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Rates and controls of nitrogen fixation in postfire lodgepole pine forests

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Abstract

Severe, stand-replacing wildfire substantially depletes nitrogen (N) stocks in subalpine conifer forests, potentially exacerbating N limitation of net primary productivity in many forested regions where fire frequency is increasing. In lodgepole pine (*Pinus contorta* var. *latifolia*) forests in the Greater Yellowstone Ecosystem (GYE), long-term data show surface soil and biomass N stocks are replenished during the first few decades following wildfire, but the source(s) of that N are unclear. We measured acetylene reduction rates in multiple cryptic niches (i.e., lichen, moss, pine litter, dead wood, and mineral soil) in 34-year-old lodgepole pine stands in the GYE to explore the rates, temporal patterns, and climate controls on cryptic N fixation. Acetylene reduction rates were highest in late May (0.376 nmol C_2H_4 g^{-1} h^{-1}) when moisture availability was high compared with early August and mid-October when moisture was relatively low (0.112 and 0.002 nmol C_2H_4 g^{-1} h^{-1}, respectively). We observed modest rates of nitrogenase activity in a few niches following a mid-summer rain event, suggesting that moisture is an important factor regulating field-based N fixation rates. In a laboratory experiment, moss responded more strongly to temperature and moisture variation than all other niches. Acetylene reduction rates in dead wood increased with temperature but not moisture content. No other niches showed clear responses to either moisture or temperature manipulation. Together, the field and laboratory results suggest that frequent asynchrony between favorable temperature and moisture conditions may limit N fixation rates in the field. Overall, total annual cryptic N fixation inputs (mean: 0.26; range: 0.07–2.9 kg N ha^{-1} $year^{-1}$) represented <10% of the postfire biomass and surface soil N accumulation in the same stands (39.4 kg N ha^{-1} $year^{-1}$), pointing to a still unknown source of ecosystem N following fire.

KEYWORDS
associative, asymbiotic, biogeochemistry, cryptic, disturbance ecology, free-living, Greater Yellowstone Ecosystem, seasonal variation

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INTRODUCTION

Disturbances often alter nitrogen (N) cycling in forested ecosystems. For example, logging and timber harvest, insect outbreaks, and wildfire, among others, drive substantial forest N losses, potentially exacerbating N limitation in forests across the world (Aber et al., 2002; Griffin et al., 2011; Mason et al., 2022; Vitousek & Matson, 1985). Globally, coniferous forests are experiencing larger, more frequent, and more severe wildfire (Flannigan et al., 2009; Higuera et al., 2021; Van Wees et al., 2021). During high-intensity fires, large quantities of N are lost primarily via direct combustion of live tree foliage, fine fuels, and litter (Maynard et al., 2014). Increases in forest wildfire frequency and severity are projected to continue (Abatzoglou et al., 2021; Higuera et al., 2021), perhaps enhancing N losses, amplifying N limitation, and slowing forest regeneration. However, following disturbance like fire, some studies show that regenerating forests rapidly accumulate soil and biomass N (Binkley et al., 2000; Johnson & Turner, 2014; Turner, Whitby, et al., 2019). Yet, we lack a clear understanding of the processes and mechanisms that replenish N losses following disturbances like wildfires.

Biological nitrogen (N) fixation is the largest source of new N in natural terrestrial ecosystems (Vitousek et al., 2013). This fundamental biological process is carried out by a variety of symbiotic and nonsymbiotic (e.g., free-living or associative) bacteria and archaea known as diazotrophs. Symbioses between N-fixing bacteria and vascular plants have been the focus of much research on N fixation, but plant–diazotroph symbioses are relatively rare in many ecosystems (Menge et al., 2017, 2019). By contrast, more cryptic forms of N fixation (e.g., fixation by soil, leaf litter, wood, lichens, mosses, etc.) are much more ubiquitous, and may be the dominant source of fixed N in many terrestrial ecosystems (Cleveland et al., 2022; Davies-Barnard & Friedlingstein, 2020). Studies of cryptic N fixation commonly focus on N inputs via soil and/or litter, but few have measured N fixation by the full suite of possible cryptic N-fixing niches present in ecosystems (Cleveland et al., 2022; Davies-Barnard & Friedlingstein, 2020). This represents a major gap in our understanding of the patterns and rates of N fixation at the global scale, especially in ecosystems where plant–diazotroph symbioses are rare (Cleveland et al., 2022; Reed et al., 2011).

Despite strong evidence of N limitation in some northern temperate and boreal forest ecosystems (Elser et al., 2007; LeBauer & Treseder, 2008), plant–diazotroph symbioses are rare in northern conifer forests (Menge et al., 2014, 2017). Even when present, common plant species associated with diazotrophs (e.g., legumes or actinorhizal N-fixing species) are often patchy across the landscape and fix N at modest rates (Fahey et al., 1985; Halvorson et al., 1992; Tierney et al., 2019; Turner, Whitby, et al., 2019). Thus, cryptic forms of N fixation may be vital for sustaining the N economy of N-poor ecosystems where symbiotic N fixers are rare. Yet, the rates and patterns of cryptic N fixation are not well characterized in northern conifer forests despite the potentially large role cryptic N fixation may play during postfire regeneration.

The Greater Yellowstone Ecosystem (GYE) provides an excellent opportunity to examine both the patterns and rates of N fixation in subalpine conifer forests following wildfire. In lodgepole pine (*Pinus contorta* var. *latifolia*) forests that regenerated after the 1988 Yellowstone fires, mass balance estimates show large increases in ecosystem N pools between 15 and 25 years postfire (Turner, Whitby, et al., 2019). During that time, soil total N stocks (0–15 cm) increased from 857 to 1200 kg N ha^{-1}, and total live lodgepole pine biomass N (foliage, wood, and roots) increased from 55 to 106 kg N ha^{-1}. The increase in N stocks coincided with rapid increases in lodgepole pine biomass, net primary production (NPP), and thus N demand, with no evidence of a compensatory decline in surface soil N as live biomass N accumulated (Turner, Whitby, et al., 2019). Atmospheric N deposition rates in the GYE are low (2–3 kg N ha^{-1} year^{-1}; NADP, 2022) and represent only a small fraction of N demand. The large increases in N stocks—despite substantial N losses during fire and the paucity of known N fixers in these stands—suggest that cryptic N fixation may represent a critical but unknown N source during forest regeneration.

Evaluating the role of cryptic N fixation in regenerating coniferous forests is hindered by a poor mechanistic understanding of how environmental controls interact to regulate the process. Previous research has shown that N fixation rates vary through time (e.g., seasonally), suggesting strong climate controls on rates (Antoine, 2004; Caputa et al., 2013; Hicks et al., 2003; Lett & Michelsen, 2014). Broadly, cryptic N fixation is controlled by both biophysical and biogeochemical factors (Cleveland et al., 2022; Reed et al., 2011). Cleveland et al. (2022) proposed a hierarchy of controls where temperature and moisture play strong direct and interacting roles. Temperature is posited to have a positive effect on N fixation across all N-fixing niches up to a theoretical optimum of ~25°C (Houlton et al., 2008). However, this optimum differs for some niches (Bytnerowicz et al., 2022; Rousk et al., 2018). Substrate moisture content also exerts varying control over N fixation across a range of N-fixing niches, with rates generally increasing with moisture content following either controlled hydration treatments or increases in field moisture conditions (e.g., rainfall or snowmelt) (Antoine, 2004; Austin et al., 2004; Hicks et al., 2003;

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Jackson et al., 2011; Rousk et al., 2017). However, few studies have explored the effects of both temperature and moisture on N fixation rates across multiple niches. A deeper understanding of mechanistic drivers would enhance the understanding of N fixation dynamics in forests and the ability to better predict how climatic variation may influence N fixation rates.

Here, our overarching goal was to explore the patterns and rates of cryptic N fixation during postfire forest stand development and how temperature and moisture regulate rates. More specifically, we addressed three primary research questions: (1) What is the magnitude of N fixation inputs via cryptic N fixers? (2) How do cryptic N fixation rates vary temporally? (3) How do temperature and moisture regulate cryptic N fixation rates? To address these questions, we measured N fixation rates in a set of regenerating lodgepole pine sites four times during the growing season over one year. We also performed a series of experimental laboratory incubations under controlled climate conditions to explore mechanistic controls on N fixation rates. We hypothesized that the highest rates of cryptic N fixation in the field would occur in the spring months when moisture availability and temperature are both relatively high, and lower when temperature and/or moisture are relatively low. Under controlled conditions, we hypothesized that N fixation rates would increase as gravimetric water content (i.e., % of dry mass) increased from ~10% to ~100% and from 5 to 35°C, with rates peaking at the theoretical N fixation optimum temperature of 25°C and a saturated hydration state (~100% gravimetric water content).

METHODS

Study sites

The study was conducted in a subset (five) of 14 forest sites previously established in Yellowstone National Park (YNP) (Figure 1). The plots are all dominated by lodgepole pine that regenerated following stand-replacing fires in 1988 and are in a phase of rapid biomass accumulation (Turner et al. 2007; Turner, Whitby, et al., 2019). All five sites are characterized by similar topography and elevation (~2100 m). Soils at all sites are shallow Inceptisols formed in Quaternary rhyolitic bedrock and rhyolite-dominated glacial deposits (Hechtman Soil Series, USDA, 2008). The mean annual temperature (MAT) is 4.6°C and the mean annual precipitation (MAP) is 391 mm. On an annual basis, the climate in Yellowstone is cold and wet during the winter (January mean temperature $= -10°C$; mean precipitation $= 53.6$ mm) and warm and dry in the summer (July mean temperature $= 14°C$ and mean precipitation $= 39.4$ mm) (1981–2010 normals, Western Regional Climate Center, 2023). Mean monthly temperature and precipitation during the study period were similar to 30-year averages albeit for a brief period of unusually warm and wet conditions in late June 2022 during which no measurements were made. The study plots included five of the most productive plots (average aboveground NPP $= 12.4$ Mg ha^{-1} year^{-1}; Appendix S1: Table S1) from Turner, Whitby, et al. (2019). Each of the 50×50 m plots at each site has been monitored extensively since 1988 (Turner et al. 2007, 2016; Turner, Whitby, et al., 2019).

Cryptic N fixation rate measurements

We measured N fixation in five distinct cryptic niches, including free-living N fixation by asymbiotic heterotrophic bacteria in pine litter (Oi horizon), dead wood, and surface mineral soil (upper 2 cm of A horizon), and associative N fixation in ground mosses (consortia of *Polytrichum* and/or *Pleurozium* species on the soil surface) and lichens (consortia of *Peltigera* or *Cladonia* species on the soil surface). Associative N fixation here refers to N fixation by bacteria that do not require a specific host to fix N and that occur as nonsymbiotic associations with lichen and mosses. We collected samples of all five niches from each plot in May, August, and October 2022 and measured in situ actual rates (no water added) of ethylene production using a modified acetylene reduction assay (ARA) (Hardy et al., 1968; Reed et al., 2007). When exposed to acetylene gas, the enzyme nitrogenase, which is responsible for the reduction of N_2 into ammonia, converts acetylene to ethylene gas, thus providing a measurable proxy for N fixation activity (Hardy et al., 1968). During each sampling period, at least five samples of each niche were collected from each plot and sealed in clear 50-mL acrylic tubes. Soil, lichen, and moss samples were collected using the acrylic tubes as corers to collect soil from a consistent depth (2 cm) and a consistent area (4.91 cm^2—area of tube face) for lichen and moss. Litter and wood samples were collected by hand and placed directly into tubes. Immediately after collection, 10% of the headspace within each sample tube was replaced with acetylene gas and mixed well with a syringe. To capture changes in nitrogenase enzyme efficiency under different field temperatures throughout the year, samples were incubated in the field for roughly the same duration of time regardless of season. There was some variation in time due to logistical limitations during some of the sampling periods, resulting in incubation times ranging from 19 to 24 h. After incubation, 15 mL of headspace was removed from each tube and transferred to an air-tight evacuated glass vial (Becton-Dickinson, Inc., Franklin Lakes, NJ, USA). Vials were transported to the University

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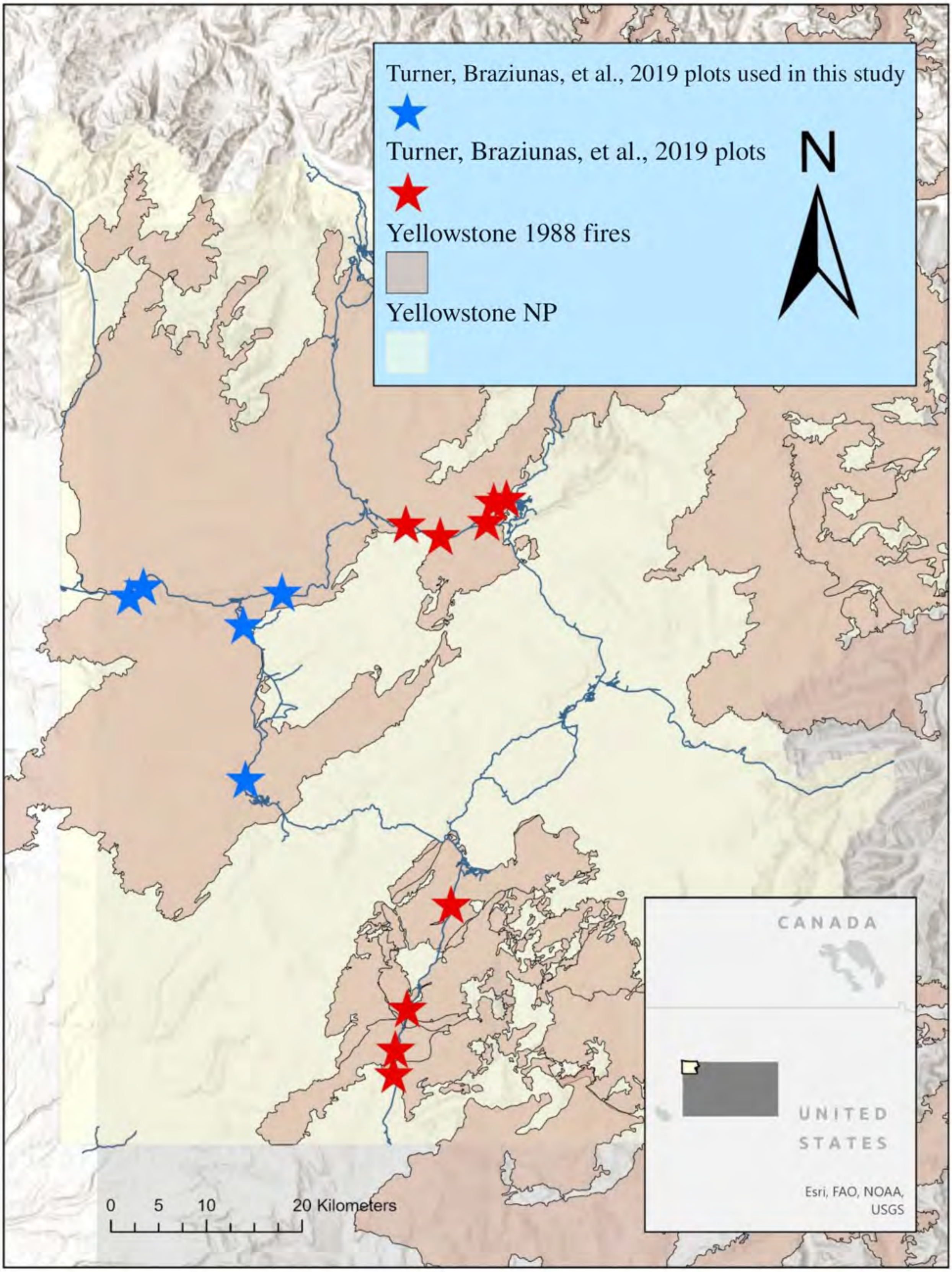

FIGURE 1 Map of the study sites in Yellowstone National Park (NP) in Wyoming, USA. Blue stars represent the location of plots measured in this study. Red and blue stars represent all plots in which forest N pools have been monitored since severe stand-replacing fire in 1988 (Turner, Whitby, et al., 2019).

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of Montana and refrigerated until ethylene content was analyzed using a Shimadzu GC-2014 gas chromatograph equipped with a flame ionization detector (Shimadzu Inc., Kyoto, Japan). Vegetation samples were oven-dried at 65°C, and soil samples were dried at 105°C for 48 h and reweighed to determine gravimetric moisture content (Topp, 1993). The ratios of acetylene reduced to N_2 reduced by nitrogenase (*R* ratio) vary widely among cryptic niches (Soper et al., 2021). Therefore, to convert acetylene reduction (AR) rates to N fixation rates, we used the geometric mean of $^{15}N_2$-calibrated *R* ratios for each niche as well as the minimum and maximum *R* ratios published in Soper et al. (2021) to calculate the ranges of N fixation inputs (Table 1).

Scaling field-based N fixation rate measurements

We used plot-level abundance, biomass, or mass/area data for each niche paired with ARA measurements to generate niche-specific spatial N fixation rates. For lichen and moss, we measured percent cover in 25 0.25-m^2 quadrats distributed equally along three parallel 50-m transects in each plot. Moss and lichen N fixation rates were calculated by pairing N fixation rates as measured by ARA with the known sample area (4.91 cm^2) and plot-level percent cover data. For litter, we systematically collected all forest floor litter within three 900-cm^2 squares placed in each of four quadrants within each plot. Litter samples were composited by quadrant and oven-dried to obtain dry biomass/area estimates per plot. For soil, scaled N fixation rates (in kilograms of N per hectare per year) were calculated using the rate of N fixed per unit dry mass of soil and average sample bulk density (in grams per cubic centimeter) in each plot measured to a depth of 2 cm. However, we assumed an active soil N fixation depth of 10 cm in all spatially scaled estimates. For wood, we measured the diameter of both ends of coarse woody debris that intersected three transects oriented at 0°, 120°, and 240° off the southern, eastern, and western edges of the plots (Turner, Braziunas, et al., 2019). Nitrogen fixation in dead wood is typically highest in more decayed wood (Jurgensen et al., 1984; Wei & Kimmins, 1998). Thus, we limited sampling to fully grounded, easily punctured, decaying dead wood that lacked bark and needles (decay class 4–5; Brown, 1974). We calculated plot-level dead wood biomass estimates for both decay classes using established protocols (Brown, 1974; Turner, Braziunas, et al., 2019) to upscale N fixation rates in decaying wood. The estimates of biomass for each niche used to scale up rates are presented in Appendix S1: Table S1.

The snow-free season in the five GYE sites is typically a 6-month period from May to October. We measured N fixation rates every two months within the snow-free period to calculate seasonal ("spring," "summer," "fall") and ultimately annual N fixation rates. However, for the summer N fixation estimate, the study plots received 4.17 mm of rain the day after our initial summer sampling concluded (NOAA, 2022). Thus, we used this opportunity to explore the effects of a mid-summer pulse of rain on N fixation by resampling all plots/niches within 24 h of the rain event. As a result, the summer N fixation estimate represents the mean of N fixation rates measured during the two sampling periods ("summer before rain event" and "summer after rain event"—Table 1). Annual N fixation rates were calculated first using mean rates at the plot level scaled on an hourly basis using 1460 h as a two-month long "seasonal" period. The seasonal rates were then summed to estimate an annual rate. We assumed that N fixation occurred at negligible rates in all niches from November to May due to the freezing or near-freezing soil temperatures that persist in YNP under snow (USDA, 2023), and evidence that N fixation rates at freezing temperatures

TABLE 1 Mean, median, and estimated range of annual N fixation rates ($n = 5$) among five cryptic niches, with *R* ratios from Soper et al. (2021) used to calculate the mean and range of N fixation rates.

	Annual N fixation rate (kg N ha^{-1} $year^{-1}$)			*R* ratio		
Niche	**Mean (±1 SE)**	**Median**	**Range**	**Geometric mean**	**Minimum**	**Maximum**
Lichen	0.1 ± 0.073	0.031	0.042–0.28	4.5	1.6	10.8
Litter	0.003 ± 0.001	0.003	0.001–0.012	3.5	0.8	13.3
Moss	0.006 ± 0.002	0.005	0.003–1.694	[a]3	0.01	5.4
Soil	0.136 ± 0.036	0.112	0.019–0.843	3.1	0.5	22
Wood	0.019 ± 0.006	0.019	0.008–0.07	4.1	1.1	10.2
Total	0.263 ± 0.119	0.169	0.072–2.9			

[a] No geometric mean for moss was reported in Soper et al. (2021), so we used the standard 3:1 *R* ratio.

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are generally very low (Hicks et al., 2003; Hobara et al., 2006; Rousk et al., 2017).

Temperature and moisture control on cryptic N fixation

Associative N fixation—To explore the effects of temperature and moisture on associative N fixation rates (lichen and moss), we conducted a full-factorial (moisture × temperature) incubation experiment in the laboratory. In May 2022, we collected moss and lichen samples (96 per niche) from the five study sites. Samples were transported to the laboratory at the University of Montana within 24 h of sampling, randomly organized into groups of six replicates, and assigned one of four moisture treatments: no treatment, 2 h of drying at 35°C, 3.5 h of drying at 35°C, or treated with 1.2 mL of de-ionized water. The treatments were designed to create a gradient of moisture levels across all samples in each niche using the upper range of daily maximum temperatures in YNP (35°C). After treatment, samples were kept in partial sunlight in a greenhouse for 24–48 h until the experiment began. Groups of 24 samples from each niche were then incubated in controlled environmental chambers at 5, 15, 25, or 35°C, with 154–159 μmol m^{-2} s^{-1} of photosynthetically active radiation to simulate understory light (e.g., Gundale et al., 2012). This was performed to acclimate the sample to their experimental treatments prior to ARA. Our primary aim is to measure the change in enzyme activity under different sustained biophysical conditions, not the immediate response to new conditions. Following a 24-h incubation period, 10% of the headspace from five of the six sample tubes in each moisture group was replaced with acetylene gas and mixed. Twenty-four hours after the acetylene was introduced, tube headspaces were sampled, stored in vacuum-sealed vials (Becton-Dickinson Inc., Franklin Lakes, NJ, USA), and refrigerated until analysis. Following the experiment, gravimetric moisture contents were obtained for each sample. Samples were then sorted into groups of 3–5 samples based on their actual moisture contents for analysis of ethylene production rates across the different levels of actual moisture content and temperature.

Heterotrophic N fixation—To explore the effects of temperature and moisture on heterotrophic N fixation rates (soil, litter, and wood), we conducted a similar but separate incubation experiment. In August 2022, we collected samples of mineral soil, decomposing plant litter, and decaying wood (160 per niche) from our field sites. Samples were transported to the University of Montana within 48 h of sample collection. Given the dry conditions at the time of sampling, the collected samples were used to represent the lowest moisture conditions. Dry samples were treated with four different levels of de-ionized water to create a range of moisture contents. Samples were incubated at the same temperatures described above. Following the experiment, gravimetric moisture contents were obtained for each sample. Following incubation, sample headspace was collected and analyzed as described above. Experimental data are reported as hourly rates of AR per treatment; we did not attempt to convert or scale measurements.

Statistical analysis

Field-based N fixation rates (by season) were calculated using plot level ($n = 5$ per niche) means for each niche. For all niches, we report arithmetic means, medians, and ranges (Table 1). While we used our seasonal sampling design to scale up N fixation rates to the annual scale, we did not test for statistical differences in scaled seasonal rates as our measurement frequency within each season was not enough to justify this. Instead, we only test for differences in AR rates between sampling periods. We analyzed the distribution of our AR data using the Shapiro–Wilk test and Q–Q plots. We found our data did not meet normality assumptions. Therefore, we used nonparametric Kruskal–Wallis tests followed by Dunn's post hoc tests to examine AR rate differences, and average sample moisture content differences between sampling periods (including before and after rain comparisons) and niches ($\alpha = 0.05$).

To analyze rates in the incubation experiments, we used AR rates as metrics of nitrogenase activity. Acetylene reduction data from these experiments were zero-inflated and strongly right-skewed with many outliers in each niche. Additionally, the moisture content data were slightly right-skewed. However, we did not remove outliers from our dataset because skewness is typical of N fixation data, and the outlying data points represent the specific microbial activity we were trying to capture (Soper et al., 2021). Using Box–Cox log-likelihood analysis, we determined that normality assumptions could not be met via log or square root transformations of the AR data due to their zero-inflated distribution. So, we first assessed whether temperature, moisture, and their interaction increased the probability of a sample being nondetectable ("inactive") or active by fitting a negative binomial generalized linear model using a logit link function, with AR rate as a binary response (0 or >0) and temperature and moisture content as covariates. Only AR rates among lichen were more likely to be above zero at higher temperatures; other niches showed no increase in nonzero probability

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with higher temperatures or moisture contents. As we could not account for other factors that may drive zero versus nonzero data, we excluded inactive samples from our analysis. “Active” (nonzero) samples met the assumptions of normality when ln-transformed. Thus, we tested the effects of temperature as a second-order polynomial on active AR rates, with square root-transformed moisture content as a continuous covariate, using analysis of covariance (ANCOVA, $\alpha = 0.05$). All analyses were performed using R statistical software (R version 4.2.2, packages: ggplot2, Wickham, 2016; multcompView, Graves et al., 2019; ggpubr, Kassambara, 2020; dplyr, Wickham et al., 2023; nlme, Pinheiro & Bates, 2024).

RESULTS

Annual N fixation rates

Across all plots, mean N inputs via fixation ranged from 0 to 0.14 kg N ha^{-1} $year^{-1}$ for each niche (Figure 2), and total cryptic N input (the sum of mean rates in all niches)

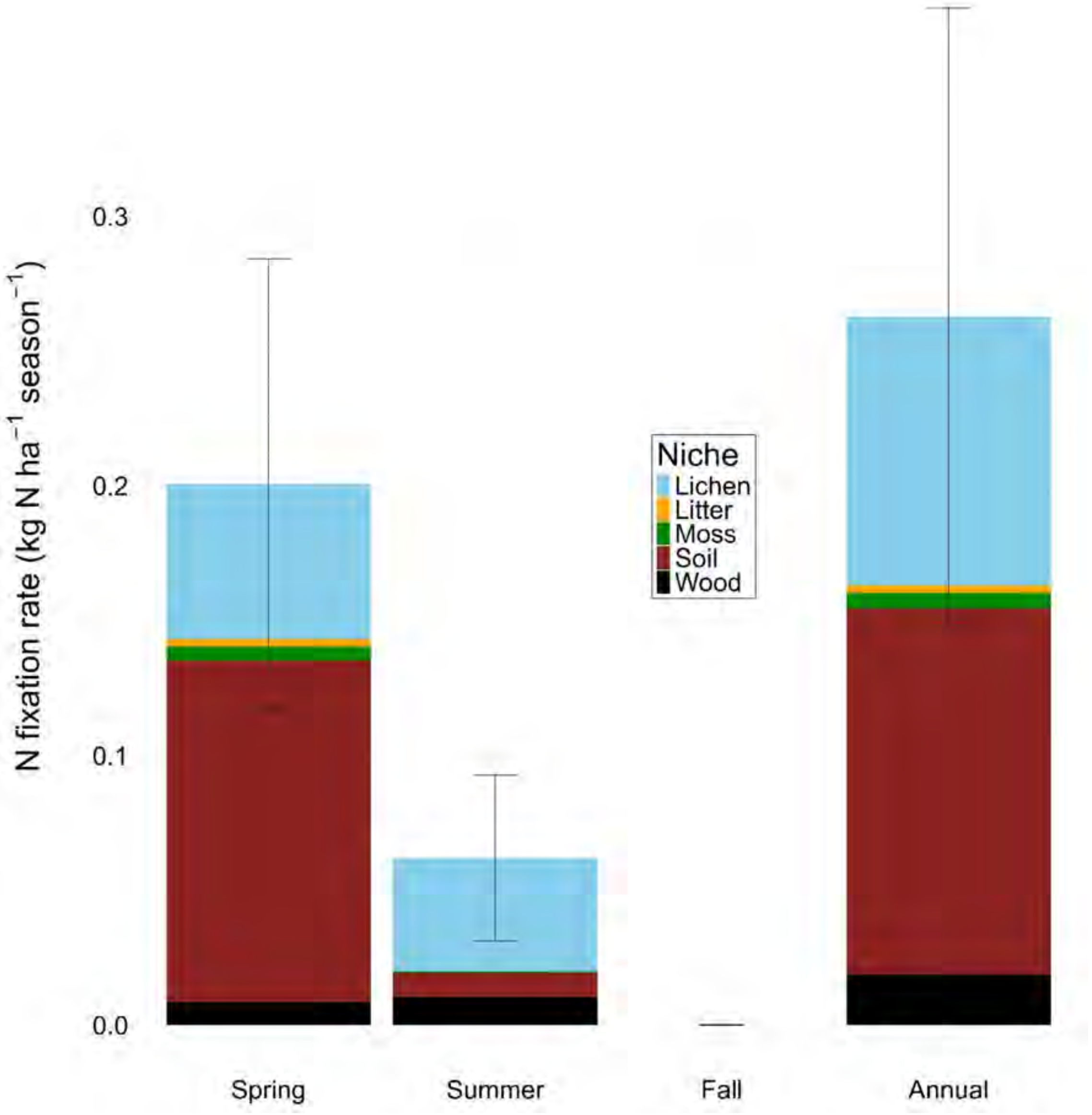

FIGURE 2 Seasonal (May–June, July–August, and September–October) rates of cryptic N fixation. Rates were calculated using mean acetylene reduction rates for each niche during each sampling period among five 34-year-old lodgepole pine-dominated study plots ($n = 5$), geometric mean R ratios listed in Table 1, and the biomass estimates in Appendix S1: Table S1. Different colors represent rates measured in the different cryptic niches. Error bars represent ±1 SE.

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was 0.26 ± 0.12 kg N ha^{-1} $year^{-1}$ (Table 1). Using minimum and maximum ratios of acetylene reduced to N_2 fixed (*R* ratios; Soper et al., 2021), we calculated a broader cryptic N fixation input range of 0.072–2.9 kg ha^{-1} $year^{-1}$ (Table 1). Soil, lichen, and wood had the highest mean rates of N fixation among the niches (0.14 ± 0.04 kg ha^{-1} $year^{-1}$; 0.1 ± 0.07 kg ha^{-1} $year^{-1}$; 0.02 ± 0.01 kg ha^{-1} $year^{-1}$, respectively; Table 1; Appendix S1: Figure S1). Combined N fixation rates via soil, lichen, and wood accounted for ~98% of total annual cryptic N fixation inputs. Soil N fixation alone accounted for ~52% of annual cryptic N fixation, whereas lichen and wood accounted for ~38% and ~8%, respectively. Kruskal–Wallis tests showed that N fixation rates did not differ among plots. Therefore, we did not further investigate other potential relationships between site-specific characteristics (e.g., soil properties, vegetation structure) and N fixation.

N fixation rates across sampling periods

Acetylene reduction rates were highly variable during each sampling period except for October, when rates were consistently low among all niches (mean among niches: 0.002 nmol C_2H_4 g^{-1} h^{-1}). The highest mean rates among all niches occurred during either after the rain event in August (3.46 nmol C_2H_4 g^{-1} h^{-1}) or during the May sampling period (0.376 nmol C_2H_4 g^{-1} h^{-1}). Elevated AR rates during these two time periods coincided with the highest average moisture contents among niches during the measurement period (Appendix S1 Table S2). Lichen and moss had the highest AR rates in both the May and August post-rain sampling period while mean rates in soil, litter, and low were orders of magnitude lower throughout the sampling period (Figure 3).

Rainfall effects on in situ N fixation rates

The "summer" N fixation rate (Figure 2) represents the average of two measurements taken on August 1 and August 3, 2022 (Appendix S1: Table S3). The first set of samples was collected following roughly three weeks with no rain, and the second set of samples was taken within 24 h of a 4.17-mm rainfall event. The rainfall event increased the average moisture content in all niches (Appendix S1: Table S4). The increases in moisture were paralleled by an increase in average AR rates across niches. This result was largely driven by increases in AR rates in lichen, moss, and wood after rain (Figure 3) and a few high rates in the post-rain measurements in the other niches. No changes in N fixation rates were measured on a niche-specific basis in soil or litter after rain.

Temperature and moisture regulation of cryptic N fixation

In the growth chamber experiment, 10% or less of samples from each niche were not active at 5°C (Appendix S1: Figure S2). Soil and litter samples rarely had more than 10% of total samples being active in any of the temperature treatments, whereas moss, lichen, and wood generally had more active samples across temperature treatments (Appendix S1: Figure S2). Overall, temperature alone increased the probability of lichen samples being active, but not the probability of any other niches showing detectable rates. Moisture was not important in increasing the probability of any of the niches showing detectable rates of AR (Appendix S1: Table S6).

Active lichen samples had considerably higher AR rates than other niches but showed no consistent response to temperature and moisture treatments (Figure 4). In active moss samples, AR rates decreased with temperature from 15 to 25°C, whereas AR rates in active wood samples increased across the same temperature gradient (Figure 4; Appendix S1: Table S7). Among active moss samples, AR rates also increased with increasing moisture content ($F_{1,15} = 8.64$, $p = 0.01$). No other niche responded to increasing moisture content or to the interaction of temperature and moisture content.

DISCUSSION

Cryptic N fixation inputs

This study is among the first to comprehensively assess a full suite of cryptic N fixation niches in a site, including multiple autotrophic and heterotrophic niches four times throughout a full growing season (Cleveland et al., 2022). Despite evidence of rapid postfire N accumulation in biomass and surface soils at the study sites (Turner, Whitby, et al., 2019), measured annual N inputs via cryptic N-fixing niches were low. The measured rates of N fixation by all cryptic niches (0.26 kg N ha^{-1} $year^{-1}$) were much lower than mass balance-based estimates of post-fire ecosystem N pool increases since 1988 (~ 39 kg N ha^{-1} $year^{-1}$) (Turner, Whitby, et al., 2019). These results suggest that even when considering our estimated range of cryptic N fixation input (0.07–2.9 kg N ha^{-1} $year^{-1}$), N fixation alone does not replenish fire-driven N losses. Our measured N fixation

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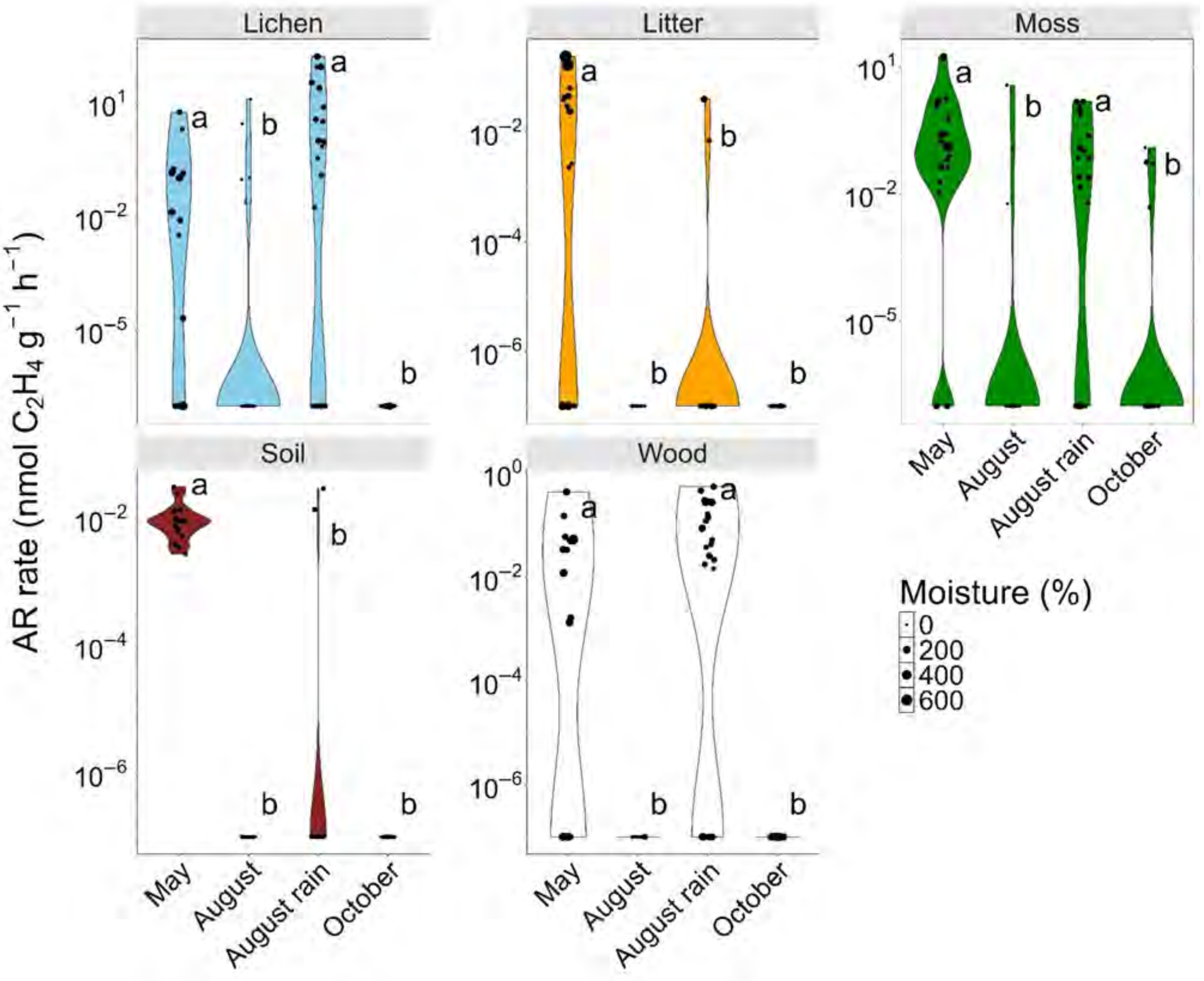

FIGURE 3 Violin plot showing the distributions of individual acetylene reduction (AR) rates among the five measured cryptic niches in 34-year-old lodgepole pine forests at four different time periods in the Greater Yellowstone Ecosystem (GYE). Individual dot sizes correspond to gravimetric moisture contents (% of dry mass). Lowercase letters correspond to significant ($p < 0.05$) pairwise differences among sampling periods specific to each niche (Appendix S1: Table S5).

rates are generally consistent with previous results in other ecosystems showing similarly low rates of N fixation following fire, although our results are among the first to explore responses to high-severity, stand-replacing burns (Tierney et al., 2019; Wong et al., 2020). Thus, the primary source of N accumulating in biomass and surface soils in these regenerating forests remains unclear.

Nitrogen fixation in mineral soil (0–10 cm), lichen, and dead wood represented the majority (98%) of the combined cryptic N fixation inputs in the sites. Total N inputs in the GYE sites were slightly higher than cryptic N fixation inputs in southern longleaf pine savannas that experience more frequent, low-intensity fire (Tierney et al., 2019), well within the range of previous estimates of cryptic N fixation inputs measured in northern temperate forests (0.01–12 kg N ha^{-1} $year^{-1}$; Son, 2001), and very similar to rates measured in a lodgepole pine forest in Wyoming (Fahey et al., 1985). On a niche-specific basis, measured N fixation rates fell on the lower end of the range of estimates reported for the same niches across various studies (Cleveland et al., 2022; Davies-Barnard & Friedlingstein, 2020; Reed et al., 2011). We posit that frequent asynchrony in near-optimal temperature and moisture conditions may limit high rates of fixation in our sites (Cleveland et al., 2022).

Temporal patterns of cryptic N fixation

Previous research has shown strong biophysical controls on cryptic N fixation, particularly temperature and moisture (Cleveland et al., 2022; Houlton et al., 2008; Reed

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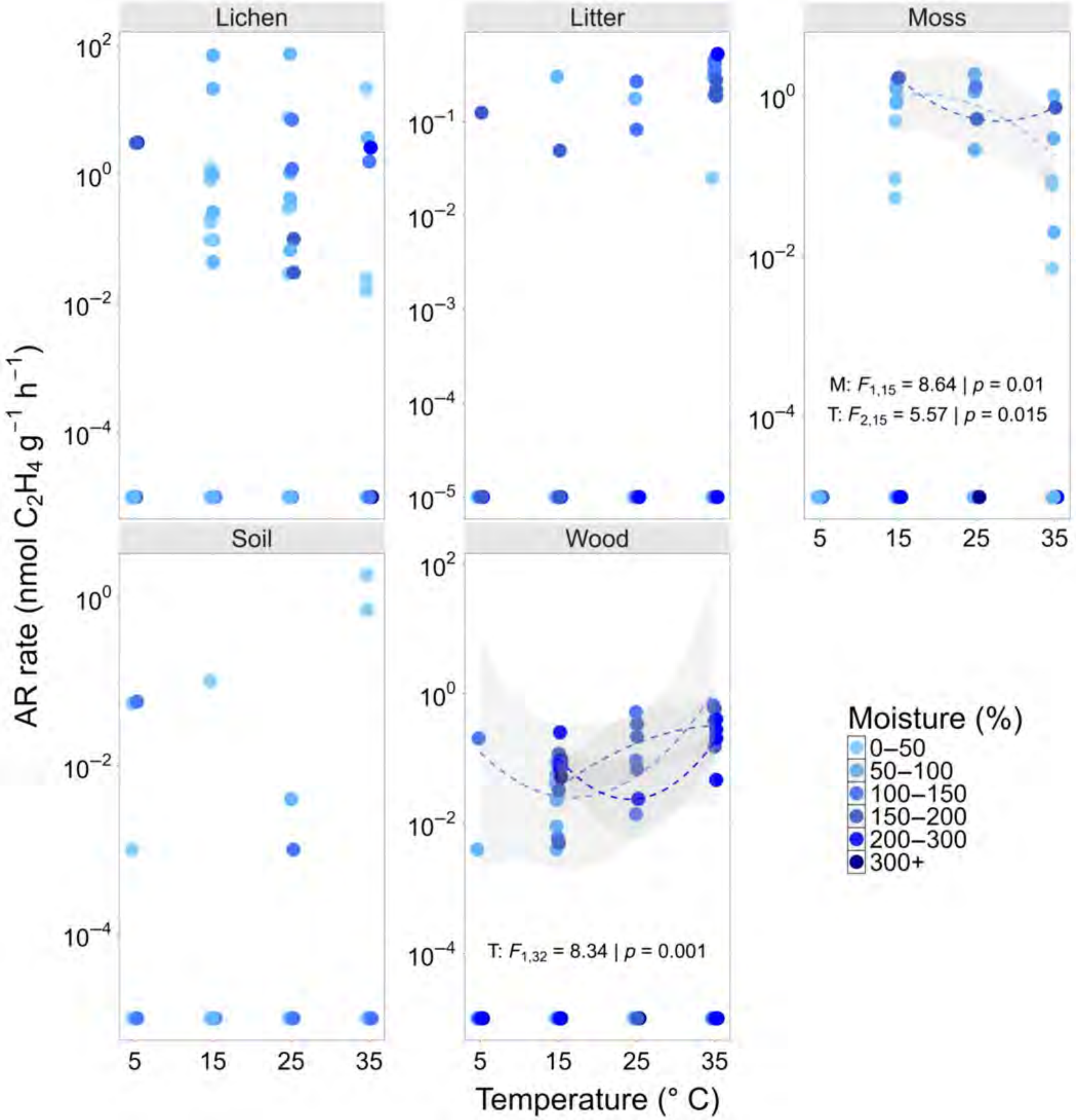

FIGURE 4 Scatter plot showing acetylene reduction (AR) rates among the five different measured cryptic niches incubated at four temperature levels ($n = 20$ for Moss and Lichen; $n = 36$ otherwise) and at varying moisture contents. Dashed lines represent linear models used in analyses of covariance (ANCOVAs) of active AR rates in response to temperature and moisture content as a covariate (with a 90% CI in gray). Niches with significant changes in AR rates contain the statistical results from ANCOVAs in their respective panel (T = temperature as a second-order polynomial, M = moisture content). Note the log scale on the *y* axes and that *y* axes are different for some panels. A complete summary of the ANCOVA results is provided in Appendix S1: Table S7.

et al., 2011; Rousk, 2022). Thus, we hypothesized that the highest rates of cryptic N fixation in the field would occur in the spring months when moisture availability and temperature are both relatively high and lower when temperature and/or moisture are relatively low. Consistent with our hypothesis, the highest measured rates of AR occurred during the May sampling period (during snowmelt) when moisture availability was relatively

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high (Figure 3). During the August sampling period, AR rates were lower compared with the May sampling period, but increased in response to a small pulse of moisture. During the October sampling period, both temperature and moisture were relatively low, and AR rates were often undetectable (Table 1). This observation is consistent with our hypothesis that both moisture availability and suitably warm temperatures are needed to sustain fixation.

We leveraged a mid-summer rainfall event (4.17 mm in 24 h) to explore the effects of episodic precipitation on N fixation rates. In most niches, the rainfall event only drove increases in AR rates in a few samples. However, dead wood, and lichen and mosses to a lesser degree, showed more consistent increases in fixation rates in response to rain (Figure 3). Wood, mosses, and lichens are known to be highly responsive to short-term increases in moisture availability (Hicks et al., 2003; Jackson et al., 2011; Larson, 1981; Woodall et al., 2020). The sensitivity to precipitation in mosses and lichen creates episodic pulses of biogeochemical activity, which have been observed in arid desert and shrubland ecosystems (e.g., Austin et al., 2004; Belnap, 2002). Consistent with the notion of biogeochemical "hot spots" and "hot moments" (McClain et al., 2003), the variable N fixation responses observed across niches after the rainfall event in this study demonstrates both the episodic nature of cryptic N fixation in semiarid systems like the GYE, as well as the sensitivity of cryptic N-fixing niches to short-term changes in environmental conditions. Importantly, this phenomenon of N fixation pulses during warm and wet periods contributes large uncertainty to our estimates of N fixation inputs on a seasonal to annual scale.

Moisture and temperature controls on nitrogenase activity

Under controlled laboratory conditions, we hypothesized that AR rates would increase with temperature and moisture content, with rates peaking near the theoretical N fixation optimum temperature of 25°C and increasing moisture contents to 100% gravimetric content. However, the effects of moisture (alone) and the interactive effects of moisture and temperature on AR rates were weak in the experiment. Nitrogenase activity was highly variable across niches in response to moisture and temperature variation, with large portions of samples in each temperature treatment showing no detectable rates of AR (Appendix S1: Figure S2). Because the diazotrophic community in each niche experiences slightly different biogeochemical environments (e.g., associations with photoautotrophic lichen or mosses vs. free-living heterotrophic bacteria in mineral soil), factors other than temperature and moisture may explain the lack of detectable nitrogenase activity and high variability among rates when activity was detected.

Certain biogeochemical factors such as substrate stoichiometry or microbial community characteristics that are known to limit nitrogenase activity in cryptic niches may have contributed to both the lack of detectable rates and/or the low rates of active samples we observed (Reed et al., 2011). For example, work in tropical forests showed that "hotspots" of N fixation activity were characterized by more diverse microbial communities and higher abundance of the *nifH* gene compared with zones of average N fixation activity (Reed et al., 2010). Additionally, phosphorus amendments have been shown to stimulate N fixation in soil, litter, and lichen niches, suggesting phosphorus may limit cryptic N fixation (Benner et al., 2007; Reed et al., 2007). Perhaps in our broad approach to sampling cryptic niches, we unknowingly collected samples of substrates that lacked the microbial characteristics or available nutrients to fix N at detectable rates. Future work should focus on interactions between biogeochemical factors and the biophysical factors examined in this study.

The relatively high rates of nitrogenase activity in lichen and moss in the 15 and 25°C incubations are consistent with the proposed ~25°C theoretical optimum for N fixation (Houlton et al., 2008). By contrast, nitrogenase activity in litter, wood, and soil showed no evidence of an optimum temperature (Figure 4). Nitrogenase activity was detected at very low rates in one to three samples among each niche except moss at 5°C (Figure 4), suggesting that N fixation activity does not occur at appreciable rates during the winter when soil temperatures are below 5°C. Of the few studies that have explored nitrogenase activity at low temperatures, Antoine (2004) found that activity in arboreal lichen ceased between 0 and 2°C, and Hobara et al. (2006) measured extremely low rates of N fixation in plant–soil cores at 6–8°C. Interestingly, the highest cryptic N fixation rates we observed in the field occurred when 24-h average temperatures were 4.6°C. However, the experimental samples experienced a constant temperature for 48 h. During the spring field incubations, temperatures ranged from 12 to −7°C within a 24-h period (NOAA, 2022). The combination of warming daytime temperatures and relatively plentiful moisture during the spring sampling event may explain the apparent discrepancy between our field and laboratory results.

Overall, our results suggest a more complex relationship between temperature, moisture, and nitrogenase activity than has been recently posited (Cleveland et al., 2022). For example, Caputa et al. (2013) found that

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relative nitrogenase activity in grassland biocrusts increased sharply from 5% to 20% moisture content (w/w) but remained constant at moisture contents >30%. Hicks et al. (2003) found that relative N fixation activity in decaying wood increased sharply from 0% to 100% moisture content but remained relatively constant from 150% to 800%. In our study, nitrogenase activity in wood was highest at moisture contents between 100% and 300%, nitrogenase activity in lichen and moss was highest at moisture contents between 50% and 100% moisture content, and nitrogenase activity in litter was highest between 100% and 300% moisture content (Figure 4). These results suggest that the relationship between moisture content and nitrogenase activity may be niche specific, and that "optimal" moisture conditions may vary among different niches. Here, the growth chamber experiment was designed to test the effects of a broad range of moisture conditions, precluding the ability to precisely identify possible critical moisture thresholds for nitrogenase activity across niches. However, future experiments should explore whether moisture thresholds exist for N fixation activity.

Our growth chamber experiment was designed to explore the effects of moisture and temperature on nitrogenase activity. However, as photoautotrophs, lichens and mosses also need light to fix the carbon that fuels N fixation (Gundale et al., 2012; Zielke et al., 2002). In this study, light levels were held constant in the moisture experiment and light levels were not measured in the field. Therefore, we cannot assess whether or how light availability may have affected rates. Additionally, different species of moss and lichen are known to fix N at differing rates (Bjerke et al., 2013; Hobara et al., 2006; Rousk et al., 2017). Measuring N fixation rates in specific lichen and moss species was beyond the scope of this study. The lichen samples we measured consisted entirely of conglomerates of *Peltigera* and *Cladonia* species, of which the former is known to sometimes fix N at very high rates (Hobara et al., 2006). However, a more species-specific investigation of N fixation in cryptic niches would likely improve the accuracy of cryptic N fixation inputs like those generated in this study.

Altogether, the sum of measured new N inputs from cryptic N fixation and N deposition rates were an order of magnitude lower than estimated increases in N pools following severe stand-replacing fire in the GYE. While we did not directly measure symbiotic N fixation, these results and others (e.g., Halvorson et al., 1992; Tierney et al., 2019) suggest that cryptic N fixation and symbiotic N fixation together are not sufficient to explain rapid increases in N pools during early phases of forest regeneration. For example, in similar lodgepole pine stands, Fahey et al. (1985) estimated the combined rates of N fixation by nodulated *Lupinus argenteus* and asymbiotic bacteria in litter to be <0.3 kg N ha^{-1} year^{-1}, with fixation by *L. argentus* adding 0.1 kg N ha^{-1} year^{-1}. Halvorson et al. (1992) estimated the rates of fixation by *Lupinus lepidus* and *Lupinus latifolius* in early successional volcanic sites on Mt. St Helens to be <0.05 kg N ha^{-1} year^{-1}. The apparent disconnect between measured N inputs and increases in soil and biomass N pools is not unique to the GYE. For example, N fixation has been found to contribute only a small proportion of N lost during fire in both temperate pine savannas (Tierney et al., 2019) and boreal pine forests (Palviainen et al., 2017). Similarly, many published studies have inferred high N fixation using mass balance-based estimates of N accretion in regenerating ecosystems, despite little to no evidence of high N fixation rates (Binkley et al., 2000; Johnson & Turner, 2014).

If N fixation rates are indeed very low in our study sites, how do we explain the rapid increases in N stocks in forests regenerating postfire? While we measured the suite of known cryptic fixers in this ecosystem, other N-fixing niches may be present. For example, N-fixing bacteria have been found in tubercles in ectomycorrhizal fungi associated with lodgepole pine roots in N-poor soils in British Columbia (Paul et al., 2007). Likewise, other pine species have also been found to contain endophytic N-fixing bacteria in their foliage that can fix N at detectable but relatively low rates (Moyes et al., 2016). However, we were unable to locate tubercles in our plots and nitrogenase activity in lodgepole pine foliage samples was below detection limits during our preliminary sampling (data not shown). Therefore, N fixation in tubercles or foliar endophytes is unlikely to be a major N source in our study sites.

Instead, other N sources may subsidize the postfire biomass N recovery. As has been hypothesized in other ecosystems, soil mineral horizons may provide a larger reservoir of N for plant uptake than previously recognized (Compton et al., 2007; Lovett et al., 2018). The bulk of the observed change in N pools from 10 to 25 years postfire in our study sites occurred in the upper 15 cm of mineral soil (Turner, Whitby, et al., 2019). However, changes in N pools below 15 cm in soil were not measured in our plots. Mineral soil horizons in similar lodgepole pine forests are known to extend well past 15 cm in depth and can contain vast amounts of N (Fahey et al., 1985; Smithwick et al., 2009). Furthermore, lodgepole pine in the GYE is known to associate with *Suillus* fungi, a genus of long-range foraging ectomycorrhizal fungi (Douglas et al., 2005; Hobbie & Högberg, 2012). In boreal forests, higher rates of colonization by *Suillus* fungi have been found on root tips deeper in the mineral soil horizon compared with root tips in the organic layer of soil and shallower mineral horizons (Rosling et al., 2003), suggesting that important microbial activity and potential

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nutrient uptake may be occurring below surface (0–15 cm) soil. Future research into the mechanisms by which forests recover N during postfire regeneration should consider such mycorrhizal associations and their influence on N dynamics throughout the entire soil column. Addressing these remaining unknowns will be critical in improving our ability to assess the resilience of N-limited systems experiencing dramatic disturbance regime changes.

AUTHOR CONTRIBUTIONS

Robert E. Heumann, Cory C. Cleveland, and Monica G. Turner designed the study. Robert E. Heumann, Cory C. Cleveland, and Monica G. Turner collected field data. Robert E. Heumann conducted the laboratory analyses. Robert E. Heumann analyzed the data and wrote the manuscript. Cory C. Cleveland and Monica G. Turner contributed to revisions.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data and code (Heumann et al., 2024) are available in the Environmental Data Initiative's EDI Data Portal at https://doi.org/10.6073/pasta/1297c769e0b6a20880d59452cc944ec9.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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