Open Access Articles

Response of the nitrogen-fixing lichen Lobaria pulmonaria to phosphorus, molybdenum, and vanadium

Oregon State
UNIVERSITY

ECOSPHERE

Response of the nitrogen-fixing lichen *Lobaria pulmonaria* to phosphorus, molybdenum, and vanadium

Jade A. Marks,[1,3,†] Julie C. Pett-Ridge,[1] Steven S. Perakis,[2] Jessica L. Allen,[3,4] and Bruce McCune[3]

[1]*Department of Crop and Soil Science, Oregon State University, Corvallis, Oregon 97331 USA*
[2]*U.S. Geological Survey, Forest and Range Ecosystem Science Center, 3200 SW Jefferson Way, Corvallis, Oregon 97331 USA*
[3]*Department of Botany and Plant Pathology, Oregon State University, Corvallis, Oregon 97331 USA*
[4]*Institute of Systematic Botany, The New York Botanical Garden, Bronx, New York 10458-5126 USA*

Abstract. Nitrogen-fixing lichens (cyanolichens) are an important source of nitrogen (N) in Pacific Northwest forests, but limitation of lichen growth by elements essential for N fixation is poorly understood. To investigate how nutrient limitation may affect cyanolichen growth rates, we fertilized a tripartite cyanobacterial lichen (*Lobaria pulmonaria*) and a green algal non-nitrogen fixing lichen (*Usnea longissima*) with the micronutrients molybdenum (Mo) and vanadium (V), both known cofactors for enzymes involved in N fixation, and the macronutrient phosphorus (P). We then grew treated lichens in the field for one year in western Oregon, USA. Lichen growth was very rapid for both species and did not differ across treatments, despite a previous demonstration of P-limitation in *L. pulmonaria* at a nearby location. To reconcile these disparate findings, we analyzed P, Mo, and V concentrations, natural abundance $\delta^{15}N$ isotopes, %N and change in thallus N in *Lobaria pulmonaria* from both growth experiments. Nitrogen levels in deposition and in lichens could not explain the large difference in growth or P limitation observed between the two studies. Instead, we provide evidence that local differences in P availability may have caused site-specific responses of *Lobaria* to P fertilization. In the previous experiment, *Lobaria* had low background levels of P, and treatment with P more than doubled growth. In contrast, *Lobaria* from the current experiment had much higher background P concentrations, similar to P-treated lichens in the previous experiment, consistent with the idea that ambient variation in P availability influences the degree of P limitation in cyanolichens. We conclude that insufficient P, Mo, and V did not limit the growth of either cyanolichens or chlorolichens at the site of the current experiment. Our findings point to the need to understand landscape-scale variation in P availability to cyanolichens, and its effect on spatial patterns of cyanolichen nutrient limitation and N fixation.

Key words: lichen growth; *Lobaria pulmonaria*; molybdenum; nitrogen; nitrogen fixation; Pacific Northwest; transplant experiment; vanadium.

† **E-mail:** marksjad@onid.oregonstate.edu

INTRODUCTION

Biological fixation is the primary mechanism by which new nitrogen (N) becomes available to most ecosystems (Vitousek et al. 2002, 2013). Nitrogen availability influences primary productivity and carbon exchange, plant growth, community composition, and carbon sequestration in

both soil and aboveground biomass (Galloway et al. 2003, van Groenigen et al. 2006, LeBauer and Treseder 2008). N-fixers (bacteria, archaea, and cyanobacteria), whether symbiotic or not, contribute available N using the nitrogenase enzyme to convert atmospheric N_2 to NH_3. N-fixation is often considered to be metabolically expensive, requiring 15 molecules of ATP per molecule of fixed N_2 (Simpson and Burris 1984), and thus large amounts of phosphorus (P). The nitrogenase enzyme also requires iron (Fe), molybdenum (Mo), or vanadium (V) as an essential cofactor (Eady 1996, Einsle et al. 2002, Bellenger et al. 2011). Although a single N-fixer may produce nitrogenase isoenzymes with all three cofactors, Mo-nitrogenase is thought to be used preferentially when Mo is available (Einsle et al. 2002, Bellenger et al. 2011). Recent evidence, however, suggests a strong affinity for V (Darnajoux et al. 2014, Hodkinson et al. 2014). Variation in the availability of Mo, V, P, or other elements may therefore limit the growth and reproduction of N-fixing organisms, as well as N-fixation inputs that shape a range of ecosystem and global processes.

Nitrogen fixing cyanolichens can be bipartite symbioses between a cyanobacteria and a fungus or they can be tripartite symbioses ("cephalolichens") composed of a fungus and green alga with pockets containing cyanobacteria (cephalodia) throughout the thallus. Cyanolichens are an important source of fixed N, particularly in old forests in wet temperate climates (Pike et al. 1972, Denison 1979, Antoine 2004, Holub and Lajtha 2004), in arctic ecosystems (Gunther 1989), and on freshly exposed substrates where they act as primary colonizers (Crittenden and Kershaw 1978). Recent estimates of N-fixation in cryptogamic ground covers (including lichen, bryophytes, and free-living cyanobacteria and algae) suggest that 46% of biological N fixation on land (49 Tg N uptake per year globally) occurs in these cryptogamic communities (Elbert et al. 2012). Cyanolichen N input can exceed atmospheric deposition and asymbiotic fixation, making it an important long-term N source (Pike et al. 1972, Antoine 2004, Holub and Lajtha 2004, Giesen et al. 2008). Limitation by Mo and P as well as Mo-P co-limitation of asymbiotic N-fixation has been demonstrated in field and laboratory experiments (Silvester 1989, Barron et al. 2008, Wurz-

burger et al. 2012, Jean et al. 2013, Reed et al. 2013); the extent, however, of Mo and P limitation in symbiotic N-fixing lichens is not known.

Epiphytic lichens accumulate nutrients passively through a combination of atmospheric deposition (Stewart et al. 1995, Clark et al. 1998) and canopy throughfall (Goward and Arsenault 2000, Campbell et al. 2010). Their inability to directly access soil reservoirs of rock-derived nutrients, such as Mo and P, results in the potential for limitation by these elements. Indeed, several studies have demonstrated P limitation in N-fixing lichens on the oldest part of the Hawaiian archipelago and in the Pacific Northwest of North America (Kurina and Vitousek 1999, Benner et al. 2007, Benner and Vitousek 2007, McCune and Caldwell 2009). Others, however, have found no response to P-fertilization (Johansson et al. 2011), or no consistent differences in lichen N-fixation rates (Bidussi et al. 2013) or tripartite cyanolichen abundance (Campbell et al. 2010) across P gradients. When P-fertilized *L. pulmonaria* were examined across an elevation gradient in British Columbia, direct and indirect light exposure and bark pH, but not P treatment, affected relative growth rates (Bidussi et al. 2013). Phosphorus fertilization of lichen communities in the arctic tundra increased N-fixation rates in the cyanolichens *Peltigera aphthosa* and *P. polydactyla* but did not correspond to a change in abundance of these lichens at the plot-level (Weiss et al. 2005).

While the influence of P on lichen growth has received some attention, the role of Mo and V in cyanolichen nutrient requirements is largely unexplored. Fertilization with Mo has been attempted in only one study of cyanolichens (Horstmann et al. 1982), and to our knowledge, co-limitation of cyanolichens by V, Mo, and P has not been studied. The discovery that many commercial P-fertilizers contain Mo as a contaminant (McBride and Spiers 2001, Molina et al. 2009) has raised concern that the positive growth and fixation responses of early P-fertilization studies were actually the result of increased Mo (Barron et al. 2008). The only existing controlled study of Mo effects on cyanolichens found that the addition of 1 μg/g Mo increased nitrogenase activity, as measured by acetylene reduction, by 180% in *L. pulmonaria* and by 50% in *Lobaria*

oregana (Horstmann et al. 1982). No one has demonstrated Mo limitation of cyanolichen growth and N fixation under natural conditions.

To better understand the effects of these nutrients and their interactions on lichen growth rates, we tested for P, Mo, and V limitation in the N-fixing cyanolichen *L. pulmonaria* with a one-year field experiment. We chose our study species, *Lobaria pulmonaria* (henceforth "*Lobaria*"), because it is ecologically significant, widely distributed, well studied, and easily transplanted. Not only is it of conservation concern as an old-growth associate in Europe, but also, it is sensitive to atmospheric deposition of sulfur and N (e.g., Geiser and Neitlich 2007). By determining fertilization effects on concentrations of Mo, V, and P in the lichen thallus, as well as thallus mass, natural abundance $\delta^{15}N$, total %N, and change in thallus N content over the 1-year experiment, we aimed to determine if Mo, V, or P limited or co-limited growth and N fixation in *Lobaria*. We also fertilized the non-N fixing green algal lichen *Usnea longissima* Ach. (henceforth "*Usnea*") to distinguish nutrient limitation patterns between fixing and non-fixing taxa. Tissue nutrient values were used to evaluate whether one-time applications of Mo, V, and P were retained by the lichen thallus for one year in the field. Natural abundance $\delta^{15}N$ values were used to assess the degree to which N in *Lobaria* originated from fixed atmospheric N_2 vs. ambient N in the environment. The few existing cyanolichen Mo limitation studies have applied micronutrients in the field or lab and measured acetylene reduction rates (a proxy for N-fixation) within 13 hours of treatment. This study is the first to document retention of a one-time application Mo and V over the course of a year-long field transplant.

Analysis of *Lobaria* growth rates in the current experiment revealed much faster growth than expected compared to a previous experiment from a nearby site. To reconcile this difference between studies, we compared climate and N deposition at the two study sites, and also analyzed archived samples from the prior experiment (2009) for P, Mo, V and N, to compare to our 2010–2011 data. These comparisons allow us to determine which factor(s) may explain wide differences in growth rates and nutrient responses in the two experiments.

METHODS

Specimen collection, fertilization, and transplant

Lobaria samples were collected at the experimental site where a *Quercus–Pseudotsuga* woodland transitions to grassland in the Coast Range foothills of Douglas County, Oregon (43.08226° N, 123.52207° W), southwest of Roseburg, near the town of Tenmile. Because *Usnea* was not present at the Tenmile site, this species was collected from an area with a similar climate in Polk County, Oregon (44.78688° N, 123.41364° W), in openings along a low-traffic country road. The road traverses foothills of the Coast Range, slightly above the Willamette Valley through forest dominated by *Pseudotsuga menziesii* with patches of hardwoods such as *Acer macrophyllum*, *Crataegus* spp. and *Cornus nuttallii*. Although we were unable to collect *Usnea* at Tenmile, the experimental site is within the natural range of habitat conditions for this species. *Usnea* transplants from an earlier study are thriving near the Tenmile site, even ten years after the initial transplant, and natural populations exist within 1 km of the site. We treated detached lobes of *Lobaria* and detached *Usnea* segments of uniform length with Mo, V, and P, then transplanted these specimens to the study site in Douglas County, Oregon, U.S.A. (43.08226° N, 123.52207° W), where they grew for one year with no further treatment.

Terminal lobes of similar size (3–6 cm long) were clipped from healthy *Lobaria* thalli lacking apothecia prior to treatment, resulting in air-dry pre-transplant weights of 0.1–0.4 g. *Usnea* strands were clipped to lengths of 20 cm prior to treatment, with weights of 0.1–0.2 g. All air-dried specimens were weighed before treatment to obtain initial biomass, using additional sacrificial samples to correct to an oven-dry basis following McCune et al. (1996). Both species were affixed to monofilament loops with clear silicone sealant and small plastic tags (Fig. 1).

Because commercial P fertilizer has been shown to contain Mo as a trace contaminant (McBride and Spiers 2001), we tested Mo and V concentrations in three replicates each of three types of P fertilizer; commercial grade triple superphosphate, reagent grade KH_2PO_4, and TraceSelect metal-free K_2HPO_4 before selecting a P salt for fertilization. Our direct measurements found that triple superphosphate contained on
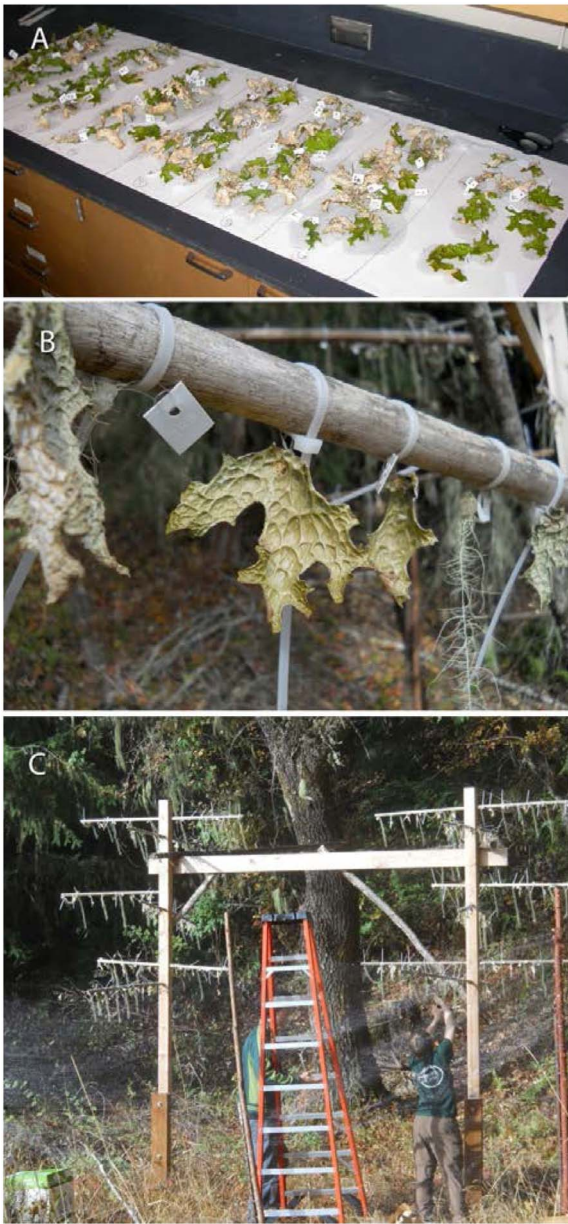
Fig. 1. Transplant construction and deployment. (A) Post-treatment *Lobaria* transplants air drying in the lab. (B) Field-dry *Lobaria* and *Usnea* transplants attached to wooden rack with zip ties. (C) Transplant racks at the Tenmile field site, October 2012.

average 9.2 µg/g Mo, while reagent grade and TraceSelect K_2HPO_4 contained 0.21 and 0.13 µg Mo/g, respectively. On average, triple superphosphate contained 597 µg/g V, while reagent grade and TraceSelect K_2HPO_4 contained 0.26 µg/g and 0.21 µg/V g, respectively. Thus, the TraceSelect

K_2HPO_4 used in this study contributed 0.0038 µM Mo and 0.012 µM V contamination in the P nutrient solutions. These trace contamination levels in the phosphorus treatments are 541 and 1037 times lower than our deliberate Mo and V additions respectively.

Treatments of Mo, V, P, Mo + P or V + P were applied to 20 randomly selected samples of each lichen species. Concentrations of Mo and V were selected in proportion to naturally occurring ratios of Mo:P or V:P reported for each lichen species (see "Clean Site Thresholds" of Geiser 1996). All treatments were applied by immersing the dry lichen lobes for 20 minutes in a nutrient solution made with de-ionized water. Phosphorous was applied as a 0.016 M solution of TraceSelect K_2HPO_4. Molybdenum was applied as a 2.1 µM solution of ACS Reagent Grade $Na_2MoO_42H_2O$. Vanadium was applied as 12.1 µM solution of ACS Reagent Grade Na_3VO_4. Combination treatments were produced by immersing lichens in a solution consisting of 2.1 µM Mo or 12.1 µM V in 0.016 M K_2HPO_4. Thirty control specimens of *Usnea* and 32 control specimens of *Lobaria* were submerged in de-ionized water in the same manner, for a total of 262 specimens (McCune et al. 1996; Fig. 1A).

After fertilization, samples were air-dried indoors overnight and transplanted into the field the following day. Three additional treated, air-dried specimens each of Mo, P, V and control *Lobaria* and three specimens each of Mo, P, and control *Usnea* were sacrificed and analyzed to determine if the nutrient additions had been retained. Specimens were transplanted outdoors on October 15, 2011 to the Tenmile site described above, where they were hung in a random configuration on wooden racks (Fig. 1B). Transplants racks were constructed from a central post and several outward radiating 2.5 cm diameter dowels positioned in all compass directions from the posts (Fig. 1C). Transplant racks were fenced to discourage herbivory by deer. Lichen transplants were recovered one year later, on October 13, 2012. Of the original 262 specimens, four *Lobaria* (2 Mo + P, 1 V + P, and 1 P-treated) were either missing entirely or visibly fragmented. The remaining 258 lichen transplants were retrieved in dry weather and transported to Oregon State University on the same day. Specimens were air-dried overnight and re-weighed.

Growth rates

Annual percent growth was calculated based on initial (M_0) and post-transplant (M_1) biomass measurements as $G = 100 \times (M_1 - M_0)/M_0$. Because lichen masses are highly sensitive to changes in humidity, moisture correction data were obtained during weighing by reweighing the same two specimens of each species after every tenth specimen. This allowed us to correct for changes in mass due to changes in humidity during the weighing process. After subtracting out the mass of the inert apparatus and correcting for ambient moisture, pre- and post-transplant weights were adjusted to a dry-mass basis using three oven-dried sacrificial lichens of each species (McCune et al. 1996). Chemical analyses were performed on subsets of the replicates used in the field transplant, after growth rates had been determined.

Total nitrogen and $\delta^{15}N$ analysis

Twenty-four control and 19 P-treated *Lobaria* and 12 control and 9 P-treated *Usnea* transplants from Tenmile were analyzed for stable isotope $\delta^{15}N$ and percent nitrogen (%N) at the Colorado Plateau Stable Isotope Laboratory, Northern Arizona University. Two specimens of Mo, V, V + P, and Mo + P treatments of each species were also measured for $\delta^{15}N$ and %N. Change in N content (ΔN content) was calculated using the measured final %N for each specimen and the average initial %N of archived untreated specimens from the 2011 field collection, along with the final and initial masses:

$$\Delta N \text{ content } = M_1(\text{final } \%N) \\ - M_0(\text{average initial } \%N).$$

An additional set of eight control and 23 P-treated *Lobaria* samples archived from McCune and Caldwell's (2009) P-fertilization experiment were analyzed for $\delta^{15}N$ and %N to allow cross-site comparisons and to compare N-fixation responses between transplant locations. In the 2009 study, *Lobaria* were collected and transplanted for a 1-year growth experiment near Wren, OR, approximately 193 km north of our transplant site near Tenmile, OR. Specimens from that study were stored in a cool, dark, dry cabinet after their final growth rates were measured in 2008.

We compared $\delta^{15}N$ and %N (as well as Mo, V, and P concentrations as described below) in three sets of un-fertilized *Lobaria*: (1) Tenmile control specimens from this study, transplanted in 2011 but receiving no nutrient additions; (2) non-transplanted, non-treated ("natural") Tenmile specimens, collected in 2011 but never returned to the field; and (3) Wren control specimens from McCune and Caldwell (2009). The comparison of nutrient concentrations in untreated *Lobaria* from both Tenmile and Wren allowed us to identify site-specific differences in nutrient availability that may have influenced treatment effects. To assess inter-annual variability in Mo, V, and P concentrations at an individual site, we collected new samples from Wren in 2012 and compared them to Wren samples collected in 2009.

Elemental analysis for Mo, V, and P

A subset of treated lichens from Tenmile (2–3 per treatment, per species for a total of 32) was analyzed for elemental concentrations after 1 year in the field (see Appendix: Table A1 for sample sizes). We also analyzed 3 P-treated and 5 control *Lobaria* archived from the 2009 Wren study, and 8 *Lobaria* collected from Wren in 2012. The small subsample size provides low power for detecting statistically significant differences, but does provide some indication of the degree to which nutrients provided by the initial treatments persisted through a year in the field.

In preparation for elemental analyses, all transplanted lichens were oven-dried overnight at 60°C and ground to a fine powder using a Retsch PM 100 agate ball-mill to avoid metal contamination. Before acquiring the agate ball mill, we also tested two other commonly-used grinding methods for potential Mo and V contamination; a Wiley mill and a steel bar roller mill. Mo concentrations in roller mill ground lichens were between 25 and 390 times higher than those ground by Wiley mill, which in turn had Mo concentrations double those of un-ground lichens (Appendix: Table A2). Both roller mill and Wiley mill ground *Lobaria* had more Mo contamination than did ground *Usnea,* most likely because *Lobaria* required longer grinding times. Neither Wiley nor roller mill produced V contamination for *Usnea.* The Wiley mill did not introduce V contamination for *Lobaria*, but *Lobaria* ground by roller mill had V concentrations 1.7 times higher than that of intact

specimens (Appendix: Table A2). We concluded it was necessary to use a grinder without steel components for studies of Mo and V in plant material.

All specimens were pre-digested at room temperature in microwave vessels for 12 hours prior to a heated, pressurized digestion at 240°C and 45 bar. Approximately 200 mg of lichen powder was microwave digested via Anton Paar Multiwave 3000 in 2 mL 30% Aristar Ultra-pure H_2O_2 and 6mL 70% Aristar Ultra-pure HNO_3 (Moreira et al. 2005). Molybdenum and V concentrations were measured by Inductively Coupled Plasma Mass Spectrometry (ICP-MS), and P concentrations were measured by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) at the W.M. Keck Collaboratory for Plasma Spectrometry at Oregon State University. Analyses were performed after digests were diluted 25-fold with 1% quartz distilled HNO_3. Stock solutions of Mo, V, and P were used as calibration standards. Two separate plant standards were measured during each analysis for quality control; one US. National Institute of Standards and Technology (NIST) plant standard reference material (SRM1515 apple leaf) and one International Atomic Energy Agency (IAEA) plant standard (IAEA-336 lichen). Analytical uncertainties for P, V, and Mo concentrations were approximately 5%.

Climate and nitrogen deposition

Both the Tenmile and Wren field sites are located in the Coast Range of Oregon and share a semi-Mediterranean climate defined by cool, wet winters and warm, dry summers. We used gridded weather interpolations (PRISM Climate Group 2014) for the 12 months corresponding to each transplant experiment to determine if temperature, precipitation, or dew point might relate to different growth responses and patterns of nutrient limitation.

Because N deposition rates can interact with P cycling in lichens (Hogan et al. 2010, Sparrius et al. 2013), we estimated total N deposition at the sites of both experiments. In particular, we expected that elevated N deposition might suppress N-fixation and growth at the Wren field site, given its closer proximity to developed areas of the Willamette Valley. To estimate N deposition we applied the Forest Inventory and

Analysis (FIA) lichen indicator method (Geiser and Neitlich 2007, Jovan 2008). Macrolichens were inventoried in one 0.4 ha plot centered on each transplant site and mean annual deposition of N was estimated from the resulting list of eutrophic and oligotrophic lichens, based on the tables and regression models summarized in McCune and Geiser (2009).

Statistical analysis

Treatment effects on growth rate, %N, ΔN content, and $\delta^{15}N$ were determined using ANOVA in R version 3.1.0 (R Core Team 2014). Because growth rates depended on initial mass for both species, we removed the effects of initial mass prior to testing for differences among treatments. Combining data from all treatments, growth rate (G) had a strong negative exponential relationship to initial mass (M_0), a trend not seen by McCune and Caldwell (2009). Thus, in the current study, thalli that were smaller prior to transplanting grew faster than thalli that were initially larger. This has been reported previously for *Lobaria* (Denison 1979) and other lichen species (Rhoades 1977, Benedict and Nash 1990). Initial and final masses of the lichen thalli were log transformed to linearize and to remove heteroscedasticity in that relationship. We then regressed final mass (log M_1) on initial mass (log M_0), which eliminated the curvilinear relationship between G and M_0 (*Lobaria*, $r^2 = 0.87$; *Usnea*, $r^2 = 0.95$). The residuals of those regressions were then used as adjusted growth rates. Adjusted growth rate can be thought of as the difference between observed and expected values of log(growth), based on initial thallus size. For example, an adjusted growth rate that is a difference of 0.05 corresponds to a growth rate that is 1.12 times (or 12%) greater than expected based on initial size alone. We tested for treatment differences with simple one-way ANOVA using the adjusted growth rates. Although we made no formal tests of normality and homogeneity of variances, adjusted growth rates were similarly variable within groups; boxplots of other response variables showed no extreme outliers or skewness.

When comparing Mo, V, and P concentrations, %N, and $\delta^{15}N$ among treatments and across the two transplant locations, small and variable sample sizes (2–3 lichens per treatment) do not allow for robust statistical analyses. For several

statistical analyses, lichens receiving a given treatment have been pooled together (as indicated in figures and figure captions). This increases sample size when comparing retention of an individual nutrient. For example, when assessing the retention of Mo in Mo-fertilized *Lobaria*, we considered Mo and Mo + P *Lobaria* together as "Mo-treated," while C, V, P, and V + P *Lobaria* were "non-Mo-treated." We therefore evaluated these data using one-way ANOVA.

RESULTS

Efficacy and retention of nutrient treatments

Immediately following nutrient application, P treatment increased P concentrations in *Lobaria* from medians of 2226 to 3002 µg/g (Fig. 2). Starting concentrations of P were much lower in *Usnea* than in *Lobaria*, and P treatment increased median P concentrations in *Usnea* from 458 to 1045 µg/g. Molybdenum treatment increased Mo concentrations from medians of 59 to 1252 ng/g in *Lobaria* and from 68 to 362 ng/g in *Usnea*. Vanadium treatment increased V concentrations in *Lobaria* from a median of 736–6662 ng/g. Post-fertilization V concentrations were not measured in treated *Usnea*. To ensure that the treatment handling did not introduce Mo, V, or P to control *Lobaria*, we also measured nutrient concentrations in natural samples that were not handled for the experiment (Fig. 2). We found no difference in Mo, V, or P concentrations in control and natural *Lobaria* ($p > 0.35$).

After 1 year in the field, P, Mo, and V concentrations increased further in treated and control specimens of both species (Fig. 2), rather than being diluted as the thalli grew ($p < 0.001$). Both P-treated and non P-treated *Lobaria* exhibited P gains of ~8% on average compared to pre-transplant concentrations, while both Mo and non-Mo treated *Lobaria* exhibited Mo gains of ~70% on average. *Lobaria* that were not treated with V exhibited average V increases of ~50% compared to pre-transplant concentrations, while V-treated *Lobaria* exhibited average V increases of ~30%.

P-treated *Lobaria* remained higher in P (median = 3347 µg P/g) than non-P-treated *Lobaria* (median = 2303 µg P/g; Fig. 2). On average, P-treated *Lobaria* contained 33% more P than their non-treated counterparts. Similarly, P concentra-

tions in P-treated *Usnea* were on average 131% higher than non-P treated *Usnea* (medians of 1431 µg P/g and 483 µg P/g, respectively). After one year, Mo-treated *Lobaria* contained 17 times more Mo on average (median = 4663 ng Mo/g) than non-Mo treated *Lobaria* (202 ng Mo/g). Mo–treated *Usnea* also remained higher (median = 698 ng Mo/g) than non-Mo-treated *Usnea* (320 ng Mo/g). Mo-treated *Usnea* contained nearly 3 times more Mo on average than their non-treated counterparts. Finally, V-treated *Lobaria* displayed higher V after one year in the field (median = 9838 ng V/g) than non-V-treated *Lobaria* (1292 ng V/g). Vanadium treated *Lobaria* and *Usnea* contained an average of five and two times more V respectively than non-treated counterparts of the same species.

Annual average growth rates

Unadjusted growth rates for all lichens were high regardless of treatment, averaging 83% for *Lobaria* and 108% for *Usnea* over the course of the 1-year experiment. All 130 *Usnea* and 32 of 128 *Lobaria* at least doubled in size over the course of the year. These high growth rates were evaluated with a repeat experiment using new transplants collected at Tenmile without nutrient treatment, but on the same racks; these showed considerably lower growth rates during October 2013–October 2014 than at the same site 2 years earlier (*Lobaria* mean growth rate: 20%, $n = 24$; *Usnea* 29%, $n = 18$).

Adjusted growth rates did not differ significantly for control, P, Mo, Mo + P, and V treatments of *Lobaria* (*p*-value for all comparisons > 0.07). Neither Mo + P nor V + P treated *Lobaria* grew faster than the controls (Fig. 3). V+P treated *Lobaria* grew less than control *Lobaria* ($p = 0.005$), but the growth rate of that treatment was only 3% less than the overall average growth rate (Fig. 3). No treatment effect on adjusted growth rate was observed for *Usnea* ($p = 0.89$; Fig. 3).

Thallus nitrogen and $\delta^{15}N$

Lobaria and *Usnea* differed in $\delta^{15}N$ by 10‰ ($p < 0.001$), with *Lobaria* averaging −1.60‰ (±SD = 1.23‰) and *Usnea* averaging −11.69‰ (±SD = 5.04‰). $\delta^{15}N$ values of *Lobaria* at Tenmile did not differ between treatments ($p = 0.60$; Fig. 4), nor did total thallus N ($p = 0.95$; averages ranged from 2.1% to 2.2% N). We also found no
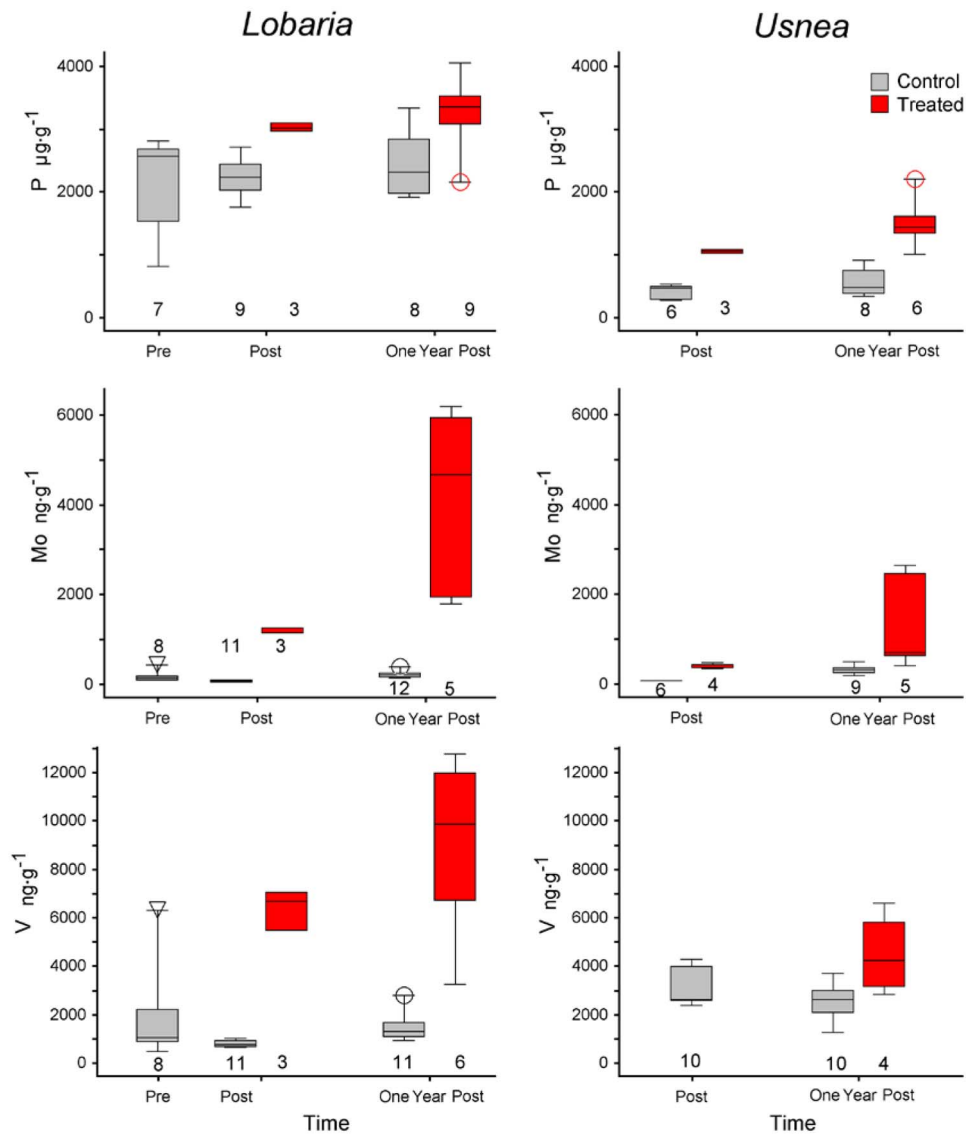
Fig. 2. Efficacy and retention of nutrient treatments. Concentrations of P, Mo, and V in nutrient-treated (red) and controls (gray) for *Lobaria* (left) and *Usnea* (right) at three dates. "Pre" was after preparation of transplants but before treatment; "Post" was immediately after treatment with the indicated nutrient, and demonstrates the initial efficacy of the treatment; "One Year Post" was after one year in the field and demonstrates retention of the treatments. Sample sizes are indicated below each box. Pre-treatment data were not obtained for *Usnea*. Boxes represent the 25th and 75th percentiles, horizontal lines within each box represent medians, and whiskers indicate the 5th and 95th percentiles; symbols indicate outliers that fall more than 1.5 times the inter-quartile range from the median.

difference in ΔN content between control and P-treated *Lobaria* at Tenmile ($p = 0.16$). To understand the lack of definitive growth and ΔN content responses among treatments, we compared $\delta^{15}N$ and %N in our study at Tenmile

with *Lobaria* samples from the McCune and Caldwell (2009) study at Wren, where P treatment doubled *Lobaria* growth. Average $\delta^{15}N$ values in control and P-treated *Lobaria* from Tenmile were similar (Fig. 4; control = −1.7‰,
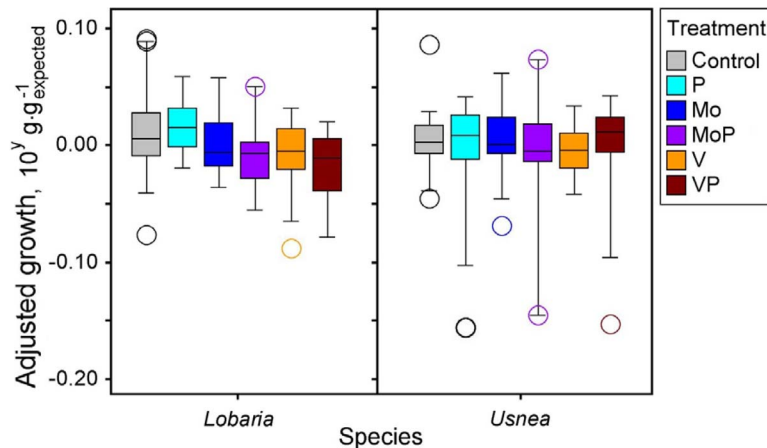
Fig. 3. Adjusted growth rates of *Lobaria* and *Usnea* for each treatment. Growth residuals are equivalent to 10^y where y is the ratio of final mass to expected final mass, based on initial mass. For example, a residual value of 0.05 corresponds to a growth rate that is 1.12 times (or 12%) greater than expected based on initial size alone. Boxes represent the 25th and 75th percentiles, horizontal lines within each box represent medians, and whiskers indicate the 5th and 95th percentiles; symbols indicate outliers that fall more than 1.5 times the inter-quartile range from the median.

P-treated = −1.5‰) but slightly higher (i.e., closer to the atmospheric value of 0‰) than both control and P-treated *Lobaria* from the Wren study (control = −1.9‰, P-treated = −2.1‰; $p < 0.002$ for differences among the four groups). P-treated and control *Lobaria* did not differ in $\delta^{15}N$ within each study site (Wren, $p = 0.52$; Tenmile, $p = 0.53$).

Total thallus N (%) in *Lobaria* was generally similar across all treatments and sites. In *Lobaria* at Wren however, %N averaged slightly (0.2%) higher in controls than with P treatment ($p = 0.030$).

Local climate and nitrogen deposition rates

Estimated average monthly dew point and average monthly maximum and minimum temperatures for Wren (1 October 2007–31 October 2008) and Tenmile (1 October 2011–31 October 2012) were similar between sites. Total precipitation for the 13 month periods was nearly the same (Wren, 1077 mm; Tenmile, 1122 mm), but some potentially important seasonal differences emerged. Previous work near the Wren site found that growth of *Lobaria* peaked in March and April, while no consistent growth occurred during the dry summer months and early autumn (Muir et al. 1997). For February through

May, total precipitation at Wren was 257 mm in 2007–2008, while Tenmile received 511 mm during February–May of 2010–2011.

We estimated total annual N deposition for Tenmile and Wren sites at 3.6 and 3.0 kg N·ha^{-1}·yr^{-1}, respectively, based on lichen surveys of communities containing 45 macrolichen species at Wren and 38 species at Tenmile. McCune and Geiser (2009) estimated confidence limits for this method as a 70% chance of being within 1 kg N·ha^{-1}·yr^{-1} of the total N deposition. The two sites shared 26 species. Both sites supported a preponderance of oligotrophic (N-intolerant) and mesotrophic species, with a few eutrophic species. Thus, based on this method, atmospheric N deposition was similar between the two sites.

Naturally occurring nutrient concentrations

Control *Lobaria* from Tenmile averaged 60% higher P concentrations than control *Lobaria* from Wren (2431 µg/g versus 1474 µg/g, $p = 0.024$; Fig. 5). Similarly, natural *Lobaria* from Tenmile (2043 µg/g, $n = 5$) were higher in P than natural *Lobaria* from Wren (1299 µg/g, $n = 5$; $p = 0.018$). However, natural and control *Lobaria* P concentrations from Tenmile were similar to P-fertilized *Lobaria* from Wren (McCune and Caldwell 2009; both $p > 0.27$) and were at least twice those of untreated
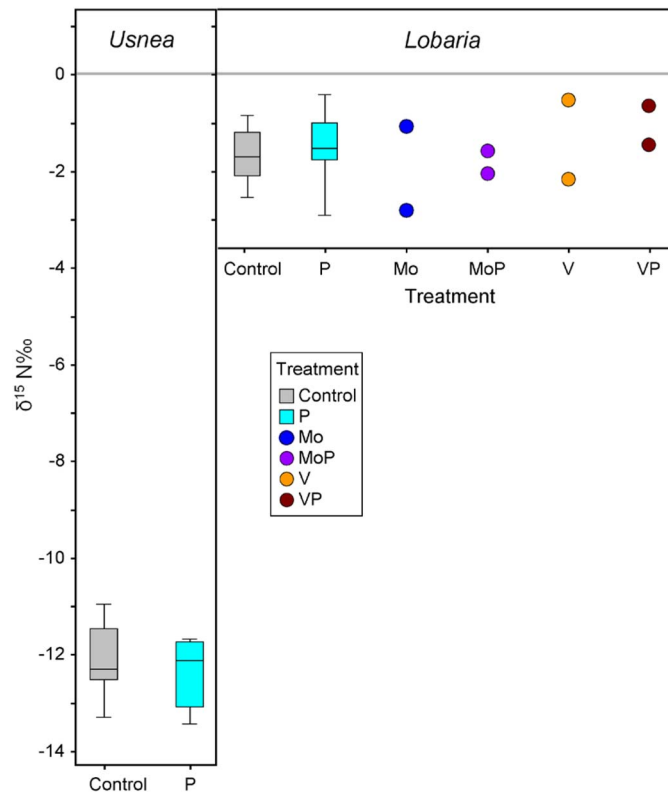
Fig. 4. Thallus δ^{15}N for *Lobaria* and *Usnea* at Tenmile. Treatments for *Lobaria* include control ($n = 24$), phosphorus ($n = 19$), molybdenum (Mo), vanadium (V), and the combinations Mo + P and V + P ($n = 2$ each). Treatments for *Usnea* include control ($n = 12$) and phosphorus ($n = 8$). Boxplots represent the 25th and 75th percentiles, horizontal lines within each box represent medians, and whiskers indicate the 5th and 95th percentiles; no outliers fall more than 1.5 times the inter-quartile range from the median. Individual data points instead of boxplots are shown for treatments with $n = 2$.

specimens from that prior study ($p = 0.005$). Mean concentration of P in control *Lobaria* from Tenmile did not differ from natural, un-transplanted archived *Lobaria*, which were stored in the lab ($p = 0.658$). Nor did mean P concentration of natural *Lobaria* collected from Wren in 2012 differ from controls from the 2007 field study ($p = 0.508$).

Molybdenum concentrations were similar between sites but higher in control than natural (untreated) *Lobaria* at Wren ($p = 0.02$). Control and natural Mo concentrations in *Lobaria* from Tenmile were 185 and 168 µg/g, respectively; from Wren, 207 and 70 µg/g, respectively. Vanadium concentrations in *Lobaria* were similar among controls and natural specimens from both sites (Tenmile controls = 1537; Tenmile naturals = 1230 µg/g; Wren naturals = 1589 µg/g), except

that V in Wren controls (2994 µg/g) was notably high ($p < 0.001$).

DISCUSSION

The lack of a strong growth response for fertilized *Lobaria* in our experiment at Tenmile contrasts with the doubling of *Lobaria* growth rates with P fertilization observed previously at the Wren site by McCune and Caldwell (2009), using the same P-treatment concentrations, on the same species, in the same climate regime. Although the favorable weather and consistently high growth rates we observed at Tenmile should correspond with high P-demand, P did not limit *Lobaria* growth in the current experiment. We attribute the fast growth and lack of a P limitation observed in our Tenmile experiment
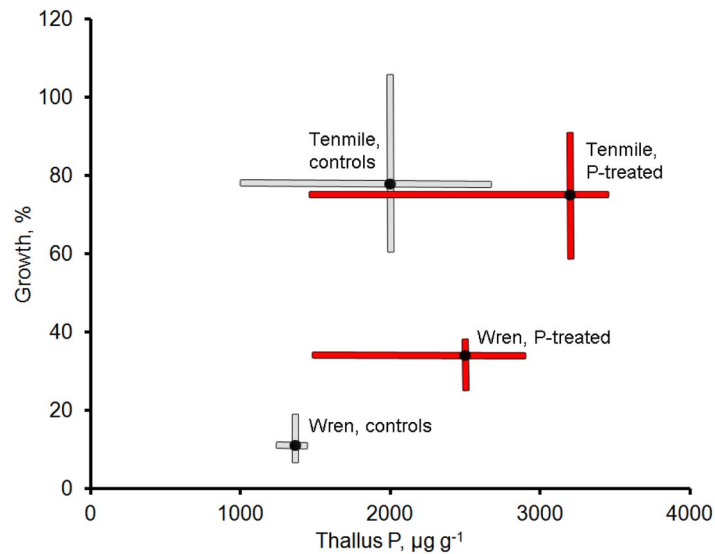
Fig. 5. Annual percent growth of *Lobaria* in relation to thallus P concentration (μg/g), combining results from two experiments in different locations: Tenmile (this paper) and Wren (McCune and Caldwell 2009). Bars indicate interquartile ranges; black dots are medians. Phosphorus treatments (red bars) were the same for both experiments. Wren specimens are from the 2009 season only and do not include 2012 data for which growth rates are not available.

to favorable weather conditions during the transplant year, and high levels of available P at the Tenmile site. Our results provide evidence that annual growth rates of *Lobaria* depend on both site-specific differences in background P and weather conditions in particular years.

Nutrient retention

After one year in the field, fertilization treatments were retained in the lichen thallus in both *Lobaria* and *Usnea*. Furthermore, additional Mo, V, and P accumulated over the duration of the transplant in both species. The retention and accumulation of Mo, V, and P does not explain why these nutrients failed to stimulate *Lobaria* growth, but it does suggest nutrient assimilation regardless of the lichen's need for nitrogenase co-factors. The passive accumulation of metals in lichen thalli is well documented in studies used to monitor pollution (e.g., Addison and Puckett 1980, Garty 2001, and others).

Effects of climate and N deposition

We considered differences in climate and N deposition as among the leading possible explanations for the difference in results of the two experiments. Differences in N deposition could influence lichen growth directly due to cyano-lichen sensitivity to ambient N, as well as indirectly via interactions between N and P (e.g., Hogan et al. 2010, Johansson et al. 2011, Sparrius et al. 2013). Although high ambient N can cause down-regulation of N-fixation, both sites had similar estimated N deposition, suggesting that deposition did not alter response to P-fertilization at one site versus the other. We also found that $\delta^{15}N$ of *Lobaria* at both Tenmile and Wren field sites was close to the atmospheric value of 0‰, consistent with physiological studies indicating that *Lobaria* can meet much of its N demand via fixation (Antoine 2004). If we assume that *Usnea* records the $\delta^{15}N$ of ambient deposition and *Lobaria* fixes N_2 without fraction-ation, then simple end-member mixing suggests that *Lobaria* derives ~86% of its N from fixation at Tenmile. Because *Usnea* was not included in the 2009 study at Wren we are unable to create a similar mixing model for that site. Small differences in $\delta^{15}N$ values of *Lobaria* at the two sites may reflect differences in N fixation, but additional information on $\delta^{15}N$ of other potential N sources is needed to evaluate this possibility. In

contrast, non-N-fixing *Usnea* displayed low $\delta^{15}N$ values ($\sim-12‰$), which probably reflect uptake of NH_3 emissions that are relatively common in this agricultural region (USDA Forest Service 2002, Felix et al. 2013, Felix et al. 2014).

Faster growth of *Lobaria* at Tenmile than Wren may be attributable to differences in growing-season precipitation. Although temperature, dew point, and total annual precipitation were comparable, the two sites differed in the distribution of annual rainfall. The scale and duration of individual rain events cannot be assessed. However, the Tenmile site received about 511 mm of precipitation during the peak growing season (February–May) of that year, about twice as much as during the corresponding period at Wren (257 mm) during that experiment. Overall, mean annual growth rates for *Lobaria* of all treatments from the Tenmile experiment are among the highest reported for *Lobaria* in Pacific Northwest of North America. The growth rates reported in this study are 20–50% higher than growth rates of P-treated *Lobaria* reported for the Wren site (McCune and Caldwell 2009). They are also higher than the annual growth rates two years later at the same site, and higher than growth rates reported for any other year-long study in this region (e.g., McCune et al. 1996, Muir et al. 1997, Antoine and McCune 2004). The high growth rates in the Tenmile experiment suggest that minimal environmental constraints (including nutrients) limited the expression of a fertilizer response at this site.

Environmental and physiological effects

Lobaria growth rates can be affected by light and moisture (Gauslaa et al. 2006, Gauslaa 2014). Although we did not measure incident light at either site, we assume that our artificial transplant racks were exposed to more direct light than epiphytes growing on branches. Thus, it is possible that the difference in light regime between Tenmile (where lichens were hung from racks) and Wren (where lichens were hung from tree branches) contributed to the difference in growth response between sites. Additionally, canopy cover can reduce nocturnal cooling and increase dewfall in branch microenvironments, both of which have been show to increase growth in *Lobaria* (Bidussi et al. 2013). However, our results show higher growth rates in Tenmile, where lichens were more exposed on transplant racks. We cannot, therefore, rule out the possibility that the overall faster growth at Tenmile than Wren was a result of differences in light environment and moisture retention between experiments. Neither of these, however, can explain the lack of a P treatment effect at Tenmile versus a strong P effect at Wren.

Recent research suggests that there is a trade-off in *Lobaria* between thallus growth, measured both as dry matter gain and increased surface area, and the production of asexual reproductive structures (soralia and/or isidia; Gauslaa 2006). Although we were able to limit our transplant specimens to those lobes lacking sexual reproductive structures (apothecia), the quantity of soralia varied in our specimens. We cannot rule out the influence of soralia production on the observed growth rates, though our large sample size and random application of treatments should have served to minimize that effect.

Molybdenum retention and potential sources

The lack of a significant effect of V and Mo, whether alone or in combination with P, on *Lobaria* growth rates suggests that neither micronutrient was limiting at Tenmile. However, the gains in P, Mo, and V in *Lobaria* transplants after one-year at the Tenmile site suggest that this environment is naturally rich in those nutrients. The average concentration of Mo in non-Mo-fertilized *Lobaria* from Tenmile was 227 ng/g 1 yr after transplanting. When this concentration is distributed over the thallus of a lichen with an average surface area of 24 cm^2 and an average dry mass of 0.3–0.5 g, Tenmile lichens contained 2.8–4.7 ng Mo per cm^2 of thallus. Using the average Mo concentration in openfall rainwater measured across the Coast Range (17 pg/g; Marks 2015) and the approximately 1122 mm of precipitation that occurred from 2011–2012 at the Tenmile site, we determined that this site could have received 1.9 ng Mo per cm^2 of thallus in that year. Although cyanolichens beneath the forest canopy may not directly intercept all of the incoming rainfall, Mo concentrations of this magnitude could be an important Mo source over several years of thallus growth, in addition to an unknown amount of Mo derived from dry deposition or leached from the canopy as part of throughfall. Variations in the quantity and

distribution of annual rainfall may affect not only thallus hydration, but also the amount of Mo deposition and retention.

Variations in phosphorus availability

Our data clearly show that naturally available P is more abundant at Tenmile than at Wren. Phosphorus concentrations in natural and control samples of *Lobaria* at Tenmile were on average 1.67 times higher than in natural and control samples from Wren. Large differences in naturally available P between sites could easily affect responses to P fertilization. P fertilization at Wren considerably boosted P in lichen thalli relative to controls, yet the fertilized P values were similar to those occurring naturally at Tenmile. Combining results from the two experiments, *Lobaria* grew fastest when internal P concentrations reached ~2000 μg/g. Above this concentration, additional P did not result in additional growth, suggesting that any P limitation had been overcome. It therefore appears that growth of *Lobaria* at Wren was P-limited, while growth at Tenmile was not.

Lichens are subjected to potential P sources that vary greatly in magnitude, including local and regional atmospheric deposition, and canopy leachate. Differences in P availability among sites may be caused by P recycling in organic matter of the dominant tree species, geomorphology, canopy cover, the presence or absence of edge effects, proximity to intensive agriculture, or P content of soil and bedrock (Walker and Syers 1976, Benner 2011, Cross and Perakis 2011, Mage and Porder 2013). More research would be necessary to determine the factors responsible for the differences in available P levels at our sites. In addition to geologic differences among sites, atmospheric inputs of P are both spatially and temporally variable in western Oregon (Fredriksen 1975); preliminary data suggest that differences in soil P between sites were not responsible for differences in lichen P contents. Analysis of lichen P concentrations may help assess whether P is locally limiting in cyanolichens and reconcile the differing results of existing cyanolichen fertilization experiments.

Literature Cited

Addison, P. A., and K. J. Puckett. 1980. Deposition of atmospheric pollutants as measured by lichen element content in the Athabasca oil sands area. Canadian Journal of Botany 58:2323–2334.

Antoine, M. E. 2004. An ecophysiological approach to quantifying nitrogen fixation by *Lobaria oregana*. Bryologist 107:82–87.

Antoine, M. E., and B. McCune. 2004. Contrasting fundamental and realized ecological niches with epiphytic lichen transplants in an old-growth *Pseudotsuga* forest. Bryologist 107:163–172.

Barron, A. R., N. Wurzburger, J. P. Bellenger, S. J. Wright, A. M. L. Kraepiel, and L. O. Hedin. 2008. Molybdenum limitation of asymbiotic nitrogen fixation in tropical forest soils. Nature Geoscience 2:42–45.

Bellenger, J. P., T. Wichard, Y. Xu, and A. M. Kraepiel. 2011. Essential metals for nitrogen fixation in a free-living N_2-fixing bacterium: chelation, homeostasis and high use efficiency. Environmental Microbiology 13:1395–1411.

Benedict, J. B., and T. H. Nash. 1990. Radial growth and habitat selection by morphologically similar chemotypes of *Xanthoparmelia*. Bryologist 93:319–327.

Benner, J. W. 2011. Epiphytes preferentially colonize high-phosphorus host trees in unfertilized Hawaiian montane forests. Bryologist 114:335–345.

Benner, J. W., S. Conroy, C. K. Lunch, N. Toyoda, and P. M. Vitousek. 2007. Phosphorus fertilization increases the abundance and nitrogenase activity of the cyanolichen *Pseudocyphellaria crocata* in Hawaiian montane forests. Biotropica 39:400–405.

Benner, J. W., and P. M. Vitousek. 2007. Development of a diverse epiphyte community in response to phosphorus fertilization. Ecology Letters 10:628–636.

Bidussi, M., Y. Gauslaa, and K. A. Solhaug. 2013. Prolonging the hydration and active metabolism from light periods into nights substantially enhances lichen growth. Planta 237:1359–1366.

Bidussi, M., T. Goward, and Y. Gauslaa. 2013. Growth and secondary compound investments in the epiphytic lichens *Lobaria pulmonaria* and *Hypogymnia occidentalis* transplanted along an altitudinal gradient in British Columbia. Botany 91:621–630.

Campbell, J., G. E. Bradfield, C. E. Prescott, and A. L. Fredeen. 2010. The influence of overstory *Populus* on epiphytic lichens in subboreal spruce forests of British Columbia. Canadian Journal of Forest Research 40:143–154.

Clark, K. L., N. M. Nadkarni, D. Schaefer, and H. L. Gholz. 1998. Atmospheric deposition and net retention of ions by the canopy in a tropical montane forest, Monteverde, Costa Rica. Journal of Tropical Ecology 14:27–45.

Crittenden, P. D., and K. A. Kershaw. 1978. Discovering role of lichens in nitrogen cycle in boreal arctic ecosystems. Bryologist 81:258–267.

Cross, A., and S. S. Perakis. 2011. Tree species and soil nutrient profiles in old-growth forests of the Oregon Coast Range. Canadian Journal of Forest Research 41:195–210.

Darnajoux, R., J. Constantin, J. Miadlikowska, F. Lutzoni, and J.-P. Bellenger. 2014. Is vanadium a biometal for boreal cyanolichens? New Phytologist 202:1469–8137.

Denison, W. C. 1979. *Lobaria oregana*, a nitrogen-fixing lichen in old-growth Douglas fir forests. Pages 266–275 *in* J. C. Gordon, C. T. W., , D. A. Perry, editors. Symbiotic nitrogen fixation in the management of temperate forests. Oregon State University School of Forestry Oregon State University, Corvallis, Oregon, USA.

Eady, R. R. 1996. Structure-function relationships of alternative nitrogenases. Chemical Reviews 96:3013–3030.

Einsle, O., F. A. Tezcan, S. L. A. Andrade, B. Schmid, M. Yoshida, J. B. Howard, and D. C. Rees. 2002. Nitrogenase MoFe-protein at 1.16 angstrom resolution: a central ligand in the FeMo-cofactor. Science 297:1696–1700.

Elbert, W., B. Weber, S. Burrows, J. Steinkamp, B. Budel, M. O. Andreae, and U. Poschl. 2012. Contribution of cryptogamic covers to the global cycles of carbon and nitrogen. Nature Geoscience 5:459–462.

Felix, J. D., E. M. Elliott, T. J. Gish, R. Maghirang, L. Cambal, and J. Clougherty. 2014. Examining the transport of ammonia emissions across landscapes using nitrogen isotope ratios. Atmospheric Environment 95:563–570.

Felix, J. D., E. M. Elliott, T. J. Gish, L. L. McConnell, and S. L. Shaw. 2013. Characterizing the isotopic composition of atmospheric ammonia emission sources using passive samplers and a combined oxidation-bacterial denitrifier approach. Rapid Communications in Mass Spectrometry 27:2239–

2246.

Fredriksen, R. L. 1975. Nitrogen, phosphorus and particulate matter budgets of five coniferous forest ecosystems in the western Cascades Range, Oregon. Dissertation. Oregon State University, Corvallis, Oregon, USA.

Galloway, J. N., et al. 2003. The nitrogen cascade. Bioscience 53:341–356.

Garty, J. 2001. Biomonitoring atmospheric heavy metals with lichens: theory and application. Critical Reviews in Plant Sciences 4:20.

Gauslaa, Y. 2006. Trade-off between reproduction and growth in the foliose old forest lichen *Lobaria pulmonaria*. Basic and Applied Ecology 7:455–460.

Gauslaa, Y. 2014. Rain, dew, and humid air as drivers of morphology, function and spatial distribution in epiphytic lichens. Lichenologist 46:1–16.

Gauslaa, Y., M. Lie, K. A. Solhaug, and M. Ohlson. 2006. Growth and ecophysiological acclimation of the foliose lichen *Lobaria pulmonaria* in forests with contrasting light climates. Oecologia 147:406–416.

Geiser, L. H. 1996. Provisional element analysis thresholds. USDA Forest Service National Lichens and Air Quality Database and Clearinghouse. http://gis.nacse.org/lichenair/index.php?page=cleansite

Geiser, L. H., and P. N. Neitlich. 2007. Air pollution and climate gradients in western Oregon and Washington indicated by epiphytic macrolichens. Environmental Pollution 145:203–218.

Giesen, T. W., S. S. Perakis, and K. Cromack, Jr. 2008. Four centuries of soil carbon and nitrogen change after stand-replacing fire in a forest landscape in the western Cascade Range of Oregon. Canadian Journal of Forest Research 38:2455–2464.

Goward, T., and A. Arsenault. 2000. Cyanolichen distribution in young unmanaged forests: A drip-zone effect? Bryologist 103:28–37.

Gunther, A. J. 1989. Nitrogen fixation by lichens in a subarctic Alaskan watershed. Bryologist 92:202–208.

Hodkinson, B. P., J. L. Allen, L. L. Forrest, B. Goffinet, E. Sérusiaux, Ó. S. Andrésson, V. Miao, J.-P. Bellenger, and F. Lutzoni. 2014. Lichen-symbiotic cyanobacteria associated with *Peltigera* have an alternative vanadium-dependent nitrogen fixation system. European Journal of Phycology 49:11–19.

Hogan, E. J., G. Minnullina, R. L. Smith, and P. D. Crittenden. 2010. Effects of nitrogen enrichment on phosphatase activity and nitrogen: phosphorus relationships in *Cladonia portentosa*. New Phytologist 186:911–925.

Holub, S. M., and K. Lajtha. 2004. The fate and retention of organic and inorganic ^{15}N nitrogen in an old-growth forest soil in western Oregon. Ecosystems 7:368–380.

Horstmann, J. L., W. C. Denison, and W. B. Silvester.

1982. $^{15}N_2$ fixation and molybdenum enhancement of acetylene-reduction by *Lobaria* spp. New Phytologist 92:235–241.

Jean, M.-E., K. Phalyvong, J. Forest-Drolet, and J. P. Bellenger. 2013. Molybdenum and phosphorus limitation of asymbiotic nitrogen fixation in forests of eastern Canada: influence of vegetative cover and seasonal variability. Soil Biology and Biochemistry 67:140–146.

Johansson, O., J. Olofsson, R. Giesler, and K. Palmqvist. 2011. Lichen responses to nitrogen and phosphorus additions can be explained by the different symbiont responses. New Phytologist 191:795–805.

Jovan, S. 2008. Lichen bioindication of biodiversity, air quality, and climate: baseline results from monitoring in Washington, Oregon, and California. General Technical Report PNW-GTR-737. USDA Forest Service, Pacific Northwest Research Station, Portland, Oregon, USA.

Kurina, L. M., and P. M. Vitousek. 1999. Controls over the accumulation and decline of a nitrogen-fixing lichen, *Stereocaulon vulcani*, on young Hawaiian lava flows. Journal of Ecology 87:784–799.

LeBauer, D. S., and K. K. Treseder. 2008. Nitrogen limitation of net primary productivity in terrestrial ecosystems is globally distributed. Ecology 89(2):371–379.

Mage, S. M., and S. Porder. 2013. Parent material and topography determine soil phosphorus status in the Luquillo Mountains of Puerto Rico. Ecosystems16:284-294.

Marks, J. A., S. S. Perakis, E. K. King, and J. Pett-Ridge. 2015. Soil organic matter regulates molybdenum storage and mobility in forests. Biogeochemistry. doi: 10.1007/s10533-015-0121-4

McBride, M. B., and G. Spiers. 2001. Trace element content of selected fertilizers and dairy manures as determined by ICP-MS. Communications in Soil Science and Plant Analysis 32:139–156.

McCune, B., and B. A. Caldwell. 2009. A single phosphorus treatment doubles growth of cyanobacterial lichen transplants. Ecology 90:567–570.

McCune, B., C. C. Derr, P. S. Muir, A. Shirazi, S. C. Sillett, and W. J. Daly. 1996. Lichen pendants for transplant and growth experiments. Lichenologist 28:161–169.

McCune, B., and L. Geiser. 2009. Macrolichens of the Pacific Northwest. Second edition. Oregon State University Press, Corvallis, Oregon, USA.

Molina, M., F. Aburto, R. Calderon, M. Cazanga, and M. Escudey. 2009. Trace element composition of selected fertilizers used in Chile: phosphorus fertilizers as a source of long-term soil contamination. Soil and Sediment Contamination 18:497–511.

Moreira, F. R., R. M. Borges, and R. M. Oliveira. 2005. Comparison of two digestion procedures for the determination of lead in lichens by electrothermal

atomic absorption spectrometry. Spectrochimica Acta Part B: Atomic Spectroscopy 60:755–758.

Muir, P. S., A. M. Shirazi, and J. Patrie. 1997. Seasonal growth dynamics in the lichen *Lobaria pulmonaria*. Bryologist 100:458–464.

Pike, L. H., D. M. Tracy, M. A. Sherwood, and D. Nielsen. 1972. Estimates of biomass and fixed nitrogen of epiphytes from old-growth Douglas fir. Pages 177–187 *in* J. F. Franklin, L. J. Dempster, and R. H. Waring, editors. Research on coniferous forest ecosystems. Pacific Northwest Forest and Range Experiment Station, Portland, Oregon, USA.

PRISM Climate Group. 2014. Oregon State University. http://prism.oregonstate.edu

R Core Team. 2014. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Reed, S. C., C. C. Cleveland, and A. R. Townsend. 2013. Relationships among phosphorus, molybdenum and free-living nitrogen fixation in tropical rain forests: results from observational and experimental analyses. Biogeochemistry 114:135–147.

Rhoades, F. M. 1977. Growth-rates of lichen *Lobaria oregana* as determined from sequential photographs. Canadian Journal of Botany 55:2226–2233.

Silvester, W. B. 1989. Molybdenum limitation of asymbiotic nitrogen-fixation in forests of Pacific Northwest America. Soil Biology and Biochemistry 21:283–289.

Simpson, F. B., and R. H. Burris. 1984. A nitrogen pressure of 50 atmospheres does not prevent evolution of hydrogen by nitrogenase. Science 224:1095–1097.

Sparrius, L. B., A. M. Kooijman, and J. Sevink. 2013. Response of inland dune vegetation to increased nitrogen and phosphorus levels. Applied Vegetation Science 16:40–50.

Stewart, G. R., S. Schmidt, L. L. Handley, M. H. Turnbull, P. D. Erskine, and C. A. Joly. 1995. ^{15}N natural-abundance of vascular rain-forest epiphytes: implications for nitrogen-source and acquisition. Plant Cell and Environment 18:85–90.

USDA Forest Service. 2002. Understanding air pollutants and air pollution effects on lichens: a Pacific Northwest perspective. Depositional nitrogen. National Lichens and Air Quality Database and Clearinghouse. http://gis.nacse.org/lichenair/?page=airpollution#Nemissions

van Groenigen, K. J., J. Six, B. A. Hungate, M. A. de Graaff, N. van Breemen, and C. van Kessel. 2006. Element interactions limit soil carbon storage. Proceedings of the National Academy of Sciences 103:6571–6574.

Vitousek, P. M., K. Cassman, C. Cleveland, T. Crews, C. B. Field, N. B. Grimm, R. W. Howarth, R. Marino, L. Martinelli, E. B. Rastetter, and J. I. Sprent. 2002. Towards an ecological understanding

of biological nitrogen fixation. Biogeochemistry 57:1–45.

Vitousek, P. M., D. N. L. Menge, S. C. Reed, and C. C. Cleveland. 2013. Biological nitrogen fixation: rates, patterns, and ecological controls in terrestrial ecosystems. Philosophical Transactions of the Royal Society B 368(1621):20130119.

Walker, T. W., and J. K. Syers. 1976. Fate of phosphorus during pedogenesis. Geoderma 15:1–19.

Weiss, M., S. E. Hobbie, and G. M. Gettel. 2005. Contrasting responses of nitrogen-fixation in arctic lichens to experimental and ambient nitrogen and phosphorus availability. Arctic Antarctic and Alpine Research 37:396–401.

Wurzburger, N., J. P. Bellenger, A. M. Kraepiel, and L. O. Hedin. 2012. Molybdenum and phosphorus interact to constrain asymbiotic nitrogen fixation in tropical forests. PLoS ONE 7:e33710.

SUPPLEMENTAL MATERIAL

APPENDIX

Table A1. Sample size of growth rate and chemical analyses for treated (Mo, V, P, Mo + P, V + P, and C) and naturally occurring (N) *Lobaria* (*L*) and *Usnea* (*U*) collected from Tenmile and Wren (2009 and 2012). For some statistical analyses, replicates for multiple treatments are pooled. Under these circumstances, sample size is indicated in figures or figure captions. The designation "n/a" indicates that samples were not collected for that combination of species and treatment, or analysis. Results are reported by species as *Lobaria*; *Usnea*.

Site and treatment	Growth rate	%N	$\delta^{15}N$	P	Mo	V
Tenmile						
C	30; 32	24; 12	24; 12	3; 3	3; 3	3; 3
P	19; 20	19; 9	19; 9	3; 2	3; 2	3; 2
Mo + P	18; 20	2; 2	2; 2	3; 3	3; 3	3; 3
Mo	20; 20	2; 2	2; 2	2; 3	2; 3	2; 3
V + P	19; 20	2; 2	2; 2	3; 2	3; 2	3; 2
V	20; 20	2; 2	2; 2	3; 2	3; 2	3; 2
Wren 2009						
C	11;† n/a	8; n/a	8; n/a	5; n/a	5; n/a	5; n/a
P	25;† n/a	23; n/a	23; n/a	3; n/a	3; n/a	3; n/a
Wren 2012						
N	n/a; n/a	0; n/a	0; n/a	8; n/a	8; n/a	8; n/a

† Values previously published in McCune and Caldwell (2009).

Table A2. Comparison of Mo and V concentrations in the lichens *Lobaria* and *Usnea* in unground and ground samples using a Wiley mill and a roller mill. The Wiley mill added between 50 and 100 ppb Mo per sample, while the roller mill added 3–47 ppm Mo per sample.

Treatment	Average of replicates		Average of replicates		Added by mill Mo (ppm)
	Mo (ppm)	SD	V (ppm)	SD	
Lobaria C, unground	0.05	0.00	0.94	0.09	
Lobaria C, Wiley	0.11	0.01	0.98	0.21	0.06
Lobaria C, roller	45.74	0.85	1.95	0.09	45.69
Lobaria P, unground	0.07	0.00	0.73	0.09	
Lobaria P, Wiley	0.12	0.01	0.88	0.00	0.05
Lobaria P, roller	27.91	0.25	1.48	0.03	27.84
Usnea C, unground	0.06	0.00	3.89	0.18	
Usnea C, Wiley	0.17	0.01	3.33	0.08	0.10
Usnea C, roller	6.71	0.05	3.92	0.02	6.65
Usnea P, unground	0.05	0.00	2.45	0.06	
Usnea P, Wiley	0.11	0.01	2.36	0.08	0.05
Usnea P, roller	3.48	0.10	2.39	0.10	3.43