Nitrogen induced soil carbon gains are resistant to loss after the cessation of excess nitrogen inputs

Emel Kangi[1], Joanna R. Ridgeway[2], Hannah D. DeHetre[1], Edward R. Brzostek[1]

[1] Department of Biology, West Virginia University, West Virginia, USA.
[2] Department of Biological Sciences, Dartmouth College, New Hampshire, USA.

Abstract

Nitrogen (N) deposition has increased soil carbon (C) storage across eastern US temperate forests by reducing microbial decomposition. However, the fate of these N-induced soil C gains are uncertain given strong declines in N-deposition rates and rising soil temperatures. As N deposition has reduced soil pH and plant C investments into the rhizosphere, we compared the extent to which removing limitations to microbial decomposition by increasing soil pH, adding artificial root exudates, or elevating soil temperature would increase microbial decomposition in soils that have and have not received excess N inputs. We hypothesized that alleviating these microbial decomposition limitations would prime soil C losses from soils that have received excess N inputs. To test this hypothesis, we conducted a soil microcosm experiment where we compared microbial respiration, microbial biomass, and soil enzyme activity in soils from an unfertilized watershed and a previously N-fertilized watershed 4 years after the end of a 30-year N deposition experiment at the Fernow Experimental Forest in West Virginia. In both watersheds, we found that removing pH, plant carbon, or temperature limitations to decomposition stimulated microbial respiration. However, microbial decomposition and soil C losses were consistently lower in the previously N-fertilized watershed across all treatments. This response, coupled with a lack of differences in microbial biomass between watersheds and treatments, suggests that long-term N fertilization has fundamentally

altered soil microbial communities and has led to a sustained impairment of the ability of the microbial community to decompose soil organic matter. Collectively, our results indicate that the legacy effect of N deposition on microbial communities may influence the persistence of soil C stocks in the face of global change.

Introduction

Nitrogen (N) deposition has facilitated the land carbon (C) sink by increasing soil C storage across eastern temperate forests in the US (Averill et al., 2018; Bowden et al., 2019; Janssens et al., 2010; Pregitzer et al., 2008). N fertilization experiments suggest that soil C increases are driven by reductions in microbial decomposition (Fog, 1988; Frey et al., 2014; Zak et al., 2008). However, different mechanisms may drive the suppression of microbial decomposition by N inputs, including reductions in soil pH, base cations, and total belowground C flux, such as root exudates and C investments to rhizosphere microbes (Carrara et al., 2021; Carrara et al., 2018; Eastman et al., 2021; Lovett et al., 2015; Treseder, 2004). Moreover, evidence from N fertilization studies suggests that N inputs may alter the temperature sensitivity of soil organic matter (SOM) by increasing the accumulation of more temperature-sensitive and recalcitrant C (Cotrufo et al., 2019; Eastman et al., 2022; Georgiou et al., 2024; Lugato et al., 2021). Addressing this uncertainty is critical to understand whether this "bonus C" will persist, given both the fading N deposition in the region and rising global temperatures (Benish et al., 2022; Gilliam et al., 2019; IPCC, 2022).

Reductions in microbial decomposition may control the persistence of C gained from historical N deposition through soil acidification and base cation leaching. Soil acidification brought on by N-deposition can directly impact microbial growth, limit organic matter decomposition, and lower the availability of base cations, such as calcium (Ca) and magnesium (Mg), which are necessary to maintain microbial stoichiometry and fitness (Fernandez et al., 2003; Hemkemeyer et al., 2021; Horn et al., 2021; Johnson et al., 2014; Rousk et al., 2010). At the Fernow Experimental Forest in West Virginia, a 30-year whole-watershed N fertilization experiment resulted in increased leaching of base cations coupled with reductions in soil

respiration and soil pH in the N-fertilized watershed (Adams et al., 2007; Gilliam et al., 2020). While the bioavailability of these base cations may recover as soil pH incrementally increases, their losses during N fertilization may indefinitely inhibit microbial growth and activity. Similarly, there is the potential for declines in pH to have a persistent effect on microbial activity as soil pH is a master variable on microbial decomposition that is reduced by many N fertilization experiments in forests (Averill et al., 2018).

N inputs may also suppress microbial decomposition by lowering plant C inputs into the rhizosphere. These plant C inputs can prime SOM decomposition by subsidizing microbial enzyme production (Cheng et al., 2014). However, unlike N-induced reductions in soil pH and base cations, which may recover more slowly, plants may be able to dynamically shift belowground C allocation over much shorter timescales (Hutchinson et al., 1998). Under elevated N availability, plants reduce their C investments to free-living soil microbes and symbionts, resulting in a reduction in microbial decomposition. These reductions in microbial decomposition subsequently lead to the accumulation of C that is stored in the particulate organic matter (POM) which is thought to be more vulnerable to microbial decomposition than C stored in other soil fractions because POM is physically accessible to microbes (Carrara et al., 2023; Chen et al., 2019; Cotrufo et al., 2019; Eastman et al., 2021; Eastman et al., 2022; Gregorich et al., 2006; Treseder, 2004). However, N fertilization studies have shown that as N inputs decline, plants rapidly resume their C investments belowground (Carrara et al., 2022). This increase in C transfer to the rhizosphere may enhance microbial decomposition and prime losses of POM (Cardon et al., 2001; Chen et al., 2019; Olayemi et al., 2022). Given that the mechanism by which N fertilization inhibits decomposition could determine whether soil C retained during N

deposition will become a C source to the atmosphere, identifying the primary limitation on microbial decomposition in forest soils recovering from elevated N inputs remains critical.

N-induced soil C gains may be more vulnerable to loss with rising temperatures due to increases in the ratio of C stored in POM to the C stored in mineral associated organic matter (MAOM) (Eastman et al., 2022; Jenkinson et al., 1991). While the decomposition of the physically protected MAOM is limited by microbial access, the decomposition of POM is limited by the high activation energy of microbial decomposition (Cotrufo et al., 2019). Microbes often lack the necessary energy to decompose the complex structure of POM, leading to its persistence in the soil. However, temperature increases can alleviate the energetic limitations of microbial SOM decomposition, magnifying POM losses in N fertilized soils (Lloyd et al., 1994; McHale et al., 1998; Peterjohn et al., 1994; Raich et al., 1992; Rustad et al., 2001). Therefore, higher POM:MAOM ratios as well as the increases in soil C:N ratio in N-fertilized soils have likely made the C stored in the Eastern temperate forests more susceptible to loss with predicted increases in soil temperature (Georgiou et al., 2024; Lugato et al., 2021). Moreover, this loss will likely be exacerbated if warming is coupled with a removal of stoichiometric limitations on decomposition or a recovery of plant-microbial interactions.

To better understand the potential future persistence of N-induced soil C gains, we tested the extent to which the addition of artificial root exudates, increases in pH and base cation availability, and temperature limit microbial decomposition after long-term N fertilization. To this end, we performed a lab microcosm experiment using soils sampled from an experimental watershed and a reference watershed four years after the end of a 30-year N fertilization experiment in the Fernow Experimental Forest near Parsons, WV. As a result of heavy N fertilization, soils in the previously N-fertilized watershed store more C and have a greater

POM:MAOM ratio than the soils in the reference watershed (Eastman et al., 2021; Eastman et al., 2022). To uncover the N-induced limitations on microbial decomposition, we incubated the soils sampled from these watersheds with and without the addition of artificial root exudates or dolomitic lime (i.e., increasing pH and base cation availability) at three different temperatures (i.e., 15, 20, and 25 ˚C). We measured cumulative CO_2 respiration to quantify soil C losses, microbial biomass, and the potential activities of hydrolytic and ligninolytic oxidative enzymes. We tested the following hypotheses:

1. The experimental removal of microbial limitations, such as soil pH and belowground C investments, will increase C losses more in soils from the previously N-fertilized watershed than the unfertilized watershed.
2. Warming will increase C losses more in soils from the previously-N fertilized watershed than the unfertilized watershed.
3. When microbial limitations, such as soil pH and belowground C investments, are removed, the temperature-induced boosts in C losses will be greater in the soils from the previously-N fertilized watershed than the unfertilized watershed.

Methods

Site Description

Our study leverages a thirty-year, whole-watershed N fertilization experiment located in the Fernow Experimental Forest (herein Fernow) in the Appalachian Mountains in Parsons, West Virginia (39.03°N, 79.67°W). This work builds off of decades of research at the Fernow that has examined the impacts of N fertilization on forest ecosystem processes (summarized in Eastman et al., 2021). We collected soils from two adjacent watersheds at the Fernow: a 34 ha watershed that was fertilized at an annual rate of 35 kg N ha-1 in the form of ammonium sulfate ($(NH_4)_2SO_4$) between the years of 1989 and 2019 (previously N-fertilized), and a 24 ha reference watershed that received only ambient N deposition (reference) (Adams et al., 2006). These watersheds receive similar rates of precipitation (1425–1484 mm · yr-1), and are at similar elevation, which range from 731 to 860 m (Adams et al., 2006; Edwards et al., 2011). They contain well-drained, coarse-textured inceptisols formed from a sandstone and shale parent material, which have been classified as loamy-skeletal, mixed mesic Typic Dystrochrepts of the Berks and Calvin series (Adams et al., 2006; Gilliam et al., 1994). The dominant tree species found in these watersheds include *Acer rubrum, Prunus serotina*, and *Liriodendron tulipfera*, but other tree species (*Fagus grandifolia*, *Betula lenta* and *Quercus rubra*) are also present (Carrara et al., 2023; Eastman et al., 2021). As a result of 30-years of N fertilization, soils in the previously N-fertilized watershed have significantly lower pH and fewer base cations (Ca^{2+}, Mg^{2+}, K^{+}), but more C and N than soils in the reference watershed (Eastman et al., 2021; Gilliam et al., 2020). N fertilization also increased N mineralization and nitrification in the organic horizon of the fertilized watershed relative to the reference watershed (Carrara et al., 2018).

Experimental Design

We performed a 15-week incubation study where we compared the respiration, potential enzyme activity, and microbial biomass C, in soils sampled from two watersheds (previously N-fertilized vs. reference), three treatments (artificial root exudates, dolomitic lime, and control), and three incubation temperatures (15, 20, and 25 ˚C). Our experimental design was 2 watersheds × 3 temperatures × 3 treatments × 6 replicates for a total of 108 microcosms.

Soil Collection and Processing

We sampled soils from a network of 6 previously established 10 × 10 m plots (Carrara et al., 2023) in each watershed at the Fernow in July 2023, 4 years after the end of N fertilization treatments. We collected the top 20 cm of mineral soil using a 5 cm diameter core, combining the soils collected from all the plots within each watershed to provide one composite mineral soil sample for each watershed.

We processed soils within one week of collection and stored soils at 4 ˚C prior to the microcosm incubation. We removed roots and homogenized the soils by sieving to 2 mm. We determined gravity-drained soil water holding capacity by saturating soil subsamples (n = 6) over a Whatman #1 filter and measuring water content (Nelson et al., 2023). We also measured soil gravimetric water content by drying subsamples (n = 3) from each watershed at 65 ˚C for 72 hours.

Microcosm Setup

We established our microcosms in a closed system consisting of wide mouth glass mason jars (930 mL), to which we added 50.0 ± 0.05g of soil collected from either the previously N-fertilized (n = 54) or the reference watershed (n = 54). We adjusted these soils to 40%

gravimetric water content with deionized water (Mushinski et al., 2020). We added deionized water to the microcosms after weekly measurements using mass to keep the soils at 40% gravimetric soil water content. We incubated the microcosms at 15 °C, 20 ˚C, or 25 ˚C in dark, climate-controlled chambers (Model: I-41VL, Percival Scientific Inc., Perry, IA, USA) for 15 weeks.

Treatments

We applied an artificial root exudate solution to a third of our samples (n = 36) at the rate of 800 ug of C per g dry soil distributed over the first two weeks of the incubation. Glucose, fructose, and sucrose each contributed 25% of the C in the solution, while succinic and malic acids contributed to 12.5% (Dang et al., 2024; Griffiths et al., 1998). For our lime treatment (n = 36), we added 4 mg of finely ground dolomitic lime (calcium magnesium carbonate) (Greenway Biotech Inc., Santa Fe Springs, CA) per g dry soil, which brought the pH of the soils sampled from the previously N-fertilized watershed from 3.65 ± 0.05 to 4.97 ± 0.09. We measured soil pH using 5 g (dry weight equivalent) of fresh soil mixed with 10 mL of deionized water and 0.1 mL of 1 M $CaCl_2$ (Fisherbrand™ accumet™ AB15 Basic pH meter, Thermo Fisher Scientific, Waltham, MA, USA).

Respiration Measurements

We measured microbial respiration by sampling the microcosm headspace CO_2 and injecting 15 mL gas samples into an infrared gas analyzer (LI-850, LI-Cor Biosciences Inc., Lincoln, NE). We made these measurements on days 1, 3 and 7 of the incubation and weekly thereafter. We equilibrated the microcosms with ambient lab air for 1 hour after each measurement to facilitate gas exchange. As lime-derived abiotic CO_2 production would be

177 negligible under the conditions used in our experiment, we ignored the contribution of this
178 process to our respiration measurements (Wu et al., 2021).

179 *Extracellular Enzyme Activity*

180 At the end of the 15 week incubation, we assayed the potential hydrolytic enzyme
181 activities of nitrogen, phosphorus, and simple carbon releasing enzymes: N-
182 acetylglucosaminidase (NAG), acid phosphatase (AP), and β-glucosidase (BG) using a
183 fluorometric microplate method and 4-methylumbelliferyl-derived substrates measured at
184 365 nm excitation and 450 nm emission (Carrara et al., 2023; Saiya-Cork et al., 2002). We also
185 assayed the potential oxidative enzyme activities of phenol oxidase and peroxidase in our soil
186 samples using a colorimetric microplate method using 3,4-L-dihydroxyphenylalanine quantified
187 at 460 nm (Carrara et al., 2023; Saiya-Cork et al., 2002). For the enzyme assays, all of the plates
188 were incubated at lab temperature (20 ˚C).

189 *Microbial Biomass C*

190 We extracted microbial biomass C from a subsample of our microcosm soils using a
191 modified chloroform fumigation extraction method (Witt et al., 2000). Briefly, we made soil
192 slurries in a potassium sulfate (K_2SO_4) solution with and without chloroform to lyse microbial
193 cell membranes and extract microbial biomass in our soil samples. We then oxidized the
194 dissolved C in the extracts to CO_2 by incubating the extracts overnight at 75 ˚C with a potassium
195 persulfate ($K_2S_2O_8$) buffer (pH = 8.2) (Doyle et al., 2004). We measured the headspace CO_2
196 using a LI-Cor LI-850 infrared gas analyzer. We calculated microbial biomass C by subtracting
197 the C in non-fumigated samples from the C in chloroform-fumigated samples and scaled our data
198 by 2.64 to correct for extraction efficiency (Vance et al., 1987).

Data Analysis

To measure the effects of watershed (previously N-fertilized vs. reference), incubation temperature (15, 20, and 25 ˚C), treatment (control, artificial root exudates, and dolomitic lime), and their interaction on soil respiration, potential extracellular enzyme activities, and microbial biomass C, we used a three-way analysis of variance (ANOVA) using R version 4.3.1 (R Core Team, 2023). For soil respiration and microbial biomass C, we also performed post hoc tests (Tukey-Kramer HSD) to compare the responses of each group. For soil respiration, we adjusted our values using the ideal gas law, after which we used cumulative respiration at the end of the 15-week incubation for our analysis. In order to determine if adding C to our microcosms through artificial root exudates would produce a priming effect, we also calculated mean-scaled respired C using the following formula:

Mean-Scaled Respired C = $R_{WS,T}$ – Exudate C – mean (Control $R_{WS,T}$), where "$R_{WS,T}$" is cumulative endpoint respiration in a given microcosm with soil from a specific watershed that has been incubated at a specific temperature, "Exudate C" is the amount of C that has been added to that microcosm via artificial root exudates, and "mean (Control $R_{WS,T}$)" is the mean respiration from all of the control (i.e. no exudate addition) microcosms with the same treatment combination (watershed and temperature) as the corresponding exudate microcosm.

To highlight the differences in enzyme profiles (AP, NAG, BG, phenol oxidase, and peroxidase) between the two watersheds at each incubation temperature (15, 20, and 25 ˚C) and soil treatment (control, artificial root exudates, and dolomitic lime), we used nonmetric multidimensional scaling (NMDS) ordination plots using Bray-Curtis dissimilarity index in R package 'vegan' (Oksanen et al., 2022). We also performed a permutational multivariate analysis of variance (PERMANOVA) using the function adonis to compare the enzyme profiles of the

two watersheds. Enzyme activities (means ± standard error) and the ANOVA tables for the enzyme activities can be found in the supplementary data (SI Table 1, SI Table 2). Data used in this study is publicly available on the ESS-DIVE repository (doi: 10.15485/2466174).

Results

Soil Respiration

Overall, microbial respiration increased with temperature and the addition of dolomitic lime and artificial root exudates (Figure 1, SI Figure 1). Soils incubated at 25 ˚C respired 143% more than soils incubated at 15 ˚C ($F = 577.273$, $p < 0.001$, SI Figure 2B). The effects of adding artificial root exudates and dolomitic lime on soil respiration were similar, with a 51% increase in soil respiration with exudates and a 58% increase with dolomitic lime compared to the control ($F = 166.041$, $p < 0.001$, SI Figure 2C). However, regardless of temperature or treatment microbial respiration was always greater in the reference watershed than in the previously N-fertilized watershed. At the end of the 15-week incubation, the cumulative soil respiration was 41% greater in the soils sampled from the reference watershed than the previously N-fertilized watershed ($F = 271.942$, $p < 0.001$, SI Figure 2A).

There were also significant interactions between temperature, watershed, and treatment on soil respiration. We observed a significant interaction of watershed and incubation temperature on soil respiration ($F = 14.117$, $p < 0.001$) where the effect of watershed on microbial respiration increased with incubation temperature. The difference in soil respiration between the two watersheds at 25 ˚C was 105% greater than at 15 ˚C (SI Figure 2D). There was also a significant interaction of treatment and incubation temperature on soil respiration ($F = 9.439$, $p < 0.001$). For soils incubated at 25 ˚C, dolomitic lime addition increased soil respiration to a greater extent than the artificial exudate addition, with dolomitic lime promoting a 52% increase and artificial root exudates promoting a 32% increase compared to the control (SI Figure 2E).

We also found differences between the previously fertilized watershed and the reference watershed in the ability of the artificial root exudates to prime soil organic matter decomposition at different incubation temperatures (Figure 2). To calculate this effect, we first subtracted the amount of C that we added to soils in the form of root exudates from the microbially respired C for each watershed at each temperature treatment. We then scaled this value using the mean of the control treatment of the corresponding incubation temperature and watershed to determine a priming effect. We observed a significant interaction of watershed and incubation temperature on our calculated priming effect ($F = 20.31$, $p < 0.001$) where at higher incubation temperatures, priming of soil organic matter was greater in the reference watershed than in the previously N-fertilized watershed (Figure 2). For the previously N-fertilized watershed we found that there was a significant positive priming effect at 15 ˚C ($F = 6.885$, $p = 0.028$, SI Figure 3). However, for the reference watershed, we observed a positive priming effect at 25 ˚C ($F = 4.168$, $p = 0.081$, SI Figure 3).

Extracellular Enzymes

The soil enzyme profiles of the previously N-fertilized watershed and the reference watershed differed in 6 out of 9 treatment combinations (Figure 3). For both the control soils and the soils supplemented with artificial exudates, extracellular soil enzyme profiles were significantly different between the previously N-fertilized watershed and the reference watershed at 15 and 20 ˚C incubation ($F_A = 3.347$, $p_A = 0.059$, $F_B = 6.463$, $p_B = 0.004$, $F_D = 5.795$, $p_D = 0.007$, $F_E = 5.280$, $p_E = 0.007$, Figure 3A, B, D, E). However, soil enzyme profiles were not different between the two watersheds at 25 ˚C for either treatment (Figure 3C, F). For soils treated with dolomitic lime, soil enzyme profiles were different between the two watersheds at

both the lowest (15 ˚C) and the highest (25 ˚C) incubation temperature ($F_G = 21.488$, $p_G = 0.005$, $F_I = 6.125$, $p_I = 0.014$, Figure 3G, I).

Microbial Biomass

There were no differences in microbial biomass between the previously N-fertilized watershed and the reference watershed (Figure 4). We only found a significant effect of incubation temperature on microbial biomass in our soils, where microbial biomass increased with temperature ($F = 6.946$, $p = 0.002$, Figure 4). Compared to the soils incubated at 15 ˚C, microbial biomass was 72% higher in soils incubated at 25 ˚C.

Discussion

Given the declining rates of N deposition and the rising global temperatures, identifying the limitations on microbial decomposition in Eastern temperate forests recovering from elevated N inputs is critical to uncovering the fate of the soil C in the region (Benish et al., 2022; Gilliam et al., 2019; IPCC, 2022). Here, we show that even with the removal of chemical and energetic constraints on microbes, microbial respiration was still lower in soils from the previously N-fertilized watershed than soils from the reference watershed (Figure 1). Soil enzyme profiles mirrored this result with overall enzyme investment differing between the previously N-fertilized watershed and the reference watershed for the majority of treatment combinations (Figure 3). In contrast to microbial respiration and enzyme profiles, we found no difference in microbial biomass between the two watersheds (Figure 4). This result indicates that the differences we observed in microbial respiration and extracellular enzyme activity are likely caused by a shift in microbial community structure and function that impairs their ability to decompose SOM even four years after fertilization has ended (Carrara et al., 2021; Fierer et al., 2012; Piñeiro et al., 2023). Thus, our results suggest that there is a sustained legacy effect of excess N inputs on microbial community composition and function, which at a broader scale may act to preserve the "bonus" soil C in eastern US temperate forests that have received historically high rates of atmospheric N deposition.

Our first hypothesis that raising soil pH and adding in artificial root exudates would result in a greater increase in microbial decomposition in the previously N-fertilized watershed compared to the reference watershed was not supported (Figure 1). Several mechanisms can explain the absence of a stronger priming effect with exudates or greater C losses with lime in the previously N-fertilized watershed. While N inputs can change the composition of SOM in

favor of the more vulnerable POM, N additions in our fertilized watershed may have resulted in the accumulation of very recalcitrant POM. Therefore, despite our experimental treatments lowering the activation energy required for the decomposition of recalcitrant compounds, microbial communities may have still been unable to overcome the energetic requirements of decomposition in the previously N-fertilized watershed. Indeed, during ongoing N-fertilization at the Fernow, SOM in the fertilized watershed was observed to be more recalcitrant, having a higher C:N ratio than the reference watershed (Eastman et al., 2021). N inputs may have also resulted in a general suppression of microbial biomass, or a microbial community shift resulting in loss of function. However, we did not find support for a N-induced suppression of microbial biomass in our study. Microbial biomass did not differ between the watersheds or with the addition of dolomitic lime or root exudates (Figure 4). As such, similar to the findings of previous studies at the Fernow, the increases in soil respiration that we observed in our microcosm study were not linked to concomitant increases in microbial biomass (Piñeiro et al., 2023). Therefore, the mechanism behind the lack of support for our first hypothesis is likely a legacy effect of N fertilization on the microbial community composition that led to a persistent loss of the ability of soil microbes to decompose soil organic matter (Fierer et al., 2012; Ramirez et al., 2010). In support, previous work at the Fernow has shown that N fertilization led to strong shifts in the microbial community composition of bacteria and fungi that resulted in declines in enzyme activity, C and N uptake, and priming (Carrara et al., 2018; Piñeiro et al., 2023). As such, our observation that the previously N-fertilized watershed had consistently lower soil respiration than the reference watershed suggests that these microbial community shifts may persist even 4 years after N fertilization has ended, which may have important implications for predicting the trajectories of soil C gains after N deposition declines. Future studies should

evaluate the taxonomic and functional composition of soil microbial communities in these watersheds several years post N fertilization to understand the degree to which microbial community shifts drive the watershed differences in SOM decomposition at the Fernow.

The lack of a strong positive priming effect in the previously N-fertilized watershed at higher incubation temperatures (Figure 2, SI Figure 3) suggests that the legacy of N fertilization impeded the ability of microbes to leverage root exudates to fuel decomposition (Allison et al., 2013; Carrara et al., 2018; Leff et al., 2015; Liu et al., 2017). In the previously N-fertilized watershed, the addition of artificial root exudates led to an increase in soil respiration that was less than the amount of carbon that we added. As such, it appears that the additional C led primarily to waste metabolism instead of growth and enhanced decomposition by the soil microbial community in the previously N-fertilized watershed. This response is usually seen when microbes are limited by N (Schimel et al., 2003). While observed increases in the soil C:N ratio in the previously N-fertilized watershed at the Fernow (Eastman et al., 2022) suggest that microbial N limitation may play a role in the lower priming effect we observed in this watershed (Piñeiro et al., 2023), it is more likely that the legacy of N fertilization gave rise to a microbial community that is unable to use root exudates to fuel growth and decomposition. During ongoing N-fertilization at the Fernow, plant belowground C investments were found to be lower in the fertilized watershed (Carrara et al., 2023; Eastman et al., 2021). Our findings suggest that there is a continued N-induced suppression of microbial ability to leverage root exudates to drive decomposition. If this legacy effect of N fertilization holds, decomposition in the previously N-fertilized watershed may not surpass the rates observed in reference watershed even after trees resume investing C belowground to gain N.

Interestingly, temperature had opposing effects on priming in the two watersheds. In the reference watershed, the increase in priming with increasing temperatures follows theory (Figure 2). At warmer temperatures, carbon additions stimulate more microbial activity likely due to temperature enhancing the activity of microbial enzymes (Baath, 2018; Stone et al., 2011; Walker et al., 2018). On the other hand, in the previously fertilized watershed, we observed the opposite pattern. At higher temperatures, we observed more negative priming (Figure 2). While the mechanism driving this result is highly uncertain, the implications are less so. It appears that the legacy of N fertilization reduces the susceptibility of C loss at higher temperatures even with the addition of artificial root exudates.

The enzyme data provides further support that there is a decline of microbial ability to decompose SOM or leverage root exudates in the previously N-fertilized watershed. We found that the majority of the extracellular enzyme profiles differed by watershed across the various treatments, particularly for the incubations at 15 ˚C and 20 ˚C (Figure 3). This result suggests that microbial enzyme investment in the previously N-fertilized watershed is still largely different from the reference watershed (Carrara et al., 2023). As such, it appears that the legacy of N fertilization may have led to a microbial community that has entered a new state in the previously N-fertilized watershed where they either lack the resources or capability to produce enzymes at the same level as the reference watershed. In support, several previous long-term N fertilization experiments have also observed strong effects on soil microbes, such as shifts in microbial community composition after N additions (Carrara et al., 2018; Fierer et al., 2012; Freedman et al., 2015). As such, microbial communities that have been altered by N fertilization may have decreased ability to decompose complex C compounds (Carrara et al., 2018). This idea that N fertilization lowers the ligninolytic potential of soil microbial communities and thereby

hinders the decomposition of complex C compounds is also supported at the Fernow as the SOM in the N-fertilized watershed has greater C:N than reference soils (Eastman et al., 2021).

Despite the lack of support for our first hypothesis, the effect of the dolomitic lime addition on microbial respiration exceeded the effect of the root exudate treatment for both watersheds. However, the lack of a greater stimulation in the previously fertilized watershed than the reference watershed suggests that pH was not the primary driver of the consistent suppression of soil respiration that we observed. N fertilization studies in other sites have often shown that soil pH is a master regulator of microbial growth, community composition, and activity (Fierer et al., 2012; Johnson et al., 2014; Kaiser et al., 2016; Rousk et al., 2010). By contrast, at the Fernow, the lack of a stronger dolomitic lime response in the previously N-fertilized watershed likely reflects that 30-years of ammonium sulfate addition only led to a marginal decrease in pH in the previously N-fertilized watershed (3.65 ± 0.05) compared to the reference watershed (3.78 ± 0.02). In support, previous research during ongoing N-fertilization in these watersheds has shown that oxidative soil enzyme activity (i.e., phenol oxidase and peroxidase) was largely insensitive to pH manipulations in the lab (Carrara et al., 2018).

Given the greater C:N ratio and POM:MAOM ratio of SOM in the previously N-fertilized watershed (Eastman et al., 2021; Eastman et al., 2022), we expected the decomposition of soil organic matter in this watershed to be more responsive to the temperature treatments than the reference watershed (Fierer et al., 2005). Our expectation reflected the C quality-temperature hypothesis which suggests the decomposition of lower quality and more chemically complex organic matter is more temperature sensitive (Bosatta et al., 1999; Davidson et al., 2006). However, in our study, warming did not induce larger increases in microbial decomposition in the previously N-fertilized watershed compared to the reference watershed. This finding

391 indicates that when energetic limitations are removed, microbes in the previously N-fertilized
392 watershed were still unable to accelerate the decomposition of chemically complex POM. At a
393 broader scale, this finding also raises the possibility that the soil C gains resulting from excess N
394 inputs to temperate forest ecosystems in the eastern US may persist despite predictions of
395 warmer temperatures for the region.

396 We acknowledge that this microcosm study has some limitations inherent to our
397 experimental design and methodology. However, microcosm experiments can be instrumental in
398 providing a mechanistic understanding of responses otherwise difficult to observe in field
399 settings, such as SOM priming, pH, and temperature effects (Benton et al., 2007; Teuben et al.,
400 1992). While we performed our soil incubations in controlled temperature and moisture
401 conditions in the absence of living roots or mycorrhizal symbionts, we added root exudates to
402 simulate the presence of roots in this controlled environment (Phillips et al., 2007). Although we
403 acknowledge that a controlled addition of artificial root exudates is unlikely to fully mimic the
404 more dynamic root exudation process that occurs in the field, this treatment likely simulated a
405 similar effect in our microcosm study. In addition, although we did not measure soil organic
406 matter pools in our soils prior to conducting our incubations, we used heterotrophic respiration as
407 a proxy of soil organic matter decomposition and C losses. Finally, while our data indicates that
408 shifts in microbial community and function are an important driver, we did not assess the
409 taxonomic or functional composition of the microbial communities in our soils. Nonetheless, our
410 results identify this as an important avenue of future research that could connect the responses
411 that we observed in this microcosm study to shifts in microbial community composition and
412 functional traits.

Research at the Fernow and across the US has shown that N deposition increased soil C storage through reductions in microbial decomposition (Averill et al., 2018; Eastman et al., 2021; Eastman et al., 2022). Here, we show that these soil C gains may be resistant to loss after excess N inputs to forests decline. Our results suggest that four years after the end of N-fertilization, the C gains in the fertilized soils were not more sensitive to loss from priming by root exudates or soil warming. Mechanistically, these findings point to a legacy effect of long-term N-fertilization on soil microbial communities, which likely experience strong losses of functional traits that impair their ability to decompose SOM. Broadly, our study suggests that the N-induced microbial community shifts may lead to the persistence of N-induced soil C gains across Eastern temperate forests of the US.

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Figures and Tables

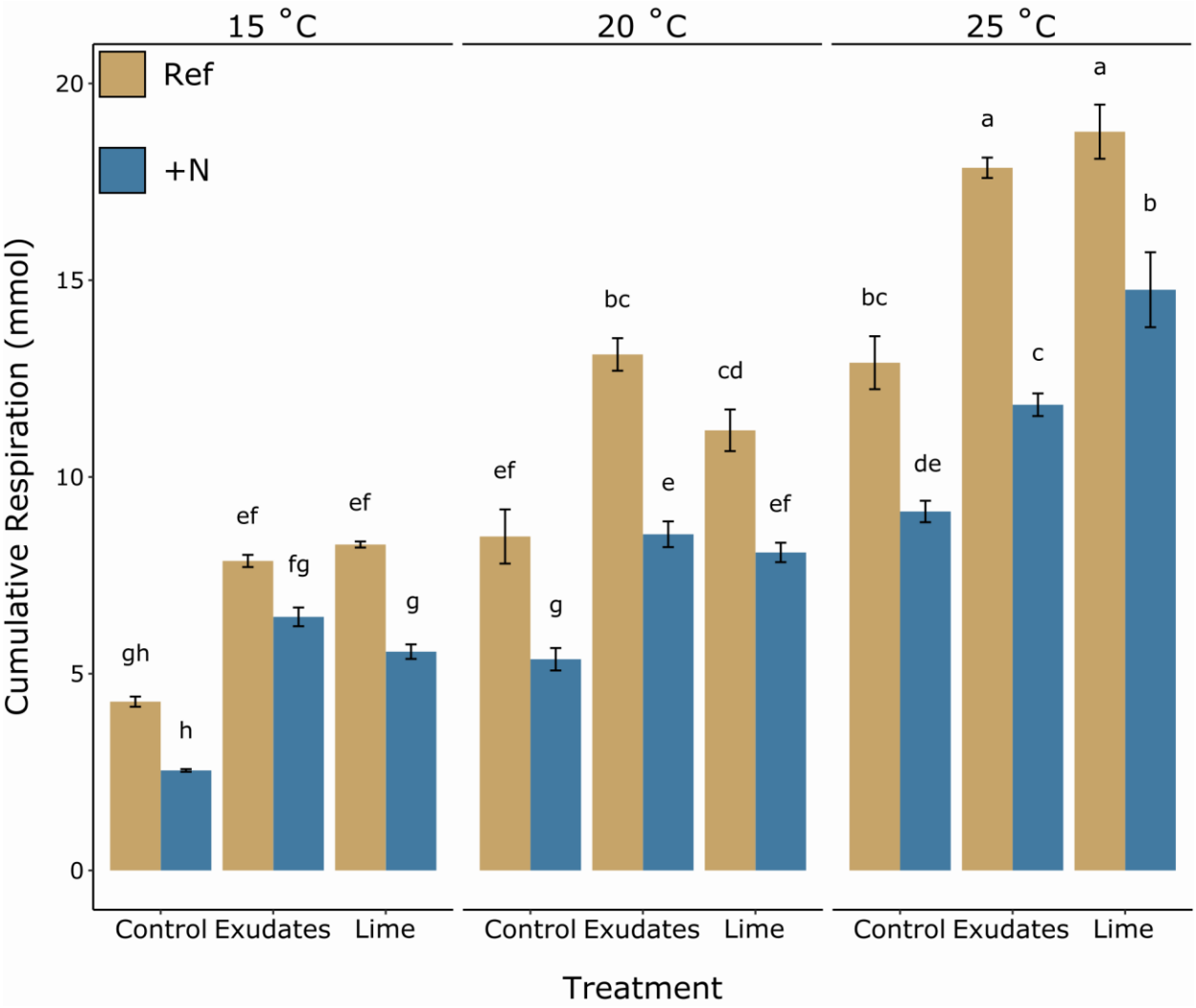
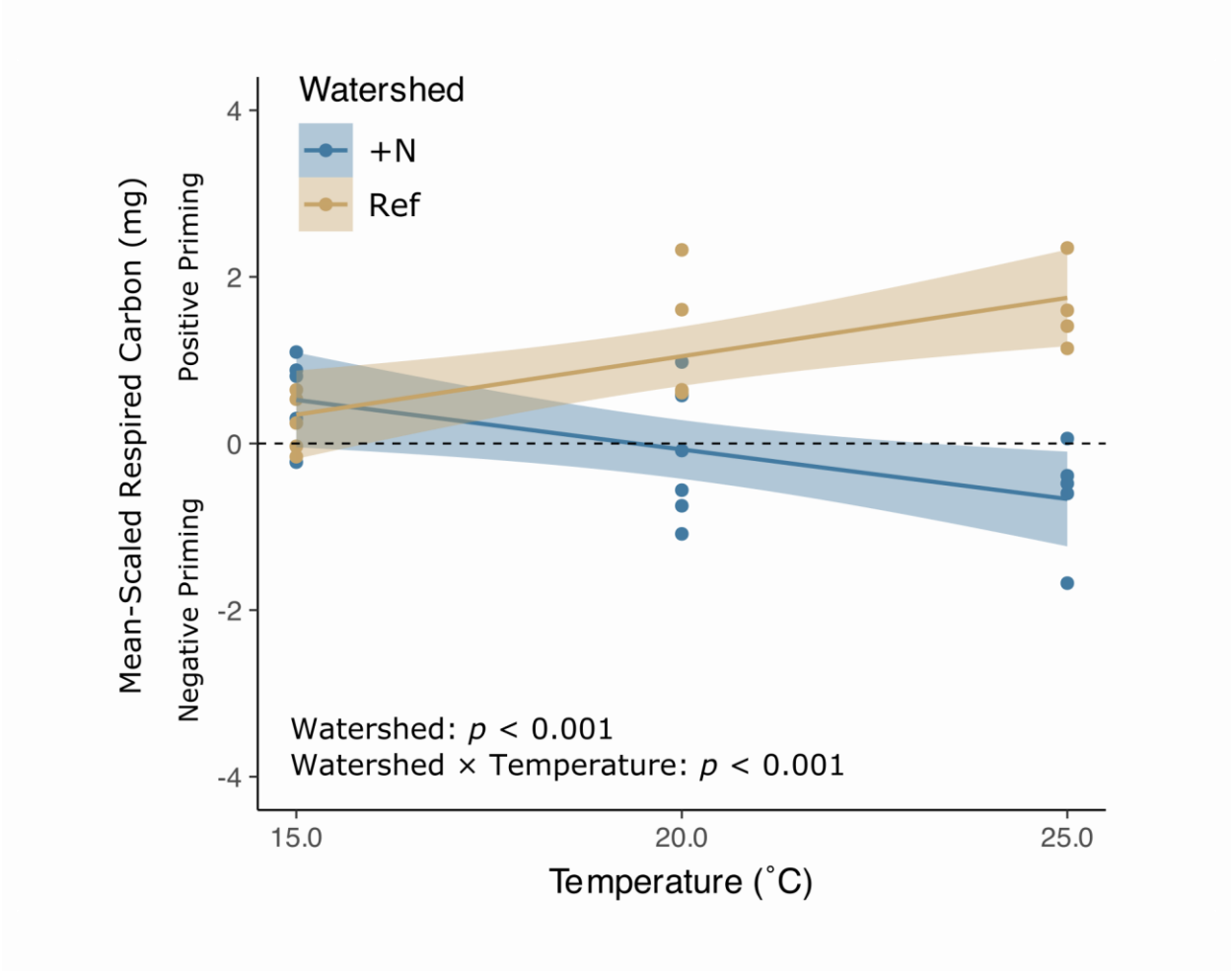
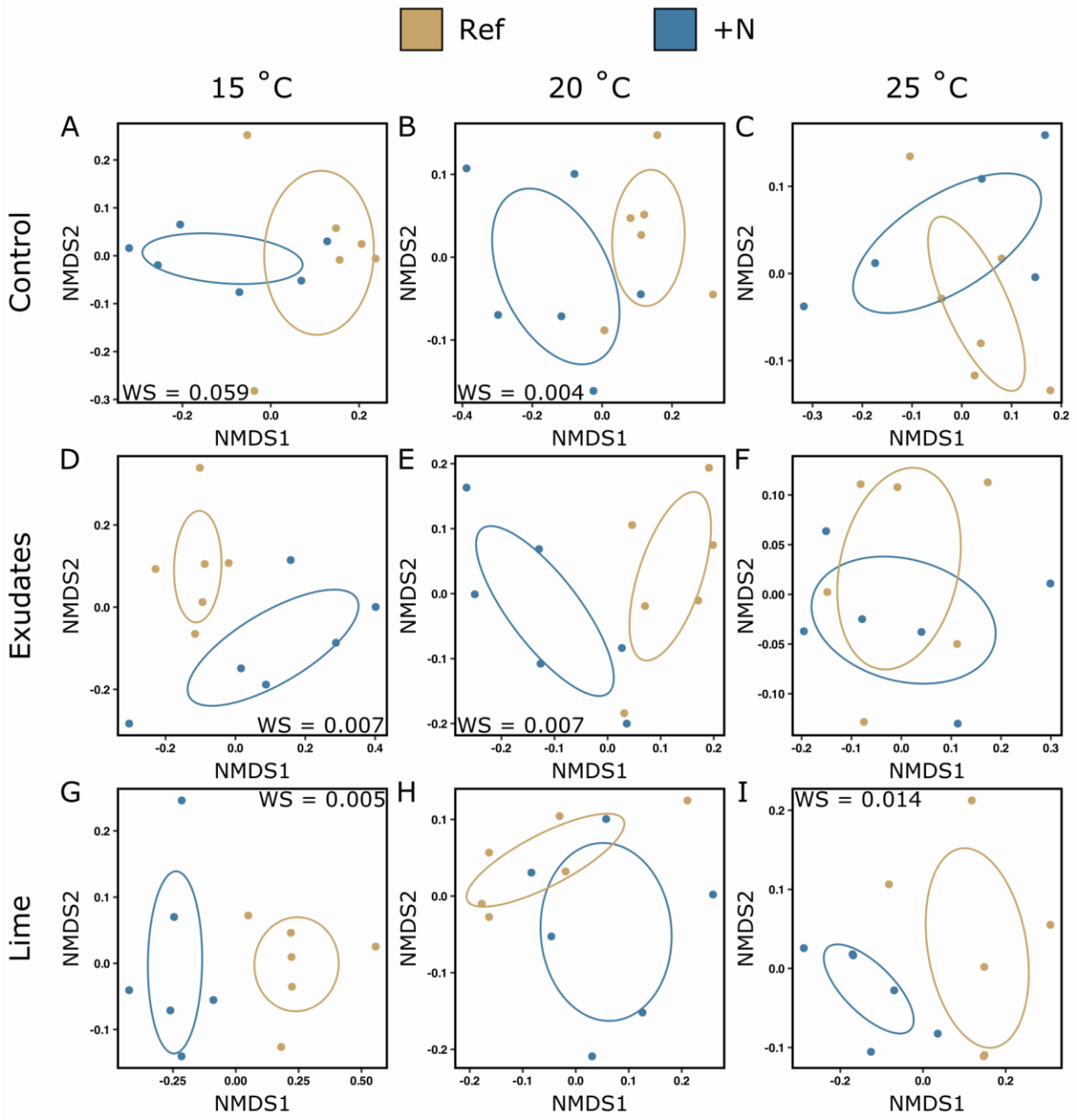


Figure 1. Endpoint cumulative respiration (mmol) of soils incubated at 15, 20, and 25 ˚C with and without the addition of artificial root exudates and lime in the reference (yellow) and previously N-fertilized (blue) watersheds. Error bars represent ± standard error. Treatment groups connected by overlapping letters are not significantly different ($p < 0.05$, Tukey-Kramer HSD). Respiration was consistently lower in the previously N-fertilized watershed.



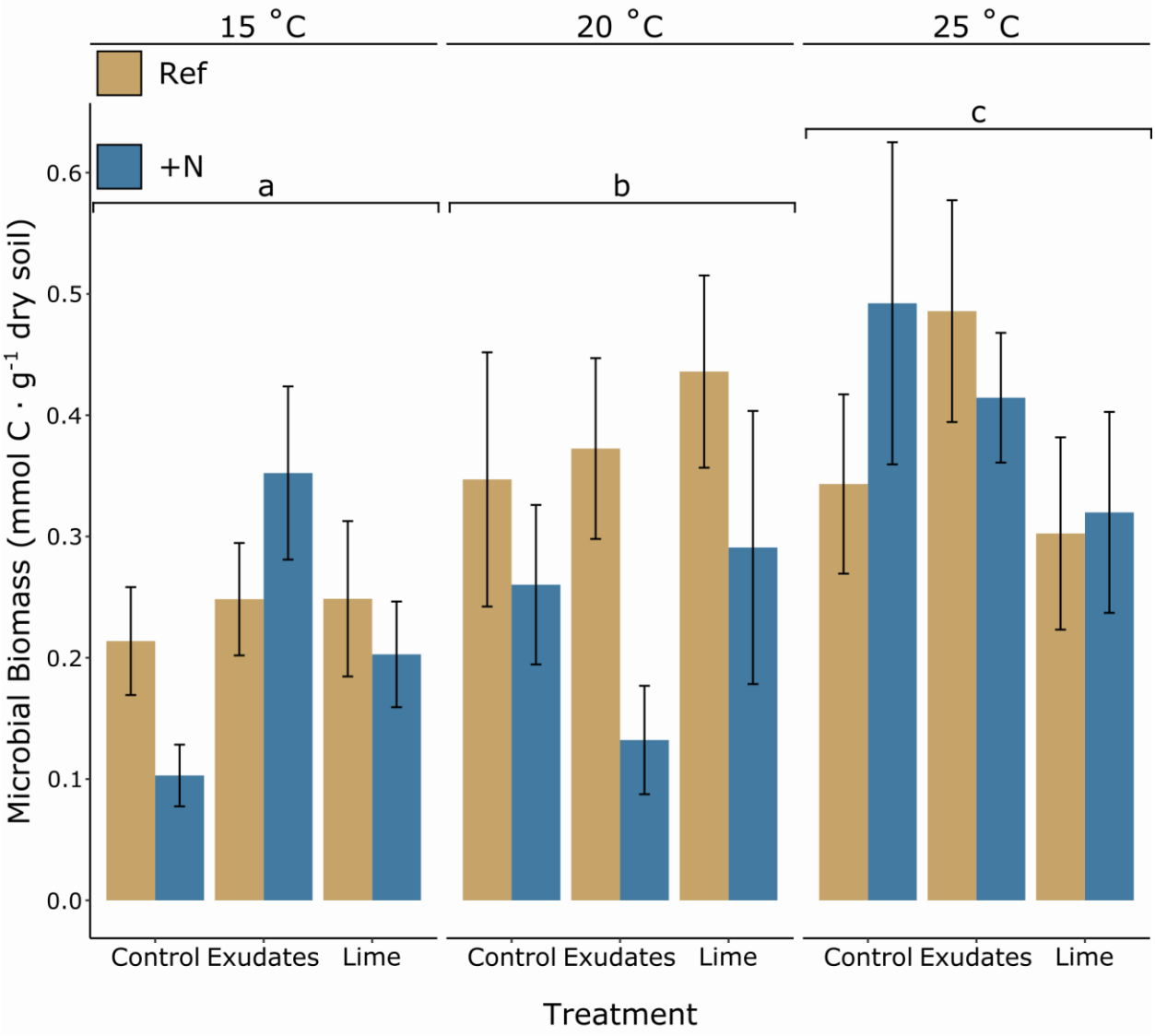
759

760 *Figure 2*. Respired carbon (mg) after artificial exudate additions, adjusted for the added C and
761 scaled by means, in the previously N-fertilized (+N, blue, adjusted $R^2 = 0.324$, p = 0.013) and
762 reference (Ref, yellow, adjusted $R^2 = 0.502$, p = 0.004) watersheds incubated at 15, 20, and 25
763 ˚C. At higher incubation temperatures, priming of soil organic matter is greater in the reference
764 watershed.



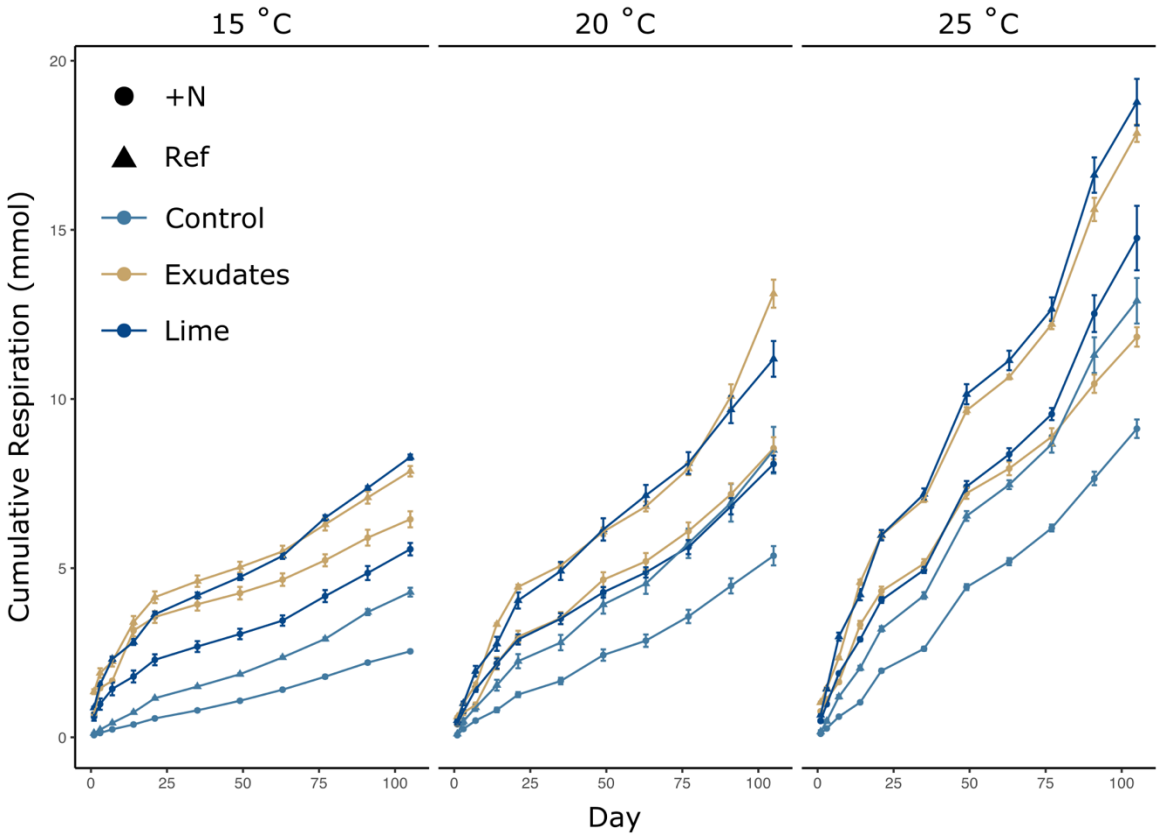
765

766 *Figure 3*. NMDS of the enzyme profiles of soils from the reference (yellow) and previously N-
767 fertilized (blue) watersheds incubated at 15 (left column), 20 (middle column), and 25 ˚C (right
768 column). Different treatments are shown on different rows: control (top row), exudate treatment
769 (middle row) and lime treatment (bottom row). PERMANOVA results of the watershed (WS)
770 effect is shown for each plot where $p < 0.1$. Enzyme profiles of the two watersheds were
771 significantly different in 6 out of 9 treatments.

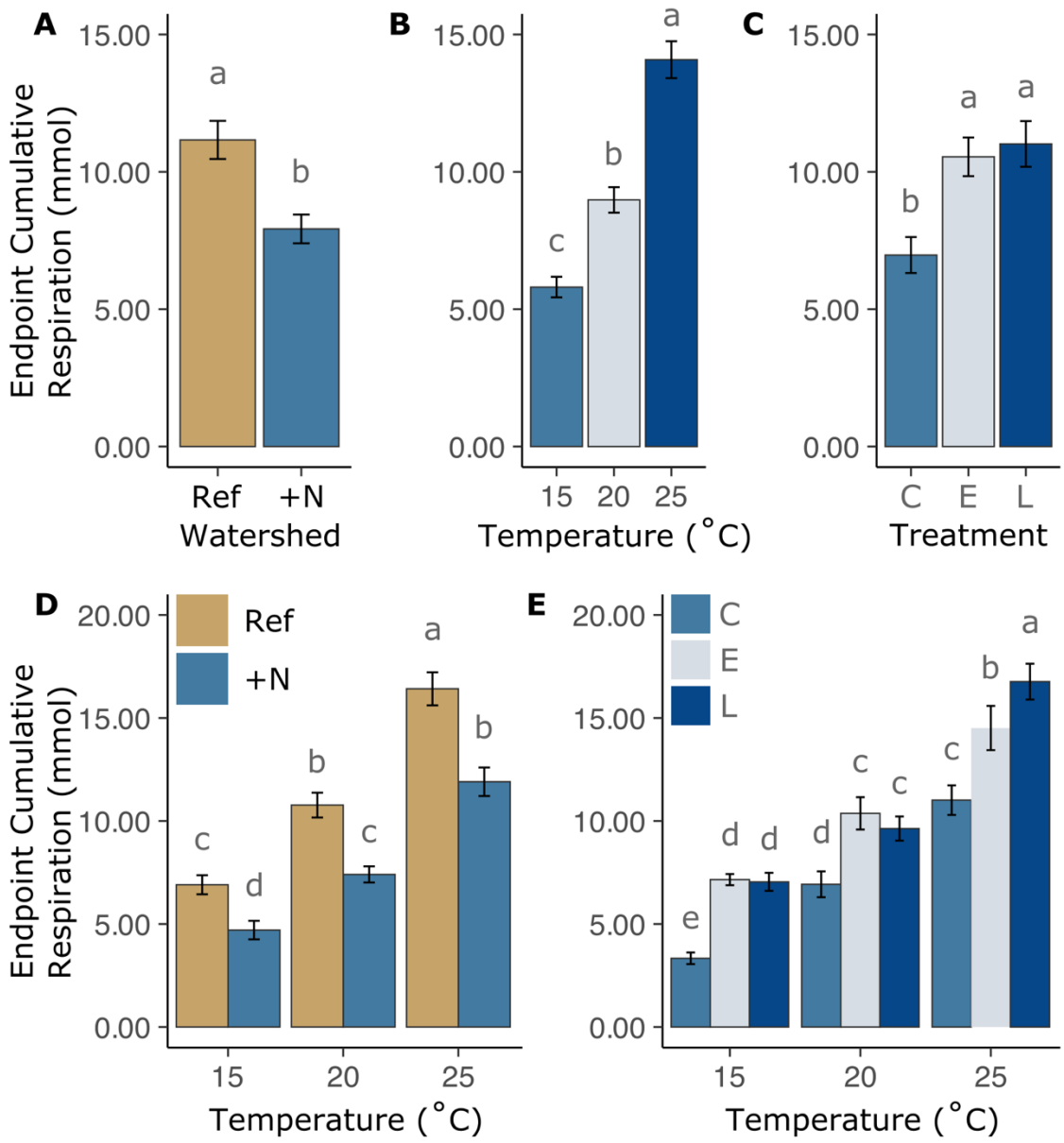


772

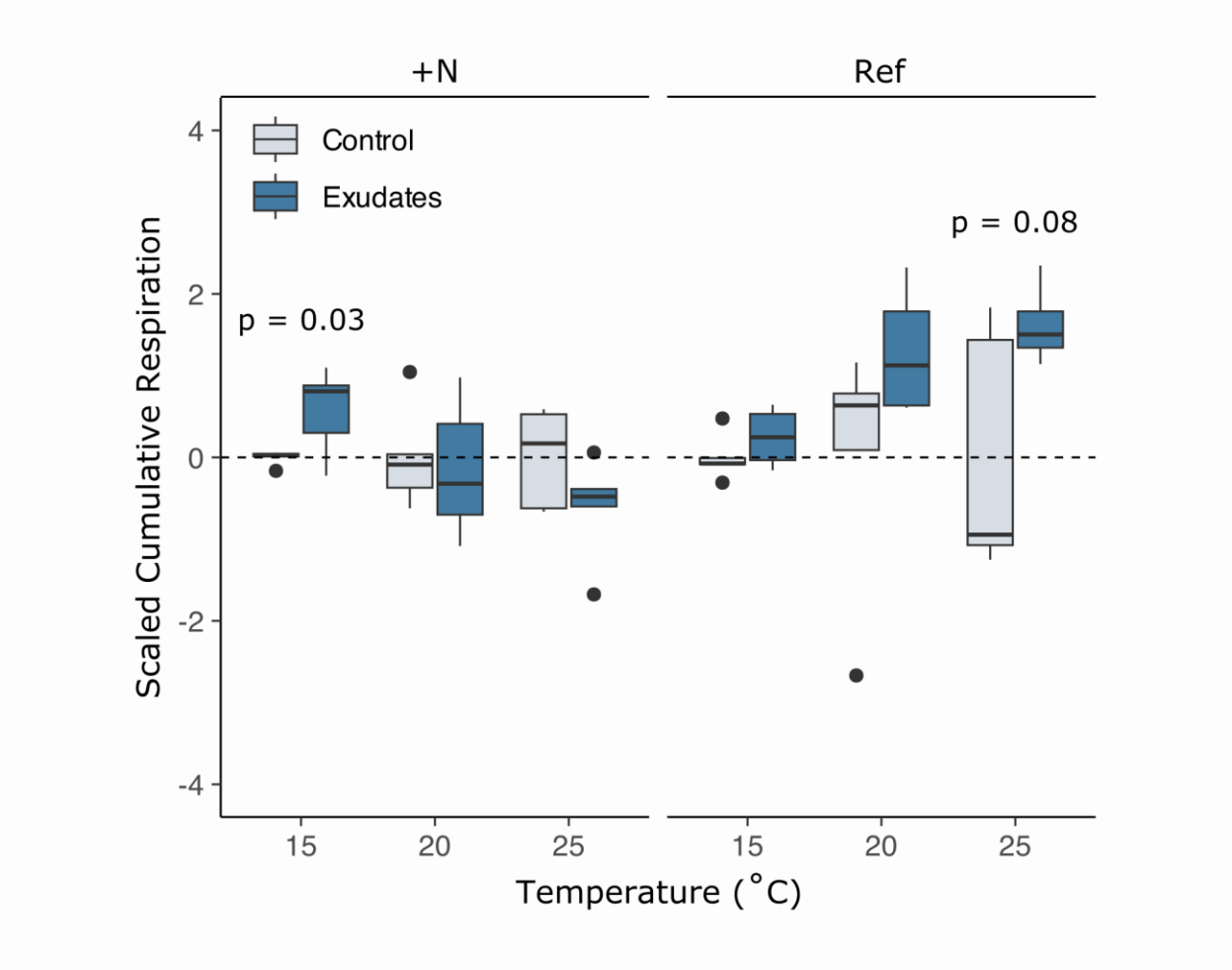
773 *Figure 4*. Microbial biomass (mmol C · g^{-1} dry soil) of soils incubated at 15, 20, and 25 ˚C with
774 and without the addition of artificial root exudates and lime in the reference (yellow) and
775 previously N-fertilized (blue) watersheds. Error bars represent ± standard error. Treatment
776 groups connected by overlapping letters are not significantly different ($p < 0.05$, Tukey-Kramer
777 HSD). Microbial biomass only varied by temperature across different treatments.



SI Figure 1. Cumulative respiration (mmol) of soils incubated at 15, 20, and 25 ˚C with and without the addition of artificial root exudates and lime in the reference and previously N-fertilized watersheds over time. Error bars represent ± standard error.



SI Figure 2. Mean endpoint cumulative respiration (mmol) by (A) watershed (Ref, yellow = reference, and +N, blue = previously N-fertilized), (B) temperature, (C) treatment (C = control, E = exudates, L = lime), (D) temperature × watershed (Ref, yellow = reference, and +N, blue = previously N-fertilized), and (E) temperature × treatment (C = control, E = exudates, L = lime). Error bars represent ± standard error. Treatment groups connected by overlapping letters are not significantly different ($p < 0.05$, Tukey-Kramer HSD).



SI Figure 3. Scaled cumulative soil respiration in the previously N-fertilized (+N) and reference (Ref) watersheds, after incubation at 15, 20, and 25 ˚C with (blue) and without (gray) the addition of artificial root exudates. Differences between the control and the exudate treatments are only shown for $p < 0.1$ within each treatment group (watershed × temperature). Priming effect is greatest in the microcosms incubated at 15 ˚C for the previously N-fertilized soils, and 25 ˚C for the reference soils.

SI Table 1. Extracellular enzyme activities (mean ± standard error) across three incubation temperatures (15, 20, and 25 ˚C) and three treatments (control, exudates and lime) in soils sampled from the previously N-fertilized watershed and the reference watershed.

		Control		Exudates		Lime	
Extracellular enzymes	Temp (˚C)	Reference Watershed	Prev. N-Fertilized	Reference Watershed	Prev. N-Fertilized	Reference Watershed	Prev. N-Fertilized
Acid phosphatase	15	0.59 ± 0.10	0.53 ± 0.05	1.01 ± 0.08	0.74 ± 0.09	1.31 ± 0.11	0.45 ± 0.08
($\mu mol \cdot g^{-1} \cdot hr^{-1}$)	20	0.54 ± 0.05	0.38 ± 0.05	0.86 ± 0.11	0.49 ± 0.04	0.90 ± 0.11	0.79 ± 0.08
	25	0.54 ± 0.07	0.45 ± 0.05	0.38 ± 0.04	0.42 ± 0.05	0.47 ± 0.03	0.28 ± 0.01
β-glucosidase	15	0.08 ± 0.00	0.06 ± 0.00	0.09 ± 0.00	0.06 ± 0.01	0.09 ± 0.01	0.06 ± 0.00
($\mu mol \cdot g^{-1} \cdot hr^{-1}$)	20	0.21 ± 0.01	0.19 ± 0.01	0.24 ± 0.02	0.15 ± 0.02	0.26 ± 0.02	0.22 ± 0.01
	25	0.18 ± 0.01	0.12 ± 0.01	0.17 ± 0.01	0.14 ± 0.01	0.18 ± 0.01	0.13 ± 0.01
N-acetyl-β- glucosaminidase	15	0.34 ± 0.08	0.15 ± 0.01	0.30 ± 0.02	0.14 ± 0.02	1.33 ± 0.35	0.32 ± 0.11
(10^{-1} $\mu mol \cdot g^{-1} \cdot hr^{-1}$)	20	0.05 ± 0.00	0.04 ± 0.00	0.09 ± 0.01	0.03 ± 0.00	0.12 ± 0.01	0.05 ± 0.01
	25	0.07 ± 0.01	0.05 ± 0.01	0.06 ± 0.01	0.05 ± 0.00	0.05 ± 0.00	0.02 ± 0.00
Phenol Oxidase	15	1.26 ± 0.09	0.86 ± 0.09	0.96 ± 0.09	0.89 ± 0.10	1.04 ± 0.08	0.84 ± 0.03
($mmol \cdot g^{-1} \cdot hr^{-1}$)	20	0.96 ± 0.06	1.17 ± 0.15	0.98 ± 0.05	0.82 ± 0.09	0.88 ± 0.03	0.85 ± 0.08
	25	0.83 ± 0.06	0.76 ± 0.05	0.75 ± 0.07	0.65 ± 0.04	0.89 ± 0.07	0.70 ± 0.02
Peroxidase	15	1.08 ± 0.09	1.02 ± 0.10	0.64 ± 0.04	1.18 ± 0.18	0.74 ± 0.08	0.98 ± 0.08
($mmol \cdot g^{-1} \cdot hr^{-1}$)	20	1.04 ± 0.09	1.63 ± 0.16	0.80 ± 0.06	0.83 ± 0.08	0.74 ± 0.10	1.04 ± 0.12
	25	0.05 ± 0.05	0.12 ± 0.12	0.06 ± 0.06	0.10 ± 0.10	0.10 ± 0.10	0.07 ± 0.07

SI Table 2. Significant main effects of watershed, temperature, treatment, and their interactions are shown for each extracellular enzyme ($p < 0.5$, ANOVA).

	AP (μmol · g^{-1} · hr^{-1})		BG (μmol · g^{-1} · hr^{-1})		NAG (μmol · g^{-1} · hr^{-1})		Phenol Oxidase (mmol · g^{-1} · hr^{-1})		Peroxidase (mmol · g^{-1} · hr^{-1})	
Effects	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Watershed	46.433	*< 0.001*	50.614	*< 0.001*	16.918	*< 0.001*	10.140	*0.002*	11.557	*0.001*
Temperature	36.782	*< 0.001*	173.740	*< 0.001*	34.470	*< 0.001*	13.688	*< 0.001*	7.681	*< 0.001*
Treatment	11.895	*< 0.001*	4.232	*0.018*	10.277	*< 0.001*	4.936	*0.009*	10.672	*< 0.001*
Watershed × Temperature	7.350	*0.001*	2.424	*0.094*	10.965	*< 0.001*	3.558	*0.033*	6.280	*0.003*
Watershed × Treatment	5.857	*0.004*	0.882	*0.418*	5.341	*0.006*	0.180	*0.835*	0.489	*0.615*
Temperature × Treatment	8.504	*< 0.001*	1.929	*0.112*	9.518	*< 0.001*	0.933	*0.449*	4.324	*0.003*
Watershed × Temperature × Treatment	6.939	*< 0.001*	2.423	*0.054*	4.621	*0.002*	2.705	*0.035*	4.808	*0.001*