



Repeated fire shifts carbon and nitrogen cycling by changing plant inputs and soil decomposition across ecosystems

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Abstract. Fires shape the biogeochemistry and functioning of many ecosystems, and fire frequencies are changing across much of the globe. Frequent fires can change soil carbon (C) and nitrogen (N) storage by altering the quantity and chemistry of plant inputs through changes in plant biomass and composition as well as the decomposition of soil organic matter. How decomposition rates change with shifting inputs remains uncertain because most studies focus on the effects of single fires, where transient responses may not reflect responses to decadal changes in burning frequencies. Here, we sampled seven sites exposed to different fire frequencies. In four of the sites, we intensively sampled both soils and plant communities across four ecosystems in North America and Africa spanning tropical savanna, temperate coniferous savanna, temperate broadleaf savanna, and temperate coniferous forest ecosystems. Each site contained multiple plots burned frequently for 33–61 years and nearby plots that had remained unburned over the same period replicated at the landscape scale. Across all sites, repeatedly burned plots had 25–185% lower bulk soil C and N concentrations but also 2–10-fold lower potential decomposition of organic matter compared to unburned sites. Soil C and N concentrations and extracellular enzyme activities declined with frequent fire because fire reduced both plant biomass inputs into soils and dampened the localized enrichment effect of tree canopies. Examination of soil extracellular enzyme activities revealed that fire decreased the potential turnover of organic matter in the forms of cellulose, starch, and chitin ($P < 0.0001$) but not polyphenol and lignin ($P = 0.09$), suggesting a shift in soil C and N cycling. Inclusion of $\delta^{13}\text{C}$ data from three additional savanna sites (19–60 years of altered fire frequencies) showed that soil C losses were largest in sites where estimated tree inputs into soils declined the most ($r^2 = 0.91$, $P < 0.01$). In conclusion, repeated burning reduced C and N storage, consistent with previous studies, but fire also reduced potential decomposition, likely contributing to slower C and N cycling. Trees were important in shaping soil C and N responses across sites, but the magnitude of tree effects differed and depended on how tree biomass inputs into soil responded to fire.

Key words: carbon cycling; coniferous forest; extracellular enzymes; fire frequency; nitrogen cycling; plant communities; repeated burning; savanna; soil.

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INTRODUCTION

Fires burn ~570 million hectares of land globally each year, affecting carbon (C) and nutrient storage and cycling and the species composition of ecosystems (Bond-Lamberty et al. 2007, Bowman et al. 2009, van der Werf et al. 2017, Pellegrini et al. 2018). Many ecosystems

burn regularly, with mean fire-return intervals ranging from two years in tropical savannas to several hundred years in boreal forests (Archibald et al. 2013, Andela et al. 2017). However, fire frequency, size, and severity are increasing due to climate change in some regions and decreasing due to anthropogenic fire suppression in other regions (Westerling et al. 2006, Miller et al. 2009, Dennison et al. 2014, Andela et al. 2017). Increasing fire frequencies can shift ecosystem C and nutrient cycles in soils by reducing inputs of organic matter to soils through repeated combustion of plant and litter biomass and associated volatilization of C and nutrients in plant biomass before they are decomposed into soils, as well as by combusting soil organic matter during intense wildfires (e.g., Ojima et al. 1994, Kauffman et al. 1995, Baird et al. 1999, Pellegrini et al. 2014, Muqaddas et al. 2015). In addition to altering detrital inputs to soils, fire can shift decomposition rates by increasing or decreasing microbial activity and nutrient availability (Kaye and Hart 1998, Wang et al. 2012, Dove et al. 2020). However, within ecosystems, it is unclear how changes in inputs are coupled with shifts in decomposition.

Recent work has demonstrated that repeated burning depletes soil C and N in most ecosystems, although there are exceptions (Pellegrini et al. 2018). Soil C and N stocks are partly regulated by fire-driven losses of plant biomass inputs, with larger losses of plant biomass expected to lead to larger reductions in soil C and N (Pellegrini et al. 2014, Kowaljew et al. 2018). For example, frequent burning can reduce tree biomass in savannas (Moreira 2000, Higgins et al. 2007, Burrows et al. 2010), which, in general, experience large concomitant soil C and N losses (Pellegrini et al. 2018). In other systems, such as coniferous forests that experience surface fires, tree mortality varies depending on tree species composition (Haase and Sackett 1998, Schwilk et al. 2009), and in many cases, these systems tend to lose less C and N (Pellegrini et al. 2018). In certain coniferous forests that experience severe stand-replacing fires where nearly all trees die, fire can cause large losses of soil organic matter from the organic and mineral horizons (Walker et al. 2019, Dove et al. 2020). Consequently, anecdotal evidence suggests that variability in fire effects on plant biomass inputs to soil, especially from trees, may partly explain long-term differences in soil responses.

In addition to shifting inputs, fire can change the decomposition of soil organic matter, but the direction of change is variable. Fire may accelerate soil C and N turnover by stimulating decomposition activity, potentially through pulsed availability of organic matter inputs after fire (Boerner et al. 2006, Rietl and Jackson 2012) or by promoting more favorable microclimate conditions (e.g., warmer conditions; Kaye and Hart 1998). Alternatively, fire may reduce decomposition by lowering microbial biomass through heat-induced mortality (Pressler et al. 2018), formation of more recalcitrant aromatics (Certini 2005, Knicker 2007), pyrogenic C (Santin et al. 2016), and changing

microbial community composition (Boerner et al. 2005, Waldrop and Harden 2008), especially in high severity fires (Dove et al. 2020).

Decomposition changes are nuanced, however, because enzymes associated with the catabolism of different molecular forms of organic matter can vary in their sensitivity to fire, which may contribute to discrepancies among reported fire effects on decomposition (Certini 2005, Knicker 2007). For example, repeated burning can reduce the potential decomposition of cellulosic compounds relative to unburned controls due to a reduction in hydrolytic enzyme activity (Eivazi and Bayan 1996, Ajwa et al. 1999, Boerner et al. 2005). However, changes in hydrolytic enzymes may be relatively unimportant in regulating soil organic matter losses because fire can enrich organic matter in aromatic and pyrogenic compounds (Certini 2005, Knicker 2007), whose decomposition depends on oxidative enzymes. Prolonged effects of fire on enzyme activity in soil may also arise because repeated burning can reduce organic matter and N availability within the soil profile and shift tree cover, both of which could either reduce or accelerate decomposition (Köster et al. 2016). These substrate and vegetation driven changes can further be exaggerated by increases of soil-inhabiting microbial communities that positively respond to repeated fires (Oliver et al. 2015), which may possess distinct decomposition capabilities.

Aside from shifting organic matter inputs to soils because of changes in total plant biomass, fire may affect soils through changes in tree cover and species composition and localized (i.e., spatially restricted to under the canopy) effects of trees on soils, such as higher soil C and N content and N mineralization under canopies (Belsky et al. 1989, Ludwig et al. 2004, Dijkstra et al. 2006). Reduced tree cover and changes in species composition due to repeated burning (Higgins et al. 2007, Peterson et al. 2007, Holdo et al. 2009) can thereby change soil properties at the landscape scale through aggregated changes in local effects (e.g., Coetsee et al. 2010, Holdo et al. 2012). Tree canopies have been shown to increase decomposition (e.g., mass-based losses; Norris et al. 2013), but how they change extracellular enzyme activities, and whether tree canopy effects on enzymes change with fire is unclear.

In this study, we tested how fire-driven changes in plant inputs, shifts in localized effects of trees on soils, and their interaction altered the storage and decomposition of soil C and N. We first focus our analysis on four sites that we intensively sampled and then perform a broader analysis using three additional sites to test the generality of the potential role of tree biomass inputs. For the first analysis, we focus on four distinct ecosystems to evaluate the generality of fire and tree cover effects by testing (1) whether fire and tree canopies change soil total C and N, (2) how extracellular enzymes involved in decomposition respond to fire and tree canopies, and (3) how C and N losses are correlated with

changes in inorganic N and extracellular enzyme activities. We hypothesized that repeated burning would reduce soil C and N stocks by reducing plant biomass inputs through volatilization and declines in the localized effects of trees. We expected that soil organic matter decomposition rates would also decline with fire because of a reduction in microbial biomass and activity, and that there would be a shift to a higher relative investment in N-acquisition enzyme activity to compensate for lower soil N.

We next incorporated data from three other decadal fire frequency manipulation experiments to evaluate mechanisms explaining variability in the effect of fire on soils across sites with different ecological and environmental conditions by testing (4) how changes in tree biomass inputs explain fire-driven changes in soil C across sites. We hypothesized that the effects of fire on soil C and N pools and microbial activity would be largest in ecosystems with the greatest losses of tree biomass inputs.

METHODS

Our site selection was meant to capture a broad range of ecosystem types that experience repeated burning on decadal timescales. Furthermore, the sites span a gradient in the effect of fire on woody biomass inputs into soils, allowing us to test the hypothesis that below-ground changes arise via fire-driven shifts in woody biomass inputs.

We sampled sites in seven locations (four were studied in greater detail, which we refer to as our main sites) spanning a range of forests and savannas that experience frequent (0.1–1 fires/year) and, in most cases, prescribed burning: temperate coniferous savanna, tropical broadleaf savanna, temperate broadleaf savanna, and temperate coniferous forest (Fig. 1, also see this figure for climate data). In four of the sites, we analyzed bulk soil C, N, $\delta^{13}\text{C}$, and extracellular enzyme activity, whereas in three additional sites from temperate and tropical savannas (South Africa, Brazil, and the United States), we analyzed bulk soil C and $\delta^{13}\text{C}$ to expand our test of whether and how changes in inputs explain changes in soil C. In the four sites for which we performed complete analyses, we sampled three replicate plots of each fire treatment (plots where fire was excluded and those burned repeatedly that ranged in size from 0.1–10 ha). In all four of our main sites except the temperate coniferous savanna, the replicates were separated by fire breaks. Within each plot, we sampled both directly underneath individual tree canopies and away from canopies to test the impact of changes in tree canopy cover (which increases under fire exclusion and decreases with more frequent burning). Where more than one tree species was locally abundant, we replicated our sampling across different tree species to test for interspecific variability in tree cover effects on soils, as described below. We sampled three to nine individuals for each tree species within

each fire treatment depending on the abundance of each species.

Main site descriptions

The tropical broadleaf savanna site was in Kruger National Park, South Africa, in the Experimental Burn Plots, which have received different fire frequency treatments since the mid-1950s (1956 at the particular site we sampled in 2017). The large-scale plots are ~5–7 ha in size and are replicated across the park in blocked designs (i.e., the different fire manipulations all occur adjacent to one another in a “block,” which is replicated across the park; Biggs et al. 2003). We sampled plots located in the Skukuza area (latitude 25.10° S, longitude 31.45° E). The soils are sandy, well drained, and derived from granitic parent material, with duplex and sodic soils covering large areas (Venter and Govender 2012). In March of 2017, we sampled soils on plots burned annually at the end of the dry season in August, when fires are most intense; the annual fire frequency represents a higher-than-historical frequency of once every 3 year (Govender et al. 2006). We compared the annually burned plots to plots unburned since the onset of the experiment.

The Kruger plots are dominated by broadleaf woody plant species in the genera *Combretum* and *Terminalia*, common savanna tree species in the region. The fire manipulation plots span a large fire-driven gradient in tree biomass, and grass cover is substantial across all plots, even in those protected from fire. We sampled soils in open areas dominated by grasses (C_4 grasses are the dominant functional type in Kruger) as well as under canopies of four different tree species: *Combretum apiculatum*, *Combretum collinum*, *Combretum hereroense*, and *Terminalia sericea*.

The temperate broadleaf savanna was in the Cedar Creek Ecosystem Science Reserve in Minnesota, USA (latitude 45.40° N, longitude 93.19° W). The fire frequency manipulation experiment was established in 1964 on a landscape that was primarily savanna and woodland (ranging from 17% to 39% tree canopy cover with an extensive herbaceous layer; Faber-Langendoen and Davis 1995). Areas of several hectares were delineated with fire breaks and assigned to different fire frequency treatments ranging from complete exclusion to burning every 3 out of 4 years. Plots were burned in the spring (April or May, depending on conditions). Soils are fine to medium sands (<3% clay) and well drained. The parent material is dominated by quartzite and the major soil associations include the Sartell and Zimmerman soil series (Grigal et al. 1974).

The tree communities in Cedar Creek are dominated by broadleaf species including bur oak and northern pin oak (*Quercus macrocarpa* and *Q. ellipsoidalis*, respectively). The plots we sampled in August of 2017 spanned a fire-driven ecotone between open savanna, with continuous grass cover in the high frequency plots, to closed-canopy forest without a continuous grassy layer

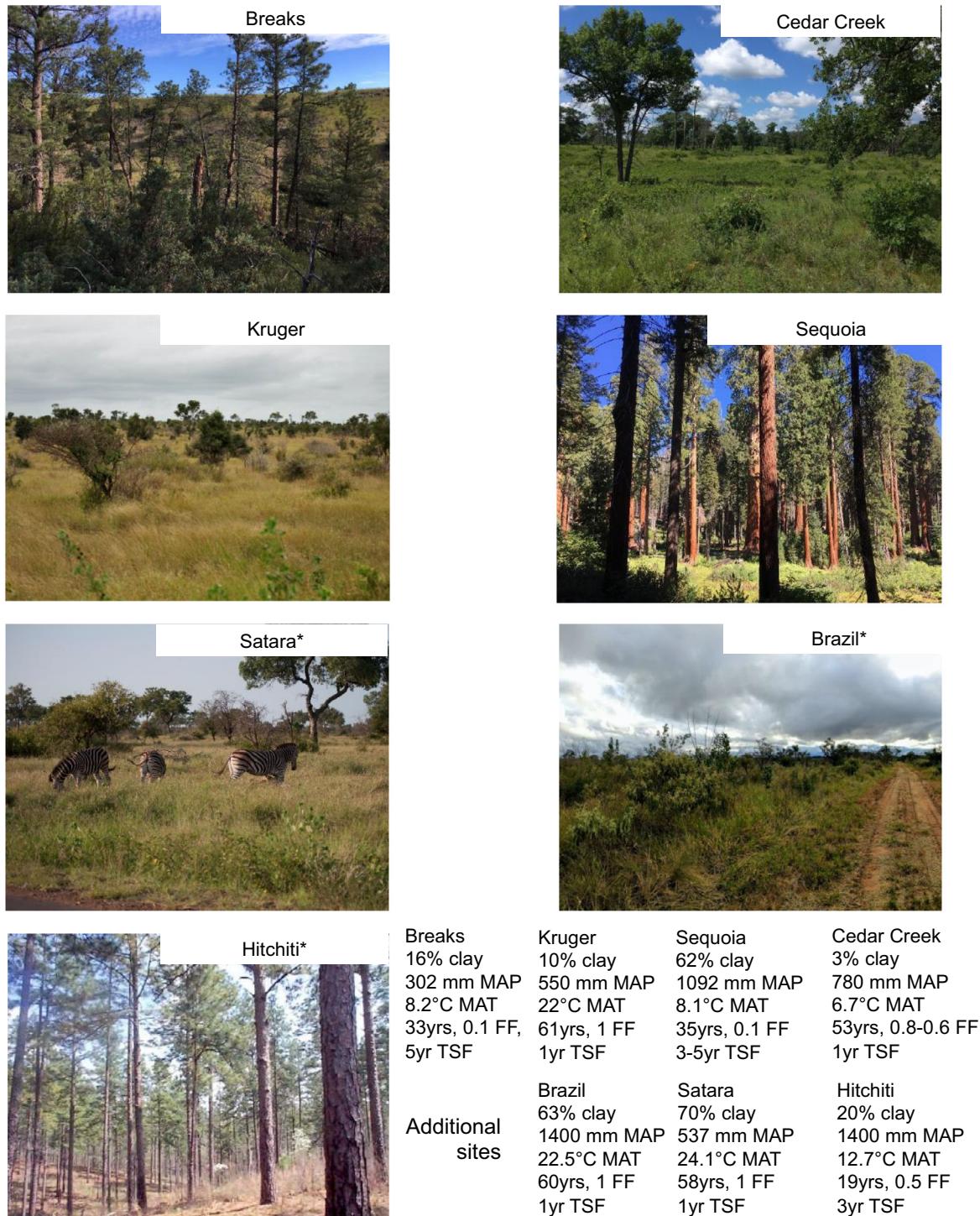


FIG. 1. Sites sampled partitioned into the main sites comprising a temperate coniferous savanna (Missouri Breaks), tropical broadleaf savanna (Kruger), temperate broadleaf savanna, (Cedar Creek), and a temperate coniferous forest (Sequoia). The sites vary in the amount of tree cover and climate but have all burned repeatedly at frequencies from annual to decadal for 33–61 year with adjacent control plots that have not burned over the same period. The additional savanna sites are denoted with a dagger † and comprise two tropical broadleaf savannas (Satara and Brazil) and a temperate coniferous savanna (Hitchiti) that were used for the ¹³C analysis. The values beneath the pictures show the percent clay of the soil, mean annual precipitation (MAP), temperature (MAT), length of time plots have differed in fire history (year), and the fire frequency of the burned plots (FF). Photo credit Adam Pellegrini except for Hitchiti, Mac Callahan.

under trees in the unburned plots (Peterson and Reich 2001). The grassy community in savannas at Cedar Creek is a mix of C_3 (17% of ground cover) and C_4 (38% of ground cover) species (surveys from 1985 to 2015; Pellegrini et al. 2020). We sampled under *Q. macrocarpa* and *Q. ellipsoidalis* and in grassy areas outside of tree canopies. In the unburned plots where forests have formed, we sampled in areas not yet encroached by forest, and have also remained unburned, to obtain the samples outside of tree canopies.

The temperate coniferous savanna was in the Missouri Breaks region in central Montana, USA. These plots do not receive direct fire manipulations, so we used variability in wildfire history to examine different fire regimes. Using remote-sensing records dating from 1984 (Monitoring Trends in Burn Severity [MTBS] product, which has been used to establish trends in wildfire; Dennison et al. 2014) we identified sites that have similar underlying geologies and climates (latitude 47.43° N, longitude 108.17° W) but different fire histories over the past 34 years. We identified a large location that had burned repeatedly (each decade since 1984 with the last fire in 2012) by overlapping individual fire events from the MTBS product. In the unburned plots, we confirmed they had not burned since at least 1984, but do not know the date of the last fire prior to that time. Areas in the Missouri Breaks typically burn in the late summer. The landscape contains bare areas, rock outcrops, and shrublands, which we avoided in our sampling. Soil properties can vary substantially but in our plots soils contained low clay and silt (16% and 3%, respectively) but high sand (81%) content.

In the Breaks, we sampled under the main woody plant species, *Pinus ponderosa* and *Juniperus scopulorum*. In the unburned plots, thick litter layers can accumulate under trees, excluding understory grasses, but these thick litter layers were largely absent in the burned plots; however, the “open” areas we sampled were always positioned in grassy areas. The grassy community is dominated by C_3 species; however, two C_4 species, *Bouteloua gracilis* and *Muhlenbergia cuspidata*, also occur in the area (Anderson 2003), which we assume likely contribute substantially to soil C pools given the relatively high soil $\delta^{13}C$ (-16‰) in the area. These sites were sampled in June 2017. The large areas containing different fire histories (distributed across a 15-ha area for the unburned “treatment” and across a separate 10-ha area for the burned “treatment”) allowed us to sample plots within each different fire frequency “treatment” that were 100–400 m apart from one another but not separated by distinct fire breaks.

The temperate coniferous forest site was in Sequoia and Kings Canyon National Parks in California, USA. We sampled areas in a network of permanent monitoring plots 0.1 ha in size that were established in the 1980s and were exposed to different fire regimes (e.g., Schwilk and Caprio 2011). We sampled three replicate plots within two fire treatments where fire has been excluded

for >150 year (Caprio and Swetnam 1995, Swetnam et al. 2009) and where prescribed burns have been conducted every 10–15 year starting in 1983–1986 (plots located in Giant Forest; latitude 36.60° N, longitude 118.73° W).

We sampled plots in a forested landscape near Giant Forest currently dominated by *Abies concolor* and *Sequoiadendron giganteum*. In each plot, we sampled underneath three individuals of *A. concolor* and *S. giganteum* as well as in “open” areas not directly adjacent to a tree trunk. There is little understory vegetation in plots where fire has been excluded, which have deep accumulations of litter, duff, and coarse wood debris, but understory plant species such as *Ceanothus*, *Lupinus*, and various grasses occur more frequently in plots repeatedly burned. Consequently, the “open” samples in the unburned plots are under thick layers of litter and duff, whereas those in the burned plots are under herbaceous and shrubby species lacking a thick litter layer. Plots have usually been burned by prescribed fire during either the spring or fall (May–June or September–October) depending on suitable conditions.

Plots sampled in Sequoia experience a Mediterranean climate, with the sites sampled at ~2,100 m elevation receiving on average 1,092 mm in precipitation (1920–2017 at Giant Forest USACE station). Most precipitation occurs during the winter (~50% as snow) with dry summers that have a distinct water deficit (Stephenson 1988). Average annual air temperatures range from 1.9 to 13.6° C in Grant Grove at a similar elevation to Giant Forest (van Mantgem et al. 2016). Soils in our sites contain a low sand (9%) content, a medium silt (29%) content, and a high clay (62%) content. These sites were sampled in September 2017.

Soil sampling

Within plots, we designed our sampling to minimize the effect of variability in topography and soil type on soil properties by avoiding sampling on slopes, rocky areas or in riparian zones and where the mineral soil horizon was <5 cm deep. For the sampling under tree canopies, we chose large trees within each site, but the exact tree size differed based on species and location (e.g., in Kruger the trees had small (~10 cm) stem diameters (taken at a breast height of 1.3 m) while, in Cedar Creek, diameters were 20–30 cm and in Sequoia they could be several meters). The soil cores were distributed over ~12.5 m² under the base of a tree (cores taken within 2 m of a tree trunk) and avoided coarse roots. In the “open” areas, we tried to maximize distance from canopies. Samples were chosen as randomly as possible within these areas.

At each sampling location within a site (e.g., under an individual tree), we aggregated soil cores from the top 5 cm of the mineral horizon sampled in five separate 125-cm³ cubes (5 × 5 × 5 cm for each sample, total volume of 625 cm³ per sampling location). We identified

the mineral horizon by first digging a hole >20 cm in depth and removing the organic horizon from the profile, when present, and then sampled the top 0–5 cm of the mineral horizon. We focused on changes in the mineral horizon because of the lack of distinct organic horizons in the savanna sites. We focused on the upper soil horizons because they are potentially most responsive to fire and are most biologically active.

Soils were hand homogenized and passed through a 2-mm sieve to remove large particles and divided into three subsamples: (1) fresh soil for soil moisture and inorganic N analyses, (2) frozen soil for enzyme analyses (see *Enzyme activity*), and (3) dried soil for total soil C, N, and $\delta^{13}\text{C}$ analyses. Gravimetric soil moisture was determined by drying soils at 105°C until they reached a constant mass (24–48 h). We used these values of moisture content to adjust all analytical chemistry concentrations to dry mass of soil.

Analyses of soil C, N, and $\delta^{13}\text{C}$

Total soil C, N, and $\delta^{13}\text{C}$ were measured by combusting samples using an elemental analyzer at Stanford University and a Costech coupled elemental analyzer and mass spectrophotometer at the University of Maryland (Costech, Valencia, CA, USA). The mass combusted per sample was optimized to maximize analytical accuracy of measurements. Duplicates were run for 10% of all samples to ensure analytical precision, which we defined to be an error of <5%.

We measured inorganic N (IN) on soils within 48 h of collection. Inorganic N was measured by extracting ~5 g of the freshly sieved homogenized soil via shaking in 50 mL of 1 mol/L KCl. Following shaking, the samples were centrifuged and filtered through leached Grade 41 ashless Whatman filters. Inorganic N was analyzed on an automated spectrophotometer WestCo SmartChem 200 discrete analyzer at Stanford University (Unity Scientific, Milford, MA, USA). Nitrate was analyzed using a cadmium column reduction, followed by a diazotization with sulfanilamide coupled with N-(1-naphthyl) ethylenediamine dihydrochloride, which was analyzed colorimetrically at 550 nm. Ammonium was analyzed using the indophenol blue method, where ammonium reacts with salicylate and hypochlorite in the presence of sodium nitroferricyanide to form the salicylic acid analog of indophenol blue in a buffered alkaline solution (pH 12.8–13), which was analyzed colorimetrically at 660 nm.

Enzyme activity

To assess potential C and N decomposition, we measured hydrolytic and oxidative extracellular enzyme activity in a subset of samples at each site. The hydrolytic enzymes were cellobiohydrolase (EC 3.2.1.91-CBH, degrades cellulose), β -glucosidase (EC 3.2.1.21-BG, degrades cellulose), α -glucosidase (EC 3.2.1.20-AG, degrades starch), β -

xylosidase (EC 3.2.1.37-BX, degrades hemicellulose), and N-acetyl- β -D-glucosaminidase (EC 3.2.1.30-NAG, degrades chitin). The oxidative enzymes were phenol oxidase (EC 1.10.3.2-PO, degrades polyphenols), and peroxidase (EC 1.11.1.7-PX, degrades polyphenols/lignin). The enzyme activity analyses were performed using methods presented in Hobbie et al. (2012), which used slightly modified methods of Sinsabaugh et al. (1992) and Saiya-Cork et al. (2002), and were performed at the University of Minnesota.

Enzyme activities were analyzed both individually and by summing all hydrolytic enzymes (excluding NAG) and the oxidative enzymes separately. We refer to the group of hydrolytic enzymes as those degrading cellulosic substrates and the oxidative enzymes as those degrading aromatic substrates. We also analyzed the ratios of enzyme activities to test how fire and tree canopies may be altering the relative activity of enzymes.

Soil CO₂ flux

To link enzyme activity measurements with soil C losses, we took advantage of previous in situ measurements on soil CO₂ flux across the temperate broadleaf savanna experimental plots at Cedar Creek conducted from 1999 to 2005, which includes both auto- and heterotrophic respiration. We assume that the variability across plots from 1999 to 2005 is representative of present-day conditions because there was no significant change in soil CO₂ flux through time. These measurements were made throughout the growing season (April–October) across eight points containing a 5 cm permanent soil collar within each replicate plot. Aboveground biomass was removed above each point prior to measurements made using a Li-Cor 6400-09 soil respiration chamber attached to a Li-Cor 6200 gas exchange system (Li-Cor, Lincoln, Nebraska, USA). For our analyses, we rescaled the data to fluxes per degree Celsius because of the high correlation between respiration and temperature ($F_{1,493} = 1,240$, $P < 0.0001$, $r^2 = 0.75$, model included the effect of fire frequency as well).

Analyses of $\delta^{13}\text{C}$ and incorporation of additional sites

We used $\delta^{13}\text{C}$ to infer the contribution of C₃ vs. C₄ plant biomass inputs to soil organic matter, an approach often used in savannas (Tieszen et al. 1979, Boutton et al. 1998). The strong fractionation differences between C₃ trees (more negative $\delta^{13}\text{C}$) and C₄ grasses (less negative $\delta^{13}\text{C}$) makes $\delta^{13}\text{C}$ a useful tracer for understanding how fire-driven reductions in tree biomass contribute to losses of soil C. While pyrolysis of organic matter can also result in $\delta^{13}\text{C}$ fractionation (Bird and Ascoug 2012), these effects are thought to be relatively small compared to fractionation via the C₄ vs. C₃ photosynthetic pathway. One limitation to using $\delta^{13}\text{C}$ to infer the different contributions of trees vs. grasses in northern latitude savannas is that several grass species use the C₃

photosynthetic pathway, and thus have similar isotopic signatures as trees. Consequently, changes in $\delta^{13}\text{C}$ could overestimate tree contributions to changing soil C. We are more confident that soil $\delta^{13}\text{C}$ is a reliable indicator of C_4 grass vs. tree contributions in Cedar Creek because a previous study found a strong positive correlation between C_4 grass biomass and soil $\delta^{13}\text{C}$ in the top 0–10 cm of soil (Pellegrini et al. 2020). Previous studies in Kruger and Cedar Creek have assumed isotope values of -13‰ for C_4 grasses and -27‰ for C_3 trees (Nelson et al. 2004, February and Higgins 2010). We analyzed $\delta^{13}\text{C}$ on samples taken under canopies and in the open separately, allowing us to evaluate the local effect of tree canopies on soil $\delta^{13}\text{C}$.

To more broadly test how fire-driven changes in tree biomass inputs regulated changes in soil C pools across sites, we incorporated data on soil C and $\delta^{13}\text{C}$ from three additional sites, expanding our comparisons from four to seven sites (Fig. 1). These additional sites share similar experimental designs to the four main sites in that they have experienced altered fire frequencies (fire exclusion vs. repeated burning at different frequencies) for several decades. Detailed descriptions of the sites have been previously published, but here we describe their relevant main designs.

Two of the sites are in tropical savannas: one in Brazil and the other in South Africa. In Brazil, the plots were located in the Reserva Ecologica do Instituto Brasileiro de Geografia e Estatística and Jardim Botânico de Brasília in the Federal District of Brazil (Pellegrini et al. 2014). The reserves contain plots that have burned at several different fire frequencies since the 1960s (resulting in roughly 60 year of altered fire frequencies), which has resulted in a large gradient in woody plant biomass (open savanna in the frequently burned plots and closed-canopy forest in fire exclusion plots covering a range of broadleaf tree species and C_4 grasses). We compared soil C and $\delta^{13}\text{C}$ in two plots burned roughly every 2 year and two plots excluded from fire since at least the 1960s (plots 1, 2, 6, and 7 in Pellegrini et al. [2014]). The site in South Africa is also in Kruger National Park (the same park as our tropical broadleaf savanna), but occurs in a drier climate with different broadleaf tree species. Experimental design is similar to the one described for our main site in Skukuza, with the sampling scheme outlined in Pellegrini et al. 2015. At the time of sampling, the plots had experienced 58 year of altered fire frequencies. We compared plots burned annually with those under fire exclusion ($n = 3$ replicates for each fire treatment).

The final site was a coniferous savanna in the southeastern United States in the Hitchiti Experimental Forest in Georgia. In Hitchiti, the plots had not been burned for at least 50 year before the onset of the fire experiment in 1989, where replicate plots received different fire frequency treatments. We compared unburned plots with those burned biennially (four replicates of each treatment) sampled 19 year after the onset of the experiment. The plots contain both hardwood and pine tree species, but loblolly

pine (*Pinus taeda*) dominates the overstory (Oliver et al. 2015).

Data analysis

To test the effect of fire and tree canopies on soil chemistry, we used mixed-effects models to accommodate the hierarchical design of sampling replicate individuals of different tree species and patches away from trees across the different sites (Bates et al. 2015; R package lme4). Because sites differed in the number of co-occurring tree species, the sampling design was unbalanced, which is a potential source of error in statistical inference (Harrison et al. 2018). Consequently, in order to test for overall effects of fire and tree canopies, we constructed a balanced sampling design by averaging values within each replicate plot for open and tree canopy samples separately. We then fit mixed-effects models using site as a random intercept including both independent and interactive fixed effects of fire and tree canopy. Site was included as a random intercept because the mean soil chemistry differed across sites. Significance of terms was evaluated using the package lmerTest that approximates the degrees of freedom using Satterthwaite's method (Kuznetsova et al. 2017).

If neither main effect of fire or tree canopy was significant, we tested if the effects were contingent on site, i.e., whether sites differed in their sensitivities to either fire and/or tree canopies. For these models, we included replicate plot as a random intercept and site as a fixed effect to test for interactions. Because of the many potential variable combinations, we first determined the variables in the top model using model selection via AIC with a threshold of two. When AIC values did not differ by >2 and the lower AIC model was more complex, we used a χ^2 test to determine whether the more complex model added significant explanatory power. If the test was not significant, we used the model with the fewest terms. Significance of terms was also evaluated using lmerTest (Kuznetsova et al. 2017). Where a significant interaction occurred, we fit mixed-effects models within each individual site with replicate plot as a random intercept (to account for the non-independence of the replicate soil samples taken within a replicate plot) to test for significant effects of fire and tree canopies. In this last analysis, we used data from all the soil samples taken within a site (i.e., not using plot-level averages).

To visualize the overall effects of fire and tree canopies on soil variables across all sites, we used values averaged across the replicate samples within each replicate plot in a site (i.e., the three patches away from tree canopies, and three patches within each separate tree species in each replicate plot). Each graph displays the effects of tree cover and fire conditionally (i.e., tree cover effect in unburned plots and fire treatment effect underneath tree canopies).

We refer to the tree canopy effect as the contrast between soils taken from under tree canopies vs. outside of the canopy. We refer to the fire effect as the contrast

between soils in the burned vs. unburned plots either under and away from tree canopies. All analyses were performed in R version 3.6.1 (R Development Core Team 2010).

RESULTS

Total soil C and N are influenced by both fire and tree cover

Across the four main sites (Fig. 1, which we focus on until the section comparing $\delta^{13}\text{C}$ across sites), fire and tree canopies both helped predict bulk soil C concentrations in the top 0–5 cm; unburned plots had $48\% \pm 22\%$ (mean \pm SE) higher soil C concentrations than repeatedly burned plots (all sites in a mixed-effects model, $F_{1,41} = 7.4$, $P = 0.01$, Fig. 2, Table 1, Appendix S1: Table S1), and soils under tree canopies had $55\% \pm 18\%$ higher bulk C concentrations than soils in open areas ($F_{1,41} = 10.7$, $P = 0.002$, Fig. 2, Table 1, Appendix S1: Table S1; each mean and SE calculated from the values across the sites [$n = 4$], while the mixed-effects models are based on plot-level averages with site as a random intercept). Changes in bulk soil N concentrations followed similar trends to C: unburned plots had 69% ($\pm 35\%$) higher soil total N concentrations than repeatedly burned plots ($F_{1,41} = 15.9$, $P < 0.001$, Table 1, Appendix S1: Table S1). There was not an overall enrichment effect of tree canopies on soil N across sites ($F_{1,41} = 2.5$, $P = 0.12$), despite a trend towards tree canopies having $26\% \pm 20\%$ higher total N concentrations than open areas. Further analyses revealed that the lack of an overall trend was because two sites had enriched N under canopies while one site had lower N under canopies (site \times canopy interaction $F_{3,136} = 10.8$, $P < 0.001$; Fig. 2, Tables 1 and 2).

Taken together, repeated burning depleted soil C and N both in the open and under tree canopies, but tree canopies generally maintained higher C than open areas, with more variable canopy effects on N across sites. We found no evidence for significant interactive effects between fire and tree canopies on soil C and N (C $F_{1,41} = 1$, $P = 0.033$ and N $F_{1,41} = 0.1$, $P > 0.5$, Appendix S1: Fig. S2), suggesting that soils under and away from canopies responded to fire similarly and vice versa. We evaluate the factors that contribute to different responses of soil variables across sites below.

Extracellular enzyme activity is lower in burned plots and away from tree canopies

Given the changes in bulk soil C and N, we next sought to test how potential decomposition activity, quantified using extracellular enzyme assays, responded to fire and tree canopies. Higher enzyme activity in burned plots and open areas would suggest microbial activity is higher and may have contributed to losses of soil C, while lower activity would suggest a potential buffering mechanism against losses. We investigated potential changes in

turnover of cellulose-, hemicellulose-, and starch-based and aromatic-based organic matter separately.

Frequently burned plots had lower potential activity of enzymes that break down cellulose, hemicellulose, and starch. The total potential activity of the hydrolytic enzymes processing these compounds was $187\% \pm 106\%$ higher in soils from unburned than burned plots ($F_{1,40} = 25.6$, $P < 0.001$, Fig. 3, Table 1). Moreover, when hydrolytic enzymes were examined individually, all enzymes had significantly lower activity in the burned than unburned plots (CBH $P < 0.001$, AG $P < 0.001$, BX $P < 0.001$, BG $P < 0.001$; Appendix S1: Table S1), illustrating that fire reduces the potential turnover of cellulose, hemicellulose, and starch. Although the potential activity of all enzymes was tightly and positively correlated with soil %C ($P < 0.0001$ for all comparisons, Appendix S1: Fig. S1), the inclusion of %C into the plot-averaged statistical models did not change the result that fire reduced either total enzyme activity (fire effect: $F_{1,40.5} = 15.3$, $P < 0.001$) or the activity of individual enzymes (fire effect: CBH $P < 0.001$, AG $P = 0.014$, BX $P < 0.001$, BG $P = 0.005$). Consequently, although we did not quantify microbial biomass directly, we found that the effects of fire were robust to considering the correlation between enzyme activity and soil %C, which can be a proxy for microbial biomass (McLauchlan and Hobbie 2004).

Fire had no significant overall effect on the total activity of oxidative enzymes that process aromatic lignin and polyphenol compounds ($F_{1,40} = 0.2$, $P > 0.5$; Table 1, Fig. 3). In contrast, tree canopies had significant effects on oxidative enzyme activity ($F_{1,40} = 7.2$, $P = 0.01$; Fig. 3), with $74\% \pm 39\%$ higher activity under trees (Table 1, Fig. 3). Both peroxidase and phenol oxidase increased under canopies ($F_{1,40} = 4.3$, $P = 0.045$ and $F_{1,40.2} = 12.8$, $P < 0.001$, respectively, Appendix S1: Table S1), but unlike any of the other enzymes, peroxidase activity actually increased with burning, but only in the open areas (fire \times canopy interaction $F_{1,40} = 5.7$, $P = 0.02$, Appendix S1: Table S1). Consequently, oxidative enzymes had a higher dependency on the proximity to trees but were less limited by fire, either not changing or even increasing in burned plots. The oxidative enzymes were not as well correlated with soil %C as the hydrolytic enzymes: there was no significant relationship with total oxidative enzyme activity ($F_{1,38.5} = 1.9$, $P = 0.18$) or peroxidase ($F_{1,38.3} = 1.0$, $P = 0.33$), but there was with phenol oxidase ($F_{1,15.7} = 5.5$, $P = 0.032$).

We did find evidence for significant fire \times canopy interactive effects for the sum of oxidative but not hydrolytic enzyme activity ($F_{1,40.1} = 6.7$, $P = 0.013$ and $F_{1,40} = 1.9$, $P = 0.17$, respectively). The interaction revealed that the enrichment of oxidative enzyme activity under trees relative to away from trees was greatest in unburned plots (Appendix S1: Fig. S3).

Overall, fire reduced the potential turnover of organic matter but only of forms targeted by hydrolytic enzymes (cellulose, hemicellulose, and starch); contrastingly, the potential turnover of aromatic organic matter was

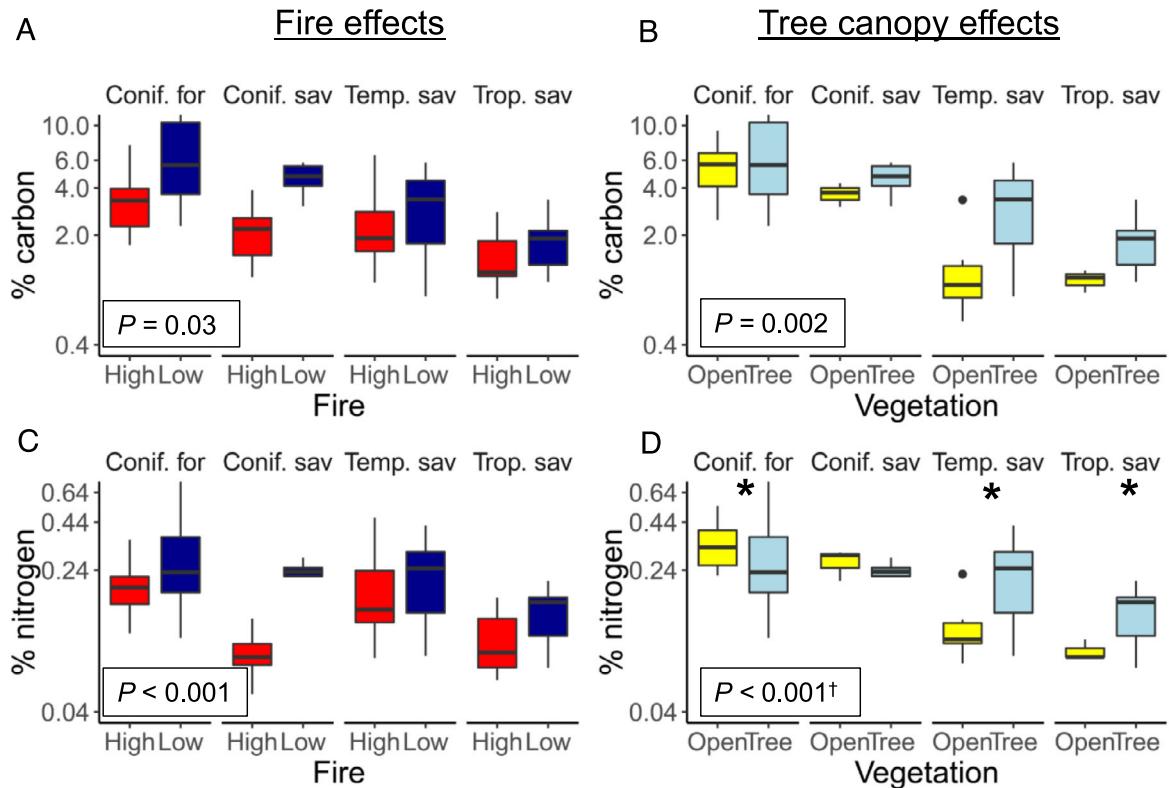


Fig. 2. Box plots of soil carbon (C) and nitrogen (N) for fire treatments (fire effects) and tree canopies (tree canopy effects) and plot-averaged values across sites. This figure and Figs. 3, 4, and 7 are the conditional values of the relative effects of the other variable (i.e., values displayed for tree cover effects are from unburned plots and those for fire effects are from under tree canopies). For fire effects, high and low delineate the frequently burned and the unburned treatments, respectively, and for tree canopy effects, open and tree delineate the samples away from tree canopies and under canopies, respectively. The y -axis is on a log scale. Box plots display medians (50th percentile), 25th and 75th percentiles, and approximate 95% confidence intervals. Inserted boxes display P values from the replicate plot-averaged mixed-effects model, with full statistics in Table 1. A dagger † next to the P value indicates that the effect is contingent on site (see Appendix S1: Table S3 for the site-level statistics). Asterisks above boxes indicate significant differences ($P \leq 0.05$) between canopy positions. Colors indicate fire treatment on the left-hand panel (red, burned; dark blue, unburned) or canopy position (yellow, in the open; light blue, under a tree canopy). Sites are temperate coniferous savanna (Conif. sav), tropical broadleaf savanna (Trop. sav), temperate broadleaf savanna (Temp. sav), and a temperate coniferous forest (Temp. for).

relatively unchanged by fire but reduced in areas away from tree canopies. The different sensitivities of hydrolytic vs. oxidative enzymes resulted in shifts in their relative ratio. Unburned plots had $165\% \pm 32\%$ higher hydrolytic:oxidative ratios than the frequently burned plots ($F_{1,40} = 25.0$, $P < 0.001$; Fig. 3, Table 1). In contrast, the effect of tree canopies was site-specific (site \times canopy interaction $F_{3,97} = 5.7$, $P = 0.001$; Table 2), with significantly lower ratios in two sites ($P < 0.01$) and a marginally significant higher ratio in one site ($P = 0.08$; Fig. 3). Consequently, burning changed the potential decomposition of organic matter in complex ways by decreasing the losses of cellulose, hemicellulose, and starch but leaving potential losses of aromatics unchanged.

Fire reduces inorganic N and microbial acquisition of N

We next sought to understand whether potential changes in N availability were related to the effects of fire and tree canopies on microbial decomposition activity. Lower soil N may result in lower potential C-

acquisition activity, but upregulation of microbial N-acquisition activity may compensate for the reduction in soil N. To ascertain how changes in soil N influenced the trends in N-acquisition activity, we quantified (1) the effect of fire on inorganic N and the activity of the enzyme N-acetyl-b-D-glucosaminidase (NAG), which targets chitin allowing microbes to access bulk and inorganic N and C in organic matter, (2) how changes in bulk and inorganic N related to changes in NAG activity, and (3) whether correlations between N and enzyme activity varied across the different C-acquisition enzymes. For the last two comparisons, we always tested whether %C was a better explanatory variable than %N using model selection.

Changes in bulk soil N were accompanied by changes in the availability and potential turnover of N, estimated by inorganic N and NAG activity, respectively. To approximate the availability of N for plant uptake (referred to as bioavailability), we analyzed inorganic N (IN) concentrations, which were $148\% \pm 100\%$ higher in unburned plots ($F_{1,41} = 14.7$, $P < 0.001$; Fig. 4,

TABLE 1. Results comparing the relative effect of tree canopies and burning treatment on soil chemistry variables.

Variable and comparison	Mean	SEM	df	<i>F</i>	<i>P</i>
%C					
Tree/Open	1.55	0.18	1,41	10.7	0.002
Unb/Burn	1.48	0.22	1,41	7.4	0.010
%N					
Tree/Open†	1.26	0.20	1,41	2.5	0.119
Unb/Burn	1.69	0.35	1,41	15.9	<0.001
C:N					
Tree/Open	1.24	0.08	1,41	10.6	0.002
Unb/Burn	0.92	0.06	1,41	2.6	0.112
NO ₃ ⁻					
Tree/Open†	1.31	0.46	1,41	0.0	0.858
Unb/Burn	1.18	0.22	1,41	0.0	0.862
NH ₄ ⁺					
Tree/Open	1.09	0.23	1,41	0.3	0.603
Unb/Burn†	2.90	1.24	1,41	17.2	<0.001
Total IN					
Tree/Open†	1.05	0.21	1,41	0.0	0.875
Unb/Burn	2.48	1.00	1,41	14.7	<0.001
NAG					
Tree/Open	2.00	0.31	1,40	5.8	0.021
Unb/Burn	4.13	1.85	1,40	22.5	<0.001
C-hydrolytic					
Tree/Open	1.45	0.11	1,40	3.5	0.070
Unb/Burn	2.87	1.06	1,40	25.6	<0.001
C-oxidative					
Tree/Open	1.74	0.39	1,40.1	7.2	0.010
Unb/Burn†	1.18	0.20	1,40.1	0.2	0.674
C-hydrolytic:C-oxidative					
Tree/Open†	1.07	0.28	1,40.0	0.0	0.945
Unb/Burn	2.65	0.32	1,40.0	25.0	<0.001
C-hydrolytic:NAG					
Tree/Open	0.93	0.06	1,40.1	1.5	0.224
Unb/Burn	0.83	0.08	1,40.1	4.8	0.034
C-oxidative:NAG					
Tree/Open†	1.12	0.18	1,40.0	0.2	0.645
Unb/Burn	0.41	0.09	1,40.0	30.3	<0.001

Notes: The mean and standard error (SE) are calculated across the site means and are a ratio. "Tree/Open" is calculated by first averaging the mean values under tree canopies in both burn treatments and dividing by the mean values away from tree canopies in both burn treatments. "Unb/Burn" is calculated by first averaging the mean values in unburned plots in both under and away from tree canopies and dividing by the mean values in the burned plots under and away from tree canopies. The statistical tests are from the mixed-effects models performed on values averaged within replicate plots using site as a random intercept. The site-by-fire and canopy interactions are given in Table 2. Individual site descriptive and test statistics are in Appendix S1: Tables S3–S7.

† In the case of a nonsignificant effect, we tested whether this was due to conflicting responses of site, and if so it is designated as site-specific.

Table 1). There were no overall effects of tree canopies on IN, and instead, the tree cover effect was site-specific (site × canopy interaction $F_{3,140} = 5.8$, $P < 0.001$; Fig. 4, Tables 1, 2). The higher IN concentrations in unburned plots on average were largely attributable to

TABLE 2. Statistical results from the interactions between site and fire and canopy from the mixed-effects models with site as a fixed effect interacting with fire and canopy, and replicate plot as a random effect.

Variable and comparison	df	<i>F</i>	<i>P</i>
%C			
S × Canopy	3,135.2	7.7	<0.001
S × Fire	3,17.8	1.4	0.274
%N			
S × Canopy	3,135.8	10.8	<0.001
S × Fire	3,18.5	3.3	0.043
C:N			
S × Canopy	3,135.2	0.9	0.438
S × Fire	3,15.0	1.4	0.279
NO ₃			
S × Canopy	3,135.9	5.6	0.001
S × Fire	3,20.9	2.8	0.065
NH ₄ ⁺			
S × Canopy	3,139.8	4.3	0.006
S × Fire	3,19.7	6.9	0.002
Tot In			
S × Canopy	3,140.0	5.8	<0.001
S × Fire	3,19.8	7.0	0.002
NAG			
S × Canopy	3,97.5	1.7	0.171
S × Fire	3,15.6	6.2	0.006
C-hyd			
S × Canopy	3,97.8	1.4	0.259
S × Fire	3,16.9	6.9	0.003
C-oxi			
S × Canopy	3,100.9	3.8	0.013
S × Fire	3,18.9	0.7	0.567
Hyd/Oxi			
S × Canopy	3,97.0	5.7	0.001
S × Fire	NC		
Hyd/NAG			
S × Canopy	3,96.4	1.2	0.299
S × Fire	3,15.7	2.1	0.143
Oxi/NAG			
S × Canopy	3,97.3	2.4	0.073
S × Fire	3,15.8	1.4	0.282

Notes: Only the ANOVA results from the interactions are displayed. NC indicates the model did not converge when the variable was included in the model.

higher NH₄⁺ concentrations (+190% ± 125%; $F_{1,41} = 17.2$, $P < 0.001$; Fig. 4, Table 1) as was the variability in tree canopy effects across sites (site × tree canopy interaction $F_{3,139.8} = 4.3$, $P = 0.006$; Fig. 4, Tables 1,2); there was no overall effect of fire or tree canopies on NO₃⁻ across sites ($P > 0.50$ for fire and tree canopies, Table 1). Consequently, fire effects on bulk soil N were consistent with changes in inorganic N, especially NH₄⁺, while tree canopy effects depended on site.

The activity of the enzyme involved in N acquisition (NAG) was 313% ± 185% higher in unburned plots than repeatedly burned plots ($F_{1,40} = 22.5$, $P < 0.001$; Fig. 4, Table 1) and 100% ± 31% higher under trees than in the

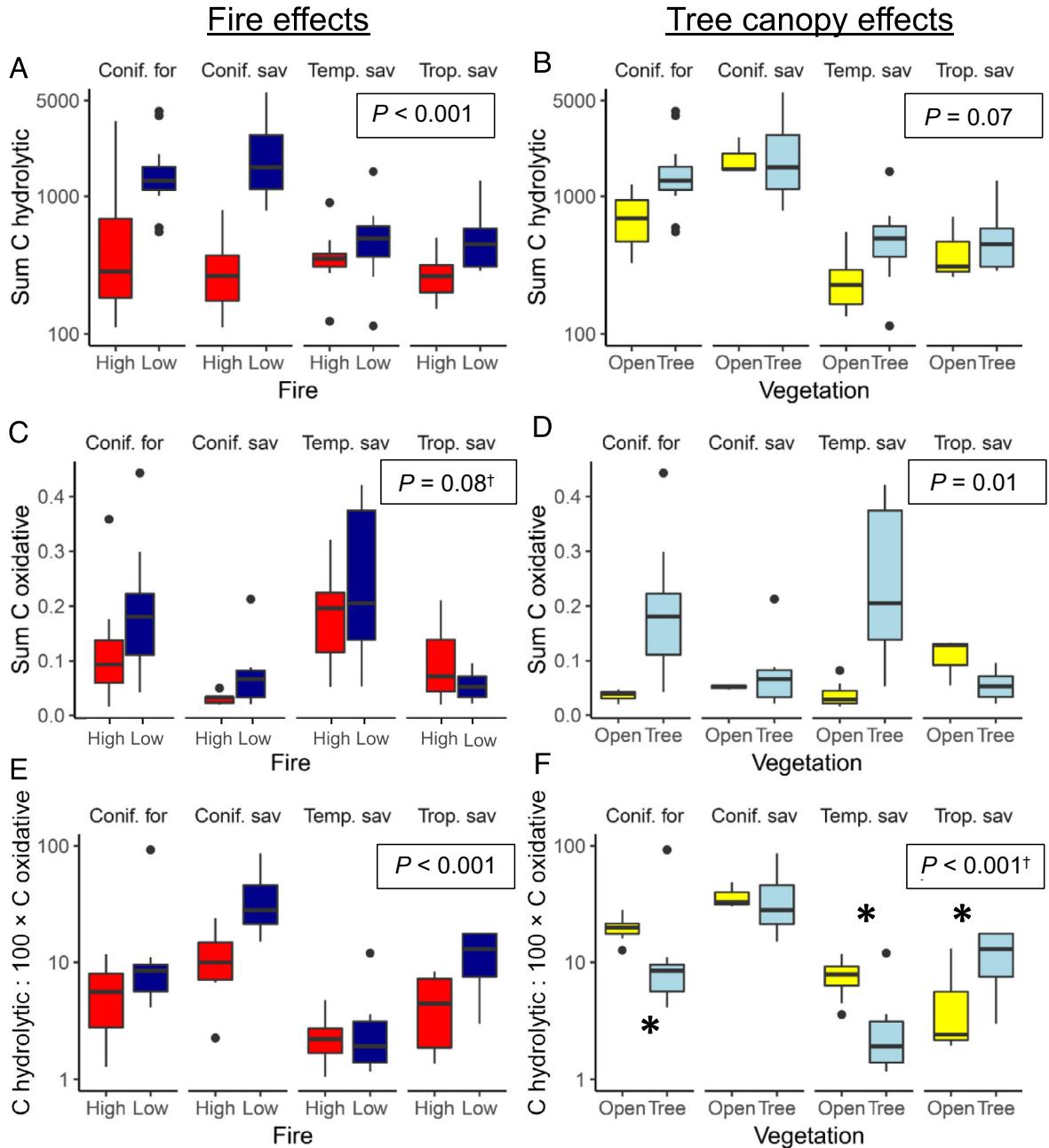


FIG. 3. Box plots of extracellular enzyme activity for (A, B) total carbon-acquisition hydrolytic and (C, D) oxidative enzymes, and (E, F) their ratio between fire treatments (fire effects) and tree canopies (tree canopy effects) for plot-averaged values across sites. The y-axis is on a log scale. Each box plot displays medians (50th percentile), 25th and 75th percentiles, and approximate 95% confidence intervals. Inserted boxes display P values from the replicate plot-averaged mixed-effects model, with full statistics in Table 1. A dagger † next to the P value indicates that the effect is contingent on site (see Appendix S1: Table S3 for the site-level statistics). Asterisks above boxes indicate significant differences ($P \leq 0.05$) between canopy positions. Colors indicate fire treatment on the left-hand panel (red, burned; dark blue, unburned) or canopy position (yellow, in the open; light blue, under a tree canopy).

open ($F_{1,40} = 5.8$, $P = 0.021$; Fig. 4, Table 1); suggesting areas burned repeatedly and away from tree canopies had lower potential turnover of chitin (a microbial N and C source), consistent with the negative effects of fire on inorganic N.

Bulk soil N was related to both inorganic N and enzyme activity across fire frequencies, canopies, and sites. In our model selection process, we considered the potential main effects of fire and canopy location as well as soil %C and C:N when evaluating the correlations

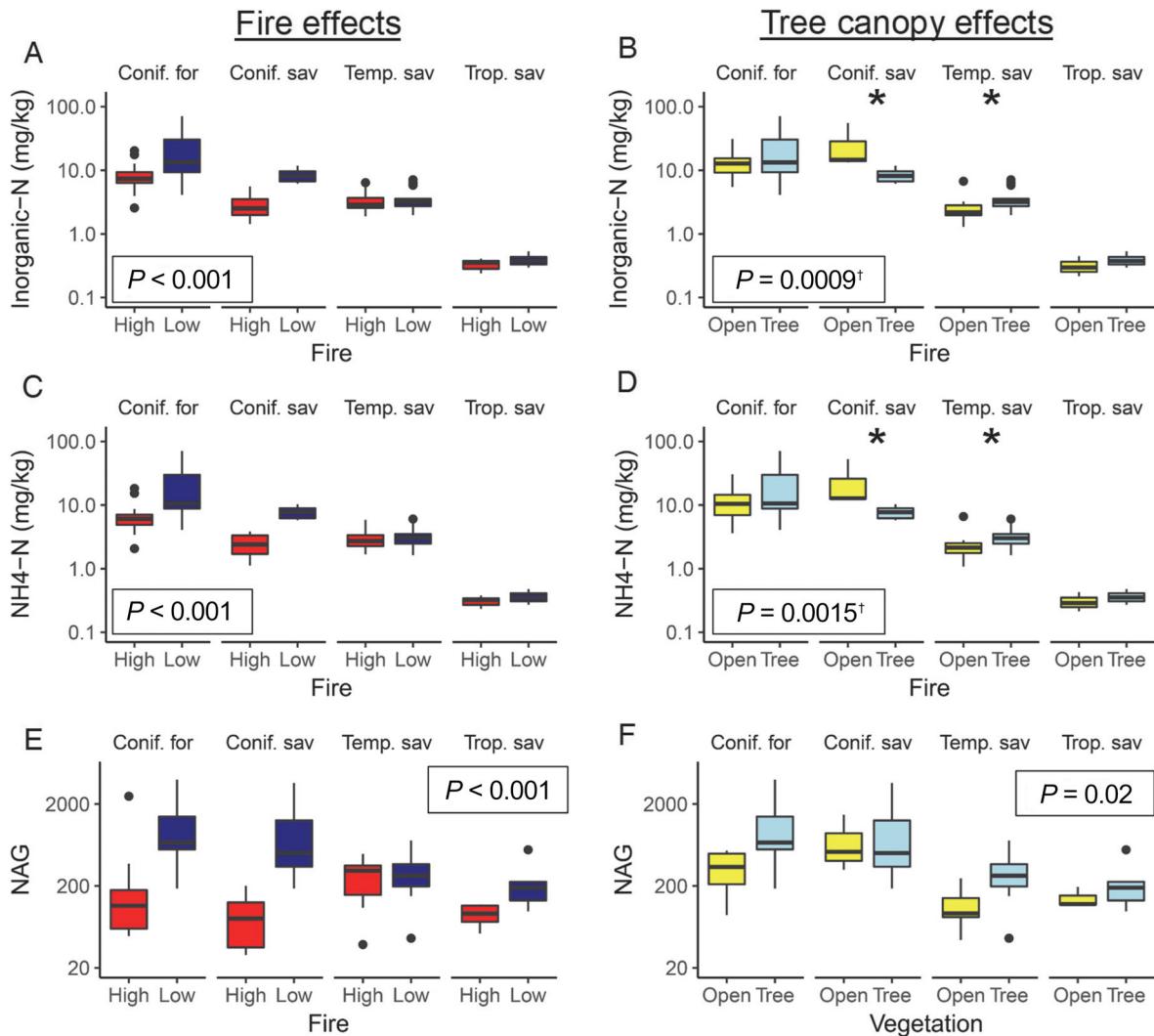


FIG. 4. Box plots of soil nitrogen availability and turnover both as (A, B) total inorganic N, (C, D) ammonium and (E, F) NAG (N-acetyl- β -D-glucosaminidase) enzyme activity for plot-averaged values across sites. The y-axis is on a log scale. Box plots display medians (50th percentile), 25th and 75th percentiles, and approximate 95% confidence intervals. Inserted boxes display P values from the replicate plot-averaged mixed-effects model, with full statistics in Table 1. A dagger † next to the P value indicates that the effect is contingent on site (see Appendix S1: Table S3 for the site-level statistics). Asterisks above boxes indicate significant differences ($P \leq 0.05$) between canopy positions. Colors indicate fire treatment on the left-hand panel (red, burned; dark blue, unburned) or canopy position (yellow, in the open; light blue, under a tree canopy).

between %N and inorganic N and enzyme activity. Bulk soil N was positively related to IN and NH_4^+ even when fire and canopy location were included in the model ($F_{1,90.5} = 770.3$, $P < 0.0001$ and $F_{1,130.5} = 66.1$, $P < 0.0001$, respectively; Fig. 5), but not to NO_3^- ($P > 0.50$, Fig. 5). NAG was also significantly correlated with total soil N ($F_{1,42} = 31.4$, $P < 0.001$; Fig. 6). Importantly, models considered the potential main effects of fire and canopy location as well as soil %C and C:N when evaluating the correlations between %N and inorganic N and enzyme activity. Consequently, fire-driven losses of soil N were associated with lower potential microbial acquisition of N and C and bioavailable IN.

Effects of C and N losses on potential organic matter decomposition activity

Total soil C and N were significantly positively correlated with potential hydrolytic C-acquisition enzyme activity for both the total activity and that of each individual enzyme (Fig. 6 and Appendix S1: Fig. S1, $F_{1,35.9} = 17.2$, $P < 0.001$). Because soil C and N were collinear predictors, we were unable to completely disentangle their relative influence on enzyme activities; however, using model selection, we evaluated which variable had the most explanatory power and whether both soil C and N should be included in the model,

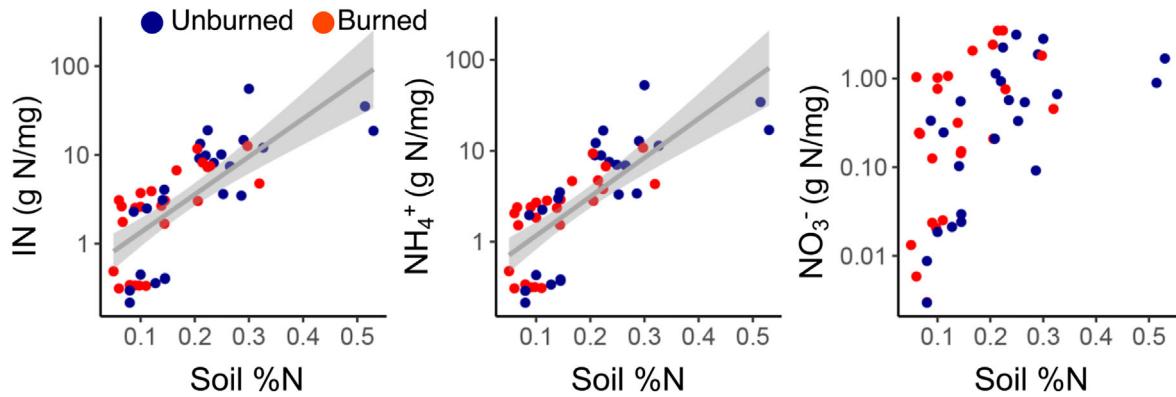


FIG. 5. Scatter plots and regressions between soil total nitrogen (N) and inorganic N (IN; total inorganic N, ammonium, and nitrate). The points are plot-averaged values across all sites and colored based on fire treatment (red, burned; dark blue, unburned). The y-axes are on a log scale. Bands indicate the standard error around the fitted model and only significant trends are displayed.

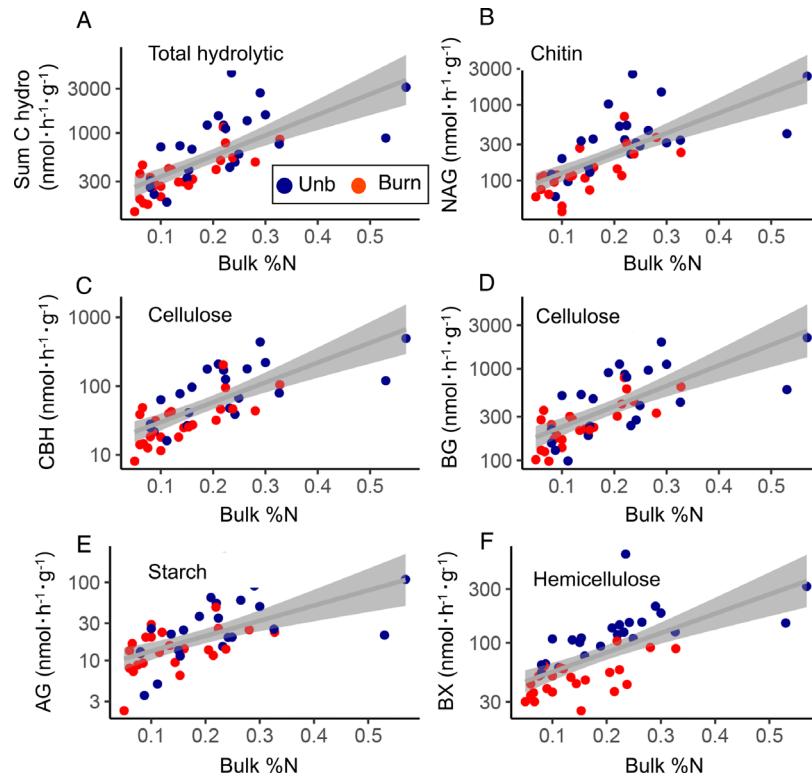


FIG. 6. Relationships between soil N and the activity of hydrolytic enzymes. (A) Sum of all hydrolytic enzymes except NAG (CBH + BG+AG + BX), (B) N-acquisition enzyme (NAG), and (C–F) individual C-acquisition enzymes that degrade cellulosic compounds (compounds given in upper left-hand corner). The points are plot-averaged values across all sites and colored based on fire treatment (red, burned; dark blue, unburned). The y-axes are on a logarithmic scale. For panels C–F: CBH $F_{1,80.5} = 34.6$, $P < 0.0001$; BG $F_{1,79.6} = 35.2$, $P < 0.0001$; AG $F_{1,103.9} = 52.6$, $P < 0.0001$; BX $F_{1,43.8} = 57.0$, $P < 0.0001$. Bands indicate the standard errors of the model fit. CBH: cellobiohydrolase, BG: β -glucosidase, AG: α -glucosidase, BX: β -xylosidase, NAG: N-acetyl- β -D-glucosaminidase.

which illustrated that soil N was the best predictor (but see *Extracellular enzyme activity is lower in burned plots and away from tree canopies* for the correlations with soil C, and Appendix S1: Fig. S1), albeit its inclusion in the top model incorporated its collinearity with soil C. In contrast, total oxidative activity did not

significantly correlate with total C or N (C $F_{1,15.7} = 5.5$, $P = 0.03$; N $F_{1,39.5} = 1.4$, $P = 0.25$). Consequently, losses of soil C and N were correlated with lower potential decomposition of cellulose, hemicellulose, and starch in burned plots and in areas away from tree canopies.

Fire decreased NAG to a greater extent than potential hydrolytic and oxidative C acquisition (analyses of the ratios $F_{1,40.1} = 4.8$, $P = 0.034$ and $F_{1,40.0} = 30.3$, $P < 0.001$, respectively; Table 1). Consequently, fire is potentially shifting the relative turnover of C and N, where fire protection increases N turnover to a greater extent than C turnover.

Coupled changes in hydrolytic enzymes and soil CO₂ flux

To test the validity of our inferences about lower enzyme activity indicating potential soil C losses via decomposition, we compared enzyme activity levels and in situ soil CO₂ flux measurements in the temperate broadleaf savanna at Cedar Creek ($n = 6$ plots measured over the growing season from 1999–2005). We found two lines of evidence that fire reduced soil CO₂ flux and that soil CO₂ flux was correlated with extracellular enzyme activities: (1) soil CO₂ flux rates were significantly lower in the burned plots than in unburned plots when controlling for soil temperature $F_{1,4} = 13.9$, $P = 0.02$; Fig. 7), and (2) total hydrolytic C-acquisition enzyme activities were positively correlated with soil CO₂ flux ($F_{1,4} = 7.9$, $P = 0.048$, $r^2 = 0.58$; Fig. 7). Like hydrolytic enzyme activity, soil CO₂ flux was significantly correlated to bulk soil %C ($r^2 = 0.72$ and $P = 0.0005$; Norris 2008). Oxidative enzymes were not correlated with soil CO₂ flux ($P > 0.5$). Consequently, independent measurements of soil C losses support our inference based on enzyme activity that fire reduces decomposition at this site.

Cross-site variability in tree biomass inputs determine soil C responses

We found support for the hypothesis that fire-driven changes in tree biomass inputs to soils were key drivers of soil C responses using $\delta^{13}\text{C}$. First, analyses across the

four sites sampled here illustrated $\delta^{13}\text{C}$ was higher in burned plots and away from tree canopies ($F_{1,41} = 16.6$, $P < 0.001$ and $F_{1,41} = 3.4$, $P = 0.072$, respectively; Fig. 8; with no overall interaction, $P > 0.5$). In the savanna sites, which contained C₄ grasses and C₃ trees (Breaks, Cedar Creek, and Kruger), fire reduced the contribution of tree biomass to soils, with $\delta^{13}\text{C}$ being 2.3–8.3‰ higher in burned relative to unburned plots (Appendix S1: Tables S2 and S4). In these savanna sites, greater inputs from C₃ plants, which are primarily trees in these ecosystems, tended to be associated with higher soil C content (temperate broadleaf savanna $F_{1,51.2} = 40.0$, $P < 0.0001$; temperate conifer savanna $F_{1,13.9} = 6.8$, $P = 0.021$; tropical savanna $F_{1,28} = 5.3$, $P = 0.029$).

When three additional savanna sites were incorporated, we found a significant positive correlation between the absolute change in percent soil total C (difference between burned and unburned) and the absolute change in $\delta^{13}\text{C}$ ($r^2 = 0.91$, Fig. 9) across all six savanna sites. Only savanna sites were analyzed here because the coniferous forest did not contain a C₄ grassy layer allowing us to determine changes in tree inputs.

DISCUSSION

Our results demonstrate that repeated burning reduced total C and N in soils due to reduced plant biomass inputs and through decreased tree abundance (Fig. 2). The correlation among fire treatments across six savanna sites suggests the impact of fire on soils is due to losses of woody plant biomass inputs (Fig. 9). In addition to changes in total C and N, both fire and the presence of trees modified potential decomposition of organic matter but in complex ways: potential decomposition of cellulose, hemicellulose, and starch compounds was reduced by repeated burning and the absence of tree

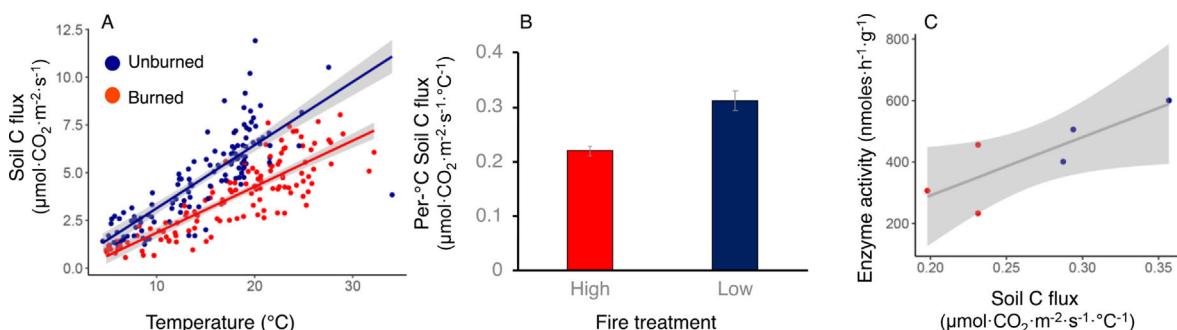


FIG. 7. Soil respiration across the fire treatment plots and its relationship with enzyme activity for the temperate broadleaf savanna in Cedar Creek. (A) Regression between soil C flux and soil temperature for all sampling points averaged within a plot for that time (51 sampling times from May 1999–September 2005). (B) Mean soil C flux (measured as $\mu\text{mol}\cdot\text{CO}_2\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) rescaled to per °C across plots within treatments (error bars indicate standard errors around the mean). (C) Regression between soil respiration (averaged within plots across all sampling times and rescaled to mean temperature during the measurement) and extracellular enzyme activity averaged within plots in the temperate broadleaf savanna at Cedar Creek. Lines indicate significant linear regression with shaded bands illustrating the standard error of the fit. Colors indicated fire treatment groups with red, burned and dark blue, unburned.

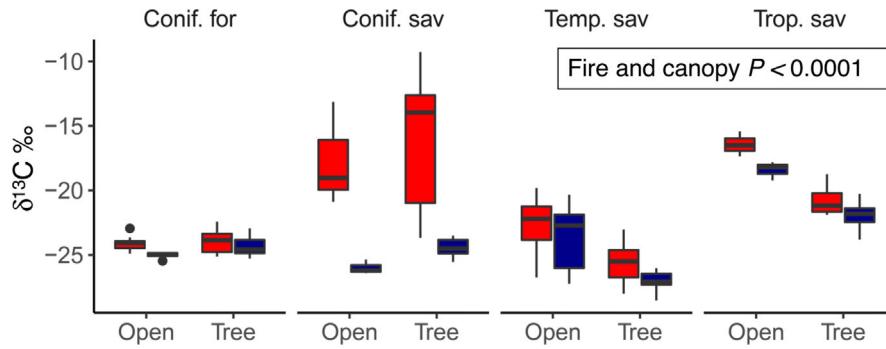


FIG. 8. Responses of $\delta^{13}\text{C}$ to fire and tree canopy. Box plots of $\delta^{13}\text{C}$ across fire treatments within different canopy position (x-axis panels), with the P values indicating significant individual effects of both fire and canopy from the full model but no significant interaction ($P > 0.5$). Box plots display medians (50th percentile), 25th and 75th percentiles, and approximate 95% confidence intervals. Colors indicated fire treatment groups with red, burned and dark blue, unburned.

canopies, while potential decomposition of aromatic compounds was only reduced by losses of tree cover and not fire (Fig. 3). Burning and tree canopies also affected the N cycle, with areas burned and away from tree canopies tending to have less inorganic N and lower potential NAG activity, which is one way microbes can acquire N (Fig. 4). Furthermore, losses of soil C and N were correlated with lower C and N turnover but only for hydrolytic enzymes, and not oxidative enzymes that target aromatic compounds (Fig. 6).

Role of changes in tree cover and its variability across sites

Local effects of trees on soil properties under the canopy are a well-documented phenomenon in ecosystems and have been linked to both changes in microclimate and plant traits (Belsky et al. 1989, Knops et al. 2002). While not all variables were significantly different under vs. away from tree canopies, in all the significant cases, elemental concentrations and enzyme activities tended to be higher under trees. We found evidence for several nuances, however, such as tree cover having a greater effect on oxidative enzyme activity than fire

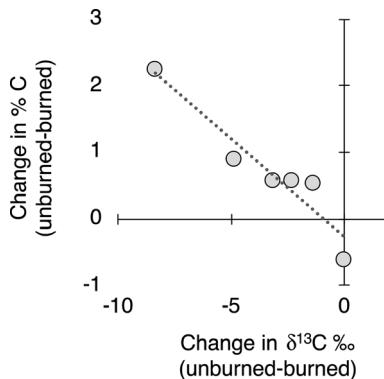


FIG. 9. Scatter plot of the differences between burned and unburned plots for $\delta^{13}\text{C}$ and bulk soil %C for six savanna sites ($r^2 = 0.91$).

frequency. Moreover, our results illustrated significant interactions between fire and tree cover effects on oxidative enzyme activity, demonstrating that the local effects of trees can depend on fire treatment. In all the cases of significant interactions, tree canopy effects were greatest in the unburned plots (Appendix S1: Fig. S3), supporting the hypothesis that fire exclusion can increase the localized impact of tree canopies (e.g., Pellegrini et al. 2015).

The significant effects of tree canopies on soils in unburned plots, which tended to have higher percent tree cover (e.g., in Cedar Creek tree cover was 81% in unburned plots and 40% in burned plots; Pellegrini et al. 2020), suggest that increasing with fire exclusion tree cover does not diminish the local effects of canopies on soils in these ecosystems. It is possible that the local effects of trees on soils decline as tree cover with fire exclusion increases in fire exclusion plots; yet in the coniferous forest, we still found effects of tree canopies on extracellular enzyme activity, suggesting persistent localized enrichment effects. Canopy \times fire interactions may potentially arise because tree canopies could modify biomass combustion during fire such as by creating a moister microclimate, thereby reducing losses, a potential effect we were unable to evaluate in our analyses. However, in the savannas, we hypothesize the local effect of tree canopies on fire behavior was relatively limited given that trees tended to be isolated, have continuous grassy layers for fuel underneath their canopies, and experience hot enough fires to be topkilled (Hoffmann et al. 2009). Alternatively, tree cover effects may be greatest in unburned plots where the lack of fire allows for larger inputs of tree litter to soils.

Further work is needed to understand the extent that trees impact soils away from their canopies. At Kruger, where the majority of herbaceous biomass is C_4 grass, soil $\delta^{13}\text{C}$ away from canopies is much closer to the value for C_4 grass than soil under canopies, but remains more depleted than expected if C was from C_4 biomass alone (February and Higgins 2010). This pattern suggests C_3 biomass inputs contribute to soil organic matter away

from tree canopies. Furthermore, at Cedar Creek, soil $\delta^{13}\text{C}$ was significantly higher away from canopies than under canopies (Fig. 8), but remained relatively low (-23.1‰), providing evidence of C_3 biomass inputs away from canopies. Both of these trends may be due to herbaceous C_3 species (Peterson et al. 2007), which we are unable to evaluate in our current analysis.

The importance of changes in trees was also apparent at the landscape scale. Across all six savanna sites, the variability in soil C losses in response to fire was significantly related to changes in soil $\delta^{13}\text{C}$, which we used to infer tree biomass contributions to soil C. To our knowledge, this is the first cross-site evaluation of the degree to which fire-driven changes in soil C depends on changes in tree biomass inputs. Previous studies have focused on spatial gradients in woody cover rather than changes within a site (Jackson et al. 2002, Lloyd et al. 2008). However, a useful future expansion should consider deeper soil depths because deep-rooted grasses can influence soil C (Fisher et al. 1994, Jackson et al. 2002, Schenk and Jackson 2002).

Persistent effects of repeated burning on decomposition

Losses of soil organic matter primarily occur through microbial respiration, leaching, or erosion. Although intense fires can combust organic matter in the upper soil horizons (Wardle et al. 2003), these direct combustive losses occur mostly from the organic horizon, with little effect on mineral soils except in intense wildfires (Wan et al. 2001, Nave et al. 2011). In fact, studies propose that single fires can lead to higher soil C because of transformations (e.g., formation of pyrogenic C) that stabilize C, transport of detritus to the mineral horizon and sorption to minerals, and a resurgence of plant productivity (Neary et al. 1999, González-Pérez et al. 2004, Certini 2005). Given the role of microbial decomposition in regulating the long-term storage of soil organic matter in ecosystems, the uncertainty in how repeated burning alters decomposition is a key knowledge gap. After a single fire, vertical transport of pyromineralized organic matter and ash can produce transient increases in dissolved organic C and microbial activity (Choromanska and DeLuca 2002, Wang et al. 2012). We propose, however, that repeated burning can result in a net-decline in organic matter decomposition by microbes (Figs. 3–4), which may reduce potential soil C fluxes (Fig. 7). Other enzymes besides the ones measured here are important for microbial N acquisition and N turnover (e.g., urease) and may respond differently, but without N manipulation we are unable to infer causality. The general decline in microbial activity in the repeatedly burned plots is consistent with studies proposing a single fire reduces microbial activity for several years and even decades via heat-induced mortality (Dooley and Treseder 2012, Metcalfe et al. 2018). Consequently, repeated burning is proposed to lead to compounding declines in microbial activity over decadal timescales, consistent with our results.

Fire has long been hypothesized to trigger a transient increase in inorganic N and cycling which then diminishes over time (Blair 1997). For example, transient increases in N cycling are largely supported by syntheses demonstrating a peak in decomposition and mineralization immediately following fire, which then decline below pre-fire levels before recovering after 1–2 year (Wan et al. 2001, Wang et al. 2012). The reduction in soil enzyme activity and inorganic N content in burned sites (Figs. 3–4) is unlikely to be a transient response to the most recent fire given that (1) the last fire occurred more than three years ago in two sites that exhibited large declines in extracellular enzyme activities (the coniferous forest in Sequoia and savanna in the Breaks) and (2) in the sites where fire occurred less than two years ago (temperate and tropical broadleaved savannas in Cedar Creek and Kruger, respectively) the burned plots had significantly lower extracellular enzyme activities, albeit the exact enzyme that responded differed. The lower enzyme activities outside of tree canopies also suggest that losses of tree cover, which generally occur over decadal timescales (Higgins et al. 2000, Moreira 2000), lead to legacy effects on decomposition activity even when fire is excluded because it can take several decades for tree populations to potentially recover.

Our results suggest that over decadal alterations of fire frequency, decomposition is likely regulated by factors such as the availability of organic matter and N, which were strongly and positively related to enzyme activity (Fig. 6, Appendix S1: Fig. S1). Changes in total soil organic matter that occur over decadal alterations in fire frequencies (Pellegrini et al. 2018) are likely contributing to the significant changes in enzymes that persist for several years postfire. Through comparisons across a range of sites that have different responses of woody biomass and underlying environmental conditions, we demonstrate that the reduction in extracellular enzyme activities is one of the more robust responses to repeated burning. Consequently, the potential for fire to result in elevated microbial activity and thus produce a transient maximum is likely reduced by high fire frequencies. The declines observed here may partly be amplified because we sampled within a few years following fire, but in the savanna systems where fires recur every 1–3 year, our sampling scheme is most representative of the net effects of fire on soil C and N availability and enzyme activity. Understanding how the differences in enzyme activity between burned and unburned plots change with time since fire would be a useful future research avenue.

Potential changes in organic matter decomposition

In all sites, fire reduced potential extracellular enzyme activity (Fig. 3), which could result in several changes in the turnover of soil organic matter. First, when hydrolytic enzymes that process cellulose, hemicellulose, and starch were analyzed individually, fire and/or the absence of trees reduced the activity of at least one

enzyme in all four sites. Second, fire never increased the activity of groups of enzymes (total hydrolytic, total oxidative, and NAG, Figs. 3 and 4), contrasting with previous studies (Boerner et al. 2000, 2006, Rietl and Jackson 2012). Third, soil CO₂ fluxes in the temperate broadleaf savanna at Cedar Creek declined with fire and followed similar trends to hydrolytic enzyme activity across the replicate plots (Fig. 7). At Cedar Creek, the soil CO₂ flux data cannot distinguish between heterotrophic and autotrophic respiration, such as from plant roots. However, fine root biomass, which can be a source of autotrophic respiration, was actually significantly higher in the repeatedly burned plots (+65% in the top 20 cm; Pellegrini et al. 2020), opposite to the direction of soil CO₂ flux. The decline in soil CO₂ flux rates is consistent with other studies on repeated burning (e.g., Metcalfe et al. 2018). Consequently, our results support previous studies that have found enzyme activity and decomposition is reduced by repeated burning (Eivazi and Bayan 1996, Ajwa et al. 1999, Boerner et al. 2005).

Hydrolytic enzyme activity was tightly correlated with soil C, potentially because soil C can be tightly related to microbial biomass (McLauchlan and Hobbie 2004). Nonetheless, fire and losses of tree cover reduced enzyme activity even when soil %C was included in the statistical model. More work is required to evaluate how the suppression of soil C losses via reduced decomposition may compensate for lower plant biomass inputs to affect total soil C pools. Furthermore, exploring the variety of processes that lead to soil C stability such as the formation of pyrogenic C (González-Pérez et al. 2004, Certini 2005) would also better identify mechanisms leading to changes in decomposition.

Finally, fire-driven losses of N may be one factor constraining C turnover (Fig. 6), as an alternative to lower biomass inputs leading to lower soil C, lower microbial biomass, and lower enzyme activity (Dooley and Treseder 2012, Pressler et al. 2018). We found that including %N in the model resulted in significant increases in the fit of the model (determined via an AIC threshold of two). Moreover, we observed (1) a significant negative effect of fire on soil N overall (Fig. 2), and in three of the four sites when analyzed individually (either through direct combustion effects or through fire-driven losses of tree cover, Appendix S1: Tables S3-S4), and (2) a fire-driven decline in the activity of N-acquisition enzymes in all the sites (Fig. 4) even when soil %C was incorporated into the model. Moreover, fire likely amplifies low N availability by reducing NAG activity and increasing the C_{hydro}:NAG ratio either due to lower biomass inputs or lower tree abundance (Fig. 7). Inferences based on NAG alone are limited, however, because NAG is also used by microbes to acquire C and there are other extracellular enzymes utilized by microbes to acquire N that may display different responses. However, our data support the hypothesis that repeated

burning can increase N limitation in ecosystems (Reich et al. 2001, Pellegrini 2016).

The exact mechanism leading to lower extracellular enzyme activity is difficult to determine. Changes in enzyme activity may partly be attributable to shifts in microbial demand for substrates (e.g., lower N availability would result in higher potential NAG activity, or higher C availability would result in lower potential CBH, BG, BX, and AG activities). Previous studies have shown that dissolved C and N can increase after fire (Wang et al. 2012), perhaps due to heat-induced mineralization of organic matter, which could lead to lower enzyme activity because of greater C and N availability. We were unable to directly test the role of supply compared with demand because we did not perform a substrate-addition experiment. However, the correlation data do not support the hypothesis that changes are due to increased supply: for example, assuming that microbial biomass scales with soil organic matter, we can test whether or not soil N correlates with N-acquisition activity, scaled relative to organic matter. When doing so, we found that NAG:C did not decline with either total soil N or dissolved inorganic N ($P = 0.056$ and $P = 0.35$, respectively). Declines in enzyme activity are more likely attributable to losses of microbial biomass, which has been observed at the temperate savanna in Cedar Creek (Norris 2008). However, the lack of change in oxidative enzymes may indicate a shift in the reliance of microbial communities on aromatic organic matter.

CONCLUSIONS

By sampling several sites across different ecosystems, we provide broad evidence that decadal changes in repeated burning shift soil C and N storage and potential turnover through several processes. Our results support the hypothesis that the reduction in aboveground inputs, especially of woody plants, is a key factor regulating fire's long-term effect on soils across six savanna sites. Fire also reduced decomposition potential by suppressing the activity of hydrolytic enzymes that break down organic matter, potentially reducing soil C losses. Yet the response of the different enzymes revealed a nuanced response of the C cycle, with fire shifting the potential turnover of cellulose-, hemicellulose-, and starch-based compounds but not the turnover of aromatics such as polyphenols and lignin, potentially allowing for persistent decomposition of aromatic compounds produced by burning. However, soil C accumulation under trees and in unburned plots, likely through greater biomass inputs, is not completely offset by the higher potential decomposition activity, illustrating the complex interactions between above- and belowground processes in determining the net changes of soil C and N.

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SUPPORTING INFORMATION

Additional supporting information may be found online at: <http://onlinelibrary.wiley.com/doi/10.1002/ecm.1409/full>

DATA AVAILABILITY

Data are available in the Dryad Digital Repository: <https://doi.org/10.5061/dryad.xwdbvr19n>