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An Ecophysiological Approach to Quantifying Nitrogen Fixation by *Lobaria oregana*

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Abstract. Lobaria oregana *is an epiphytic macrolichen associated with old-growth Douglas-fir forests in the Pacific Northwest. Nitrogen fixation by this often-abundant cyanolichen provides an ecologically significant input of new N to the forest ecosystem. This study estimates annual* N_2 *fixation by* L. oregana *using a model based on physiological field measurements and laboratory experiments. Meteorological data from the Wind River Canopy Crane site and the H. J. Andrews Experimental Forest are used to calculate annual* N_2 *fixation rates, assuming that hydration and temperature are the two parameters controlling nitrogenase activity. At the crane site, estimated annual* N_2 *fixation is 1.5 kg* ha^{-1}*. In the H. J. Andrews Experimental Forest,* L. oregana *may fix 2.6–16.5 kg* N_2 ha^{-1} yr^{-1} *depending on its stand-level canopy biomass. The model's predictions are checked by using published growth rates and standing* L. oregana *biomass estimates to calculate independent values for annual* N_2 *fixation at each site.*

Keywords. Cyanolichens, *Lobaria oregana*, nitrogen fixation, old-growth lichens.

Cyanolichens dominate epiphyte assemblages in wet old-growth, mid-elevation Douglas-fir forests of Oregon and Washington (Neitlich 1993; Pike et al. 1975; Sillett 1995). They are important ecosystem components because of their ability to fix atmospheric N_2 into forms of N available for plant uptake (Denison 1979). Nitrogen is tightly conserved in old-growth forests (Sollins et al. 1980), and biological N_2 fixation brings new N into the ecosystem. Nitrogenous leachates (Millbank 1982) and *in situ* decomposition of cyanolichens (Rhoades 1983) supports canopy food webs (Carroll 1979). Decomposition of litterfall releases N to plants on the forest floor (Pike 1978).

Lobaria oregana (Tuck.) Müll. Arg. is the most abundant N_2-fixing lichen in these forests, often accounting for 60–80% of the total epiphytic lichen biomass (McCune 1994; Pike et al. 1977; Sillett 1995). Studies undertaken in the H. J. Andrews Experimental Forest (HJA) during the 1970's estimate that *L. oregana* may fix up to 4.5 kg N_2 ha^{-1} yr^{-1} (Denison 1979; Pike 1978), over 50% of the annual new N input to the forest's nutrient budget (Sollins et al. 1980). No further attempts to quantify N_2 fixation by cyanolichens in Douglas-fir forests have been made since these initial estimates.

The objective of this study was to quantify annual N_2 fixation by *L. oregana*. Field measurements and laboratory experiments were conducted in order to identify which parameters influenced N_2 fixation rates, and these parameters were combined with meteorological data to model N_2 fixation. This approach was used to estimate annual N_2 fixation by *L. oregana* at the canopy crane site in the Wind River Experimental Forest and at the HJA. The model's predicted values were compared to estimates of annual N_2 fixation that were calculated independently from data on growth rates and stand-level biomass.

METHODS

Study organism.—Lobaria oregana is a foliose cyanolichen whose yellow-green thalli drape over branches or grow loosely appressed to bark. It is one of only 3–4% of lichens representing a tripartite symbiosis among a mycobiont (Ascomycota), a green algal photobiont (Chlorophyta, *Myrmecia*), and a cyanobacterial photobiont (*Nostoc*) (Honegger 1991). *Lobaria oregana* is most accurately referred to as a cephalodiate chlorolichen because the cyanobacterium is restricted to cephalodia, and the green alga does the bulk of CO_2 fixation.

Study site.—Lobaria oregana was collected and fieldwork conducted at the Wind River Canopy Crane (WRCC) Research Facility near Carson, Washington. The crane site is located within the Wind River Experimental Forest (45°49′ N, 121°55′ W) in the Gifford Pinchot National Forest. The 4-ha crane plot is centered on a Liebherr 550 HC construction crane. The crane is 87 m high, has a horizontal reach of 85 m, and provides access to 10^5 m^3 of old-growth forest canopy. The dominant canopy trees are *Pseudotsuga menziesii* (Mirb.) Franco and *Tsuga heterophylla* (Raf.) Sarg.; *Thuja plicata* Donn. is also common. Stand age is approximately 500 years, and the site displays classic old-growth characteristics (Franklin & Spies 1991). The non-vascular epiphyte community is vertically stratified, with peak abundance of *L. oregana* oc-

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curring in the ''light transition zone'' of the middle canopy (McCune et al. 1997).

Sampling protocol.—*Lobaria oregana* was collected at two-month intervals during winter and spring of 2000 and 2001 as well as fall of 2000. During each sampling trip, the canopy crane was used to access collection sites at two heights (ca 13 and 35 m) on two individuals each of *P. menziesii*, *T. heterophylla*, and *T. plicata*. Different trees were utilized on each sampling trip. Three thalli were taken from each site, for a total of 36 thalli per sampling trip.

Physiological measurements.—Nitrogenase activity was measured using the acetylene reduction assay (ARA) (Hardy et al. 1973). Acetylene was generated by reacting calcium carbide with water, then injected into 300-ml air-tight Plexiglass incubation chambers to a concentration of 10% by volume. The chambers were shaken vigorously, vented to atmospheric pressure, and internal 12-V fans were engaged. Lichen thalli were incubated for 60 min., and empty control chambers were used to quantify background levels of C_2H_4 present in the C_2H_2. After the incubation period, syringes were used to withdraw three 1-ml gas samples from each experimental and control chamber. The samples were immediately injected into septum-topped autosampler vials in order to ensure leak-proof transport back to the laboratory. Ethylene content of each sample was determined using a Varian model 330 gas chromatograph equipped with a 0.318 × 76 cm stainless steel column of Poropak N and a flame ionization detector. The theoretical 4:1 conversion ratio between C_2H_2 reduction and N_2 reduction rates was used (Crawford et al. 2000).

Field measurements.—The ARA was used to measure nitrogenase activity in each of the 36 *Lobaria oregana* thalli collected per sampling trip. These measurements were made on the ground under ambient conditions that approximated temperature and photosynthetically active radiation (PAR) recorded at the lichen collection site. PAR was measured with the External Quantum Sensor on a LiCor 6400 Portable Photosynthesis System. A digital thermometer was used to measure temperature inside the incubation chamber before and after the ARA, and directly outside the chamber at 20 and 40 min. during the incubation. These ambient conditions were not intended to represent the entire day, or the entire season. Rather, the field ARA were intended to capture ''snapshots'' of nitrogenase activity in hydrated lichens under certain temperature conditions. No attempt was made to validate the assumption of equilibrium between thallus temperature and air temperature. The four temperature measurements taken during the course of the ARA were typically within 0.5°C of each other, and the average temperature was ultimately used for a field-based temperature response curve (0–15°C) for nitrogenase activity.

Extra thalli collected in fall 2000 were randomly assigned to four groups ($n = 9$ per group) and stored in the hydrated state in the dark for 0, 6, 12, or 16 hr prior to the ARA, which was also conducted in the dark. This experiment was designed to test whether N_2 fixation in hydrated lichens assayed at 5°C occurred at the same rate during night and day.

Desiccation rates of *L. oregana* were measured in the field at 2°C, 98% RH, under overcast skies, and in still air with no measurable wind. Fully hydrated thalli were placed on a branch at a height of approximately 5 m and left to dry for 0 to 90 min. Groups of three thalli were removed and weighed at 10-min. intervals.

Laboratory experiments.—All lichens used in laboratory experiments received the same re-hydration treatment. Thalli were placed upon saturated sponges in a tray of distilled water, and then misted at 5-min. intervals using a spray bottle. Thallus water content (WC) was expressed in absolute terms, and the ''sacrificial'' method was used to correct mass to an oven-dried basis (McCune et al. 1996).

A laboratory-based temperature response curve for nitrogenase activity was constructed using lichens collected in spring 2001. Thalli were re-hydrated for 60 min. at temperatures from 0–20°C. Nitrogenase activity was measured at each 2°C interval ($n = 3$ per temperature level). PAR levels were kept constant at 100 μE m^{-2} $sec.^{-1}$ during re-hydration and subsequent ARAs.

The relationship between thallus WC and nitrogenase activity was determined using lichens collected in fall 2000. First, thalli were re-hydrated for 0, 5, 10, 30, 60, 120, and 180 min. ($n = 3$ per hydration time) to determine how fast re-hydration occurred. Next, thalli were hydrated using carefully timed periods of misting and air-drying to achieve 300, 250, 200, 150, 100, and 50% WC (actual WC was typically ±20% of target). Nitrogenase activity was measured for each WC ($n = 6$ per level). Temperature and PAR were kept constant at ca 13°C and 100 μE m^{-2} $sec.^{-1}$, respectively.

Modeling N_2 fixation.—The model rested upon the following set of simplifying assumptions derived from results of the field measurements and laboratory experiments summarized above. 1) All thalli had to be hydrated to be physiologically active. 2) Rainfall immediately hydrated and activated all thalli. 3) When rain stopped, all thalli stayed hydrated and active for 60 min. 4) N_2 fixation was independent of canopy position and host tree species. 5) N_2 fixation was not limited by light. 6) N_2 fixation rates were controlled by temperature.

Temperature-dependent N_2 fixation rates (nmol N_2 per kg dry mass of lichen) were generated for each 30-min. period in the meteorological datasets. Daily, monthly, or annual N_2 fixation was simply the sum of all 30-min. periods in which *L. oregana* was hydrated. Rates were scaled up to the stand level using the estimated canopy biomass of *L. oregana*. At WRCC, standing biomass was approximately 550 kg ha^{-1} (McCune et al. 1997). Estimates were made for sites within HJA where *L. oregana* biomass was approximately 3,523, 2,620, 1,587 (Neitlich 1993), and 500 kg ha^{-1} (Denison 1979).

The WRCC meteorological data used to drive the model were recorded at an open meteorological station on the ground near the crane tower. Detailed data from February 1998 to the present are available online at www.depts.washington.edu/wrccrf. Data from Climatic Station 2 (CS2MET), located within the old-growth forest of Watershed #2 at HJA, were obtained online at ftp.fsl.orst.edu/pub/henshaw/pinto.

Statistical analysis.—The effects of tree species, height, and dark exposure on nitrogenase activity were evaluated by separate one-way ANOVA using JMP 4.0 (SAS Institute 2001). To examine the potential effect of height, the mean nitrogenase activity of the three thalli collected at each height was used as the dependent variable ($n = 30$ trees). To examine the potential effect of tree species, the mean nitrogenase activity of the six thalli from each tree was used as the dependent variable ($n = 10$ trees). Data from all analyses satisfied the assumptions of parametric ANOVA, which were examined using diagnostic tools in JMP 4.0.

RESULTS

Model assumptions.—Nitrogenase activity in *L. oregana* increased as thallus WC increased, with

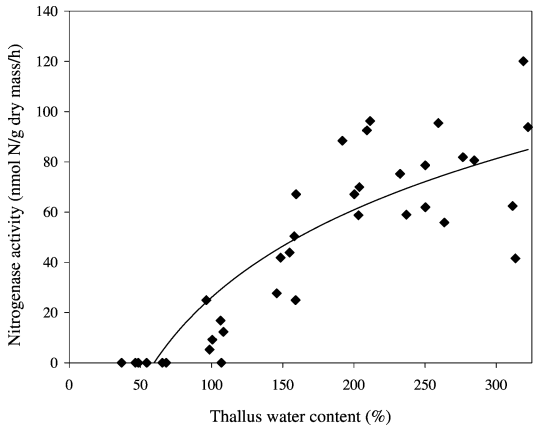

FIGURE 1. The relationship between nitrogenase activity and thallus water content of *L. oregana* (R^2 = 0.74). The ARA was used to measure nitrogenase activity of thalli in laboratory conditions of 13°C and 100 μE m^{-2} $sec.^{-1}$ PAR.

measurable activity beginning at around 100% WC (Fig. 1). When desiccated thalli were re-hydrated, they reached 200% WC within 10 minutes (Fig. 2). Since thalli quickly reached the minimum WC necessary for optimal nitrogenase activity (i.e., 150–200%), precipitation events were assumed to immediately activate N_2 fixation.

Average WC of *L. oregana* thalli dropped to below 100% after 60 min. (Fig. 3), and nitrogenase activity was negligible below 100% WC (Fig. 1). Therefore, it was assumed that *L. oregana* fixed N_2 for only 60 min. after precipitation stopped. Nitrogenase activity did not differ among groups of lichens assayed in the dark at constant temperature after exposure to 0–16 hr of darkness (p = 0.89). Therefore, since the longest nights in mid-winter at WRCC and HJA do not exceed 16 hr, light is unlikely to limit N_2 fixation.

Nitrogenase rates did not depend on the tree species from which the lichens were collected (p = 0.79). The difference in nitrogenase activity between hydrated lichens collected from the middle (35 m) and the bottom (13 m) of the canopy was not significant (p = 0.91). Therefore, canopy position and host tree species were not incorporated as variables in modeling N_2 fixation.

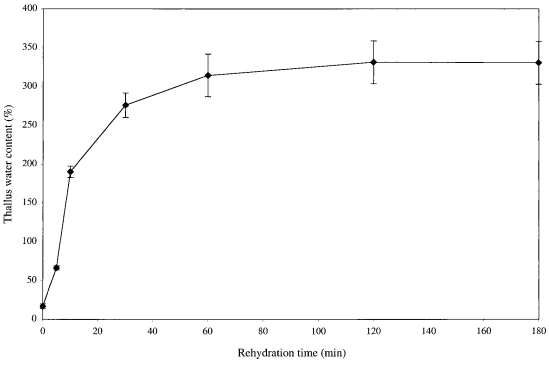

FIGURE 2. Thallus water content of *L. oregana* following various re-hydration periods. Water content is expressed on an absolute basis, with the oven-dried mass as the 0% reference point. Data are means of n = 3 ± 1 S.E.

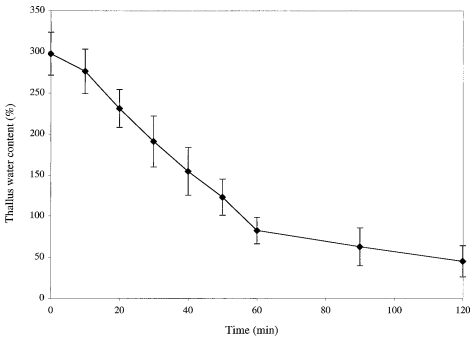

FIGURE 3. Desiccation of *L. oregana* thalli. Measurements were made under overcast skies at 98% RH and 2°C. Data are means of n = 3 ± 1 S.E.

Temperature regulated nitrogenase activity in hydrated *L. oregana* (Fig. 4). Measurable activity began at 1–2°C and increased almost linearly with increasing temperature up to 15°C. A sensitivity analysis showed less than a 10% difference between laboratory and field data in determining the slope of the linear regression of nitrogenase activity vs. temperature.

Model predictions.—Assuming a 550 kg ha^{-1} standing biomass of *L. oregana*, estimated annual N_2 fixation was 1.5 kg ha^{-1} at WRCC (Fig. 5) and 2.6 kg ha^{-1} at HJA. The high predicted N_2 fixation rates in spring and fall reflected the relatively higher rainfall and moderate temperatures during these

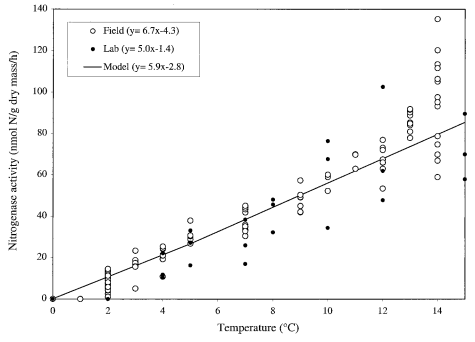

FIGURE 4. Temperature response curves for nitrogenase activity in *L. oregana*. The temperature response equation used for modeling N_2 fixation was the average of the field- and laboratory-based curves (R^2 = 0.89). Acetylene reduction assays performed in the field were at ambient PAR levels (typically 50–150 μE m^{-2} $sec.^{-1}$) and those performed in the laboratory were at 100 μE m^{-2} $sec.^{-1}$ PAR.

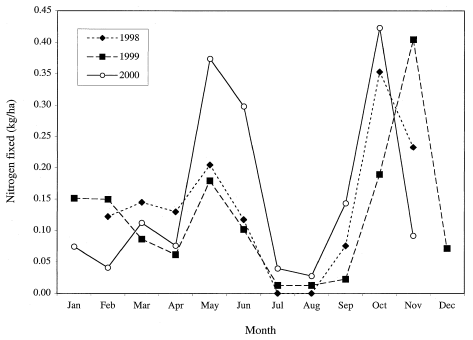

FIGURE 5. Annual trends in N_2 fixation by *L. oregana* at WRCC, as predicted by the model. Variation is based on seasonal trends in temperature and precipitation.

periods. Although 1.3 times more rain fell at WRCC, predicted N_2 fixation was 1.7 times greater at HJA (Table 1). This difference was due to longer summer drought and consistently cooler winter temperatures at WRCC.

In sites where *L. oregana* is massively abundant (e.g., Sillett 1995), predicted annual N_2 fixation increases accordingly. Based on biomass estimates for stands at HJA (Neitlich 1993), annual N_2 fixation may exceed 16 kg ha^{-1} in some areas (Table 1).

DISCUSSION

Modeling a process as complex as stand-level N_2 fixation inevitably requires a set of simplifying assumptions. The present model rests on the assumption that N_2 fixation rates are dependent on air temperature, but independent of light and canopy position. In reality, a myriad of light and temperature conditions exist within the vertical gradient of a forest canopy, and air temperature and thallus temperature may vary by several degrees (Campbell & Coxson 2001; Coxson & Coyle 2003). Furthermore, light-dark transition responses in nitrogenase activity have been observed in other cyanolichen species (Kershaw et al. 1977; Nash 1996; but also see Denison 1979).

Another necessary assumption was that precipitation acts as an ''on/off'' switch for N_2 fixation, even though synchronous hydration and desiccation of thalli of different sizes and canopy positions is unlikely (Gauslaa & Solhaug 1998; Renhorn et al. 1997). Certain environmental conditions may result in considerable lag times between re-hydration and re-activation of optimal metabolic activity (Kershaw 1985). In addition, particularly humid or very windy situations may result in desiccation times that are much slower or much faster, respectively, than 60 min. (Campbell & Coxson 2001). However, quantifying the distribution of thallus size classes throughout a forest canopy and monitoring trends in thallus hydration and desiccation within a representative matrix of light and temperature conditions will require an enormous sampling effort.

The temperature sensitivity of nitrogenase activity in cyanolichens is well established (e.g., Denison 1979; Kershaw 1985; MacFarlane & Kershaw 1977). The response curve used in the model is typical in that activity begins above a certain limiting temperature and then increases with increasing temperature within the range sampled. Variation in nitrogenase activity among replicates is likely due in part to irregular distribution of cephalodia (Millbank & Kershaw 1970), although instantaneous N_2 fixation rates also depend on coinciding environmental factors and pretreatment conditions (Crittenden & Kershaw 1978).

Another source of error lies in the conversion factor between the ARA and N_2 fixation rates. Although the theoretical ratio is 4:1, ratios as high as 25:1 have been observed in free-living cyanobacteria (Peterson & Buris 1976), which would result in serious over-estimation of annual N_2 fixation rates. Calibration of ARA results against $^{15}N_2$ incorporation by *L. oregana* would increase the robustness of the estimates (Millbank 1982). Interestingly, application of the 4:1 conversion to an earlier estimate of annual N_2 fixation by *L. oregana* (Denison 1979) produces a number nearly identical to this model's prediction for the same site.

TABLE 1. Annual N_2 fixation by *L. oregana*, as predicted by the model, in five different old-growth Douglas-fir forests. Precipitation rates are 3-year averages based on meteorological data from WRCC and CS2MET at HJA. Expected N_2 fixation rates (see Discussion) are calculated by multiplying estimated *L. oregana* biomass by a 15% annual growth rate and 2% thallus N content.

Site	Mean precipitation (mm yr^{-1})	*L. oregana* biomass (kg ha^{-1})	Source of biomass data	Predicted N_2 fixation (kg ha^{-1} yr^{-1})	Expected N_2 fixation: 15% annual growth
WRCC	1460	550	McCune et al. 1997	1.5	1.7
HJA 1	1840	550	Denison 1979	2.6	1.7
HJA 2	1840	1587	Neitlich 1993	7.5	4.8
HJA 3	1840	2620	Neitlich 1993	12.3	7.9
HJA 4	1840	3523	Neitlich 1993	16.5	10.6

A good test of any model is corroboration with independent results. This model can be checked by using estimates of standing biomass and annual lichen growth rates to calculate annual N_2 fixation. Lichen transplant experiments have shown that *L. oregana* thalli have an average annual growth rate of at least 15% (Denison 1988; Sillett & McCune 1998). The average N content of *L. oregana* tissue is 2% (Antoine 2001). Therefore, 1.7 kg ha^{-1} yr^{-1} of N_2 fixation would be needed to support a 15% growth rate at WRCC, and over 10 kg N_2 ha^{-1} yr^{-1} would be required in HJA sites where *L. oregana* is abundant (Table 1). Most of this annual productivity is invested in recovering biomass lost to physical injury, fragmentation, and *in situ* decomposition (Rhoades 1983).

The model-predicted annual N_2 fixation rate at WRCC is very close to the value calculated above. However, predicted rates for HJA are high enough to support a 24% annual growth rate. Perhaps the meteorological conditions that result in higher putative N_2 fixation at HJA also promote higher growth rates. Indeed, 23% and 30% annual growth of healthy thalli was observed in *L. oregana* at HJA by Sillett and McCune (1998) and Rhoades (1977), respectively.

''Canopy-level averaging'' may explain why this model is useful despite its many simplifications. All thalli were assumed to respond similarly to changes in hydration and temperature, that is, an ''average'' response is used as proxy for the heterogeneity that actually exists among individuals. Forest canopies have a vertical microclimate gradient where increased height means more rapid wetting and drying cycles and more extreme temperature and humidity fluctuations (Parker 1995, 1997). Uniform rates of hydration and desiccation are assumed for all thalli, with the acknowledgment that *in situ* cycles may be more or less rapid depending on local microclimate conditions and thallus size. Further replication of the experiments described here is necessary to evaluate and refine this model.

Future investigations may also elucidate the physiological basis of *L. oregana*'s landscape-level distribution. For example, the temperature restrictions on N_2 fixation may explain why this species is absent from epiphyte assemblages in higher-elevation forests (Sillett & Neitlich 1996). Perhaps rainy-season temperatures are simply too low to permit sufficient N_2 fixation for survival. An alternative explanation for low winter N_2 fixation is depletion of energy reserves due to insufficient light, especially in lower canopy thalli. Placing *L. oregana* transplants and meteorological data-loggers into higher-elevation forests, and periodically testing N_2 and CO_2 fixation rates could test these hypotheses.

Conclusions.—Site-to-site differences in *L. oregana* abundance and local patterns of temperature and precipitation determine how much N_2 is fixed in a given forest. As a result, *L. oregana*'s importance in forest nutrient cycling is heterogeneous across its landscape distribution. In areas where it reaches peak abundance, as in close proximity to major streams in old-growth Douglas-fir forests (Howe 1978; McCune et al. 2002; Sillett & Neitlich 1996), the magnitude of its contribution to the forest N budget may be higher than has previously been acknowledged. Furthermore, these estimates consider *L. oregana* alone, thereby underestimating total N_2 fixation by all cyanolichens.

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