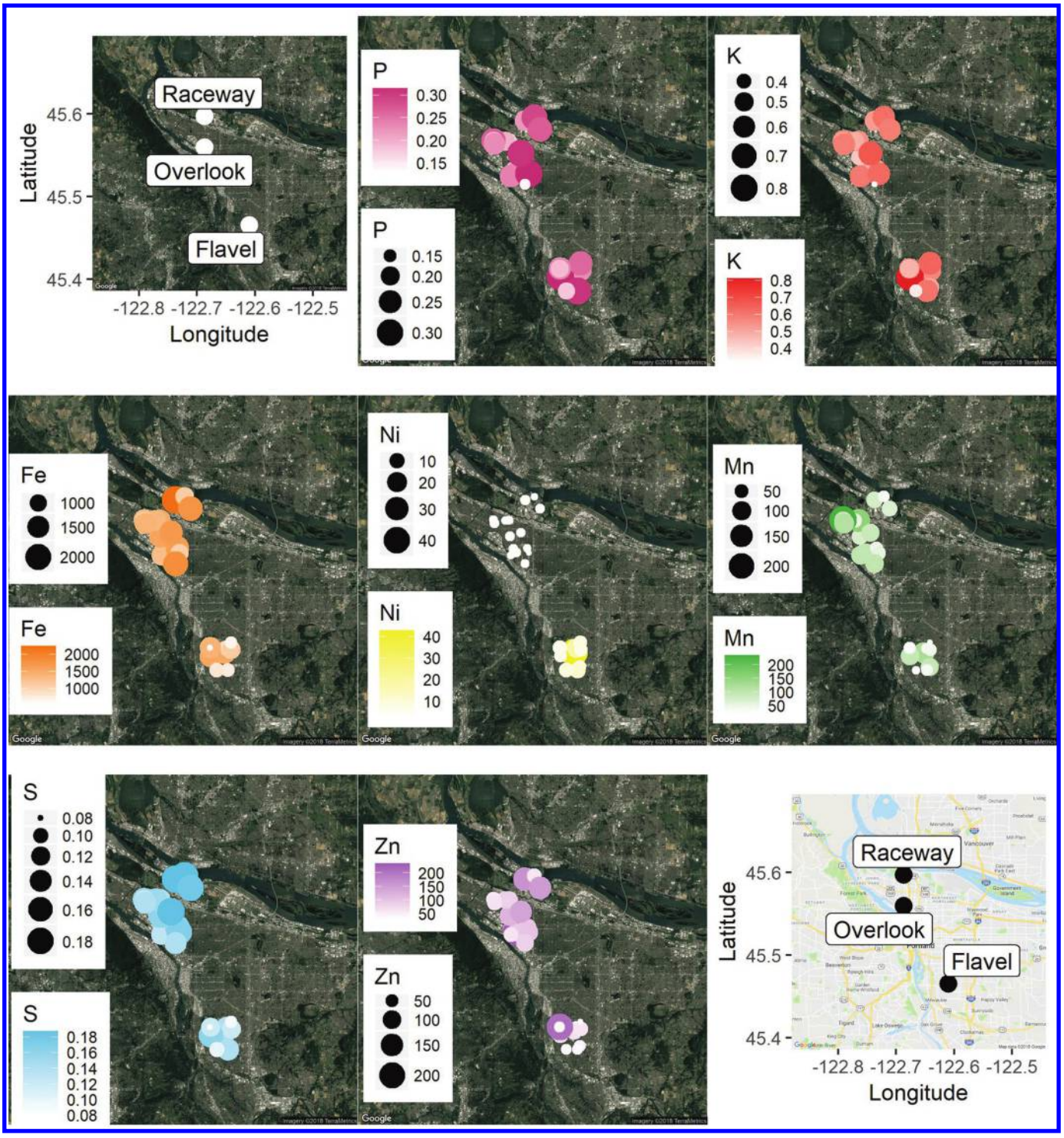
Species	Site	Indicator value	*P*
Alternaria alternata	Raceway	0.545	0.005
Alternaria arborescens	Raceway	0.520	0.020
Chaetomium sp.	Overlook	0.392	0.065
Cladosporium allicinum	Raceway & Overlook	0.598	0.005
Cladosporium sp.	Overlook & Flavel	0.423	0.095
Diaporthe cotoneastri	Tillamook	0.598	0.005
Diaporthe rudis	Flavel	0.514	0.035
Diaporthe viticola	Flavel	0.447	0.070
Ophiognomonia intermedia	Tillamook	0.505	0.035
Sordaria humana	Raceway	0.447	0.060

Table 4. ANOVA results for previously measured concentrations of elements in moss biomass, showing differences between the current sites studied.

Element	$F_{[2,26]}$	*P*
S	8.64	0.00133
Fe	5.78	0.00841
Mn	4.11	0.028
Ni	42.1	<0.0001
Zn	6.11	0.00668

at play in compositional differences, as well as other environmental factors such as elevation. If pollution is a major contributor, an obvious source of pollutants in our sampling sites is vehicle traffic; all of our sites were located along roadways, and thus represent an impact gradient for vehicle traffic, with Raceway and Overlook receiving considerably more emissions than Flavel or Tillamook because of their proximity to Interstate 5 (<1000 m). Additionally, dominance effects may have influenced diversity patterns for Tillamook samples. For example, indicator species characterized the dominant members of their respective site communities (Table 2), and three out of the five trees sampled at the Tillamook site had assemblages that were dominated by pathogenic *Diaporthe* species (Fig. 2) that may have inhibited other species from colonizing the leaf tissue; members of the genus have been shown to produce antimicrobial com-

Fig. 6. Maps of the Portland area with our three urban sites labelled (top-left, satellite image; bottom-right, street map), followed by seven panels of selected elemental concentrations (P, K, Fe, Ni, Mn, S, Zn) measured in moss biomass collected from the 10 closest locations to each of our metropolitan alder-sampling sites. [Colour online.]



pounds (Tanney et al. 2016). Fewer species were also isolated from the Overlook site, where assemblages were similarly dominated by other pathogenic fungi in *Cladosporium*, species of which have been documented as antagonistic towards other pathogens (Busby et al. 2016). Considering the high variability among leaves from the same tree, and trees in the same site, a larger survey may clarify patterns in both alpha and beta diversity.

While we did not directly measure air pollutants in our study, the publicly-available dataset provided by Gatziolis et al. (2016) uses mosses as bioindicators of air

Botany Downloaded from www.nrcresearchpress.com by 73.11.45.213 on 03/25/19
For personal use only.

Botany Downloaded from www.nrcresearchpress.com by 73.11.45.213 on 03/25/19
For personal use only.

pollution in the same metropolitan area as our study. We acknowledge that the moss bioaccumulation data were collected several years before our sample collection period; however, we contend that the trends identified by Gatziolis et al. (2016) remain applicable to our sites, and merely suggest historical environmental differences among our sites that may be underlying variation in fungal endophyte community composition, in addition to other environmental factors. Raceway and Overlook are adjacent to Interstate 5 (a major highway in the region), and mosses collected from near these sites had accumulated elevated levels of sulfur, iron, and lead relative to Flavel, the only other metropolitan site in our study. *Cladosporium* and *Alternaria* species were most common in these sites, which may suggest that these fungi are more tolerant of pollution from vehicle emissions. Likar and Regvar (2009) observed the same fungal endophytes in *Salix caprea* L. growing in both polluted and non-polluted sites, although they hypothesized that the relative tolerances of species likely differed according to the local environment. Our study was not designed to test metal tolerances of isolated fungi, but Li et al. (2012) found that an *Alternaria* isolate was not inhibited by zinc-supplemented media, which may coincide with elevated levels of zinc in mosses collected near Overlook where *Alternaria* was a dominant genus.

Conclusions

Fungal endophyte communities are often dynamic and are known to show seasonal, spatial, and functional variation in their host plants (Arnold 2007; Scholtysik et al. 2013; Younginger and Ballhorn 2017). Red alder is an economically and ecologically important tree species in the Pacific Northwest, but beyond its root-associated microbes (*Frankia* bacteria and endomycorrhizal fungi) little is known about the microbial communities it harbors and their responses to environmental conditions. Our results suggest proximity to different point sources of air pollution — potentially in conjunction with other site characteristics — partially influences fungal endophyte community composition in red alder leaves. Future studies should quantitatively examine relationships between endophyte diversity in red alder and sources of air pollution.

Acknowledgements

Funding by the National Science Foundation (NSF) to D.J.B. (grants IOS 1457369 and 1656057) is gratefully acknowledged. We further acknowledge financial support through BUILD EXITO (grants 5TL4GM118965-03, 5UL1GM118964-03, and 5RL5GM118963-03). Finally, we would like to thank six anonymous reviewers for their insightful comments.

References

Arnold, A.E. 2007. Understanding the diversity of foliar endophytic fungi: Progress, challenges, and frontiers. Fungal Biol. Rev. **21**: 51–66. doi:10.1016/j.fbr.2007.05.003.

Aschehoug, E.T., Metlen, K.L., Callaway, R.M., and Newcombe, G. 2012. Fungal endophytes directly increase the competitive effects of an invasive forb. Ecology, **93**(1): 3–8. doi:10.1890/11-1347.1. PMID:22486080.

Bennett, D.D., and Tkacz, B.M. 2008. Forest health monitoring in the United States: a program overview. Aust. For. **71**(3): 223–228. doi:10.1080/00049158.2008.10675039.

Busby, P.E., Peay, K.G., and Newcombe, G. 2016. Common foliar fungi of *Populus trichocarpa* modify *Melampsora* rust disease severity. New Phytol. **209**(4): 1681–1692. doi:10.1111/nph.13742. PMID:26565565.

Cáceres De, M., and Legendre, P. 2009. Associations between species and groups of sites: indices and statistical inference. Ecology, **90**(12): 3566–3574. doi:10.1890/08-1823.1. PMID:20120823.

Cairney, J.W.G., and Meharg, A.A. 1999. Influences of anthropogenic pollution on mycorrhizal fungal communities. Environ. Pollut. **106**(2): 169–182. doi:10.1016/S0269-7491(99)00081-0. PMID:15093044.

Campbell, S., Smith, G., Temple, P., Pronos, J., Rochefort, R., and Andersen, C. 2000. Monitoring for ozone injury in west coast (Oregon, Washington, California) forests in 1998. Gen. Tech. Rep. PNW-GTR-495. U.S. Department of Agriculture, Forest Service, Pacific Northwest Research Station Portland, Ore. https://www.fs.usda.gov/treesearch/pubs/2948 [accessed 8 January 2018].

Chen, H., and Boutros, P.C. 2011. VennDiagram: a package for the generation of highly-customizable Venn and Euler diagrams in R. BMC Bioinform. **12**(1): 35. doi:10.1186/1471-2105-12-35.

De Boeck, H.J., Lemmens, C.M.H.M., Gielen, B., Bossuyt, H., Malchair, S., Carnol, M., Merckx, R., Ceulemans, R., and Nijs, I. 2007. Combined effects of climate warming and plant diversity loss on above- and below-ground grassland productivity. Environ. Exp. Bot. **60**(1): 95–104. doi:10.1016/j.envexpbot.2006.07.001.

De Cáceres, M., Legendre, P., and Moretti, M. 2010. Improving indicator species analysis by combining groups of sites. Oikos, **119**(10): 1674–1684. doi:10.1111/j.1600-0706.2010.18334.x.

Department of Environmental Quality. 2017. State of Oregon: Programs - Bullseye Glass. Available from http://www.oregon.gov/deq/Programs/Pages/Bullseye-Glass.aspx [accessed 8 January 2018].

Donovan, G.H., Jovan, S.E., Gatziolis, D., Burstyn, I., Michael, Y.L., Amacher, M.C., and Monleon, V.J. 2016. Using an epiphytic moss to identify previously unknown sources of atmospheric cadmium pollution. Sci. Total Environ. **559**: 84–93. doi:10.1016/j.scitotenv.2016.03.182. PMID:27058127.

Dufrêne, M., and Legendre, P. 1997. Species assemblages and indicator species: The need for a flexible asymmetrical approach. Ecol. Monogr. **67**(3): 345–366. doi:10.1890/0012-9615(1997)067[0345:SAAIST]2.0.CO;2.

Ek-Ramos, M.J., Zhou, W., Valencia, C.U., Antwi, J.B., Kalns, L.L., Morgan, G.D., Kerns, D.L., and Sword, G.A. 2013. Spatial and temporal variation in fungal endophyte communities isolated from cultivated cotton (*Gossypium hirsutum*). PLoS ONE, **8**(6): e66049. doi:10.1371/journal.pone.0066049. PMID:23776604.

Fisher, P.J., Petrini, O., Petrini, L.E., and Descals, E. 1992. A preliminary study of fungi inhabiting xylem and whole stems of *Olea europaea*. Sydowia, **44**: 117–121.

Frey, S.D., Drijber, R., Smith, H., and Melillo, J. 2008. Microbial biomass, functional capacity, and community structure after 12 years of soil warming. Soil Biol. Biochem. **40**(11): 2904–2907. doi:10.1016/j.soilbio.2008.07.020.

Gatziolis, D., Jovan, S., Donovan, G., Amacher, M., and Monleon, V. 2016. Elemental atmospheric pollution assessment via moss-based measurements in Portland, Oregon. Gen. Tech. Rep. PNW-GTR-938. U.S. Department of Agriculture, Forest Service, Pacific Northwest Research Station, Portland, Ore.

https://www.fs.usda.gov/treesearch/pubs/51076 [accessed 8 January 2018].
Geiser, L.H., and Neitlich, P.N. 2007. Air pollution and climate gradients in western Oregon and Washington indicated by epiphytic macrolichens. Environ. Pollut. **145**: 203–218. doi:10.1016/j.envpol.2006.03.024. PMID:16777297.
Griffith, G.S., and Boddy, L. 1990. Fungal decomposition of attached angiosperm twigs. New Phytol. **116**(3): 407–415. doi:10.1111/j.1469-8137.1990.tb00526.x.
Grimmett, I.J., Smith, K.A., and Bärlocher, F. 2012. Tar-spot infection delays fungal colonization and decomposition of maple leaves. Freshw. Sci. **31**(4): 1088–1095. doi:10.1899/12-034.1.
Harrington, C.A., Zasada, J.C., and Allen, E.A. 1994. Biology of red alder (*Alnus rubra* Bong.). *In* The biology and management of Red Alder. *Edited by* D.E. Hibbs, D.S. DeBell, and R.F. Tarrant. Oregon State University Press, Corvallis, Ore. pp. 3–22.
He, J.-S., Bazzaz, F.A., and Schmid, B. 2002. Interactive effects of diversity, nutrients and elevated CO_2 on experimental plant communities. Oikos, **97**(3): 337–348. doi:10.1034/j.1600-0706.2002.970304.x.
Hesse, U., Schöberlein, W., Wittenmayer, L., Förster, K., Warnstorff, K., Diepenbrock, W., and Merbach, W. 2003. Effects of *Neotyphodium* endophytes on growth, reproduction and drought-stress tolerance of three *Lolium perenne* L. genotypes. Grass Forage Sci. **58**(4): 407–415. doi:10.1111/j.1365-2494.2003.00393.x.
Jumpponen, A., and Jones, K.L. 2009. Massively parallel 454 sequencing indicates hyperdiverse fungal communities in temperate *Quercus macrocarpa* phyllosphere. New Phytol. **184**(2): 438–448. doi:10.1111/j.1469-8137.2009.02990.x. PMID:19674337.
Jumpponen, A., and Jones, K.L. 2010. Seasonally dynamic fungal communities in the *Quercus macrocarpa* phyllosphere differ between urban and nonurban environments. New Phytol. **186**(2): 496–513. doi:10.1111/j.1469-8137.2010.03197.x. PMID:20180911.
Junker, C., Draeger, S., and Schulz, B. 2012. A fine line — Endophytes or pathogens in *Arabidopsis thaliana*. Fungal Ecol. **5**(6): 657–662. doi:10.1016/j.funeco.2012.05.002.
Kahle, D., and Wickham, H. 2013. ggmap: spatial visualization with ggplot2. R J. **5**(1): 144–161. R Foundation for Statistical Computing.
Khan, Z., Rho, H., Firrincieli, A., Hung, S.H., Luna, V., Masciarelli, O., Kim, S.-H., and Doty, S.L. 2016. Growth enhancement and drought tolerance of hybrid poplar upon inoculation with endophyte consortia. Curr. Plant Biol. **6**: 38–47. doi:10.1016/j.cpb.2016.08.001.
Lappalainen, J.H., Koricheva, J., Helander, M.L., and Haukioja, E. 1999. Densities of endophytic fungi and performance of leafminers (Lepidoptera: Eriocraniidae) on birch along a pollution gradient. Environ. Pollut. **104**: 99–105. doi:10.1016/S0269-7491(98)00146-8.
Lee, D.-B., Nam, W., Kwak, Y.-S., Cho, N.-H., and Lee, S.-S. 2009. Phytoremediation of heavy-metal-contaminated soil in a reclaimed dredging area using *Alnus* species. J. Ecol. Environ. **32**(4): 267–275. doi:10.5141/JEFB.2009.32.4.267.
Lemons, A., Clay, K., and Rudgers, J.A. 2005. Connecting plant-microbial interactions above and belowground: a fungal endophyte affects decomposition. Oecologia, **145**(4): 595–604. doi:10.1007/s00442-005-0163-8. PMID:16001218.
LeRoy, C.J., Fischer, D.G., Halstead, K., Pryor, M., Bailey, J.K., and Schweitzer, J.A. 2011. A fungal endophyte slows litter decomposition in streams. Freshw. Biol. **56**(7): 1426–1433. doi:10.1111/j.1365-2427.2011.02581.x.
Li, H.-Y., Li, D.-W., He, C.-M., Zhou, Z.-P., Mei, T., and Xu, H.-M. 2012. Diversity and heavy metal tolerance of endophytic fungi from six dominant plant species in a Pb–Zn mine wasteland in China. Fungal Ecol. **5**(3): 309–315. doi:10.1016/j.funeco.2011.06.002.
Likar, M., and Regvar, M. 2009. Application of temporal temperature gradient gel electrophoresis for characterisation of fungal endophyte communities of *Salix caprea* L. in a heavy metal polluted soil. Sci. Total Environ. **407**(24): 6179–6187. doi:10.1016/J.SCITOTENV.2009.08.045.
Lin, Y., He, X., Ma, T., Han, G., and Xiang, C. 2015. Priority colonization of *Cinnamomum camphora* litter by endophytes affects decomposition rate, fungal community and microbial activities under field conditions. Pedobiologia (Jena), **58**(5–6): 177–185. doi:10.1016/j.pedobi.2015.09.001.
Matsumura, E., and Fukuda, K. 2013. A comparison of fungal endophytic community diversity in tree leaves of rural and urban temperate forests of Kanto district, eastern Japan. Fungal Biol. **117**(3): 191–201. doi:10.1016/j.funbio.2013.01.007. PMID:23537876.
McCulley, R.L., Bush, L.P., Carlisle, A.E., Ji, H., and Nelson, J.A. 2014. Warming reduces tall fescue abundance but stimulates toxic alkaloid concentrations in transition zone pastures of the U.S. Front. Chem. **2**: 88. doi:10.3389/fchem.2014.00088. PMID:25374886.
McMurdie, P.J., and Holmes, S. 2013. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS ONE, **8**(4): e61217. doi:10.1371/journal.pone.0061217. PMID:23630581.
Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H., Szoecs, E., and Wagner, H. 2017. vegan: Community Ecology Package. Available from https://cran.r-project.org/package=vegan [accessed 4 August 2017].
Parks & Recreation. 2017. Tree Inventory Project. Available from https://www.portlandoregon.gov/parks/53181 [accessed 5 November 2016].
Porter, J.R., and Sheridan, R.P. 1981. Inhibition of nitrogen fixation in alfalfa by arsenate, heavy metals, fluoride, and simulated acid rain. Plant Physiol. **68**(1): 143–148. doi:10.1104/pp.68.1.143. PMID:16661858.
Printz, B., Sergeant, K., Lutts, S., Guignard, C., Renaut, J., and Hausman, J.-F. 2013. From tolerance to acute metabolic deregulation: contribution of proteomics to dig into the molecular response of alder species under a polymetallic exposure. J. Proteome Res. **12**(11): 5160–5179. doi:10.1021/pr400590d. PMID:24015726.
Rakwal, R., Agrawal, G.K., Kubo, A., Yonekura, M., Tamogami, S., Saji, H., and Iwahashi, H. 2003. Defense/stress responses elicited in rice seedlings exposed to the gaseous air pollutant sulfur dioxide. Environ. Exp. Bot. **49**(3): 223–235. doi:10.1016/S0098-8472(02)00072-2.
Ren, A., Li, C., and Gao, Y. 2011. Endophytic fungus improves growth and metal uptake of *Lolium Arundinaceum* Darbyshire Ex. Schreb. Int. J. Phytoremediation, **13**(3): 233–243. doi:10.1080/15226511003671387. PMID:21598789.
Renker, C., Blanke, V., and Buscot, F. 2005. Diversity of arbuscular mycorrhizal fungi in grassland spontaneously developed on area polluted by a fertilizer plant. Environ. Pollut. **135**(2): 255–266. doi:10.1016/j.envpol.2004.11.002. PMID:15734585.
Rodriguez, R.J., White, J.F., Arnold, A.E., and Redman, R.S. 2009. Fungal endophytes: diversity and functional roles. New Phytol. **182**(2): 314–330. doi:10.1111/j.1469-8137.2009.02773.x. PMID:19236579.
Rudgers, J.A., and Clay, K. 2007. Endophyte symbiosis with tall fescue: How strong are the impacts on communities and ecosystems? Fungal Biol. Rev. **21**(2–3): 107–124. doi:10.1016/j.fbr.2007.05.002.
Scholtysik, A., Unterseher, M., Otto, P., and Wirth, C. 2013. Spatio-temporal dynamics of endophyte diversity in the can-

Botany Downloaded from www.nrcresearchpress.com by 73.11.45.213 on 03/25/19
For personal use only.

opy of European ash (*Fraxinus excelsior*). Mycol. Prog. **12**(2): 291–304. doi:10.1007/s11557-012-0835-9.

Schützendübel, A., and Polle, A. 2002. Plant responses to abiotic stresses: heavy metal-induced oxidative stress and protection by mycorrhization. J. Exp. Bot. **53**(372): 1351–1365. doi:10.1093/jxb/53.372.1351. PMID:11997381.

Shade, A., and Handelsman, J. 2012. Beyond the Venn diagram: the hunt for a core microbiome. Environ. Microbiol. **14**(1): 4–12. doi:10.1111/j.1462-2920.2011.02585.x. PMID:22004523.

Sheik, C.S., Beasley, W.H., Elshahed, M.S., Zhou, X., Luo, Y., and Krumholz, L.R. 2011. Effect of warming and drought on grassland microbial communities. ISME J. **5**(10): 1692–1700. doi:10.1038/ismej.2011.32. PMID:21451582.

Sieber, T.N., Sieber-Canavesi, F., and Dorworth, C.E. 1991. Endophytic fungi of red alder (*Alnus rubra*) leaves and twigs in British Columbia. Can. J. Bot. **69**(2): 407–411. doi:10.1139/b91-056.

Stone, J.K., Polishook, J.O.N.D., and White, J.F. 2004. Endophytic fungi. *In* Measuring and monitoring biodiversity of fungi. *Edited by* G. Mueller, G. Bills, and M. Foster. Elsevier Academic Press, Boston, Mass. pp. 241–270.

Suryanarayanan, T.S. 2013. Endophyte research: Going beyond isolation and metabolite documentation. Fungal Ecol. **6**(6): 561–568. doi:10.1016/j.funeco.2013.09.007.

Tanney, J.B., McMullin, D.R., Green, B.D., Miller, J.D., and Seifert, K.A. 2016. Production of antifungal and antiinsectan metabolites by the *Picea* endophyte *Diaporthe maritima* sp.nov. Fungal Biol. **120**: 1448–1457. doi:10.1016/j.funbio.2016.05.007. PMID:27742098.

Transportation Data Section. 2016. 2015 Transportation Volume Tables. Oregon Department of Transportation, Salem, Ore. [accessed 8 January 2018].

United States Environmental Protection Agency. 2011. 2011 National Air Toxics Assessment https://www.oregon.gov/ODOT/Data/Documents/TVT_Complete_2015.pdf [accessed 8 January 2018].

United States Environmental Protection Agency. 2017. Query AirData. Available from https://www.epa.gov/national-air-toxics-assessment/2011-national-air-toxics-assessment [accessed 8 January 2018].

Waller, F., Achatz, B., Baltruschat, H., Fodor, J., Becker, K., Fischer, M., Heier, T., Hückelhoven, R., Neumann, C., von Wettstein, D., Franken, P., and Kogel, K.-H. 2005. The endophytic fungus *Piriformospora indica* reprograms barley to salt-stress tolerance, disease resistance, and higher yield. Proc. Natl. Acad. Sci. U.S.A. **102**(38): 13386–13391. doi:10.1073/pnas.0504423102. PMID:16174735.

Warren, D.D. 2009. Production, Prices, Employment, and Trade in Northwest Forest Industries, All Quarters 2008. Resour. Bull. PNW-RB-258. U.S. Department of Agriculture, Forest Service, Pacific Northwest Research Station, Portland, Ore. https://www.srs.fs.usda.gov/pubs/33388 [accessed 18 June 2018].

Wężowicz, K., Rozpądek, P., and Turnau, K. 2014. The diversity of endophytic fungi in *Verbascum lychnitis* from industrial areas. Symbiosis, **64**(3): 139–147. doi:10.1007/s13199-015-0312-8.

Wohlgemuth, H., Mittelstrass, K., Kschieschan, S., Bender, J., Weigel, H.-J., Overmyer, K., Kangasjarvi, J., Sandermann, H., and Langebartels, C. 2002. Activation of an oxidative burst is a general feature of sensitive plants exposed to the air pollutant ozone. Plant Cell Environ. **25**(6): 717–726. doi:10.1046/j.1365-3040.2002.00859.x.

Younginger, B.S., and Ballhorn, D.J. 2017. Fungal endophyte communities in the temperate fern *Polystichum munitum* show early colonization and extensive temporal turnover. Am. J. Bot. **104**(8): 1188–1194. doi:10.3732/ajb.1700149.

Botany Downloaded from www.nrcresearchpress.com by 73.11.45.213 on 03/25/19
For personal use only.