

Regional constraints to biological nitrogen fixation in post-fire forest communities

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Abstract. Biological nitrogen fixation (BNF) is a key ecological process that can restore nitrogen (N) lost in wildfire and shape the pace and pattern of post-fire forest recovery. To date, there is limited information on how climate and soil fertility interact to influence different pathways of BNF in early forest succession. We studied asymbiotic (forest floor and soil) and symbiotic (the shrub *Ceanothus integerrimus*) BNF rates across six sites in the Klamath National Forest, California, USA. We used combined gradient and experimental phosphorus (P) fertilization studies to explore cross-site variation in BNF rates and then related these rates to abiotic and biotic variables. We estimate that our measured BNF rates 22 years after wildfire ($6.1\text{--}12.1 \text{ kg N}\cdot\text{ha}^{-1}\cdot\text{yr}^{-1}$) are unlikely to fully replace wildfire N losses. We found that asymbiotic BNF is P limited, although this is not the case for symbiotic BNF in *Ceanothus*. In contrast, *Ceanothus* BNF is largely driven by competition from other vegetation: in high-productivity sites with high potential evapotranspiration (Et), shrub biomass is suppressed as tree biomass increases. Because shrub biomass governed cross-site variation in *Ceanothus* BNF, this competitive interaction led to lower BNF in sites with high productivity and Et. Overall, these results suggest that the effects of nutrients play a larger role in driving asymbiotic than symbiotic fixation across our post-fire sites. However, because symbiotic BNF is 8–90× greater than asymbiotic BNF, it is interspecific plant competition that governs overall BNF inputs in these forests.

Key words: biological nitrogen fixation; climate gradient; Klamath National Forest; P fertilization; plant competition; soil nitrogen.

INTRODUCTION

Biological nitrogen fixation (BNF) is the main non-anthropogenic input of nitrogen to terrestrial ecosystems worldwide (Cleveland et al. 1999, Vitousek et al. 2002). Understanding constraints on BNF is ecologically important because net primary productivity in many ecosystems is nitrogen limited (LeBauer and Treseder 2008). This is particularly true in early-successional post-fire communities, where combustion losses of N can deplete N stores needed for long-term forest recovery (Raison 1979, Wan et al. 2001). Better resolution of the factors that constrain both symbiotic and asymbiotic BNF pathways across spatially heterogeneous natural landscapes is needed (Menge and Hedin 2009), and would help predict where and when we expect N to be most limiting to primary productivity and post-disturbance ecosystem recovery (Chapin et al. 1994, Uliassi and Ruess 2002).

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The potential interactive effects of multiple ecological constraints on symbiotic and asymbiotic BNF are of particular interest in early-successional post-fire temperate forests, where rates of biomass accrual can be rapid and yet highly variable depending on local resource availability. Despite this importance, no studies explicitly test how cross-site variation in the factors known to affect BNF may differentially drive ecosystem-level N inputs from asymbiotic and symbiotic fixers.

Phosphorus (P) is well known to be limiting to BNF in agricultural ecosystems and greenhouse experiments, but its importance in natural systems is less well understood (Gates 1974, Uliassi et al. 2000). Soil P levels change over the course of ecosystem succession (Crews et al. 1995) and can also vary with soil parent material (Anderson 1988), creating a wide range of possible conditions that may shape the degree to which P limits BNF over time and space (Crews et al. 2000). Climate also regulates BNF, and at a global scale, BNF rates are highest in warm and wet biomes with high evapotranspiration (Cleveland et al. 1999). At smaller scales, seasonal and diurnal changes in temperature and moisture may also shape BNF rates in both symbiotic (Uliassi and Ruess 2002) and asymbiotic organisms (Reed et al. 2007). This suggests that cross-site variation in climate may also play a role in regulating BNF. There is nevertheless a paucity of coherent information on how

both P and climate may independently or interactively influence BNF inputs via symbiotic and asymbiotic pathways under field conditions and across sites.

Plant competition can be especially important in shaping symbiotic BNF, particularly through succession, wherein short-statured N-fixing plants drop out as they become overtapped by taller, longer-lived species (Chapin et al. 1994). Indeed, temperate N-fixing trees generally display trade-offs between the ability to fix N and persist in late successional communities (Menge et al. 2010). In this way, resource-based controls on BNF must be considered not only for their effects on the physiology and activity of symbiotic N-fixing plants, but also for how they influence competition between N-fixing and non-N-fixing species (Vitousek et al. 2002). Such competitive interactions may be further modulated by variation in climate and/or resource supply across the landscape, yielding gradients in how strongly competitive vs. physiological constraints shape symbiotic BNF.

We characterized variation in BNF across sites in the Klamath National Forest in the early successional shrub *Ceanothus integerrimus* (deerbrush), and in free-living soil N-fixers, through combined gradient and experimental studies. The mixed conifer/broadleaf forests of the Klamath/Siskiyou region are unique among western U.S. forests in their high diversity of species (Whittaker 1960), which is in part attributed to shifting resources and topographic heterogeneity, offering a model system for studying cross-site constraints on BNF. Specifically, we examined how symbiotic BNF in *Ceanothus* and asymbiotic BNF in soil varied in response to temperature and precipitation across a broad climatic gradient 22 years after wildfire. We also looked at how climate and competition between N-fixers and non-fixers interact, as we expected that more productive climates may increase BNF, but also increase the strength of competition. Along this climate gradient, we further tested whether symbiotic and asymbiotic BNF were P limited by comparing fixation rates to natural variation in soil P and in response to two years of experimental P fertilization. We hypothesized that both symbiotic and asymbiotic BNF would increase in more productive climates, and with higher soil P, but that symbiotic BNF would decrease as a function of plant competition.

METHODS

Site and experimental design

The mixed broadleaf-conifer forests of the Klamath Mountains of Southern Oregon and Northern California occupy a region of steep topography (Appendix A: Fig. A1) in close proximity to a coastal maritime climate, which together lead to sharp climatic gradients (Whittaker 1960). On average, the Klamath region has warm (15–20°C), dry summers and cold (0–3°C), wet winters, with pronounced summer drought. The area also contains a mosaic of parent materials due to a complex geologic history that contributes to wide variation in soil P supply across the region (Whittaker

1960). Thus, the Klamath region offers gradients in climate and soil biogeochemistry that can be used to ask how these variables affect BNF.

Ceanothus integerrimus is an actinorhizal species that forms symbioses with *Frankia* (Busse 2000). Although BNF in other *Ceanothus* species has been studied (McNabb and Cromack 1983, Busse 2000), rates of fixation for *C. integerrimus* have not. To examine limitations on BNF in *C. integerrimus* (*Ceanothus* hereafter) across the region, we established six south-facing sites in the Klamath National Forest (Table 1). All sites were burned in wildfires in 1987, and were 22 years old at the time of final sampling (2009). Sites were salvage logged two to three years after fire, after which conifers (Douglas-fir and Ponderosa pine) were planted. Shrubs, which included *Ceanothus integerrimus* and *Arctostaphylos viscida*, were mechanically cut, 1 foot (0.30 m) above the ground, from around conifer seedlings after two to four years of growth. After these treatments, *Ceanothus* became the more dominant species (Lopez 2007). We controlled for aspect, stand age, and salvage history because *Ceanothus* is abundant on south-facing slopes in this region (Lopez 2007). Sites were at least 1 km apart, and spanned a 37-km distance.

Sites were located in the Douglas-fir series of the Klamath Region. The region is prone to wildfires, and frequent (every 5–25 years), mixed-severity fires shape vegetation patterns (Taylor and Skinner 1998). Dominant conifers included *Pseudotsuga menziesii* (Douglas-fir), *Pinus ponderosa* (Ponderosa pine), and *Pinus lambertiana* (Sugar pine). Dominant broadleaf species included *Arbutus menziesii* (madrone), *Lithocarpus densiflorus* (tanoak), *Quercus kelloggii* (black oak), *Quercus chrysolepis* (canyon live oak), and *Salix scouleriana* (scouler willow); in addition, *Acer macrophyllum* (bigleaf maple) was also found in one site. The dominant shrub was *Ceanothus*. Together, these species made up 84–99% of the total cover in our study area (M. Lopez and J. Shatford, *unpublished data*). Sites tended to have steep topography (Fig. A1), with rocky soils that had little to no organic horizon.

We used latitude, longitude, and elevation parameters to estimate average climate variables (Table 1) for each site using ClimateWNA (Wang et al. 2006). ClimateWNA interpolates 1961–1990 climate data from the PRISM model (Daly et al. 2002) with an elevation component, and thus offers high-resolution data for mountainous regions. As is common in the region, sites located farther to the west had higher mean annual precipitation (MAP). In addition, our higher elevation sites had lower MAP and mean annual temperatures (MAT). Because we were constrained to a specific stand age and management history, our ability to select sites with strictly orthogonal variation in climate parameters was limited. As a consequence, our more westerly sites were generally lower in elevation, leading to MAP and MAT being positively correlated ($r^2 = 0.95$, $P < 0.001$). We therefore explored BNF as a function of potential

TABLE 1. Selected characteristics for the six sites in the Klamath National Forest, California, USA.

| Site | Latitude (°N) | Longitude (°W) | Elevation (m) | MAT (°C) | MAP (mm) | Et (mm/yr) |
|---------|---------------|----------------|---------------|----------|----------|------------|
| Clear | 41.721 | 123.494 | 469 | 12.2 | 1644 | 1119 |
| Cade | 41.832 | 123.351 | 634 | 11.8 | 1565 | 1092 |
| Horse | 41.776 | 123.295 | 636 | 11.2 | 1539 | 1063 |
| Seiad 3 | 41.905 | 123.129 | 1140 | 8.6 | 918 | 816 |
| Seiad 2 | 41.898 | 123.124 | 1080 | 8.6 | 860 | 857 |
| Seiad 1 | 41.907 | 123.121 | 1311 | 7.7 | 915 | 844 |

Notes: Sites are listed from west to east. Abbreviations are MAT, mean annual temperature; MAP, mean annual precipitation; Et, evapotranspiration rate.

evapotranspiration (Et) rates, which integrate MAP and MAT in a biologically meaningful way. Indeed, site-level aboveground biomass of dominant plant species (see *Shrub and tree biomass*) was positively related to Et ($r^2 = 0.83$, $P = 0.01$). Et was calculated by ClimateWNA using the Hargreave's equation (Hargreaves and Samani 1982).

We established six rectangular 4×7 m plots at each site in spring 2006, for a total of 36 plots across all sites. Plots were randomly located along three or four transects regularly placed through the stand at right angles to the stand boundary. Plots were chosen along transects with a random number generator with the caveat that plots contained *Ceanothus* and Douglas-fir. We used a field-scale ^{15}N dilution technique to quantify background BNF by *Ceanothus* across the climatic gradient and in response to experimental P additions at each site. To accomplish this, three replicate plots at each site were randomly assigned to each of the following treatments: (1) trace-level ^{15}N addition (control) or (2) trace-level ^{15}N plus P fertilizer addition. Both ^{15}N and P were added in fall of 2007 and 2008, prior to measurements in 2009. P was added as 150 kg P·ha $^{-1} \cdot \text{yr}^{-1}$ in the form of super triple phosphate. Details of ^{15}N addition are provided in the next subsection.

Quantifying *Ceanothus* BNF

We employed ^{15}N dilution to quantify N fixed by *Ceanothus*. This technique uses trace-level ^{15}N additions to isotopically enrich plant available N in soil, and then compares tissue ^{15}N enrichment of target N-fixing and non-fixing reference species to that of atmospheric N₂ to quantify the percentage of fixed N (%Ndfa) in the target N-fixer. Six experimental plots at each site received ^{15}N -labeled ammonium sulfate (10 atom%) at the rate of 0.4 g N·m $^{-2} \cdot \text{yr}^{-1}$ in fall of 2007 and 2008. We then collected foliar tissue samples for isotopic and nutrient analysis in May, June, and October of 2009 from both the target N-fixer (i.e., *Ceanothus*) and a non-N-fixing species that occurred at all sites (Douglas-fir). Foliage from two to three plants per species per plot was collected, composited by plot, dried at 65°C, ground, and analyzed for N concentrations and $^{15}\text{N} : ^{14}\text{N}$ ratios at the Colorado Plateau Stable Isotope Laboratory (Flagstaff, Arizona, USA) using a Carlo Erba NC2100 elemental analyzer (CE Elantech, Lakewood, New Jersey, USA). Isotopic

values were converted to atom% (Fry 2006) and the standard ^{15}N value for atmospheric N was subtracted to give atom% excess (AE). Foliar ^{15}N values were averaged for the three sample dates within a plot before %Ndfa was calculated as:

$$\% \text{Ndfa} = \frac{{}^{15}\text{N AE reference} - {}^{15}\text{N } Ceanothus}{{}^{15}\text{N AE reference}} \times 100. \quad (1)$$

We only used foliar material for quantifying %Ndfa because foliage has shorter turnover times than woody stem and root material, and thus would be more likely to reflect the ^{15}N addition. %Ndfa was converted to total N fixed (kg N/ha) by multiplying against *Ceanothus* N content (*Ceanothus* tissue %N \times *Ceanothus* biomass). To estimate cumulative BNF since fire, we also took into account differential N contents of *Ceanothus* tissue types, as well as annual turnover of N through foliage.

Different tissue types of *Ceanothus* had different %N (foliage 3.0% \pm 0.05%, stem 0.7% \pm 0.02% [mean \pm SE]), thus we split tissue types into root, stem, and foliage to calculate a weighted model of N fixed (root and stem tissue %N were assumed to be equivalent). We measured all stems of *Ceanothus* in each plot, live and dead, and included dead stems to better estimate cumulative *Ceanothus* BNF over succession since time of fire. Because *Ceanothus* is a deciduous shrub, we also included annual leaf litter production estimates in cumulative BNF calculations. We used a growth model for *Arctostaphylos viscida* (whiteleaf manzanita), a regional shrub species with a similar growth habit, to estimate basal stem diameter (BD) as a function of age (A) and stem density (SD) (Hanson 1997:96):

$$\ln(\text{BD}) = 2.036 + 1.104 \ln(A) - 0.0015\sqrt{\text{SD}} - 0.0006(\ln(A))^2\sqrt{\text{SD}}. \quad (2)$$

We set the stand age in 2009 to 22 years and used 2009 *Ceanothus* stem densities (Appendix A: Table A1; see methods in *Shrub and tree biomass*) in each plot to back calculate BD for every stem in every year from its 2009 BD back to initial wildfires. We then used published allometric equations (Hughes et al. 1987) to scale from BD to *Ceanothus* stem and foliar biomass (per m 2) separately, and incorporated foliar production for each year into estimates of cumulative BNF over time since fire. We included root biomass using published

root:shoot ratios (0.69) for *Ceanothus velutinus* (Busse 2000). Foliage %N was taken from the October 2009 foliage samples. Stem material for %N was collected from three *Ceanothus* plants in October 2009 within each plot, and was bulked within treatment for each site for analysis. All tissue samples were analyzed for %C and %N with a Carlo Erba NA 1500 CHN analyzer (Fisons Instruments, Beverly, Massachusetts, USA). Finally, cumulative N fixed over time (22 years since fire) was calculated as

$$\sum_{t=1}^{22} \left(\%Nd_{fa} \times [(\%N_{foliage} \times FB_t) + (\%N_{stem} \times SB_t) + (\%N_{root} \times RB_t)] \right) \quad (3)$$

where FB_t , SB_t , and RB_t are foliar, stem, and root biomass in year t , respectively.

Using Eq. 1 to quantify %Nd_{fa} led to negative values if foliar ¹⁵N of reference species was less than that of *Ceanothus*, which we interpreted to mean that *Ceanothus* was not fixing N₂ in these plots. Throughout the manuscript, BNF refers to N-fixation on a per-area basis (Eq. 3), while %Nd_{fa} refers to the percentage of tissue N that was derived from fixation (Eq. 1).

Shrub and tree biomass

We were interested in the degree to which competition from dominant non-fixing woody species drove *Ceanothus* BNF. We thus characterized the current above-ground biomass of dominant tree and shrub species using published allometric equations (Appendix A: Table A2). If basal diameters were smaller than the lower range limit for a given allometric equation, linear extrapolations were used to fit the lower range limit to zero. Within the six 4 × 7 m plots at each site, we measured diameters of all stems of dominant species at the base or breast height (1.37 m) depending on parameters needed for that species' allometric equation, and measured heights with a height pole or clinometer and meter tape.

Soil and litter C, N, and P

We characterized soil N concentrations and mineralization rates, soil P concentrations, forest floor C, N, and P concentrations, and foliar C, N, and P concentrations at all sites. All N:P ratios were calculated on a mass basis.

We sampled soil in May 2010 to 10 cm at three locations per plot, composited samples, and sieved to 2 mm. We determined gravimetric soil water content at 110°C for 48 hours. We extracted soil NH₄⁺ + NO₃⁻ (i.e., dissolved inorganic N, DIN) from 7 g wet soil with 35 mL 0.5 mol/L K₂SO₄ for 1 hour, followed by gravity filtration through prerinced Whatman No. 1 filters. We determined potential net N mineralization by incubating another set of 10 g soils at 60% water holding capacity at 25°C for 28 days in the laboratory, followed by

extraction for DIN. Potential net N mineralization was calculated as DIN_{t1} – DIN_{t0}, where t0 is the initial time point and t1 is time point 1. Bray-1 soil P was extracted from 5 g soil for 1 minute using 25 mL of 0.03 mol/L NH₄F and 0.025 mol/L HCl, followed by centrifugation at 3400 rpm for 5 minutes, then filtering the supernatant through a Whatman No. 42 filter. We also measured water-extractable total dissolved N and P in forest floor and mineral soil by shaking 20 g of soil in 100 mL of deionized water for 1 h, filtering through a Gelman GF/F filter, followed by high-temperature persulfate digestion. Nitrate, ammonium, and phosphorus in extracts were measured colorimetrically by Lachat flow injection (Lachat, Milwaukee, Wisconsin, USA).

Foliage for C, N, and P determination was collected in October 2009 from three *Ceanothus* and Douglas-fir within each plot, bulked by species per plot, and dried and ground. Tissue C and N were analyzed as for soil. For tissue P, 0.5-g dry mass sample was ashed at 500°C for 12 hours, digested with 5 mL of 5 mol/L HCl, brought up to 0.5 mol/L HCl and analyzed for total P on the Lachat. Forest floor samples collected in May 2009 were dried after acetylene reduction assays, then ground and analyzed for tissue C, N, and P using the same protocols as foliage samples.

Asymbiotic N fixation

We used acetylene reduction to quantify rates of free-living BNF in forest floor and mineral soil. Forest floor at these sites consisted primarily of standing litter above mineral soil, with minimal organic horizon development. Forest floor was sampled in May 2009 using a 0.2 × 0.2 m quadrat systematically placed within plots. Mineral soil was sampled from within the same quadrat with a trowel to a 10 cm depth. Samples were kept refrigerated and returned to the lab within one week for acetylene reduction measurements. Approximately 20 g forest floor or 100 g mineral soil at ambient moisture was placed into a 480-mL Mason jar (Ball, Muncie, Indiana, USA) whose lid was fitted with rubber septa. Acetylene was injected to create a 10% concentration by volume in the headspace and jars were incubated for 3 h at 25°C. Blanks from each plot were assayed to correct for natural ethylene production. After incubation, headspace was mixed and subsamples were injected directly into an Agilent 7890A gas chromatograph (Agilent Technologies, Santa Clara, California, USA).

Acetylene reduction rates (ARA) per gram of sample were converted to BNF (per unit area) in forest floor using masses determined from quadrat data in each plot, and in mineral soil using typical bulk density for our sites within the Klamath National Forest (1 g soil/cm³; Joseph Blanchard, *personal communication*). We used the theoretical conversion ratio of 4:1 for all ARA-to-BNF conversions. This ratio takes into account both the number of electrons used to reduce C₂H₂ to C₂H₄ vs. N₂ to NH₃, as well as H₂ production by the nitrogenase enzyme (Anderson et al. 2004). Although many studies

use a 3:1 ratio, we found 4:1 closer to published ^{15}N calibrated conversion ratios for forest floor and soil. To account for potential influence of variation in ARA to BNF conversion ratios, we also discuss our results constrained by the highest and lowest published ^{15}N calibrated ARA conversion rates for soil and forest floor/litter (Appendix B: Table B1). In addition, we note that laboratory incubations to obtain ARA values are static, single time-point measures that do not necessarily reflect in-field conditions, or temporal variability.

Data analyses

To analyze P fertilization effects on soil N or P, foliar P, or BNF, we used two-way analyses of variance (ANOVA) with site and treatment (+P or control) as fixed effects on soil P and BNF variables. Phosphorus addition did not alter *Ceanothus* BNF variables (*Ceanothus* biomass, %Ndfa, or total BNF (see *Results*); we therefore pooled data from control and P-fertilized plots in subsequent analyses of *Ceanothus* BNF.

All calculations of symbiotic and asymbiotic BNF were carried out separately for each of the 36 total plots in the study prior to estimating site- and study-level means and variances. Specifically, our plot-based calculations use a single value unique to each study plot for reference species ^{15}N , and *Ceanothus* ^{15}N , stem density, biomass, and %N when calculating %Ndfa or cumulative BNF in each plot. Given the potential for covariance among the components that contribute to BNF estimates, such independent plot-level estimates capture field-based variance in overall BNF estimates. With plot-level estimates of %Ndfa or total N fixed, we then estimated means and standard errors at the site level using $n = 6$ plots per site, and at the study level using data from all 36 plots. Because plots were replicated within sites, we used site-level estimates to examine how climate, soil and foliar nutrients, and competition shaped BNF. Due to the presence of outliers that were over two standard deviations from the mean, we used geometric means to analyze net N mineralization rates. For asymbiotic N fixation rates, we used simple linear regression to explore the effects of nutrients in forest floor and soil.

For symbiotic BNF, we first used regression techniques to ask whether %Ndfa or *Ceanothus* biomass best explained variation in total BNF. If %Ndfa explained more variance, this would suggest that the physiological ability of *Ceanothus* to up- or down-regulate fixation is more important than shrub abundance in determining overall fixation rates. We then used regression techniques to explore which exogenous variable(s) best explained each component of *Ceanothus* BNF (biomass and %Ndfa).

Results from univariate analyses led us to hypothesize that indirect effects may play a role in driving cross-site variation in symbiotic BNF. We used structural equation modeling (SEM) to confirm these indirect effects and compare the relative strength of climate, soil,

and competition on BNF. SEM are based on regression analyses, but can incorporate networks of causal relationships to confirm postulated relationships among variables (Grace 2006).

We used site averages for all variables in order to focus on cross-site, rather than within-site, variability. Due to low degrees of freedom from only $n = 6$ sites, results from these models should be interpreted with caution. However, SEM are particularly well suited to test for indirect effects in ecosystems (Grace 2006), and thus were well suited to complement our univariate analyses. Models were analyzed with AMOS 5.0 (Arbuckle 2006). We postulated a preliminary model based on our understanding of the ecosystem and a priori knowledge from univariate analyses (Grace et al. 2010). We then selected a model by deleting paths that were not significant ($P > 0.05$), and using Akaike's information criterion (AIC [Akaike 1974]) to select the model structure that best fit the empirical data (Fig. 5A). We use standardized path coefficients to compare the strength of paths in the final model. These coefficients represent how the variation in one variable depends on the variation in a second variable (Grace 2006). These can also be used to describe the indirect paths between variables.

RESULTS

Asymbiotic N fixation

Phosphorus fertilization stimulated asymbiotic BNF in forest floor and mineral soil (Fig. 1A, B). Forest floor BNF varied across sites, as did the effect of fertilization (Fig. 1A). It might be expected that sites with lower soil or litter P might show a greater response to fertilization, but this was not the case. Relative increases in forest floor acetylene reduction rates (ARA) with P fertilization were not related to background levels of P in forest floor ($r^2 < 0.01$, $P = 0.99$) nor mineral soil ($r^2 = 0.12$, $P = 0.50$) of control plots (Appendix B: Fig. B1a, b). Comparisons of forest floor ARA to C, N, and P in forest floor (Fig. B1c, d, e) failed to find a relationship between forest floor ARA and forest floor %N ($r^2 = 0.08$, $P = 0.59$), but did reveal a negative relationship between forest floor %C and forest floor ARA ($r^2 = 0.74$, $P = 0.03$) and a positive relationship between forest floor %P and ARA ($r^2 = 0.68$, $P = 0.05$).

Similar to forest floor BNF, we did not find relationships between background levels of TDN:TDP (total dissolved nitrogen and phosphorus, respectively) and the relative increase in soil ARA with P fertilization. Although there was a significant positive correlation with soil P, this seemed driven by a single point (Fig. B2a, b). Across site variation in various measures of mineral soil P, N, and C (including soil Bray-I P, net N mineralization, and %C) were unrelated to soil ARA.

Average BNF rates in the top 10 cm of mineral soil estimated by 4:1 ARA : BNF conversion ($0.84 \pm 0.16 \text{ kg N} \cdot \text{ha}^{-1} \cdot \text{yr}^{-1}$) were 10 times greater than those in the forest floor ($0.08 \pm 0.03 \text{ kg N} \cdot \text{ha}^{-1} \cdot \text{yr}^{-1}$), but both forest

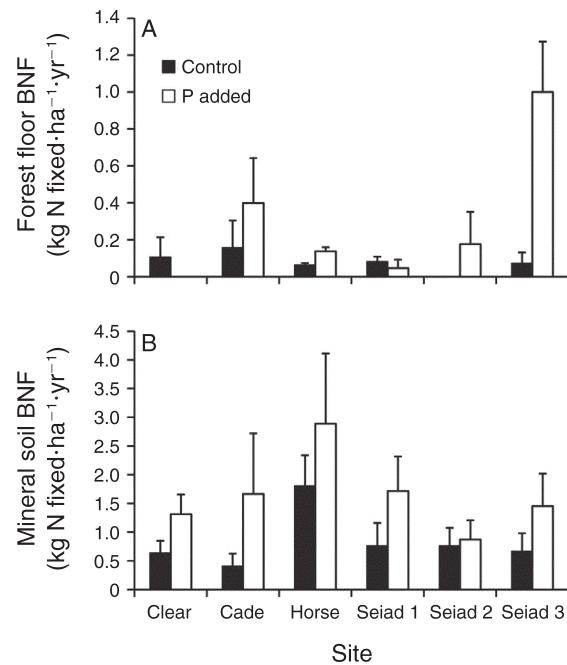


FIG. 1. Asymbiotic biological nitrogen fixation (BNF) in control and P-fertilization plots in (A) forest floor and (B) mineral soil (top 10 cm) across six different sites in the Klamath National Forest, California, USA. Two-way ANOVAs were used to test the effects of P fertilization, site, and their interaction on BNF. Results from statistical tests: forest floor fertilization, $F_{1,24} = 7.95$, $P < 0.01$; site, $F_{5,24} = 4.22$, $P < 0.01$; fertilization \times site, $F_{5,24} = 4.06$, $P < 0.01$. Mineral soil fertilization, $F_{1,24} = 5.56$, $P < 0.03$; site, $F_{5,24} = 1.74$, $P = 0.16$; fertilization \times site, $F_{5,24} = 0.23$, $P = 0.95$. Bars represent means \pm SE.

floor and mineral soil asymbiotic BNF rates were small (1% and 11%, respectively) compared to average *Ceanothus* BNF (7.46 ± 1.48 kg N·ha⁻¹·yr⁻¹). Even if we estimate BNF using the lowest published ¹⁵N calibrated conversion rates, asymbiotic N fixation in the forest floor (0.09 N·ha⁻¹·yr⁻¹) and mineral soil (2.5 kg N·ha⁻¹·yr⁻¹) remain substantially less than *Ceanothus* BNF.

Ceanothus BNF

Phosphorus fertilization did not affect *Ceanothus* BNF (two-way ANOVA; site $F_{5,29} = 0.53$, $P = 0.75$; treatment $F_{1,29} = 0.83$, $P = 0.40$), %Ndfa (two-way ANOVA; site $F_{5,29} = 2.26$, $P = 0.07$; treatment $F_{1,29} = 0.03$, $P = 0.86$), or biomass (two-way ANOVA; site $F_{5,29} = 4.43$, $P < 0.01$; treatment $F_{1,29} = 0.41$, $P = 0.53$), although fertilization did successfully increase soil P (two-way ANOVA; site $F_{5,29} = 4.17$, $P < 0.01$; treatment $F_{1,29} = 31.24$, $P < 0.01$). We therefore pooled *Ceanothus* BNF data in control and fertilization treatments. In addition, P fertilization did not affect any measure of soil N (potential net N mineralization, soil available N, total dissolved nitrogen) or foliar P of any species tested (all $P > 0.05$), but did decrease the N:P of *Ceanothus*

foliage (two-way ANOVA; site $F_{5,29} = 2.66$, $P = 0.04$; treatment $F_{1,29} = 4.71$, $P = 0.04$).

Ceanothus biomass and BNF correlated positively with potential net N mineralization (Appendix B: Fig. B3). The site with the lowest *Ceanothus* BNF (Horse Creek) had potential net N mineralization rates that were at least 10 times lower than sites with high BNF (e.g., Seiad 1 or 2). *Ceanothus* BNF tended to be lower as Et increased ($r^2 = 0.44$, $P = 0.15$), though this was nonsignificant.

While %Ndfa did not correlate with BNF, *Ceanothus* biomass was a significant predictor of *Ceanothus* BNF (Fig. 2A, B). Therefore, to further explore the influence of climate on BNF, we examined how climate was related to *Ceanothus* biomass and found that *Ceanothus* biomass decreased as Et increased (Fig. 3A). This result (less plant growth in more productive climates) could potentially be explained by tree biomass (Fig. 3B), which increased with Et. Indeed, trees negatively affected *Ceanothus* biomass (Fig. 3C), suggesting a competitive effect. Breaking down the effect of trees further, broadleaf species had a stronger negative effect (Fig. 3D) than conifers ($r^2 = 0.07$, $P = 0.62$, data not shown) on *Ceanothus* biomass. These relationships are evaluated further with SEM modeling.

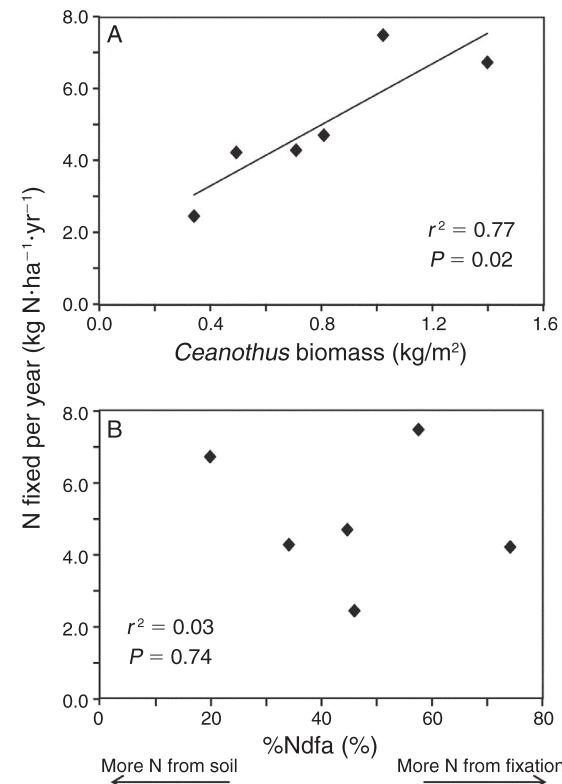


FIG. 2. Cross-site variation in *Ceanothus* BNF as a function of (A) *Ceanothus* biomass and (B) %Ndfa. %Ndfa reflects the degree to which *Ceanothus* obtains its N from atmospheric vs. soil sources. Data points represent means for each of six sites.

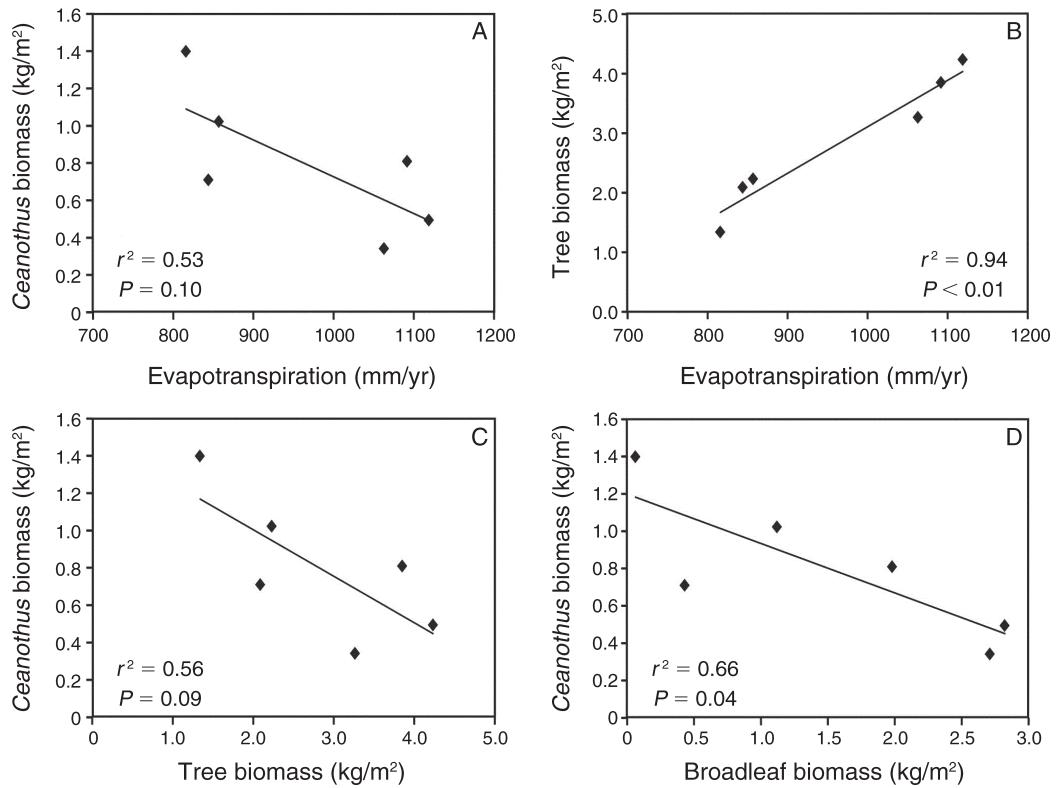


FIG. 3. Relationships among *Ceanothus* biomass, broadleaf biomass, tree biomass, and evapotranspiration (Et) in the Klamath National Forest. Data points represent means for each of six sites.

Although %Ndfa did not systematically shape patterns of symbiotic BNF across sites (Fig. 2B), it nevertheless offers insight into how different biotic and abiotic factors affect the physiological ability of shrubs to fix atmospheric N₂. Neither Et nor soil P were related to %Ndfa (Fig. 4A, B). As tree biomass increased, shrubs derived a greater proportion of N from fixation than soil sources (Fig. 4C), although this trend was not statistically significant. As noted in the methods, using Eq. 1 to quantify %Ndfa led to negative values if foliar ¹⁵N of reference species was less than that of *Ceanothus*, which we interpreted to mean that *Ceanothus* was not fixing N₂ in these plots. Relationships between %Ndfa and Et, soil P and tree biomass, however, did not change in direction or significance if %Ndfa values were used that included negative values. We also looked for relationships between %Ndfa with negative values and abiotic and biotic site values, although no significant patterns were observed.

We used SEM to understand how the univariate relationships fit together as a network of interactions. Our preliminary model (Fig. 5A) incorporated the relationships between biotic and abiotic variables that were suggested from preliminary univariate analyses. Our final model (Fig. 5B) showed correspondence between the covariance matrices implied by the model and observed with empirical data ($\chi^2 = 8.45$, $df = 5$, $P =$

0.11). The paths from Et to *Ceanothus* biomass or %Ndfa were nonsignificant and were deleted. Thus Et did not directly affect the different components of *Ceanothus* BNF. Rather, Et strongly affected tree biomass, which directly and negatively affected *Ceanothus* BNF, confirming the hypothesized indirect effects of Et on *Ceanothus* biomass and BNF as mediated through tree biomass.

DISCUSSION

Nitrogen inputs from asymbiotic and symbiotic N-fixing organisms play an important role in the succession of N-limited forests (Chapin et al. 1994, Menge and Hedin 2009). We explored the abiotic and biotic factors that drive regional variation in BNF in early-successional post-fire forest communities of the Klamath/Siskiyou region. Our results suggest that asymbiotic BNF was P-limited, whereas symbiotic BNF in *Ceanothus* was not. In contrast, *Ceanothus* BNF seemed largely driven by competition from non-fixing trees; productive sites with high Et sustained high tree biomass that suppressed *Ceanothus* biomass, leading to decreased symbiotic BNF. Overall, these results suggest that the effects of nutrients play a larger role in driving asymbiotic than symbiotic fixation. However, symbiotic fixation rates were 8–90× greater than asymbiotic rates, and symbiotic rates were controlled indirectly by tree

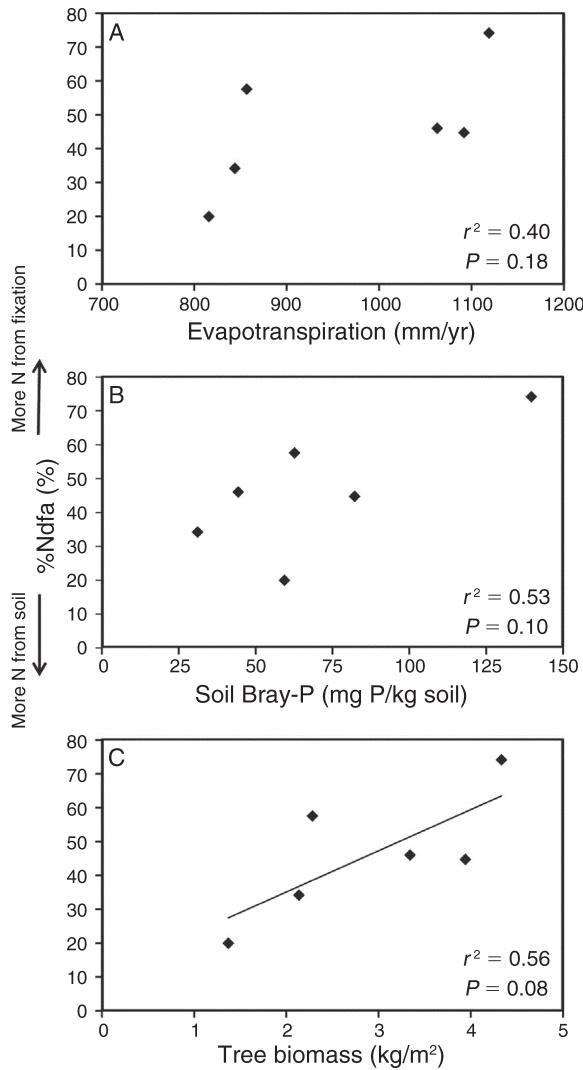


FIG. 4. Cross-site variation in %Ndfa as a function of (A) evapotranspiration, (B) soil P levels, and (C) tree biomass. Data points represent means for each of six sites.

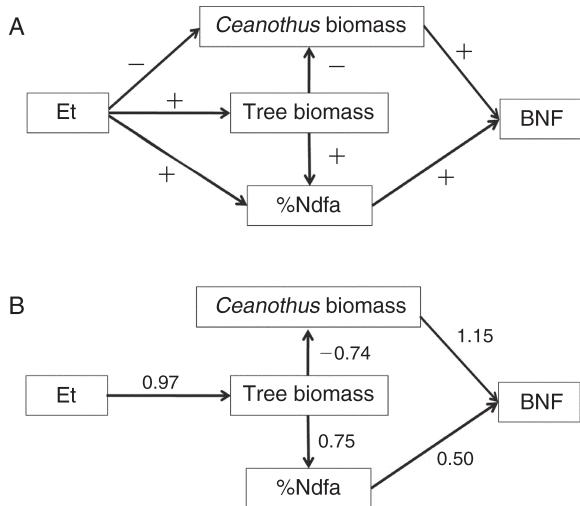
competition. Together, these results suggest that climate's effect on interspecific plant competition governs early-successional BNF inputs in these forests.

Biogeochemistry and BNF

Results from P fertilization showed that asymbiotic BNF in forest floor and mineral soil were P limited, matching results from other ecosystems (Crews et al. 2000, Vitousek and Hobbie 2000, Reed et al. 2007), although we cannot rule out the possibility that trace molybdenum in our P fertilizer contributed to this response (Silvester 1989, Barron et al. 2008). Although asymbiotic BNF varied across sites, natural regional variation in soil P availability or N:P ratios did not explain these differences, although forest floor P and C did correlate well with forest floor N-fixation rates (Appendix B: Fig. B1c, d). It is possible that low quality

carbon compounds, such as lignin, affected forest floor BNF, possibly due to site-level differences in species composition (Aber and Melillo 1982) and/or inputs of woody material and/or accumulations of partially decomposed detritus (Currie et al. 2002). Indeed, lignin has been demonstrated to substantially slow BNF in litter in Hawaiian forest ecosystems (Vitousek and Hobbie 2000). The dominant tree species in our sites (*Quercus* spp., Douglas-fir, and *Pinus* spp.) tend to have high lignin concentrations (Aber and Melillo 1982), which may help explain why forest floor ARA and BNF rates were lower in the Klamath than other ecosystems (Son 2001). Low fixation rates may have also been exacerbated by steep slopes and rocky soils, which tend to preclude the formation of soil with a deep organic horizon or forest floor layer.

Phosphorus can in some cases limit symbiotic BNF (Crews 1993, Uliassi et al. 2000, Finzi and Rodgers 2009), although we found that P fertilization did not change *Ceanothus* BNF, biomass, nor the percentage of *Ceanothus* tissue N derived from fixation. In addition, regional variation in soil P and N:P was not related to *Ceanothus* BNF rates. It is possible that P was not



Indirect effects

$$\begin{aligned}
 \text{Et} \rightarrow \text{Tree biomass} \rightarrow \text{Ceanothus biomass}: & -0.72 \\
 \text{Et} \rightarrow \text{Tree biomass} \rightarrow \text{Ceanothus biomass} \rightarrow \text{BNF}: & -0.46 \\
 \text{Tree biomass} \rightarrow \text{Ceanothus biomass} \rightarrow \text{BNF}: & -0.46
 \end{aligned}$$

FIG. 5. (A) Conceptual model of relationships between abiotic and biotic factors affecting cross-site variation in *Ceanothus* BNF. Plus and minus signs on arrows denote hypothesized relationships given univariate data. Structural equation modeling was used to analyze the conceptual model. (B) Paths that were not significant at the $P \leq 0.05$ level were deleted from the final model. Numbers denote standardized path coefficients, and below the model are standardized indirect effects and path coefficients (see Methods). R^2 , the proportion of variation explained by all paths, for different endogenous variables is, for *Ceanothus* biomass, 0.55; tree biomass, 0.94; %Ndfa, 0.56; BNF, 0.94.

limiting in these forests: soil P was high compared to other forest ecosystems (e.g., Romania et al. 1994, Perakis et al. 2006). A lack of P-limitation may have also been due to stronger limitation by other resources such as water, known to limit forest growth in the region (Whittaker 1960, Harrington and Tappeiner 2009).

There is wide variation in the degree to which symbiotic N-fixing species obtain N from fixation vs. soil. BNF is energetically costly, and high soil N supply can suppress nodule formation in N-fixers, leading to down-regulation of BNF (Ingestad 1980, Fujikake et al. 2003, Barron et al. 2011). For some genera and ecological settings, however, it appears that soil N availability under natural field conditions is rarely high enough to trigger overall suppression of plant-level BNF (Binkley et al. 1994, Menge and Hedin 2009). Indeed, we found that *Ceanothus* BNF was greatest at sites with high soil N availability (Appendix B: Fig. B3), which suggests that *Ceanothus* did not down-regulate BNF in response to the range of soil N observed in the field. Instead it appears that BNF increased soil N availability over time, as has been found for other N-fixing species (Zavitkovski and Newton 1968, Oakley et al. 2003, Erickson et al. 2005). Higher rates of external N availability than we observed in the field, such as those applied under experimental N fertilization, may be necessary to suppress nodulation and BNF in *Ceanothus* species (Thomas and Berry 1989).

Even though field rates of *Ceanothus* BNF in our study did not exhibit down-regulation at high soil N supply (Appendix B: Fig. B3), our results highlight how down-regulation of ecosystem BNF by N-fixers may instead occur via community processes (competitive exclusion of N-fixers by late successional species [Menge and Hedin 2009]). For example, it is possible that higher productivity Klamath sites (with low fixation rates) once had high *Ceanothus* BNF that already senesced. Under this scenario, we would expect that *Ceanothus* biomass was greater before tree canopy closure, and that there would be more standing dead stems of *Ceanothus* in higher productivity plots. This was not the case, as the number of dead *Ceanothus* stems was not positively related to Et ($r^2 = 0.09, P = 0.56$) or tree biomass ($r^2 = 0.13, P = 0.48$). Thus, our data suggest that, in the Klamath ecosystem, BNF is not down-regulated as soil N becomes non-limiting, but that the highest uptake of N by non-fixers potentially occurs in those sites where there is the least amount of BNF. Experimental N-fertilization across our productivity gradient would elucidate the degree to which different sites remain N-limited after the early-successional flush of BNF by *Ceanothus*.

Competition and BNF

Total BNF inputs in these 22-year-old forests were largely determined by symbiotic BNF, which was constrained by competition from later successional tree species (Fig. 5B). Broadleaf trees, which resprout after

fire to quickly achieve height and biomass (Harrington and Tappeiner 2009), were an important determinant of this pattern, as broadleaf biomass and density were negatively correlated with *Ceanothus* biomass and density (Fig. 3D, Appendix A: Table A1). In addition, resprouting oak species in our plots averaged 4.9 ± 0.9 m in height across sites, while, in contrast, *Ceanothus* heights were generally less than 2 m (personal observation). This suggests that resprouting broadleaf species easily overtopped N-fixing shrubs and caused light limitation. Species such as *Quercus chrysolepis*, *Q. kelloggii*, and *Arbutus menziesii*, dominants in our high productivity plots, are well known to exert competitive effects by lowering resources such as water and light (Pabst et al. 1990, Harrington et al. 1994). We found that competition for N may also be important: in high productivity plots, *Ceanothus* obtained a greater proportion of its N from atmospheric sources (Figs. 4c, 5b), presumably because soil N was preempted by non-N-fixing tree species.

If high productivity sites had higher BNF rates earlier in succession, we may be underestimating *Ceanothus* N-inputs. Our data suggest that this was not the case, and that *Ceanothus* biomass, and thus BNF, may remain consistently low through succession in high productivity plots due to early competition from quickly resprouting broadleaf species. In addition, results from other studies suggest that %Ndfa is unlikely to have been substantially higher earlier in succession. Busse (2000) did not find differences in *Ceanothus velutinus* %Ndfa across different-aged stands, and Kim (1987) found similarly low cumulative BNF rates in young *Ceanothus integrifolius* stands in Oregon. However, chronosequence studies or detailed remote sensing data would help resolve how succession proceeds in the region, which is important for further constraining N budgets.

Other studies have suggested or demonstrated that later successional species outcompete N-fixing species, potentially leading to forests remaining in N-limited states (Binkley et al. 1992, Chapin et al. 1994, Busse 2000, Menge et al. 2010). Indeed, Menge et al. (2010) showed that N-fixing tree species are less shade tolerant than non-fixing tree species. This suggests that phenomenological plant competition models (e.g., Lotka-Volterra) may be useful in predicting Nfixer persistence, though these types of models are not often used to predict ecosystem N inputs.

Symbiotic BNF can potentially be affected by various factors (e.g., climate, nutrients, light, competition), all of which can affect plant growth rates, or the physiology of the fixation process (Vitousek et al. 2002). Understanding the relative importance of these mechanisms will further our ability to predict N inputs over larger spatial and temporal scales. In our study, the biomass of *Ceanothus* explained a larger component of cross-site variation in BNF rates than %Ndfa, suggesting that the factors affecting shrub growth rates exert strong controls on ecosystem N inputs. Indeed, Nfixer or

nodule biomass has been shown to drive BNF rates across a wide range of ecosystems: *Acacia koa* in Hawaii (Pearson and Vitousek 2001), *Alnus tenuifolia* and *A. viridis* in Alaska (Uliassi and Ruess 2002, Mitchell and Ruess 2009), *A. rubra* and *Ceanothus velutinus* in Oregon (McNabb and Cromack 1983, Binkley et al. 1994, Busse 2000), and *Coriaria arborea* in New Zealand (Menge and Hedin 2009). Consequently, variation in annual symbiotic BNF of such species across a landscape may be readily calculated from data on N-fixer biomass, with less intensive information on %Ndfa. In this case, large-scale annual estimates of BNF, or relative differences in BNF between sites, can be derived from vegetation inventory information, with fewer cost- and labor-intensive measurements of BNF via ^{15}N dilution or acetylene reduction. Estimates of cumulative BNF over time would also have to incorporate growth models for the N-fixing species.

Interestingly, the effects of climate on BNF rates showed the opposite pattern in the Klamath/Siskiyou region as they do on a global scale. Across biomes globally, BNF tends to increase as a function of Et (Cleveland et al. 1999). In contrast, Et had an indirect negative effect on symbiotic BNF rates in the Klamath/Siskiyou because higher Et sites invoked more competition from trees (Fig. 5B). This cross-site pattern may hold true for ecosystems where BNF is governed by competitive interactions between early-successional N-fixers and non-fixing later successional species: specifically, competitive exclusion is likely to occur more quickly and thoroughly in high-productivity sites. On the other hand, at larger (global) scales, cross-site variability in plant biomass may be swamped by variability across biomes, especially as the species of N-fixer changes to ones with different NPP (e.g., *Ceanothus* vs. *Alnus rubra*).

Fire, fixation, and ecosystem N budgets

Nitrogen tends to be limiting to primary productivity in fire-prone ecosystems because fire combusts N in plant and soil pools, leading to net N losses (Raison 1979, Wan et al. 2001, Bormann et al. 2008). However, fire cycles that promote colonization by symbiotic N-fixing species can also restore N lost in fire (Johnson and Curtis 2001, Giesen et al. 2008). It is the interactive effects of fire and BNF that ultimately determine long-term forest N-budgets, and lead to the varied range of effects of fire on soil N (Johnson and Curtis 2001, Perakis et al. 2011).

Our results suggest that, unlike other *Ceanothus* species (Johnson et al. 2005), N inputs by *C. integrifolius* and asymbiotic fixation may not currently restore fire N losses. Averaged across sites, *Ceanothus* and asymbiotic fixation provided $8.3 \text{ kg N}\cdot\text{ha}^{-1}\cdot\text{yr}^{-1}$, which, over the 22 years since the 1987 wildfires would add up to $183 \text{ kg N}/\text{ha}$. Fire-driven N losses from western U.S. forests vary, but published estimates range from 300 to 700 kg N/ha (Johnson et al. 1998, 2005, Bormann et al.

2008, Giesen et al. 2008) suggesting that losses are not rapidly replenished by early-successional BNF via *Ceanothus*, and instead would require >100 -yr fire return intervals to allow eventual replenishment by asymbiotic fixers. Low rates of atmospheric N deposition across this region are also insufficient to restore fire N loss ($<1 \text{ kg N}\cdot\text{ha}^{-1}\cdot\text{yr}^{-1}$; data available online).⁴ When considered spatially across our sites, imbalances between N inputs and fire N losses may be even larger: sites with the lowest N inputs from fixation (89 kg N/ha over 22 years), had the greatest tree biomass (Fig. 3C, D), and thus stand to lose the most N capital from plant pools in wildfire events. In addition, *Ceanothus* densities increase with salvage logging and shrub management operations (Lopez 2007), suggesting that we may actually be overestimating *Ceanothus* fixation inputs for non-managed areas of the Klamath National Forest.

It is unlikely that these forests have sustained such N losses with each fire cycle over long time scales. It is possible that we have underestimated asymbiotic and/or *Ceanothus* fixation rates, possibly due to using incorrect ARA conversion rates (Appendix B: Table B1) and studying BNF close to shrub senescence (McNabb and Cromack 1983), and that we have not fully characterized alternate sources of N input from fog, dry deposition, and epiphytic cyanolichens. It is also possible that current management practices, such as fire suppression, have widened imbalances between N inputs and loss with each fire cycle. Fire suppression can lead to decreased ecosystem N in regions where early successional N-fixers result in the largest N inputs (Johnson and Curtis 2001, Oakley et al. 2003). The Klamath/Siskiyou forests have been under a policy of fire suppression since 1905, and this has continued until at least 1995 (Staus et al. 2002, Taylor and Skinner 2003). This policy reduces the ability of *Ceanothus* to colonize and fix N, and may also lead to fires with higher N losses. A return to frequent low-severity fire regimes would result in fires that promote shrub growth within intact older stands (Oakley et al. 2003, Spies et al. 2006) and may reduce N limitation in these forests.

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⁴ <http://nadp.sws.uiuc.edu/data/amaps/ndep/amaps.html>

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SUPPLEMENTAL MATERIAL

Appendix A

Supplementary study site information, including photographs, vegetation data, and allometric equations, used to obtain tree and shrub biomass ([Ecological Archives E094-064-A1](#)).

Appendix B

Supplementary results: asymbiotic N-fixation rates recalculated with a range of published conversion factors, forest floor and soil ARA rates, and N-mineralization rates ([Ecological Archives E094-064-A2](#)).