

Running Head: Ecosystem responses to repeated burning

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

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Abstract

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

24 shaping soil carbon responses across sites, but the magnitude of tree effects differed and
25 depended on how tree biomass inputs into soil responded to fire.

26

27 Keywords: carbon cycling, coniferous forest, extracellular enzymes, fire frequency, nitrogen
28 cycling, plant communities, repeated burning, soil, savanna

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30

31

32 **Introduction**

33 Fires burn ~570 million hectares of land globally each year, affecting carbon (C) and
34 nutrient storage and cycling and the species composition of ecosystems (Bond-Lamberty et al.
35 2007, Bowman et al. 2009, van der Werf et al. 2017, Pellegrini et al. 2018). Many ecosystems
36 burn regularly, with mean fire-return intervals ranging from two years in tropical savannas to
37 several hundred years in boreal forests (Archibald et al. 2013, Andela et al. 2017). However, fire
38 frequency, size, and severity is increasing due to climate change and decreasing due to land-use
39 change (Westerling et al. 2006, Miller et al. 2009, Dennison et al. 2014, Andela et al. 2017).
40 Increasing fire frequencies can shift ecosystem C and nutrient cycles in soils by reducing inputs
41 of organic matter to soils through repeated combustion of plant and litter biomass and associated
42 volatilization of C and nutrients in plant biomass before they are decomposed into soils, as well
43 as by combusting soil organic matter during intense wildfires (e.g., (Ojima et al. 1994, Kauffman
44 et al. 1995, Baird et al. 1999, Pellegrini et al. 2014, Muqaddas et al. 2015)). In addition to
45 altering detrital inputs to soils, fire can shift decomposition rates by increasing or decreasing
46 microbial activity and nutrient availability (Kaye and Hart 1998, Wang et al. 2012, Dove et al.
47 2020). However, within ecosystems, it is unclear how changes in inputs are coupled with shifts
48 in decomposition.

49 Recent work has demonstrated that repeated burning depletes soil C and N in most
50 ecosystems, although there are exceptions (Pellegrini et al. 2018). Soil C and N stocks are partly
51 regulated by fire-driven losses of plant biomass inputs, with larger losses of plant biomass
52 expected to lead to larger reductions in soil C and N (Pellegrini et al. 2014, Kowaljew et al.

53 2018). For example, frequent burning can reduce tree biomass in savannas (Moreira 2000,
54 Higgins et al. 2007, Burrows et al. 2010), which in general experience large concomitant soil C
55 and N losses (Pellegrini et al. 2018). In other systems, such as coniferous forests that experience
56 ground fires, tree mortality varies depending on tree species composition (Haase and Sackett
57 1998, Schwilk et al. 2009), and in many cases these systems tend to lose less C and N (Pellegrini
58 et al. 2018). In certain coniferous forests that experience severe stand-replacing fires, fire can
59 cause large immediate losses of soil organic matter from the organic and mineral horizons
60 (Walker et al. 2019, Dove et al. 2020). Consequently, variability in fire effects on plant biomass
61 inputs to soil, especially from trees, may partly explain long-term differences in soil responses.

62 In addition to shifting inputs, fire can change the decomposition of soil organic matter,
63 but the direction of change is not straightforward. Fire may accelerate soil C and N turnover by
64 stimulating decomposition activity, potentially through pulsed availability of organic matter
65 inputs after fire (Boerner et al. 2006, Rietl and Jackson 2012) or by promoting more favorable
66 microclimate conditions (e.g., warmer conditions (Kaye and Hart 1998)). Alternatively, fire may
67 reduce decomposition by lowering microbial biomass through heat-induced mortality, formation
68 of more recalcitrant aromatics (Certini 2005, Knicker 2007), pyrogenic C (Santin et al. 2016),
69 and changing microbial community composition (Boerner et al. 2005, Waldrop and Harden
70 2008), especially in high severity fires (Dove et al. 2020).

71 Decomposition changes are nuanced, however, because enzymes associated with the
72 catabolism of different molecular forms of organic matter can vary in their sensitivity to fire,
73 which may contribute to discrepancies among reported fire effects on decomposition (Certini
74 2005, Knicker 2007). For example, repeated burning can reduce the potential decomposition of
75 cellulosic compounds relative to unburned controls due to a reduction in hydrolytic enzyme
76 activity (Eivazi and Bayan 1996, Ajwa et al. 1999, Boerner et al. 2005). However, changes in
77 hydrolytic enzymes may be relatively unimportant in regulating soil organic matter losses because
78 fire can enrich organic matter in aromatic and pyrogenic compounds (Certini 2005, Knicker
79 2007), whose decomposition requires an upregulation of oxidative enzymes. Prolonged effects of
80 fire on enzyme activity in soil may also arise because repeated burning can reduce organic matter
81 and N availability within the soil profile and shift tree cover, both of which could either reduce
82 or accelerate decomposition (Köster et al. 2016). These substrate and vegetation driven changes
83 can further be exaggerated by increases of soil-inhabiting microbial communities that positively

84 respond to repeated fires (Oliver et al. 2015), which may possess distinct decomposition
85 capabilities.

86 Aside from shifting organic matter inputs to soils related to changes in total plant biomass
87 in a landscape, fire may affect soils through changes in tree cover and species composition and
88 localized (i.e., spatially restricted to under the canopy) effects of trees on soils, such as higher
89 soil C and N content and N mineralization under canopies (Belsky et al. 1989, Ludwig et al.
90 2004, Dijkstra et al. 2006). Reduced tree cover and changes in species composition due to
91 repeated burning (Higgins et al. 2007, Peterson et al. 2007, Holdo et al. 2009) can thereby
92 change soil properties at the landscape scale through aggregated changes in local effects (e.g.,
93 (Coetsee et al. 2010, Holdo et al. 2012)). Tree canopies have been shown to increase
94 decomposition (e.g., mass-based losses (Norris et al. 2013)), but how they change extracellular
95 enzyme activities, and whether tree canopy effects on enzymes change with fire is unclear.

96 In this study, we tested how fire-driven changes in plant inputs, changes in localized
97 effects of trees on soils, and their interaction altered the storage and decomposition of soil C and
98 N. We first focus our analysis on four sites that we intensively sampled and then perform a
99 broader analysis using three additional sites to test the generality of the potential role of inputs.
100 For the first analysis, we focus on four distinct woody ecosystem types to evaluate the generality
101 of fire and tree cover effects by testing (i) whether fire and tree canopies change soil total C and
102 N, (ii) how extracellular enzymes involved in decomposition respond to fire and tree canopies,
103 and (iii) how C and N losses are correlated with changes in inorganic N and extracellular enzyme
104 activities. We hypothesized that repeated burning would reduce soil C and N stocks by reducing
105 plant biomass inputs through volatilization and declines in the localized effects of trees. We
106 expected that soil organic matter decomposition rates would also decline with fire because of a
107 reduction in microbial biomass and activity, and that there would be a shift to a higher relative
108 investment in N acquisition enzyme activity to compensate for lower soil N.

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

115

116 Methods

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

121 We sampled sites in seven locations (four were studied in greater detail, which we refer
122 to as our main sites) spanning a range of forests and savannas that experience frequent (0.1-1
123 fires per-year) and in most cases prescribed burning: temperate coniferous savanna, tropical
124 broadleaf savanna, temperate broadleaf savanna, and temperate coniferous forest (Figure 1, also
125 see this figure for climate data). In four of the sites, we analyzed bulk soil C, N, $\delta^{13}\text{C}$, and
126 extracellular enzyme activity, whereas in three additional sites from temperate and tropical
127 savannas (South Africa, Brazil, and the United States) we analyzed bulk soil C and $\delta^{13}\text{C}$ to
128 expand our test of whether and how changes in inputs explain changes in soil C. In the four sites
129 for which we performed complete analyses, we sampled three replicate plots of each fire
130 treatment (plots where fire was excluded and those burned repeatedly that ranged in size from
131 0.1-10 ha). In all four of our main sites except the temperate coniferous savanna, the replicates
132 were separated by fire breaks. Within each plot, we sampled both directly underneath individual
133 tree canopies and away from canopies to test the impact of changes in woody plant abundance
134 and canopy cover (which increases under fire exclusion and decreases with more frequent
135 burning). Where more than one tree species was locally abundant, we replicated our sampling
136 across different tree species to test for interspecific variability in tree cover effects on soils, as
137 described below. We sampled three to nine individuals for each tree species within each fire
138 treatment depending on the abundance of each species.

139

140 Main site descriptions

141 The tropical broadleaf savanna site was located in Kruger National Park, South Africa, in
142 the 'Experimental Burn Plots,' which have received different fire frequency treatments since the
143 mid-1950s (1956 at the particular site we sampled in 2017). The large-scale plots are ~5-7 ha in
144 size and are replicated across the park in blocked designs (i.e., the different fire manipulations all
145 occur adjacent to one another in a 'block' which is replicated across the park (Biggs et al. 2003).

146 We sampled plots located in the Skukuza area (latitude: -25.10, longitude: 31.45). The soils are
147 sandy, well drained, and derived from granitic parent material, with duplex and sodic soils
148 covering large areas (Venter and Govender 2012). In March of 2017, we sampled sites on plots
149 burned annually at the end of the dry season in August, when fires are most intense; the annual
150 fire frequency represents a higher-than-historical frequency of once every three years (Govender
151 et al. 2006). We compared the annually burned plots to plots unburned since the onset of the
152 experiment.

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

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

168 The tree communities in Cedar Creek are dominated by broadleaf species including bur
169 oak and northern pin oak (*Quercus macrocarpa* and *Q. ellipsoidalis*, respectively). The plots we
170 sampled in August of 2017 spanned a fire-driven ecotone between open savanna, with
171 continuous grass cover in the high frequency plots, to closed-canopy forest without a continuous
172 grassy layer under trees in the unburned plots (Peterson and Reich 2001). The grassy community
173 in savannas at Cedar Creek is a mix of C_3 (29% of ground cover) and C_4 (38% of ground cover)
174 species (surveys from 1985-2015, Pellegrini et al. *in press*). We sampled under *Q. macrocarpa*
175 and *Q. ellipsoidalis* and in grassy areas outside of tree canopies. In the unburned plots where

176 forests have formed, we sampled in areas not yet encroached by forest to obtain the samples
177 outside of tree canopies.

178 The temperate coniferous savanna was in the Missouri Breaks region in central Montana,
179 USA. These plots do not receive direct fire manipulations, so we used variability in wildfire
180 history to examine different fire regimes. Using remote-sensing records dating from 1984
181 [Monitoring Trends in Burn Severity, MTBS, product, which has been used to establish trends in
182 wildfire, (Dennison et al. 2014)] we identified sites that have similar underlying geologies and
183 climates (latitude: 47.43, longitude: -108.17) but different fire histories (from 1984-2017). We
184 identified a large location that had burned repeatedly (each decade since 1984 with the last fire in
185 2012) by overlapping individual fire events from the MTBS product. In the unburned plots, we
186 confirmed they had not burned since at least 1984, but do not know the date of the last fire prior
187 to that time. Areas in the Missouri Breaks typically burn in the late summer. The landscape
188 contains bare areas, rock outcrops, and shrublands, which we avoided in our sampling. Soil
189 properties can vary substantially but in our plots soils contained low clay and silt (6% and 3%,
190 respectively) but high sand (81%) content.

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

202 The temperate coniferous forest site was in Sequoia and Kings Canyon National Parks in
203 California, USA. We sampled areas in a network of permanent monitoring plots 0.1 ha in size
204 that were established in the 1980s and were exposed to different historical fire regimes (e.g.,
205 (Schwilk and Caprio 2011)). We sampled three replicate plots within two fire treatments where
206 fire has been excluded for >150 years (Caprio and Swetnam 1995, Swetnam et al. 2009) and

207 where prescribed burns have been conducted every 10-15 years starting in 1983-1986 (plots
208 located in Giant Forest, latitude: 36.60, longitude: -118.73).

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

219 Plots sampled in Sequoia experience a Mediterranean climate, with the sites sampled at
220 ~2100 m elevation receiving on average 1092 mm in precipitation (1920-2017 at Giant Forest
221 USACE station). Most precipitation occurs during the winter (~50% as snow) with dry summers
222 and a distinct water deficit (Stephenson 1988). Average annual air temperatures range from 1.9-
223 13.6° C in Grant Grove at a similar elevation to Giant Forest (van Mantgem et al. 2016). Soils in
224 our sites contain a low sand (9%) content, a medium silt (29%) content, and a high clay (62%)
225 content. These sites were sampled in September 2017.

226 227 *Soil sampling*

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

237 At each sampling location within a site (e.g., under an individual tree), we aggregated soil
238 cores from the top 5 cm of the mineral horizon sampled in five separate 125 cm³ cubes
239 (5x5x5cm for each sample, total volume of 625 cm³ per-sampling location). We identified the
240 mineral horizon by first digging a hole >20 cm in depth and removing the organic horizon from
241 the profile, when present, and sampling the top 0-5 cm of the mineral horizon. We focused on
242 changes in the mineral horizon because of the lack of distinct organic horizons in the savanna
243 sites. We focused on the upper soil horizons because they are potentially most responsive to fire
244 and are most biologically active.

245 Soils were passed through a 2-mm sieve to remove coarse particles and divided into three
246 sub-samples: (i) fresh soil for soil moisture and inorganic N analyses, (ii) frozen soil for enzyme
247 analyses (see below), and (iii) dried soil for total soil C, N, and $\delta^{13}\text{C}$ analyses. Gravimetric soil
248 moisture was determined by drying soils at 105° C until they reached a constant weight (24-48
249 hours). We used these values of moisture content to adjust all analytical chemistry concentrations
250 to dry mass of soil.

251
252 *Analyses of soil C, N, and $\delta^{13}\text{C}$*

253 Total soil C, N, and $\delta^{13}\text{C}$ were measured by combusting samples using an Elemental
254 Analyzer at Stanford University and a Costech coupled Elemental Analyzer and Mass
255 Spectrophotometer at University of Maryland. Mass combusted per-sample was optimized to
256 maximize analytical accuracy of measurements. Duplicates were run for 10% of all samples to
257 ensure analytical precision which we defined to be an error of less than 5%.

258 We measured inorganic N (IN) on soils within 48 hours of collection. Inorganic N was
259 measured by extracting ~5 g of the freshly sieved homogenized soil via shaking in 50 ml of 1M
260 KCl. Following shaking, the samples were centrifuged and filtered through Grade 41 ashless
261 Whatman filters. Inorganic N was analyzed on an automated spectrophotometer WestCo
262 SmartChem 200 discrete analyzer at Stanford University. Nitrate was analyzed using a cadmium
263 column reduction, followed by a diazotization with sulfanilamide coupled with N-(1-
264 naphthyl)ethylenediamine dihydrochloride, which was analyzed colorimetrically at 550 nm.
265 Ammonium was analyzed using the indophenol blue method, where it reacts with salicylate and
266 hypochlorite in the presence of sodium nitroferricyanide to form the salicylic acid analog of

267 indophenol blue in a buffered alkaline solution (pH 12.8-13), which was analyzed
268 colorimetrically at 660 nm.

269

270 *Enzyme activity*

271 To assess potential C and N decomposition, we measured hydrolytic and oxidative
272 extracellular enzyme activity in a subset of samples at each site. The hydrolytic enzymes were:
273 cellobiohydrolase (EC 3.2.1.91 - CBH, degrades cellulose), β -glucosidase (EC 3.2.1.21 - BG,
274 degrades cellulose), α -glucosidase (EC 3.2.1.20 - AG, degrades starch), β -xylosidase (EC
275 3.2.1.37 - BX, degrades hemicellulose), and N-acetyl- β -D-glucosaminidase (EC 3.2.1.30 - NAG,
276 degrades chitin). The oxidative enzymes were: phenol oxidase (EC 1.10.3.2 - PO, degrades
277 polyphenols), and peroxidase (EC 1.11.1.7 - PX, degrades polyphenols/lignin). The enzyme
278 activity analyses were performed using methods presented in Hobbie et al. (2012), which used
279 slightly modified methods of Sinsabaugh et al. (1992) and Saiya-Cork et al. (2002), and were
280 performed at the University of Minnesota.

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

286

287 *Soil CO₂ flux*

288 To link enzyme activity measurements with soil C losses, we took advantage of previous
289 in situ measurements on soil CO₂ flux across the temperate broadleaf savanna experimental plots
290 at Cedar Creek conducted from 1999-2005, which includes both auto- and heterotrophic
291 respiration. We assume that the variability across plots from 1999-2005 is representative of
292 present-day conditions because there was no trend in soil CO₂ flux. These measurements were
293 made throughout the growing season (April-October) across eight points within each replicate
294 plot. Aboveground biomass was removed above each point prior to measurements made using a
295 Li-Cor 6400-09 soil respiration chamber attached to a Li-Cor 6200 gas exchange system over a 5
296 cm permanent soil collar. For our analyses, we re-scaled the data to fluxes per-degree Celsius

297 because of the high correlation between respiration and temperature ($F_{1,493}=1240$, $p<0.0001$,
298 $r^2=0.75$, model included the effect of fire frequency as well).

299

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

301 We used $\delta^{13}\text{C}$ to infer the contribution of C_3 vs. C_4 plant biomass inputs to soil organic
302 matter, an approach often used in savannas (Tieszen et al. 1979, Boutton et al. 1998). The strong
303 fractionation differences between C_3 trees (more negative $\delta^{13}\text{C}$) and C_4 grasses (less negative
304 $\delta^{13}\text{C}$) makes $\delta^{13}\text{C}$ a useful tracer for understanding how fire-driven reductions in tree biomass
305 contribute to losses of soil C. While pyrolysis of organic matter can also result in $\delta^{13}\text{C}$
306 fractionation (Bird and Ascough 2012), these effects are thought to be relatively small compared
307 to fractionation via the C_4 vs. C_3 photosynthetic pathway. One limitation to using $\delta^{13}\text{C}$ to infer
308 the different contributions of trees vs. grasses in northern latitude savannas is that several grass
309 species use the C_3 photosynthetic pathway, and thus have similar isotopic signatures as trees.
310 Consequently, changes in $\delta^{13}\text{C}$ could overestimate tree contributions to changing soil C. We are
311 more confident that soil $\delta^{13}\text{C}$ is a reliable indicator of C_4 grass vs. tree contributions in Cedar
312 Creek because a previous study found a strong positive correlation between C_4 grass biomass and
313 soil $\delta^{13}\text{C}$ in the top 0-10 cm of soil (Pellegrini et al. *in press*). Previous studies in Kruger and
314 Cedar Creek have assumed isotope values of -13‰ for C_4 grasses and -27‰ for C_3 trees
315 (Nelson et al. 2004, February and Higgins 2010). We analyzed $\delta^{13}\text{C}$ on samples taken under
316 canopies and in the open separately, allowing us to evaluate the local effect of tree canopies on
317 soil C.

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

325 Two of the sites are in tropical savannas: one in Brazil and the other in South Africa. In
326 Brazil, the plots were located in the Reserva Ecologica do Instituto Brasileiro de Geografia e
327 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 years 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 C₄ grasses). We compared soil C and $\delta^{13}\text{C}$ in two plots burned roughly every 2 years 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 is outlined in Pellegrini et al. 2015. At the time of sampling, the plots had experienced 58 years 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 years 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 years 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 errors 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 distribution of soil chemistry differed across sites.

359 Significance of terms was evaluated using the package *lmerTest* that approximates the degrees of
360 freedom using Satterthwaite's method (Kuznetsova et al. 2017).

361 If neither main effect was significant, we tested if the effects were contingent on site, i.e.,
362 whether sites differed in their sensitivities to either fire and/or tree canopies. For these models,
363 we included replicate plot as a random intercept and site as a fixed effect to test for interactions.
364 Because of the many potential variable combinations, we first determined the variables in the top
365 model using model selection via AIC with a threshold of two. When AIC values did not differ by
366 >2 and the lower AIC model was more complex, we used a χ^2 test to determine whether the more
367 complex model added significant explanatory power. If the test was not significant, we used the
368 model with the fewest terms. Significance of terms was also evaluated using the *lmerTest*
369 (Kuznetsova et al. 2017). Where a significant interaction occurred, we fit mixed effects models
370 within each individual site with replicate plot as a random intercept (to account for the non-
371 independence of the replicate soil samples taken within a replicate plot) to test for significant
372 effects of fire and tree canopies.

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

378 We refer to the tree canopy effect as the contrast between soils taken from under tree
379 canopies vs. outside of the canopy. We refer to the fire effects as the contrast between soils in the
380 burned vs. unburned plots either under and away from tree canopies. All analyses were
381 performed in R version 3.6.1 (R Development Core Team 2010).

382

383 **Results**

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

385 Across the four main sites (Figure 1, which we focus on until the section comparing $\delta^{13}\text{C}$
386 across sites), fire and tree canopies both helped predict bulk soil C concentrations in the top 0-5
387 cm; unburned plots had 48% (+/-22% standard error) higher soil C concentrations than
388 repeatedly burned plots (all sites in a mixed effects model, $F_{1,41}=7.4$, $p=0.01$, Figure 2, Table 1,
389 Appendix S1: Table S1), and soils under tree canopies had 55% (+/-18%) higher bulk C

390 concentrations than soils in open areas ($F_{1,41}=10.7$, $p=0.002$, Figure 2, Table 1, Appendix S1:
391 Table S1) (each mean and SE calculated from the values across the sites ($n=4$), while the mixed-
392 effects models are based on plot-level averages with site as a random intercept). Changes in bulk
393 soil N concentrations followed similar trends to C: unburned plots had 69% ($\pm 35\%$) higher soil
394 total N concentrations than repeatedly burned plots ($F_{1,41}=15.9$, $p<0.001$, Table 1, Appendix S1:
395 Table S1). There was not an overall enrichment effect of tree canopies on soil N across sites
396 ($F_{1,41}=2.5$, $p=0.12$), despite a trend towards tree canopies having 26% ($\pm 20\%$) higher total N
397 concentrations than open areas. Further analyses revealed that the lack of an overall trend was
398 because two sites had enriched N under canopies while one site had lower N under canopies
399 (significant site-canopy interaction $F_{3,136}=10.8$, $p<0.001$)(Figure 2, Tables 1,2).

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

407

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

409 Given the changes in bulk soil C and N, we next sought to test how potential
410 decomposition activity, quantified using extracellular enzyme assays, responded to fire and tree
411 canopies. Higher enzyme activity in burned plots and open areas would suggest microbial
412 activity is higher and may have contributed to losses of soil C, while lower activity would
413 suggest a potential buffering mechanism against losses. We investigated potential changes in
414 turnover of cellulose-, hemicellulose-, and starch-based and aromatic-based organic matter
415 separately.

416 Frequently burned plots had lower potential activity of enzymes that break down
417 cellulose, hemicellulose, and starch. The total potential activity of the hydrolytic enzymes
418 processing these compounds was 187% ($\pm 106\%$) higher in soils from unburned than burned
419 plots ($F_{1,40}=25.6$, $p<0.001$, Figure 3, Table 1). Moreover, when hydrolytic enzymes were
420 examined individually, three of the four enzymes had significantly lower activity in the burned

421 than unburned plots (CBH: $p < 0.001$, AG: $p < 0.001$, BX: $p < 0.001$, BG: $p < 0.001$, Appendix S1:
422 Table S1), illustrating that fire reduces the potential turnover of cellulose, hemicellulose, and
423 starch. Although the potential activity of all enzymes were tightly and positively correlated with
424 soil %C ($p < 0.0001$ for all comparisons, Appendix S1: Figure S1), the inclusion of %C into the
425 plot-averaged statistical models did not change the result that fire reduced either total enzyme
426 activity ($F_{1,40.5} = 15.3$, $p < 0.001$) or the activity of individual enzymes (CBH: $p < 0.001$, AG:
427 $p = 0.014$, BX: $p < 0.001$, BG: $p = 0.005$). Consequently, although we did not quantify microbial
428 biomass directly, we found that the effects of fire were robust to considering the correlation
429 between enzyme activity and soil %C, which can be a proxy for microbial biomass (McLauchlan
430 and Hobbie 2004).

431 Fire had no significant overall effect on the total activity of oxidative enzymes that
432 process aromatic lignin and polyphenol compounds ($F_{1,40} = 0.2$, $p > 0.5$, Table 1, Figure 3). In
433 contrast, tree canopies had significant effects on oxidative enzyme activity ($F_{1,40} = 7.2$, $p = 0.01$,
434 Figure 3), with 74% (+/-39%) higher activity under trees (Table 1, Figure 3). Both peroxidase
435 and phenol oxidase increased under canopies ($F_{1,40} = 4.3$, $p = 0.045$ and $F_{1,40.2} = 12.8$, $p < 0.001$,
436 respectively, Appendix S1: Table S1), but unlike any of the other enzymes, peroxidase activity
437 actually increased with burning, but only in the open areas (fire-canopy interaction: $F_{1,40} = 5.7$,
438 $p = 0.02$, Appendix S1: Table S1). Consequently, oxidative enzymes had a higher dependency on
439 the proximity to trees but were less limited by fire, either not changing or even increasing in
440 burned plots. The oxidative enzymes were not as well correlated with soil %C as the hydrolytic
441 enzymes: there was no significant relationship with total oxidative enzyme activity ($F_{1,38.5} = 1.9$,
442 $p = 0.18$) or peroxidase ($F_{1,38.3} = 1.0$, $p = 0.33$), but there was with phenol oxidase ($F_{1,15.7} = 5.5$,
443 $p = 0.032$).

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

448 Overall, fire reduced the potential turnover of organic matter but only of forms targeted
449 by hydrolytic enzymes (cellulose, hemicellulose, and starch); contrastingly, the potential
450 turnover of aromatic organic matter was resilient to fire but reduced in areas away from tree
451 canopies. The different sensitivities of hydrolytic vs. oxidative enzymes resulted in shifts in the

452 ratios between hydrolytic and oxidative enzymes. There was a reduction in hydrolytic:oxidative
453 ratios in the frequently burned plots ($F_{1,40}=25.0$, $p<0.001$, Figure 3, Table 1). In contrast, the
454 lower ratio under tree canopies was site-specific (interaction: $F_{3,97}=5.7$, $p=0.001$, Table 2), with
455 significant effects in two sites ($p<0.01$) and a marginally significant effect in one ($p=0.08$)
456 (Figure 3). Consequently, burning changed the potential decomposition of organic matter in
457 complex ways by decreasing the losses of cellulose, hemicellulose, and starch but leaving
458 potential losses of aromatics unchanged.

459

460 *Fire reduces inorganic N and microbial acquisition of N*

461 We next sought to understand whether potential changes in N availability were related to
462 the effects of fire and tree canopies on microbial decomposition activity. Lower soil N may result
463 in lower potential C-acquisition activity, but higher microbial N-acquisition activity may
464 compensate for the reduction in soil N. To ascertain how changes in soil N influenced the trends
465 in N-acquisition activity, we first quantified (i) the effect of fire on inorganic N and the activity
466 of the enzyme NAG which targets chitin allowing microbes to access N and C, (ii) how changes
467 in N related to changes in NAG activity, and (iii) whether correlations between N and enzyme
468 activity varied across the different C-acquisition enzymes. For the last two comparisons, we
469 always tested whether %C was a better explanatory variable than %N using model selection.

470 Changes in bulk soil N were accompanied by changes in the availability and potential
471 turnover of N, estimated by inorganic N and NAG activity, respectively. To approximate the
472 availability of N for plant uptake (referred to as bioavailability), we analyzed inorganic N (IN)
473 concentrations, which were 148% (+/-100%) higher in unburned plots ($F_{1,41}=14.7$, $p<0.001$,
474 Figure 4, Table 1). There were no overall effects of tree canopies on IN, and instead the tree
475 cover effect was site-specific (site-by-tree cover effect: $F_{3,140}=5.8$, $p<0.001$, Figure 4, Tables
476 1,2). The higher IN concentrations in unburned plots on average were largely attributable to
477 higher NH_4^+ concentrations (+190% +/-125%) (fire: $F_{1,41}=17.2$, $p<0.001$; Figure 4, Table 1) as
478 was the variability in tree canopy effects across sites (site-tree canopy effect: $F_{3,139,8}=4.3$,
479 $p=0.006$; Figure 4, Tables 1,2); there was no overall effect of fire or tree canopies on NO_3^- across
480 sites ($p>0.50$ for fire and tree canopies, Table 1). Consequently, fire effects on bulk soil N were
481 consistent with changes in inorganic N, especially NH_4^+ , while tree canopy effects depended on
482 site.

483 The activity of the enzyme involved in N acquisition (NAG, N-acetyl- β -D-
484 glucosaminidase, which breaks down chitin) was 313% (+/-185%) higher in unburned plots than
485 repeatedly burned plots ($F_{1,40}=22.5$, $p<0.001$, Figure 4, Table 1) and 100% (+/-31%) higher
486 under trees than in the open ($F_{1,40}=5.8$, $p=0.021$, Figure 4, Table 1); suggesting areas burned
487 repeatedly and away from tree canopies had lower potential turnover of chitin (a microbial N and
488 C source), consistent with the negative effects of fire on inorganic N.

489 Bulk soil N was related to both inorganic N and enzyme activity across fire frequencies,
490 canopies, and sites. In our model selection process, we considered the potential main effects of
491 fire and canopy location as well as soil %C and C:N when evaluating the correlations between
492 %N and inorganic N and enzyme activity. Bulk soil N was positively related to IN and NH_4^+
493 even when fire and canopy location were included in the model ($F_{1,90.5}=770.3$, $p<0.0001$ and
494 $F_{1,130.5}=66.1$, $p<0.0001$, respectively, Figure 5), but not to NO_3^- ($p>0.50$, Figure 5). NAG was
495 also significantly correlated with total soil N ($F_{1,42}=31.4$, $p<0.001$, Figure 6). Importantly,
496 models considered the potential main effects of fire and canopy location as well as soil %C and
497 C:N when evaluating the correlations between %N and inorganic N and enzyme activity.
498 Consequently, fire-driven losses of soil N were associated with lower potential microbial
499 acquisition of N and C and plant-available IN.

500

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

502 Total soil C and N were significantly positively correlated with potential hydrolytic C-
503 acquisition enzyme activity for both the total activity and that of each individual enzyme (Figure
504 6 and Appendix S1: Fig. S1, $F_{1,35.9}=17.2$, $p<0.001$). Because soil C and N were collinear
505 predictors, we were unable to completely disentangle their relative influence on enzyme
506 activities; however, using model selection, we evaluated which variable had the most
507 explanatory power and whether both C and N should be included in the model, which illustrated
508 that soil N was the best predictor (but see above for the correlations with %C, and Appendix S1:
509 Fig. S1), albeit its inclusion in the top model incorporated its collinearity with %C. In contrast,
510 total oxidative activity did not significantly correlate with total C or N (C: $F_{1,15.7}=5.5$, $p=0.03$, N:
511 $F_{1,39.5}=1.4$, $p=0.25$). Consequently, losses of soil C and N were correlated with lower potential
512 decomposition of cellulose, hemicellulose, and starch in burned plots and in areas away from tree
513 canopies.

514 Fire decreased NAG to a greater extent than potential hydrolytic and oxidative C
 515 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,
 516 Table 1). Consequently, fire is potentially shifting the relative turnover of C and N, where fire
 517 protection increases N turnover to a greater extent than C turnover.

518

519 *Coupled changes in hydrolytic enzymes and soil CO₂ flux*

520 To test whether our inferences about lower enzyme activity indicating potential soil C
 521 losses via decomposition, we compared enzyme activity levels and in situ soil CO₂ flux
 522 measurements in the temperate broadleaf savanna at Cedar Creek (n=6 plots measured over the
 523 growing season for 3 years). We found two lines of evidence that fire reduced soil CO₂ flux and
 524 that soil CO₂ flux was correlated with extracellular enzyme activities: (i) soil CO₂ flux rates were
 525 significantly lower in the burned plots than in unburned plots when soil temperature was
 526 controlled for (ANOVA test on re-scaled flux per-degree C: $F_{1,4}=13.9$, $p=0.02$, Figure 7), and (ii)
 527 total hydrolytic C-acquisition enzyme activity was positively correlated with respiration rates
 528 ($F_{1,4}=7.9$, $p=0.048$, $r^2=0.58$, Figure 7). Like hydrolytic enzyme activity, soil CO₂ flux was
 529 significantly correlated to bulk soil %C ($r^2=0.72$ and $p=0.0005$, (Norris 2008)). Oxidative
 530 enzymes were not correlated with soil CO₂ flux ($p>0.5$). Consequently, independent
 531 measurements of soil C losses support our inference based on enzyme activity that fire reduces
 532 decomposition at this site.

533

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

535 To explain variability in fire effects across sites, we tested the hypothesis that fire-driven
 536 changes in tree biomass inputs to soils were key drivers of soil C responses using $\delta^{13}\text{C}$. First,
 537 analyses across the four sites sampled here illustrated $\delta^{13}\text{C}$ was higher in burned plots and away
 538 from tree canopies ($F_{1,41}=16.6$, $p<0.001$ and $F_{1,41}=3.4$, $p=0.072$, respectively, Figure 8, with no
 539 overall interaction, $p>0.5$). In the savanna sites, which contained C₄ grasses and C₃ trees (Breaks,
 540 Cedar Creek, and Kruger), fire reduced the contribution of tree biomass to soils, with $\delta^{13}\text{C}$ being
 541 2.3-8.3% higher in burned relative to unburned plots (Appendix S1: Tables S2 and S4). In the
 542 four main sites, greater inputs from C₃ plants, which are primarily trees in these ecosystems,
 543 tended to be associated with higher soil C content (temperate broadleaf savanna: $F_{1,51.2}=40.0$,
 544 $p<0.0001$; temperate conifer savanna: $F_{1,13.9}=6.8$, $p=0.021$; tropical savanna: $F_{1,28}=5.3$, $p=0.029$).

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

550

551 **Discussion**

552 Our results demonstrate that repeated burning reduced total C and N in soils due to
553 reduced plant biomass inputs and through decreased tree abundance (Figure 2). The positive
554 correlation between changes in $\delta^{13}\text{C}$ and changes in total C among fire treatments across six
555 savanna sites suggests the impact of fire on soils is due to losses of woody plant biomass inputs
556 (Figure 9). In addition to changes in total C and N, both fire and the presence of trees modified
557 potential decomposition of organic matter but in complex ways: potential decomposition of
558 cellulose, hemicellulose, and starch compounds was reduced by repeated burning and the
559 absence of tree canopies, while potential decomposition of aromatic compounds was only
560 reduced by losses of tree cover and not fire (Figure 3). Burning and vegetation type also affected
561 the N cycle, with areas burned and away from tree canopies tending to have less inorganic N and
562 lower potential NAG (chitin-degrading) activity, which is one way microbes can acquire N
563 (Figure 4). Furthermore, losses of soil C and N were correlated with lower C and N turnover but
564 only for hydrolytic enzymes, and not oxidative enzymes that target aromatic compounds, when
565 accounting for fire and tree canopy effects (Figure 6).

566

567 *Role of changes in tree cover and its variability across sites*

568 Local effects of trees on soil properties under the canopy are a well documented
569 phenomenon in ecosystems and have been linked to both changes in microclimate and plant traits
570 (Belsky et al. 1989, Knops et al. 2002). While not all variables were significantly different under
571 vs. away from tree canopies, in all the significant cases, elemental concentrations and enzyme
572 activities tended to be higher under trees. We found evidence for several nuances, however, such
573 as a greater effect of tree cover on oxidative enzyme activity rather than fire effects alone.
574 Moreover, our results illustrated significant interactions between fire and tree cover on oxidative
575 enzyme activities, demonstrating that the local effects of trees can also depend on fire treatment.

576 In all the cases of significant interactions, tree canopy effects were greatest in the unburned plots
577 (Appendix S1: Fig. S3), supporting the hypothesis that fire exclusion can increase the localized
578 impact of tree canopies (e.g., (Pellegrini et al. 2015)).

579 The significant effects of tree canopies on soils in unburned plots, which tended to have
580 higher percent tree cover (e.g., in Cedar Creek tree cover was 84% in unburned plots and 46% in
581 burned plots, (Pellegrini et al. 2020)), suggest that higher tree cover does not diminish the local
582 effects of canopies on soils in these ecosystems. It is possible that the local effects of trees on
583 soils decline as tree cover increases in fire exclusion plots; yet in the coniferous forest, we still
584 found effects of tree canopies on extracellular enzyme activity, suggesting persistent localized
585 enrichment effects. Tree-fire interactions may potentially arise because tree canopies could
586 modify biomass combustion during fire such as by creating a moister microclimate, thereby
587 reducing losses, a potential effect we were unable to evaluate in our analyses. However, in the
588 savannas, we hypothesize this effect was relatively limited given that trees tended to be isolated,
589 have continuous grassy layers for fuel, and fires can get sufficiently hot to topkill trees
590 (Hoffmann et al. 2009). Alternatively, tree cover effects may be greatest in unburned plots
591 because the lack of fire allows for larger inputs of tree litter to soils.

592 Further work is needed to understand the extent that trees impact soils away from their
593 canopies. At Kruger, where the majority of herbaceous biomass is C₄ grass, soil $\delta^{13}\text{C}$ away from
594 canopies is much closer to the value for C₄ grass than soil under canopies, but remains more
595 depleted than expected if C was from C₄ biomass alone (February and Higgins 2010). This
596 pattern suggests C₃ biomass inputs away from tree canopies. Furthermore, at Cedar Creek, soil
597 $\delta^{13}\text{C}$ was significantly higher away from canopies than under canopies (Figure 8), but remained
598 relatively low (-23.1‰), providing evidence of C₃ biomass inputs away from canopies. Both of
599 these trends may be due to non-woody C₃ species (Peterson et al. 2007), which we are unable to
600 isolate in our current analysis.

601 The importance of changes in trees was also apparent at the landscape scale. Across all
602 six savanna sites, the variability in soil C losses in response to fire was significantly related to
603 changes in soil $\delta^{13}\text{C}$, which we used to infer tree biomass contributions to soil C. To our
604 knowledge, this is the first cross-site evaluation of the degree to which fire-driven changes in soil
605 C depends on changes in tree biomass inputs. Previous studies have focused on spatial gradients
606 in woody cover rather than manipulations within a site (Jackson et al. 2002, Lloyd et al. 2008).

607 However, a useful future expansion should consider deeper soil depths because deep-rooted
608 grasses can influence soil C (Fisher et al. 1994, Jackson et al. 2002, Schenk and Jackson 2002).

609

610 *Persistent effects of repeated burning on decomposition*

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

632 Fire has long been hypothesized to trigger a transient increase in inorganic N and cycling
633 which then diminishes over time (Blair 1997). For example, transient increases in N cycling are
634 largely supported by syntheses demonstrating a peak in decomposition and mineralization
635 immediately following fire, which then decline below pre-fire levels before recovering after 1-2
636 years (Wan et al. 2001, Wang et al. 2012). The reduction in soil enzyme activity and inorganic N
637 content in burned sites (Figures 3-4) is unlikely to be a transient response to the most recent fire

638 given that (i) the last fire occurred more than three years ago in two sites that exhibited large
639 declines in extracellular enzyme activities (the coniferous forest in Sequoia and savanna in the
640 Breaks), and (ii) in the sites where fire occurred less than two years ago (temperate and tropical
641 broadleaved savannas in Cedar Creek and Kruger, respectively) the burned plots had
642 significantly lower extracellular enzyme activities, albeit the exact enzyme that responded
643 differed. The lower enzyme activities outside of tree canopies also suggest that losses of tree
644 cover, which generally occur over decadal timescales (Higgins et al. 2000, Moreira 2000), lead
645 to legacy effects on decomposition activity even when fire is excluded because it can take
646 several decades for tree populations to potentially recover.

647 Our results suggest that over decadal alterations of fire frequency, decomposition is likely
648 regulated by factors such as the availability of organic matter and N, which were strongly and
649 positively related to enzyme activity (Figure 6, Appendix S1: Fig. S1). Changes in total soil
650 organic matter that occur over decadal alterations in fire frequencies (Pellegrini et al. 2018) are
651 likely contributing to the persistent effects of fire on enzymes for several years post fire. Through
652 comparisons across a range of sites that have different responses of woody biomass and
653 underlying environmental conditions, we demonstrate that the reduction in extracellular enzyme
654 activities is one of the more robust responses to repeated burning. Consequently, the potential for
655 fire to result in elevated microbial activity and thus produce a transient maximum is likely
656 reduced by high fire frequencies. The declines observed here may partly be amplified because
657 we sampled within a few years following fire, but in the savanna systems where fires recur every
658 1-3 years, our sampling scheme is most representative of the net effects of fire on soil C and N
659 availability and enzyme activity. Understanding how the differences between burned and
660 unburned plots change with time since fire would be a useful future research avenue.

661

662 *Potential changes in organic matter decomposition*

663 In all sites, fire reduced potential extracellular enzyme activity (Figure 3), which could
664 result in several changes in the turnover of soil organic matter. First, when hydrolytic enzymes
665 that process cellulose, hemicellulose, and starch were analyzed individually, fire and/or the
666 absence of trees reduced the activity of at least one enzyme in all four sites. Second, fire never
667 increased the activity of groups of enzymes (total hydrolytic, total oxidative, and NAG, Figures
668 3-4), contrasting with previous studies (Boerner et al. 2000, 2006, Rietl and Jackson 2012).

669 Third, soil CO₂ fluxes in the temperate broadleaf savanna at Cedar Creek declined with fire and
670 followed similar trends to hydrolytic enzyme activity across the replicate plots (Figure 7). At
671 Cedar Creek, the soil CO₂ flux data cannot distinguish between heterotrophic and autotrophic
672 respiration, such as from plant roots. However, fine root biomass, which can be a source of
673 autotrophic respiration, was actually significantly higher in the repeatedly burned plots (+65% in
674 the top 20 cm, (Pellegrini et al. 2020)), opposite to the direction of respiration. The decline in
675 soil CO₂ flux rates is consistent with other studies on repeated burning (e.g., (Metcalf et al.
676 2018)). Consequently, our results support previous studies that have found enzyme activity and
677 decomposition is reduced by repeated burning (Eivazi and Bayan 1996, Ajwa et al. 1999,
678 Boerner et al. 2005).

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

688 Finally, fire-driven losses of N may be one factor constraining C turnover (Figure 6), as
689 an alternative to lower biomass inputs leading to lower soil C, lower microbial biomass, and
690 lower enzyme activity (Dooley and Treseder 2012, Pressler et al. 2018). However, in our model
691 selection approach, we found that including %N in the model resulted in significant increases in
692 the fit of the model (determined via an AIC threshold of two). Moreover, we observed (i) a
693 significant negative effect of fire on soil N overall (Figure 2), and in three of the four sites when
694 analyzed individually (either through direct combustion effects or through fire-driven losses of
695 tree cover, Appendix S1: Tables S3-S4), and (ii) a fire-driven decline in the activity of N-
696 acquisition enzymes in all the sites (Figure 4) even when soil %C was incorporated into the
697 model. Moreover, fire likely amplifies low N availability by reducing NAG activity and
698 increasing the C_{hydro}:NAG ratio either due to lower biomass inputs or lower tree abundance
699 (Figure 7). Inferences based off of NAG alone are limited, however, because NAG is also used

700 by microbes to acquire C and there are other extracellular enzymes utilized by microbes to
701 acquire N that may display different responses. However, our data support the hypothesis that
702 repeated burning can increase N limitation in ecosystems (Reich et al. 2001, Pellegrini 2016).

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

719

720 **Conclusions**

721 By sampling several sites across different ecosystems, we provide broad evidence that
722 decadal changes in repeated burning shift soil C and N storage and potential turnover through
723 several processes. Our results support the hypothesis that the reduction in aboveground inputs,
724 especially of woody plants, is a key factor regulating fire's long-term effect on soils across six
725 savanna sites. Fire also reduced decomposition potential by suppressing the activity of hydrolytic
726 enzymes that break down organic matter, potentially reducing soil C losses. Yet the response of
727 the different enzymes revealed a nuanced response of the C cycle, with fire shifting the potential
728 turnover of cellulose-, hemicellulose-, and starch-based compounds but not the turnover of
729 aromatics such as polyphenols and lignin, potentially allowing for persistent decomposition of
730 aromatic compounds produced by burning. However, soil C accumulation under trees and in

731 unburned plots, likely through greater biomass inputs, is not completely offset by the higher
732 potential decomposition activity, illustrating the complex interactions between above- and
733 belowground processes in determining the net changes of soil C and N.

734

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743

744 **Literature Citations**

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998 **Data Availability:** Data are available in the Dryad Digital Repository:

999 <https://doi.org/10.5061/dryad.xwdbrv19n>

1000

1001 **Table 1:** Results comparing the relative effect of tree canopies and burning treatment on soil
1002 chemistry variables. The Mean and standard error (SEM) are calculated across the site means
1003 and are a ratio. The “Tree/Open” is calculated by first averaging the mean values under tree
1004 canopies in both burn treatments and dividing by the mean values away from tree canopies in
1005 both burn treatments. The “Unb/Burn” is calculated by first averaging the mean values in

1006 unburned plots in both under and away from tree canopies and dividing by the mean values in
 1007 the burned plots under and away from tree canopies. The statistical tests are from the mixed-
 1008 effects models performed on values averaged within replicate plots using site as a random
 1009 intercept. In the case of an insignificant effect, we explored whether this was due to conflicting
 1010 responses of site, and if so it is indicated as “site specific”. The site by fire and canopy
 1011 interactions are given in Table 2. Individual site descriptive and test statistics are in Appendix
 1012 S1: Tables S3-S7.

<i>Variable</i>	<i>Comp.</i>	<i>Mean</i>	<i>SEM</i>	<i>df</i>	<i>F</i>	<i>p</i>	<i>Comments</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	site specific
	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	site specific
	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	site specific
Tot IN	Tree/Open	1.05	0.21	1	41	0.0	0.875	site specific
	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-hyd	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-oxi	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	site specific
C-hyd:C-oxi	Tree/Open	1.07	0.28	1	40.0	0.0	0.945	site specific
	Unb/Burn	2.65	0.32	1	40.0	25.0	<0.001	
C-hyd: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-oxi:NAG	Tree/Open	1.12	0.18	1	40.0	0.2	0.645	site specific
	Unb/Burn	0.41	0.09	1	40.0	30.3	<0.001	

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1016 **Table 2:** Statistical results from the interactions between site and fire and canopy from the
 1017 mixed-effects models with site as a fixed effect interacting with fire and canopy, and replicate
 1018 plot as a random effect. Only the ANOVA results from the interactions are displayed. NC
 1019 indicates not converged when the variable was included in the model.

<i>Variable</i>	<i>Comparison</i>	<i>df</i>		<i>F</i>	<i>p</i>
<u>% C</u>	<i>S:Canopy</i>	3	135.2	7.7	<0.001
	<i>S:Fire</i>	3	17.8	1.4	0.274
<u>% N</u>	<i>S:Canopy</i>	3	135.8	10.8	<0.001
	<i>S:Fire</i>	3	18.5	3.3	0.043
<u>C:N</u>	<i>S:Canopy</i>	3	135.2	0.9	0.438
	<i>S:Fire</i>	3	15.0	1.4	0.279
<u>NO₃</u>	<i>S:Canopy</i>	3	135.9	5.6	0.001
	<i>S:Fire</i>	3	20.9	2.8	0.065
<u>NH₄⁺</u>	<i>S:Canopy</i>	3	139.8	4.3	0.006
	<i>S:Fire</i>	3	19.7	6.9	0.002
<u>Tot IN</u>	<i>S:Canopy</i>	3	140.0	5.8	<0.001
	<i>S:Fire</i>	3	19.8	7.0	0.002
<u>NAG</u>	<i>S:Canopy</i>	3	97.5	1.7	0.171
	<i>S:Fire</i>	3	15.6	6.2	0.006
<u>C hyd</u>	<i>S:Canopy</i>	3	97.8	1.4	0.259
	<i>S:Fire</i>	3	16.9	6.9	0.003
<u>C oxi</u>	<i>S:Canopy</i>	3	100.9	3.8	0.013
	<i>S:Fire</i>	3	18.9	0.7	0.567
<u>Hyd/Oxi</u>	<i>S:Canopy</i>	3	97.0	5.7	0.001
	<i>S:Fire</i>	NC			
<u>Hyd/NAG</u>	<i>S:Canopy</i>	3	96.4	1.2	0.299
	<i>S:Fire</i>	3	15.7	2.1	0.143
<u>Oxi/NAG</u>	<i>S:Canopy</i>	3	97.3	2.4	0.073

	<i>S:Fire</i>	3	15.8	1.4	0.282
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1021 **Figure legends**

1022 **Figure 1:** Sites sampled partitioned into the main sites comprising a temperate coniferous
 1023 savanna (Missouri Breaks) tropical broadleaf savanna (Kruger), temperate broadleaf savanna,
 1024 (Cedar Creek), and a temperate coniferous forest (Sequoia). The sites vary in the amount of tree
 1025 cover and climate but have all burned repeatedly at frequencies from annual to decadal for 33-61
 1026 years with adjacent control plots that have not burned over the same period. The additional
 1027 savanna sites are denoted with an asterisk and comprise two tropical broadleaf savannas (Satara
 1028 and Brazil) and a temperate coniferous savanna (Hitchiti), which were used for the ¹³C analysis.
 1029 The values beneath the pictures show the percent clay of the soil, mean annual precipitation
 1030 (MAP), temperature (MAT), length of time plots have differed in fire history (years), and the fire
 1031 frequency of the burned plots (FF). Photo credit Adam Pellegrini except for Hitchiti, Mac
 1032 Callahan.

1033 **Figure 2:** Box plots of soil carbon (C) and nitrogen (N) for fire treatments (fire effects) and tree
 1034 canopies (tree canopy effects) and plot-averaged values across sites. This figure and Figures 3-4
 1035 and 7 are the conditional values of the relative effects of the other variable (i.e., values displayed
 1036 for tree cover effects are from unburned plots and those for fire effects are from under tree
 1037 canopies). For fire effects, high and low delineate the frequently burned and the unburned
 1038 treatments, respectively, and for tree canopy effects open and tree delineate the samples away
 1039 from tree canopies and under canopies, respectively. Y axis is on a log scale. Box plots display
 1040 medians (50th percentile), 25th and 75th percentiles and approximate 95% confidence intervals.
 1041 Inserted boxes display p-values from the replicate-plot averaged mixed-effects model, with full
 1042 statistics in Table 1. An asterisk next to the p-value indicates that the effect is contingent on site
 1043 (see Appendix S1: Table S3 for the site-level statistics). Colors indicate fire treatment on the left-
 1044 hand panel (red = burned, dark blue = unburned) or canopy position (yellow = in the open, light
 1045 blue = under a tree canopy).

1046 **Figure 3:** Box plots of extracellular enzyme activity for total carbon-acquisition hydrolytic (A
 1047 and B) and oxidative (C and D) enzymes and their ratio (E and F) between fire treatments (fire
 1048 effects) and tree canopies (tree canopy effects) for plot-averaged values across sites. Y axis is on
 1049 a log scale. Each box plot displays medians (50th percentile), 25th and 75th percentiles and

1050 approximate 95% confidence intervals. Units for hydrolytic enzymes are $\text{nmoles h}^{-1} \text{g}^{-1}$ and
 1051 oxidative enzymes are $\mu\text{moles h}^{-1} \text{g}^{-1}$. Inserted boxes display p-values from the replicate-plot
 1052 averaged mixed-effects model, with full statistics in Table 1. An asterisk next to the p-value
 1053 indicates that the effect is contingent on site (see Appendix S1: Table S3 for the site-level
 1054 statistics). Colors indicate fire treatment on the left-hand panel (red = burned, dark blue =
 1055 unburned) or canopy position (yellow = in the open, light blue = under a tree canopy).

1056 **Figure 4:** Box plots of soil nitrogen availability and turnover both as total inorganic N (A-B) and
 1057 ammonium (C-D) and NAG (N-acetyl- β -D-glucosaminidase) enzyme activity (E-F) for plot-
 1058 averaged values across sites. Y axis is on a log scale. Box plots display medians (50th percentile),
 1059 25th, and 75th percentiles and approximate 95% confidence intervals. Units for NAG are nmoles
 1060 $\text{h}^{-1} \text{g}^{-1}$. Inserted boxes display p-values from the replicate-plot averaged mixed-effects model,
 1061 with full statistics in Table 1. An asterisk next to the p-value indicates that the effect is
 1062 contingent on site (see Appendix S1: Table S3 for the site-level statistics). Colors indicate fire
 1063 treatment on the left-hand panel (red = burned, dark blue = unburned) or canopy position (yellow
 1064 = in the open, light blue = under a tree canopy).

1065 **Figure 5:** Scatter plots and regressions between soil total nitrogen (N) and inorganic N (total
 1066 inorganic N, ammonium, and nitrate). The points are plot-averaged values across all sites and
 1067 colored based on fire treatment (red = burned and dark blue = unburned). Y axes are on a log
 1068 scale. Bands indicate the standard error around the fitted model and only significant trends are
 1069 displayed.

1070 **Figure 6:** Relationships between soil N and the activity of hydrolytic enzymes. A) sum of all
 1071 hydrolytic enzymes except NAG (CBH+BG+AG+BX), B) N-acquisition enzyme (NAG), C-F)
 1072 individual C-acquisition enzymes that degrade cellulosic compounds (compounds given in upper
 1073 left-hand corner). The points are plot-averaged values across all sites and colored based on fire
 1074 treatment (red = burned and dark blue = unburned). Y axes are on a logarithmic scale and units
 1075 are $\text{nmoles h}^{-1} \text{g}^{-1}$. Statistics for panels A and B are in the text. For panels C-F: CBH:
 1076 $F_{1,80.5}=34.6, p<0.0001$; BG: $F_{1,79.6}=35.2, p<0.0001$; AG: $F_{1,103.9}=52.6, p<0.0001$; BX: $F_{1,43.8}=57.0,$
 1077 $p<0.0001$. Bands indicate the standard errors of the model fit.

1078 **Figure 7:** Soil respiration across the fire treatment plots and its relationship with enzyme activity
 1079 for the temperate broadleaf savanna in Cedar Creek. A) regression between soil C flux and soil
 1080 temperature for all sampling points averaged within a plot for that time (51 sampling times from

1081 May 1999-Sept. 2005) B) mean soil C flux (measured as $\mu\text{Mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) re-scaled to per-
1082 degree C across plots within treatments (error bars indicate standard errors around the mean). C)
1083 regression between soil respiration (averaged within plots across all sampling times and re-scaled
1084 to mean temperature during the measurement) and extracellular enzyme activity averaged within
1085 plots in the temperate broadleaf savanna at Cedar Creek. Lines indicates significant linear
1086 regression with shaded bands illustrating the standard error of the fit. Colors indicated fire
1087 treatment groups with red = burned and dark blue = unburned.

1088 **Figure 8:** Responses of $\delta^{13}\text{C}$ to fire and tree canopy. Box plots of $\delta^{13}\text{C}$ across fire treatments
1089 within different canopy position (x-axis panels), with the p values indicating significant
1090 individual effects of both fire and canopy from the full model but no significant interaction
1091 ($p>0.5$). Box plots display medians (50th percentile), 25th and 75th percentiles and approximate
1092 95% confidence intervals. Colors indicated fire treatment groups with red = burned and dark blue
1093 = unburned.

1094 **Figure 9:** Scatter plot of the differences between burned and unburned plots for $\delta^{13}\text{C}$ and bulk
1095 soil %C for six savanna sites ($r^2=0.91$).



Breaks



Cedar Creek



Kruger



Sequoia



Satara*



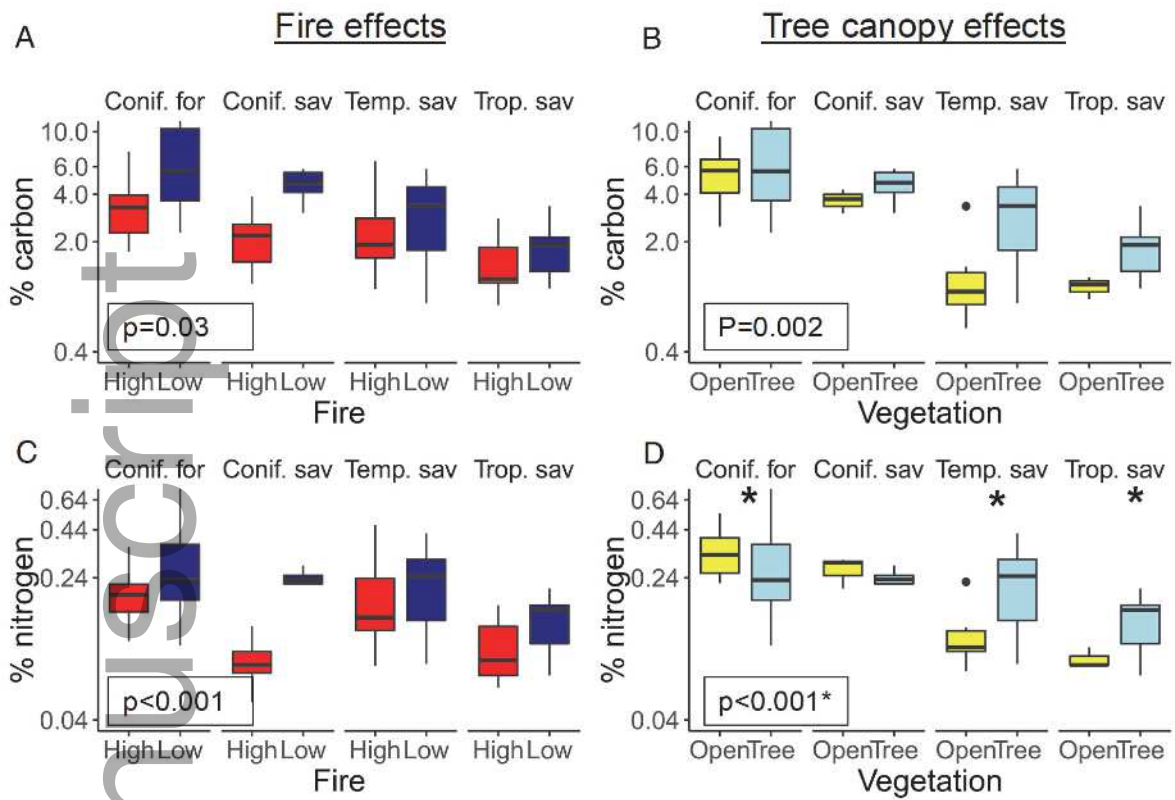
Brazil*



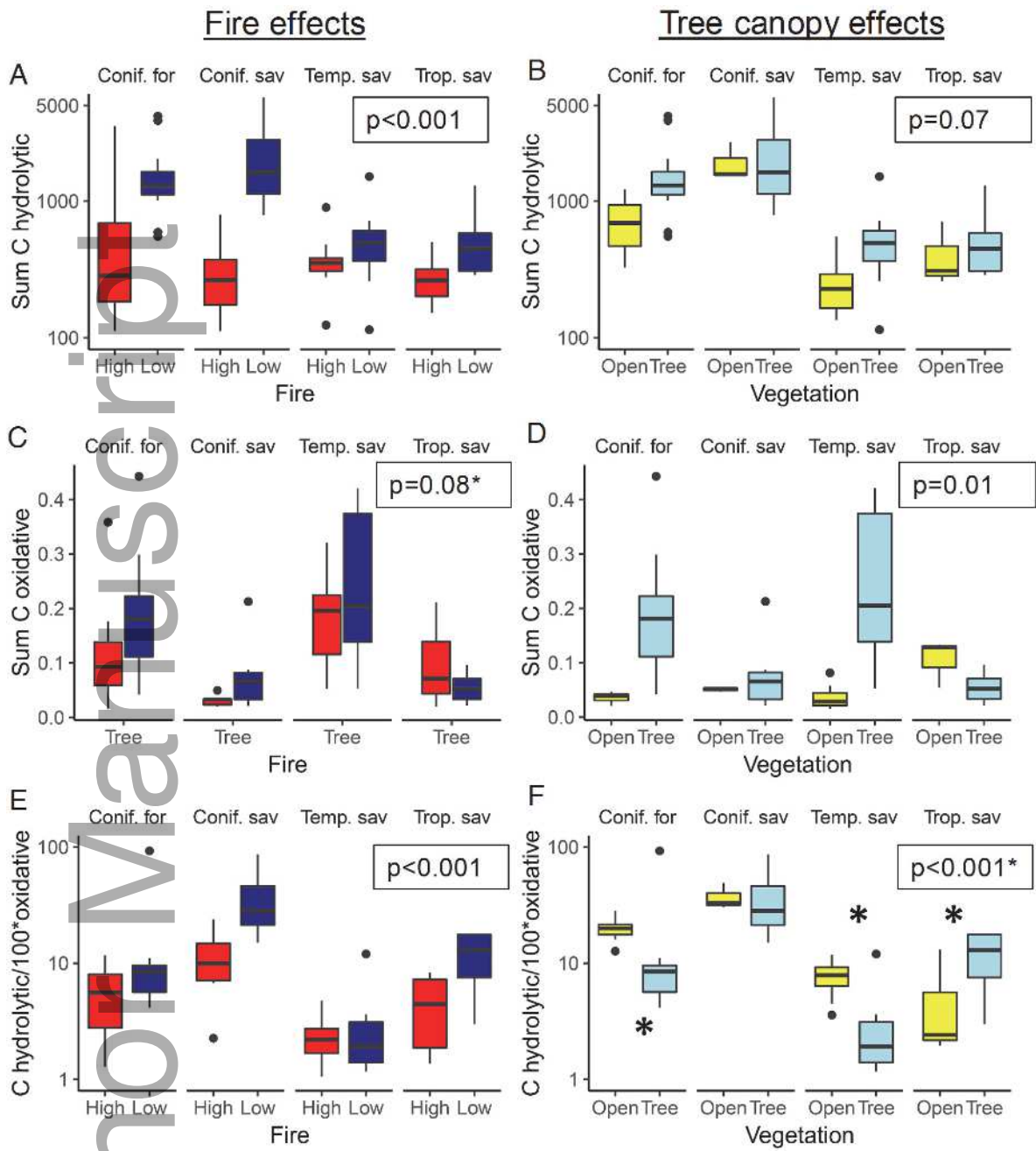
Hitchiti*

Breaks	Kruger	Sequoia	Cedar Creek
16% clay	10% clay	62% clay	3% clay
302 mm MAP	550 mm MAP	1092 mm MAP	780 mm MAP
8.2°C MAT	22°C MAT	8.1°C MAT	6.7°C MAT
33yrs, 0.1 FF,	61yrs, 1 FF	35yrs, 0.1 FF	53yrs, 0.8-0.6 FF
5yr TSF	1yr TSF	3-5yr TSF	1yr TSF
Additional sites	Brazil	Satara	Hitchiti
	% clay	70% clay	20% clay
	1400 mm MAP	537 mm MAP	1400 mm MAP
	22.5°C MAT	24.1°C MAT	12.7°C MAT
	60yrs, 1 FF	58yrs, 1 FF	19yrs, 0.5 FF
	1yr TSF	1yr TSF	3yr TSF

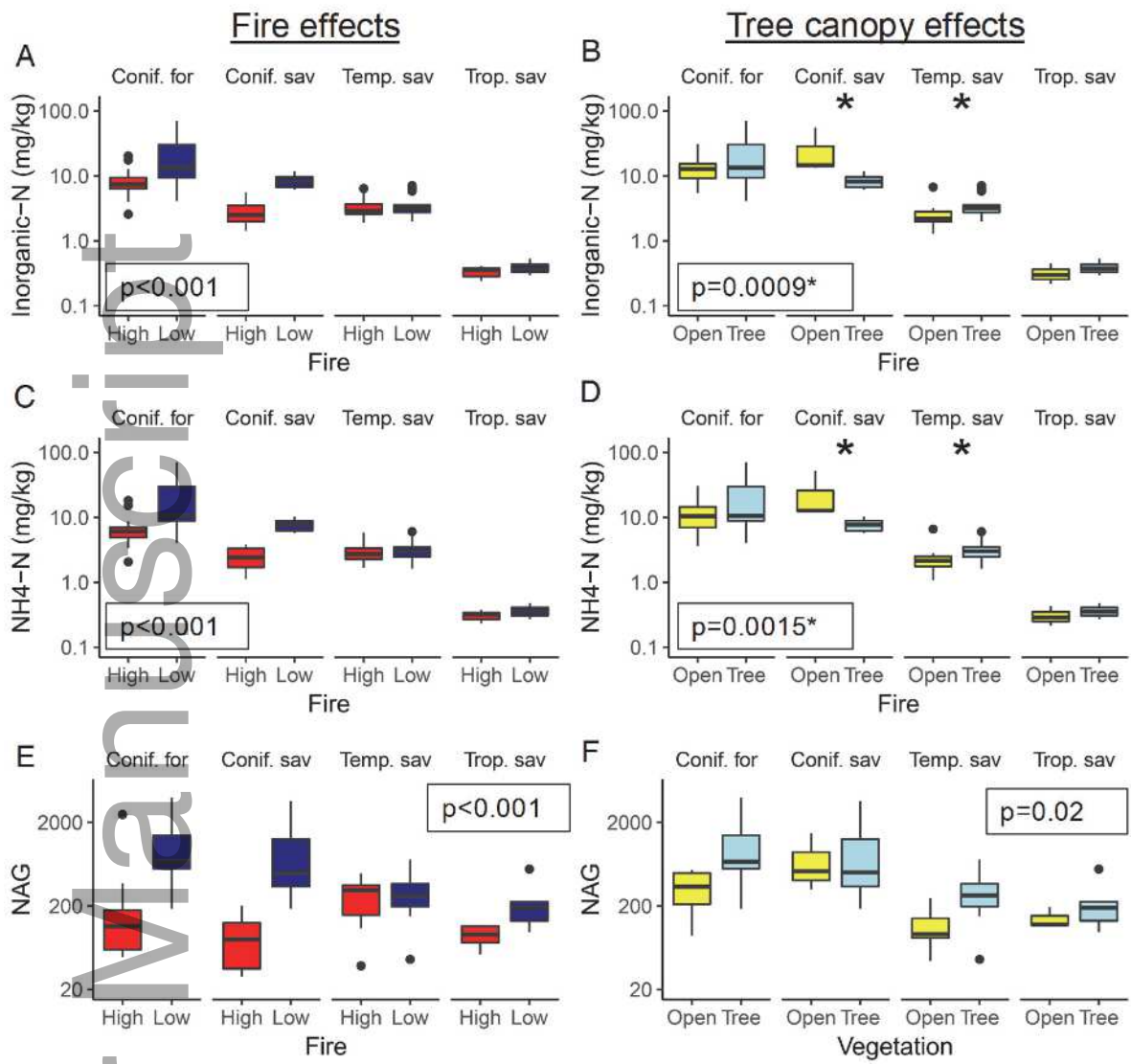
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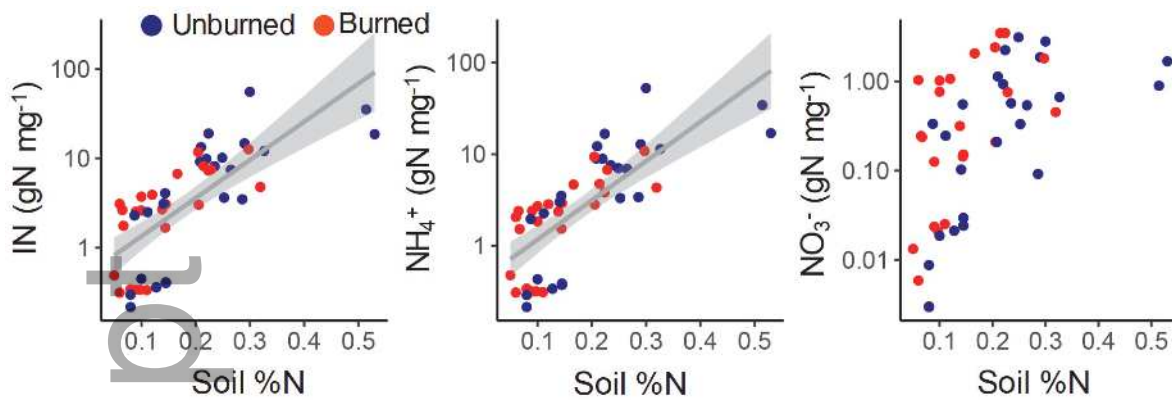
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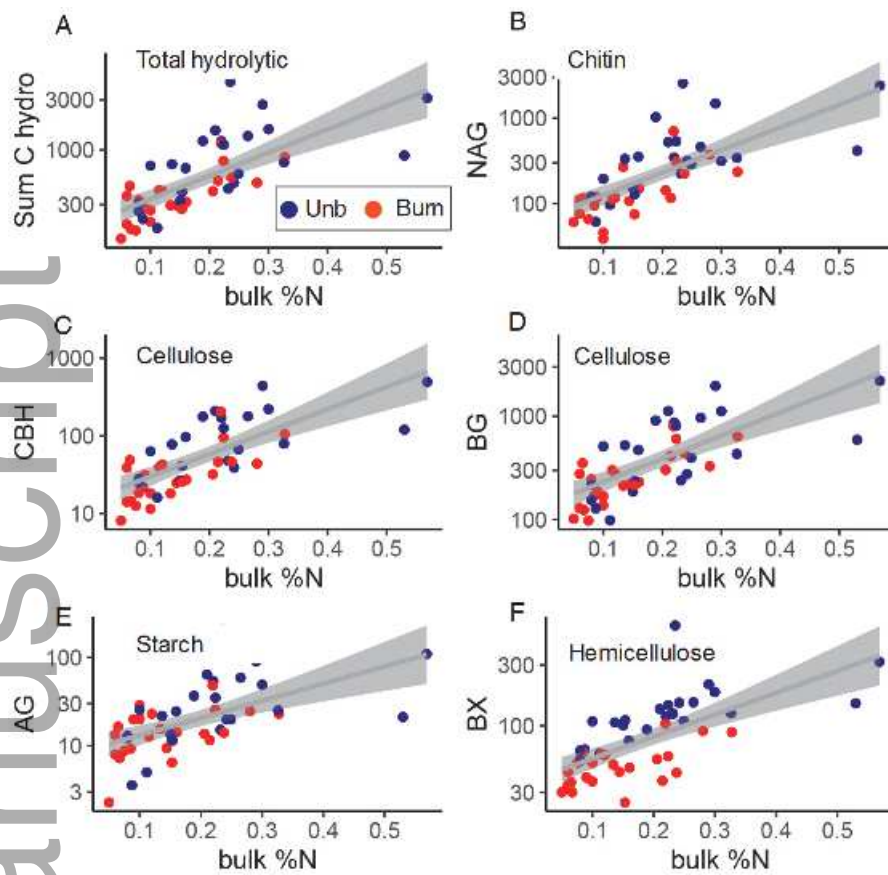


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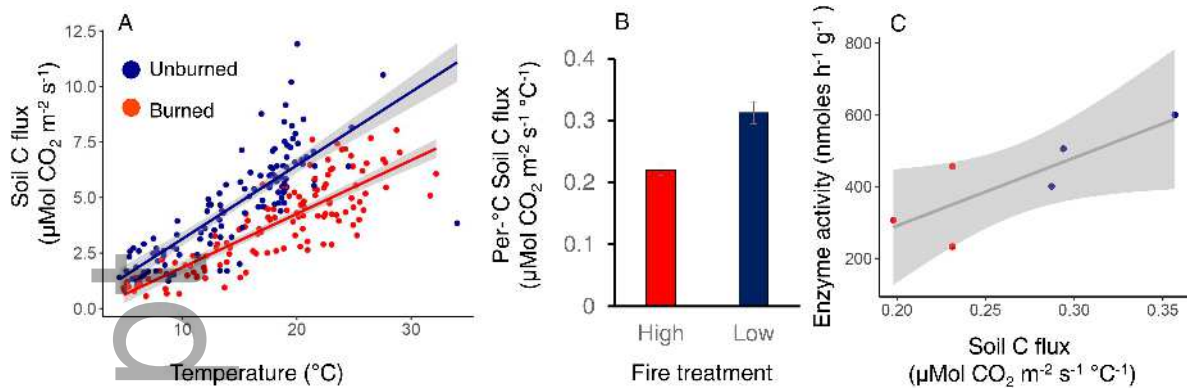


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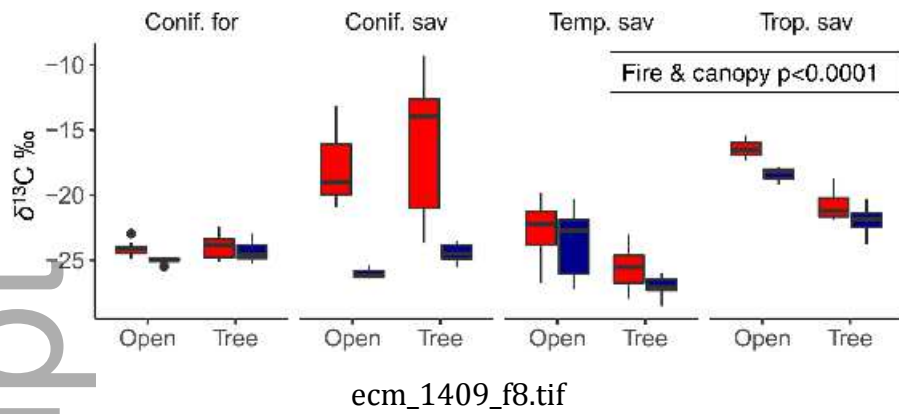
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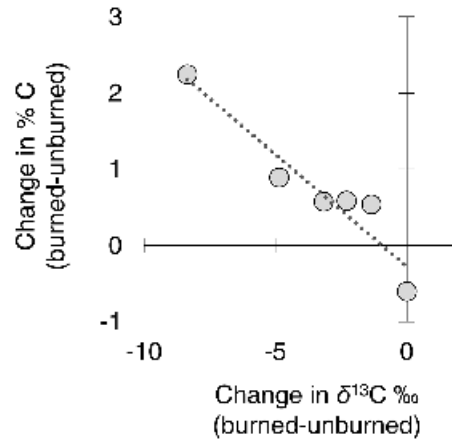


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