

# Soil microbial response to *Rhododendron* understory removal in southern Appalachian forests: Effects on extracellular enzymes

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## ABSTRACT

*Rhododendron maximum* is a native evergreen shrub that has expanded in Appalachian forests following declines of American chestnut (*Castanea dentata*) and eastern hemlock (*Tsuga canadensis*). *R. maximum* is of concern to forest managers because it suppresses hardwood tree establishment by limiting light and soil nutrient availability. We are testing *R. maximum* removal as a management strategy to promote recovery of Appalachian forests. We hypothesized that *R. maximum* removal would increase soil nitrogen (N) availability, resulting in increased microbial C-demand (i.e. increased C-acquiring enzyme activity) and a shift towards bacterial-dominated microbial communities. *R. maximum* removal treatments were applied in a  $2 \times 2$  factorial design, with two *R. maximum* canopy removal levels (removed vs not) combined with two O-horizon removal levels (burned vs unburned). Following removals, we sampled soils and found that dissolved organic carbon (DOC), N (TDN,  $\text{NO}_3^-$ ,  $\text{NH}_4^+$ ), and microbial biomass all increased with *R. maximum* canopy + O-horizon removal. Additionally, we observed increases in C-acquisition enzymes involved in degrading cellulose ( $\beta$ -glucosidase) and hemicellulose ( $\beta$ -xylosidase) with canopy + O-horizon removal. We did not see treatment effects on bacterial dominance, though F:B ratios from all treatments increased from spring to summer. Our results show that *R. maximum* removal stimulates microbial activity by increasing soil C and N availability, which may influence recovery of forests in the Appalachian region.

## 1. Introduction

In terrestrial ecosystems, plant-soil interactions regulate the structure of aboveground and belowground communities as well as rates of biogeochemical processes (Berg and Smalla, 2009; Ehrenfeld et al., 2005; Wardle et al., 2004). Plants influence soil microbial communities through their carbon (C) inputs via litterfall and root exudation (Berg and Smalla, 2009; Chapman and Newman, 2010; Wardle et al., 2006), while soil microorganisms influence plant productivity by mobilizing nutrients such as nitrogen (N), highlighting the potential for complex feedbacks between plants and belowground communities (van der Heijden et al., 2008). Such feedbacks are common in forest ecosystems, where different tree species are associated with distinct microbial communities that exhibit significant functional differences in terms of extracellular enzyme production and nutrient cycling (Ribbons et al., 2016; Weand et al., 2010). Similarly, forest understory shrubs and herbaceous vegetation can influence microbial community structure and function, even within the same forest type (Burke et al., 2011; Fu et al., 2015; Shen et al., 2018; Wurzbarger and Hendrick, 2007).

In moist cove and riparian habitats in southern Appalachian forests of the eastern US, the dominant understory species is rosebay rhododendron (*Rhododendron maximum* L.), a native evergreen shrub. *R. maximum* dominates plant-soil interactions in these forests by suppressing decomposition rates (Ball et al., 2008; Hunter et al., 2003; Strickland et al., 2009) and immobilizing N and other nutrients in complex organic compounds that are preferentially utilized by *R. maximum*'s own mycorrhizal symbionts (Wurzbarger and Hendrick, 2009, 2007). This immobilization of nutrients, along with attenuation of light, inhibits recruitment of hardwood tree seedlings, thereby influencing forest dynamics (Beckage et al., 2000; Clinton, 2003; Nilsen et al., 2001). Further, in the past century *R. maximum* has experienced a habitat expansion, due to the die-off of American chestnut (*Castanea dentata* (Marsh) Borkh) in the early 20th century (Elliott and Vose, 2012), and more recently it has increased its growth following the decline of eastern hemlock (*Tsuga canadensis* (L.) Carrière) due to hemlock wooly adelgid (*Adelges tsugae* Annand) infestation (Ford et al., 2012). Landscape-level studies also show that where *R. maximum* is present in the understory, forest trees are on average 6 m shorter than

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where it is absent (Bolstad et al., 2018). These studies suggest that riparian forest structure may be fundamentally altered in the wake of eastern hemlock decline. This has prompted forest managers to suggest aggressive management strategies involving the removal of *R. maximum* from areas impacted by hemlock die-off in order to promote forest recovery (Vose et al., 2013).

Proposed *R. maximum* management strategies include mechanical removal of the *R. maximum* understory and subsequent use of herbicides to suppress stump sprouting (Vose et al., 2013). Soil responses to understory vegetation removal are challenging to predict, with prior studies reporting positive, negative, and neutral responses of soil C and N, microbial biomass, fungal:bacterial (F:B) ratios, and extracellular enzyme activities in response to forest understory removal (Boerner et al., 2008; Gai and Boerner, 2007; Shen et al., 2018; Wu et al., 2011; Zhao et al., 2011). A prior *R. maximum* removal study in the southern Appalachian region showed modest increases in soil inorganic N with no evident effects on soil microbial biomass or invertebrate communities (Wright and Coleman, 2002; Yeakley et al., 2003). Though that study was not replicated and was confounded by a large disturbance event (hurricane) that affected the reference plot, it suggests that *R. maximum* canopy removal alone may not affect soil communities and processes in the short term.

Proposed *R. maximum* management strategies also involve the use of low-intensity prescribed fire to remove the thick soil O-horizon that develops in *R. maximum* thickets (Vose et al., 2013). Soil responses to prescribed fire in forests generally depend on vegetation type, fire frequency, and fire intensity (Certini, 2005). Though soil organic matter (SOM) often decreases following fires (Certini, 2005; González-Pérez et al., 2004), low intensity burns can increase SOM decomposability by heat-altering carbon polymers (Knicker, 2007), resulting in increased C available to soil microorganisms. Additionally, low-intensity burns can increase soil N availability by converting organic N to inorganic forms (Certini, 2005; Hernández and Hobbie, 2008). In southern Appalachian forests, low intensity prescribed fires have not significantly affected soil C and N stocks (Hubbard et al., 2004; Knoepp et al., 2009, 2004), but have increased inorganic-N transformation rates in some cases (Knoepp et al., 2004). In other forested regions, prescribed burns have resulted in increased N availability and altered activities of microbial extracellular enzymes (Boerner et al., 2008; Rietl and Jackson, 2012; Taylor and Midgley, 2018). Studies addressing the combined effects of forest understory removal and prescribed burns in eastern US forests are rare, though increased bacterial activity and altered fungal and bacterial catabolic function have been reported when understory removal and prescribed burning were combined (Gai and Boerner, 2007).

The objective of this study was to examine soil responses to *R. maximum* understory removal in combination with soil O-horizon removal via prescribed burning at the Coweeta Hydrologic Laboratory in the southern Appalachian mountains of North Carolina. We focused on responses of soil C and N pools, fungal vs bacterial dominance, and extracellular enzyme production by microbial communities following *R. maximum* removal. We hypothesized that (1) *R. maximum* + O-horizon removal would mobilize organic matter from recalcitrant *R. maximum* leaf litter, resulting in increased DOC and N availability in mineral soils and a shift towards bacterial-dominated microbial communities; (2) that increased N availability would increase microbial C demand, resulting in elevated production of extracellular enzymes associated with C acquisition; and (3) that reductions in lignin-rich *R. maximum* leaf litter in the O-horizon following burning would result in reduced activities of lignolytic enzymes.

## 2. Materials and methods

### 2.1. Site description

We conducted this study at the Coweeta Hydrologic Laboratory (CWT, latitude 35°03' N, longitude 83°25' W), a U.S. Forest Service

experimental forest located in the Nantahala Mountains of western North Carolina within the Blue Ridge physiographic province in the southern Appalachians. Soils are deep sandy loams underlain by folded schist and gneiss. Two soil orders are found within the study sites, Inceptisols and Ultisols in the Cullasaja-Tuckasegee and Edneyville-Chestnut complexes, respectively (Thomas, 1996). Soils are characterized by high organic matter in the A horizon, a clay accumulating B horizon, and depth to saprolite of 80–100 cm.

We selected areas within the Coweeta Basin in mesic, riparian areas with low-to-moderate slopes (< 30%) and elevations ranging from 760 to 1060 m. All study areas had high abundance of *R. maximum*. Mean annual temperature at Coweeta is 12.6 °C and seasonally ranges from 3.3 to 21.6 °C. While annual rainfall is usually abundant in this region, averaging ca. 1800 mm, drought years are becoming increasingly common (Laseter et al., 2012).

### 2.2. Experimental design and sample collection

We applied four *R. maximum* removal treatments to sixteen 20 m × 20 m (0.04 ha) plots located in the Coweeta Basin. Six of the sixteen plots have been monitored for vegetation dynamics, carbon and nutrient pools and fluxes, and soil solution chemistry since 2004 (Ford et al., 2012; Knoepp et al., 2011; Nuckolls et al., 2009). We established ten additional plots with similar characteristics, and then randomly selected among the sixteen plots to assign treatments, resulting in four replicates of each treatment. The four treatments were designed to remove the *R. maximum* canopy (hereafter, CR), remove the soil O-horizon (hereafter, FF), remove the *R. maximum* canopy and soil O-horizon (hereafter, CFFR), and no removal (hereafter, REF). The CR and CFFR treatments included cutting *R. maximum*, immediately followed by application of herbicide on cut stumps (Eşen and Zedaker, 2004; Harrell, 2006; Romancier, 1971). The herbicide was a triclopyr amine (Garlon 3A<sup>®</sup>, DOW Agrosiences) formulation with an aquatic label (50% triclopyr amine/50% water) to prevent stump sprouting. *R. maximum* cutting (CR, CFFR) occurred in March–May 2015. O-horizon removal in the FF and CFFR treatments involved low intensity prescribed fires, which temporarily removed the Oi (leaf litter) layer but did not consume the Oe+Oa layers (Elliott and Miniati, 2018). Fires were implemented in plots in March 2016 and were performed according to the USDA Forest Service, Nantahala National Forest Prescribed Burning Plan (USDA, 2011).

In April and July 2017, two years following *R. maximum* canopy removal and one year following partial O-horizon removal (Oi only), we took three A-horizon (0–10 cm depth) soil cores from each plot and composited samples by plot. We transported soils to the lab on ice and stored samples at 4 °C until analysis.

### 2.3. Soil pH, soil C and N, microbial biomass C and N

Gravimetric soil water content was determined by mass loss after drying at 105 °C for 24 h. Soil pH was measured in a soil:water slurry, 1:1 by volume, using a Hach Sension+ pH meter (Hach company, Loveland, CO, USA). Microbial biomass C and N were determined using a modified chloroform fumigation extraction procedure described by Fierer and Schimel (2003). Extracts were measured for extractable dissolved organic carbon (DOC), total extractable nitrogen (TDN), microbial biomass carbon (MBC) and microbial biomass nitrogen (MBN) on an Elementar vario cube TOC/TN (Elementar Americas Inc, Mt. Laurel, NJ, USA). Extracts were analyzed for extractable NH<sub>4</sub> and NO<sub>3</sub> on a Lachat QuikChem flow injection analyzer (Hach Company, Loveland, CO, USA). Dissolved organic nitrogen (DON) was calculated as TDN – (NH<sub>4</sub> + NO<sub>3</sub>).

### 2.4. Extracellular enzyme assays

We measured activities of eight extracellular enzymes involved in C,

**Table 1**

Extracellular enzymes assayed in this study, their abbreviations, and their functions.

Enzyme	Abbreviation	Enzyme Function
β-glucosidase	BG	Cellulose degradation
β-xylosidase	XYL	Hemicellulose degradation
β-D-cellubiosidase	CHB	Cellulose degradation
Acid Phosphatase	AP	Phosphorus mineralization
Leucine aminopeptidase	LAP	Protein depolymerization
N-acetyl-β-glucosaminidase	NAG	Chitin degradation
Phenol oxidase	POX	Lignin degradation
Peroxidase	PER	Lignin degradation

N, and phosphorus (P) cycling in sampled soils. For the hydrolytic enzymes (AP, LAP, NAG, BG, CHB, XYL) (Table 1), we performed fluorometric enzyme assays modified from Saiya-Cork et al. (2002). Briefly, we homogenized ~0.25 g of fresh soil in 125 ml of pH-adjusted 50 mM sodium acetate buffer and stirred homogenate continuously while 200 µl aliquots were added to a 96-well microplate containing substrates fluorescently labelled with 7-amino-4-methylcoumarin (AMC) or 4-methylumbelliferone (MUB). AMC-linked substrates were used to measure LAP activity while MUB-linked substrates were used for all other hydrolytic enzymes. We used a single concentration (10 µM) AMC or MUB standard on each plate, and each plate contained eight analytical replicates of each assay. We measured fluorescence using a Tecan infinite M200 microplate reader (Tecan Group Ltd, Mannedorf, Switzerland) with excitation and emission wavelengths of 365 nm and 450 nm, respectively.

We also measured potential enzyme activity of two oxidative enzymes, POX and PER (Table 1), using colorimetric microplate assays (Saiya-Cork et al., 2002). Oxidative enzyme activities were determined by measuring color change associated with the breakdown of the substrate 3,4-dihydroxy-L-phenylalanine (L-DOPA). We measured absorbance of microplate wells at 460 nm using a Tecan infinite M200 microplate reader (Tecan Group Ltd, Mannedorf, Switzerland).

Activities of extracellular enzymes were corrected for dry soil mass and for microbial biomass C. Prior to multivariate statistical analysis (see below), enzyme activities were relativized based on the maximum observed activity for each respective enzyme in the data set. Ratios of C and N cycling enzymes were calculated as BG:(NAG + LAP) while ratios of C and P cycling enzymes were calculated as BG:AP. These ratios are commonly employed as metrics of relative microbial nutrient demand (Sinsabaugh et al., 2008).

## 2.5. DNA extraction and qPCR

DNA was extracted from ~0.25 g of fresh soil using the DNeasy PowerSoil kit (Qiagen, Valencia, CA, USA) and extracts were quantified using a Qubit fluorometer (Thermo Fisher Inc., Waltham, MA, USA). Total bacterial abundance and total fungal abundance were estimated via qPCR amplification of the 16s rRNA gene and the internal transcriber spacer (ITS) region, respectively. For 16s rRNA gene amplification, we used the primer set EUB 518 and EUB 338, while for ITS amplification we used the primers ITS1f and 5.8s (Fierer et al., 2005). Each qPCR reaction contained 10 µl Quantitect SYBR green master mix (Qiagen, Valencia, CA, USA), 0.5 µM forward and reverse primer, 3 ng DNA template, and nuclease-free H<sub>2</sub>O to 20 µl. For both 16s and ITS, thermal cycling conditions were 15 min at 95 °C followed by 40 cycles of 15 s at 94 °C, 30 s at 55 °C and 30 s at 72 °C. Standard curves were generated by amplifying serial dilutions of plasmids containing cloned copies of the target sequences. All qPCR reactions were performed in triplicate. Amplification efficiencies ranged from 80.4 to 89.2% with R<sup>2</sup> values > 0.99. Amplification specificity was determined using melt curve analysis. 16s and ITS gene abundances were normalized per gram dry soil and F:B ratios were calculated for each sample by calculating

ratios of ITS to 16s gene copies (Fierer et al., 2005).

## 2.6. Statistical analysis

All statistical analyses were performed in R (R Core Development Team, 2017). We used principal components analysis (PCA) to visualize multivariate extracellular enzyme profiles across treatments (*princomp* function, *vegan* package). Treatment effects on multivariate enzyme profiles were determined with permutational analysis of variance (PERMANOVA) using Euclidean distance matrices. We used a nested PERMANOVA, which allowed us to account for non-independence of enzyme measurements from the same plot across sample dates (*nested.permanova* function, *biodiversityR* package). Prior to PERMANOVA, we tested for multivariate dispersion effects using the *betadis* function in the *vegan* package. Effects of treatment and sampling date on soil chemistry variables, individual enzymes, C:N and C:P enzyme ratios, and fungal/bacterial abundance were tested with linear mixed effects models using the *lme4* package. Treatment and sample date were considered fixed effects in the models while plot was considered a random effect. We compared models with treatment as the only fixed effect to models containing both treatment and sampling date as fixed effects using AIC<sub>c</sub> and selected the model with the highest AIC<sub>c</sub> weight. Model selection results are presented in Tables S1–S4. Pairwise comparisons between treatments were made with Tukey's HSD using the *lsmeans* package. Relationships between F:B ratios and C:N and C:P enzyme ratios were determined with linear regression, while relationships between fungal and bacterial abundance and individual enzymes were determined using Pearson correlation. Where necessary, values were log transformed in order to meet assumptions of normality of residuals. For visualization purposes, log-transformed values were back-transformed. Where sampling date was not included in the best-supported mixed effects model, we only show treatment comparisons to illustrate effects of *R. maximum* removal.

## 3. Results

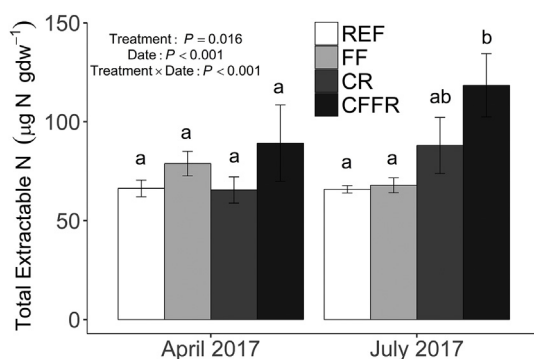
### 3.1. Soil pH, C and N pools, and microbial biomass C and N

For all soil variables except for TDN and DON, the best-supported mixed effects models had treatment as the only fixed effect (Table S1). Soil pH was not different among treatments, while DOC, MBC, MBN, NH<sub>4</sub>, and NO<sub>3</sub>, were all significantly different among treatments (Table 2). DOC was higher in CFFR plots relative to CR plots (~50% higher, *P* = 0.025) and was marginally higher in CFFR plots relative to REF plots (~33% higher, *P* = 0.089). MBC was higher in CFFR plots

**Table 2**

DOC, MBC, MBN, TDN, NH<sub>4</sub>, NO<sub>3</sub>, and pH across treatments. Means followed by one standard error are presented. Units for DOC and MBC are µg C g soil<sup>-1</sup> while units for MBN, NH<sub>4</sub>, and NO<sub>3</sub> are µg N g soil<sup>-1</sup>. Different superscript letters indicate significant pairwise differences between treatments (*P* < 0.05), while asterisks indicate marginally significant differences (*P* < 0.1). Linear mixed models with significant effects (*P* < 0.05) are presented in bold. Treatment abbreviations are as follows: reference (REF), O-horizon removal (FF), canopy removal (CR), and canopy + O-horizon removal (CFFR).

Treatment	DOC	MBC	MBN	NH <sub>4</sub>	NO <sub>3</sub>	pH
REF	697 <sup>a*</sup> (55.8)	240 <sup>a</sup> (21.6)	42.7 <sup>a</sup> (4.61)	3.70 <sup>a</sup> (0.672)	0.042 <sup>a</sup> (0.014)	4.86 (0.111)
FF	721 <sup>ab</sup> (59.9)	306 <sup>ab</sup> (35.7)	53.8 <sup>a</sup> (7.96)	3.25 <sup>a</sup> (0.526)	0.031 <sup>a</sup> (0.011)	4.89 (0.274)
CR	635 <sup>a</sup> (31.3)	315 <sup>ab</sup> (45.9)	55.9 <sup>a</sup> (6.93)	3.19 <sup>a</sup> (0.228)	0.041 <sup>a</sup> (0.039)	4.97 (0.272)
CFFR	932 <sup>b</sup> (89.9)	530 <sup>b</sup> (126)	96.9 <sup>b</sup> (13.9)	6.54 <sup>b</sup> (0.978)	0.139 <sup>b</sup> (0.035)	4.75 (0.249)
Linear Mixed Model Effects						
Treatment	<b>0.008</b>	<b>0.005</b>	<b>&lt; 0.001</b>	<b>0.001</b>	<b>&lt; 0.001</b>	0.438



**Fig. 1.** Total extractable N (TDN) across treatments and sampling dates. Bars represent means while error bars are  $\pm$  one standard error.  $P$ -values presented are linear mixed model effects of treatment, sampling date, and treatment  $\times$  date interactions. Different letters represent significant pairwise differences between treatments within a sampling date ( $P < 0.05$ ). Treatment abbreviations are as follows: reference (REF), O-horizon removal (FF), canopy removal (CR), and canopy + O-horizon removal (CFFR).

than in REF plots only ( $\sim 125\%$  higher,  $P = 0.008$ ). MBN,  $\text{NH}_4$ , and  $\text{NO}_3$  were all higher in CFFR plots than in all other treatments (all  $P < 0.05$ ). MBN increased  $\sim 125\%$ ,  $\text{NH}_4$  increased  $\sim 100\%$ , and  $\text{NO}_3$  increased  $\sim 230\%$  in CFFR plots relative to REF plots (Table 2).

The best-supported model for TDN had both treatment and sampling date as fixed effects (Table S1) and had a significant treatment  $\times$  date interaction term, where CFFR plots had approximately 100% higher TDN than REF ( $P = 0.015$ ) and FF ( $P = 0.021$ ) plots only in the July sampling (Fig. 1). Across all samples, DON comprised  $> 90\%$  of TDN, resulting in identical patterns across sampling dates and treatments for DON (data not shown).

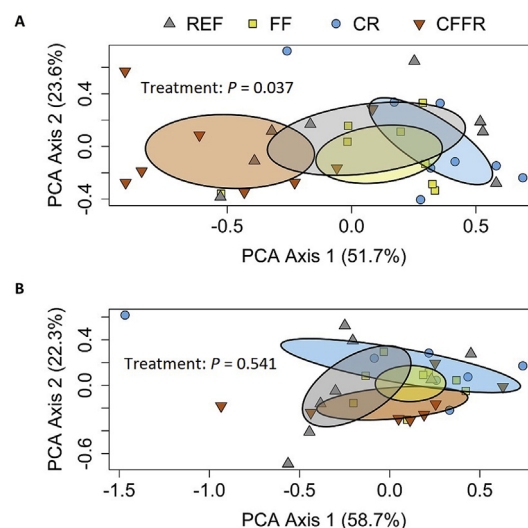
### 3.2. Extracellular enzyme activities

There were no multivariate dispersion effects on extracellular enzyme activities across treatments ( $P > 0.05$ ), indicating that PERMANOVA was able to reliably identify treatment effects. PERMANOVA showed that soil mass-corrected enzyme profiles were significantly different among treatments (Fig. 2A), while microbial biomass-corrected enzyme profiles were not (Fig. 2B).

For individual enzyme activities corrected for soil mass, all best-supported mixed models had treatment as the only fixed effect (Table S2). BG, CHB, XYL, and AP were significantly different among treatments, LAP was marginally different among treatments, and NAG, POX, and PER were not different among treatments (Fig. 3). BG activity was higher in CFFR plots than in CR plots ( $\sim 100\%$  higher,  $P = 0.02$ ) and was marginally higher in CFFR than in both REF ( $P = 0.053$ ) and FF plots ( $P = 0.059$ , Fig. 3A). CHB activity was higher in CFFR than in CR plots only ( $\sim 150\%$  higher,  $P = 0.039$ , Fig. 3B). XYL activity was higher in CFFR than in all other treatments ( $\sim 100$ – $175\%$  higher, all  $P < 0.05$ , Fig. 3C). AP activity was higher in CFFR than in CR plots only ( $\sim 130\%$  higher,  $P = 0.009$ , Fig. 3D) while LAP activity was marginally higher in CFFR than in CR plots ( $\sim 60\%$  higher,  $P = 0.073$ , Fig. 3E).

The best-supported mixed models for all microbial biomass-corrected enzyme activities had treatment as the only fixed effect (Table S3). There were no significant effects of treatment on biomass-corrected activities of any of the eight extracellular enzymes (all  $P > 0.05$ , Fig. S1).

Best-supported models for C:N and C:P enzyme ratios had both treatment and sampling date as fixed effects (Table S4). For C:N enzyme ratios, there was a significant treatment  $\times$  date interaction, where CFFR plots had marginally higher C:N enzyme ratios than REF plots ( $P = 0.065$ ) and CR plots ( $P = 0.077$ ) only in the April sampling (Fig. 4A). For C:P enzyme ratios, there was a significant effect of



**Fig. 2.** Principal components analysis (PCA) of extracellular enzyme activities corrected for dry soil mass (A). PCA axis 1 is negatively correlated with all hydrolytic enzymes (BG, CHB, XYL, AP, LAP, NAG) while PCA axis 2 is positively correlated with oxidative enzymes (POX, PER). PCA of extracellular enzyme activities corrected for microbial biomass (B). PCA axis 1 is negatively correlated with all eight enzymes while PCA axis 2 is negatively correlated with XYL and CHB and positively correlated with POX and PER.  $P$ -values presented are treatment effects from nested PERMANOVA. Ellipses are 95% confidence intervals around the centroid of each treatment. Treatment abbreviations are as follows: reference (REF), O-horizon removal (FF), canopy removal (CR), and canopy + O-horizon removal (CFFR).

sampling date and a marginal treatment  $\times$  date interaction, though no pairwise comparisons between treatments within sampling dates were significant (Fig. 4B).

### 3.3. Bacterial and fungal abundance

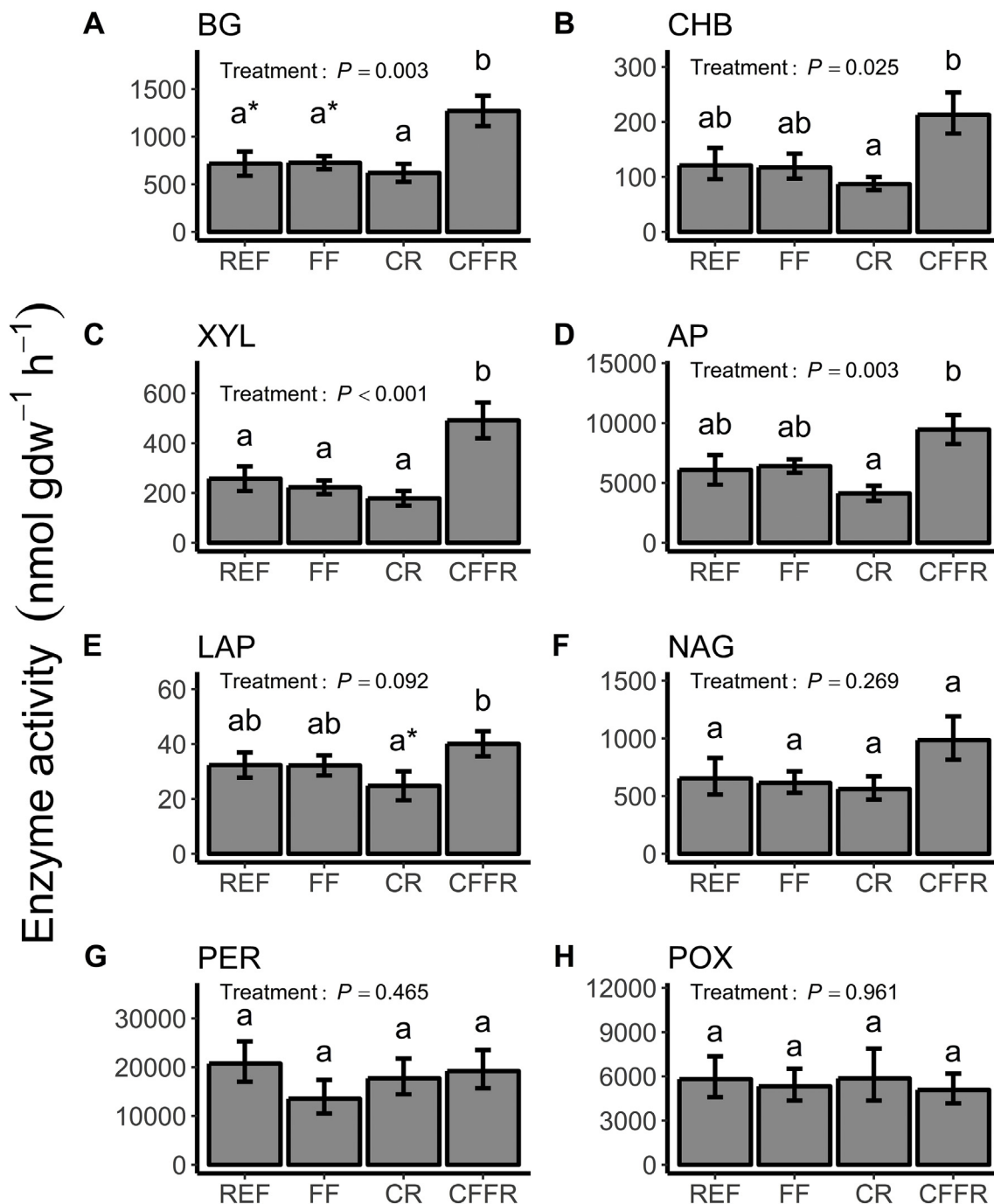
Best-supported models for bacterial abundance, fungal abundance, and F:B ratios had both treatment and sampling date as fixed effects (Table S4). Bacterial abundance was marginally higher in CFFR plots relative to CR plots in July ( $\sim 50\%$  higher,  $P = 0.097$ ) and was overall higher in the April sampling than in the July sampling ( $\sim 70\%$  higher,  $P < 0.001$ , Fig. 5A). Bacterial abundance was significantly positively correlated with the C-acquiring enzymes BG, CHB, and XYL (Table 3). Fungal abundance was higher in the July sampling than in the April sampling ( $\sim 80\%$  higher,  $P < 0.001$ ), and there were no differences in fungal abundance between treatments (Fig. 5B). There were significant positive correlations between fungal abundance and the N-acquiring enzyme NAG and the P-acquiring enzyme AP and a marginal negative correlation between fungal abundance and the lignolytic enzyme POX (Table 3).

The observed fungal and bacterial abundance patterns resulted in F:B ratios that were not significantly different among treatments, but were higher in the July sampling than in the April sampling ( $\sim 200\%$  higher,  $P < 0.001$ , Fig. 5C). There was a significant negative relationship between F:B ratios and C:N enzyme ratios (Fig. 5D) and a marginal negative relationship between F:B ratios and C:P enzyme ratios (Fig. 5E).

## 4. Discussion

*Rhododendron maximum* promotes a soil N feedback in Appalachian forests, in which soil N availability is limited by the preferential immobilization of N from *R. maximum* leaf litter by the plant's own mycorrhizal symbionts (Wurzburger and Hendrick, 2009). We predicted that the combination of *R. maximum* canopy and soil O-horizon removal

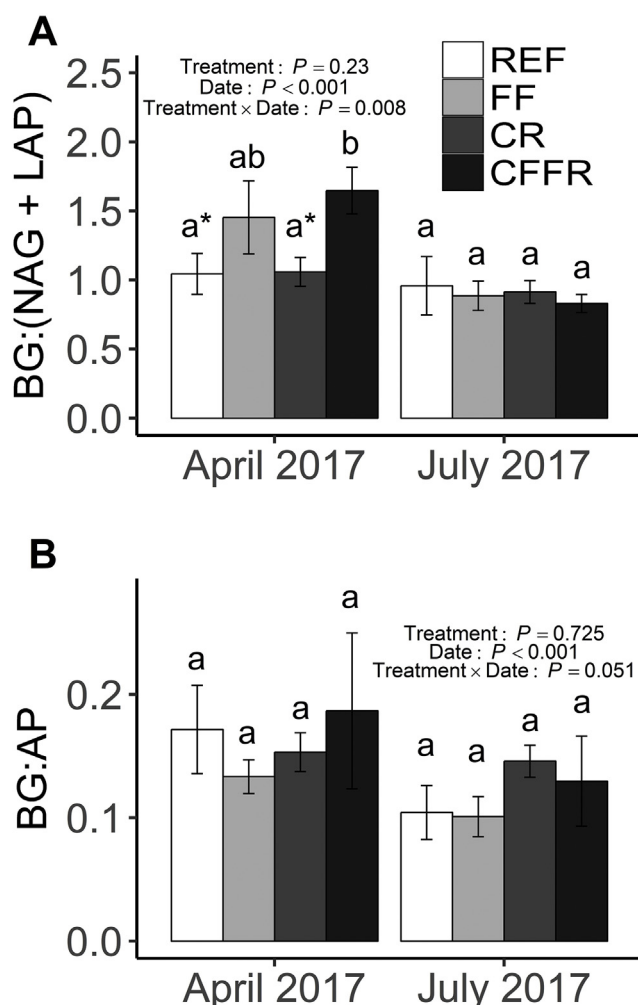




**Fig. 3.** Individual extracellular enzyme activities corrected for dry soil mass across treatments: BG (A), CHB (B), XYL (C), AP (D), LAP (E), NAG (F), POX (G), and PER (H). Bars represent means while error bars are  $\pm$  one standard error. *P*-values presented are linear mixed model effects of treatment. Different letters represent significant differences between treatments ( $P < 0.05$ ), while asterisks represent marginally significant differences ( $P < 0.1$ ). Treatment abbreviations are as follows: reference (REF), O-horizon removal (FF), canopy removal (CR), and canopy + O-horizon removal (CFFR).

would disrupt this feedback, resulting in increased soil N availability. Our results are generally consistent with this prediction, as TDN was higher in canopy + O-horizon removal plots compared with reference plots in our summer sampling (Fig. 1). We also observed increased DOC availability in canopy + O-horizon removal plots relative to reference plots (Table 2). The DOC and TDN responses may be explained by increased availability of C and N following prescribed burns. A parallel study to this one found that burning in the O-horizon removal plots and canopy + O-horizon removal plots resulted in temporary removal of the leaf litter (Oi) layer, which was replaced by litter fall the next year, with no apparent effects on Oe/a layers (Elliott and Miniati, 2018).

However, even a single low-intensity burn event may have generated the C and N responses we observed, as heat-alteration of organic compounds during low-intensity burns can promote microbial colonization of residues (Knicker, 2007), potentially mobilizing organic C and N from heat-altered *R. maximum* leaf litter. Similar responses of DOC and TDN in A-horizon soils in response to prescribed fire have been recently reported in other forested regions (Näthe et al., 2018). We also saw significantly higher TDN in canopy + O-horizon removal plots compared with O-horizon removal plots in our summer samples (Fig. 1). The lack of increase in TDN in O-horizon removal plots following burning may be due to continued N uptake by *R. maximum* roots



**Fig. 4.** C:N enzyme ratios (**A**) and C:P enzyme ratios (**B**) across treatments and sample dates.  $P$ -values presented are linear mixed model effects of treatment, sampling date, and treatment  $\times$  date interactions. Different letters represent significant differences between treatments within a sampling date ( $P < 0.05$ ), while asterisks represent marginally significant differences ( $P < 0.1$ ). Treatment abbreviations are as follows: reference (REF), O-horizon removal (FF), canopy removal (CR), and canopy + O-horizon removal (CFFR).

and associated mycorrhizae, which were still active in O-horizon removal plots, or may be due to incomplete O-horizon removal in these plots (Elliott and Miniati, 2018). The lack of treatment differences in TDN in our spring samples may have been due to delayed soil N response to removal treatments or due to N immobilization by soil bacteria, which were 70% more abundant in spring than summer and were more abundant in canopy + O-horizon removal plots than in other treatments (Fig. 5A), potentially dampening any treatment effects on TDN.

We also observed increases in inorganic N ( $\text{NH}_4$ ,  $\text{NO}_3$ ) in canopy + O-horizon removal plots compared with all other treatments (Table 2). This may be explained by direct conversion of organic N to inorganic N by combustion (Certini, 2005) or increased inorganic-N transformation rates following burns, as has been observed at other southern Appalachian sites (Knoepp et al., 2004). These effects, in combination with reduced inorganic N uptake by roots and mycorrhizae following *R. maximum* canopy removal, could have produced the observed trend, where both canopy removal and prescribed fire were necessary to increase concentrations of soil inorganic N.

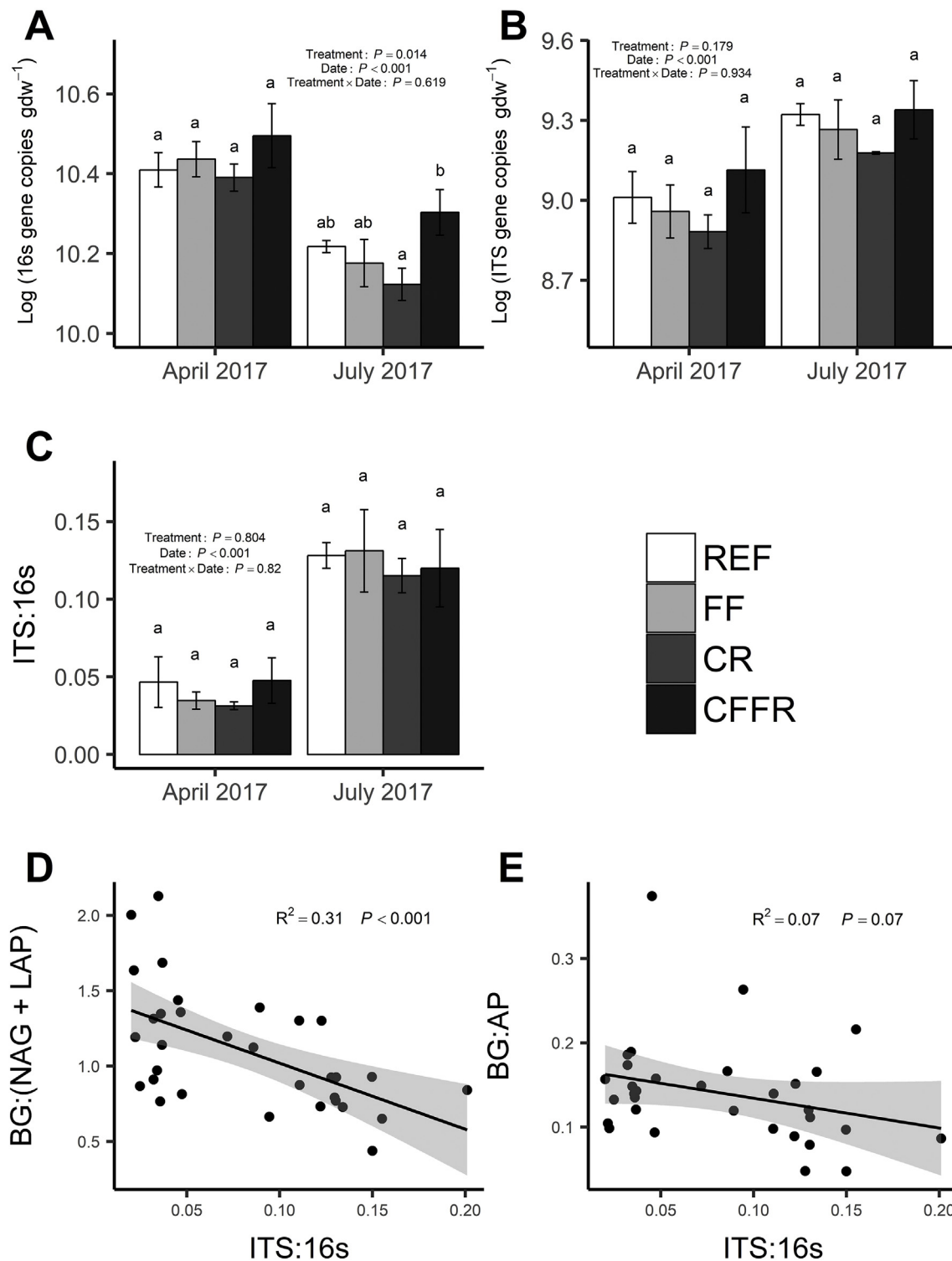
The observed increases in soil N and DOC were associated with an apparent increase in microbial C-demand, as two C-acquiring enzymes (BG, XYL) were elevated in canopy + O-horizon removal plots relative

to all other treatments (Fig. 3). Similar responses of C-acquiring enzymes to increased N availability have been observed in earlier studies in different regions (Allison and Vitousek, 2005; Geisseler and Horwath, 2009). Increased N availability can also result in reduced N-acquisition enzyme activity (Allison and Vitousek, 2005; Ramirez et al., 2012; Sinsabaugh et al., 2002), which is often explained using a resource allocation framework. Within this framework, microorganisms increase production of enzymes for acquiring scarce resources and reduce production of enzymes when resources are abundant (Allison et al., 2010). Our results appear to be inconsistent with this framework, as activity of all hydrolytic enzymes, including N-acquiring enzymes, were generally higher with *R. maximum* removal (Figs. 2A and 3). This response was likely driven by increases in microbial biomass, evidenced by the lack of treatment differences in biomass-corrected enzyme activities (Fig. 2B, Figure S1). This points to nutrient supply-driven enzyme production (i.e. biomass effects) as opposed to nutrient demand-driven enzyme production (i.e. resource allocation) in our soils, though the particularly strong response of C-acquiring enzymes to increased N suggest that some resource allocation may have occurred.

Interestingly, the largest observed differences in DOC and all hydrolytic enzyme activities were between the canopy + O-horizon removal and canopy removal only treatments (Table 2, Fig. 3). This may be explained by reduced root exudation of DOC following *R. maximum* cutting, which likely resulted in significant root die-back. Root exudation is known to stimulate microbial production of extracellular enzymes in rhizosphere soils (Brzostek et al., 2013), potentially accounting for the consistent responses of DOC and enzyme activities in this study. Though root die-back also likely occurred in canopy + O-horizon removal plots, DOC and TDN mobilized by prescribed fire may have compensated for reductions in root exudation.

Prior studies examining *R. maximum* effects on extracellular enzyme activities found elevated activities of phenol oxidase (POX) in O-horizon soils under *R. maximum* thickets (Wurzburger and Hendrick, 2007), leading us to predict that *R. maximum* removals would reduce lignolytic enzyme (POX, PER) production. Our results show no treatment effects on POX or PER activity (Fig. 3), likely because POX activity differences were previously shown in O-horizon soils of *R. maximum* thickets, while we measured enzyme activities only in A-horizon soils. The lack of treatment differences in POX and PER may also be due to incomplete O-horizon removal by fire, potentially resulting in similar availability of lignin-rich substrates across treatments.

Because bacteria are generally associated with higher growth rates and more copiotrophic lifestyles relative to fungi (Strickland and Rousk, 2010), we predicted that increases in DOC and N following *R. maximum* removal would stimulate bacterial growth and lead to bacterial-dominated microbial communities. Our results do not support this prediction; removal treatments did not result in differences in F:B ratios (Fig. 5C). Though our results do not show treatment effects on microbial community structure at this coarse scale, studies using more sophisticated molecular tools (i.e. 16s and ITS sequencing) have shown changes in bacterial and fungal communities following forest management practices (i.e. Bastida et al., 2017), highlighting the need for similar tools to be used in future studies to determine effects of *R. maximum* removal on microbial community structure. Though treatments did not affect F:B ratios in our study, we did observe a clear shift towards higher F:B ratios from spring to summer (Fig. 5C), which was due to simultaneous declines in bacterial abundance and increases in fungal abundance (Fig. 5A and B). The bacterial decline was likely linked to declines in soil moisture from spring to summer (Elliott and Miniati, 2018), while fungi are less susceptible to soil drying (Schimel et al., 2007). The increase in fungal abundance may be linked to seasonal increases in root C inputs to mineral soil, as has been shown in other forested regions (Voříšková et al., 2014). Seasonal increases in plant productivity may also have promoted growth of mycorrhizal fungi, potentially contributing to the high fungal abundance we observed in summer. This possibility is supported by the positive



**Fig. 5.** Bacterial (16s) abundance (**A**), fungal (ITS) abundance (**B**), and F:B ratios (**C**) across treatments and sample dates. Also shown are relationships between C:N enzyme ratios and F:B ratios (**D**) and relationships between C:P enzyme ratios and F:B ratios (**E**). Bars represent means while error bars are  $\pm$  one standard error.  $P$ -values presented are linear mixed model effects of treatment, sampling date, and treatment  $\times$  date interactions. Different letters represent significant differences between treatments within a sampling date ( $P < 0.05$ ). Treatment abbreviations are as follows: reference (REF), O-horizon removal (FF), canopy removal (CR), and canopy + O-horizon removal (CFFR).

correlations between fungal abundance and N- and P-acquiring enzymes (Table 3), which are known to be produced by mycorrhizae (Burke et al., 2011). Additionally, we found a negative correlation between fungal abundance and the lignolytic enzyme POX (Table 3), contrary to prior research on the lignolytic capabilities of soil fungi

(Strickland and Rousk, 2010). Other studies have reported similar negative correlations between lignolytic enzymes and soil fungi in forests (Brockett et al., 2012), and many mycorrhizal taxa may not be capable of producing lignolytic enzymes (Smith and Read, 2010), further supporting the possibility that many of the fungi we detected were

**Table 3**

Pearson correlation coefficients between 16s (bacterial) abundance, ITS (fungal) abundance, and eight extracellular enzymes. Values represent correlation coefficients (upper lines) and *P* values (lower lines). Correlation coefficients in bold represent significant relationships between variables (*P* < 0.05).

	BG	CHB	XYL	AP	LAP	NAG	POX	PER
16s Abundance	<b>0.381</b>	<b>0.500</b>	<b>0.394</b>	0.224	0.161	0.029	0.009	0.059
	0.032	0.004	0.026	0.218	0.378	0.876	0.962	0.505
ITS Abundance	0.117	0.062	0.288	<b>0.358</b>	0.264	<b>0.391</b>	−0.343	0.122
	0.523	0.736	0.110	0.044	0.144	0.027	0.055	0.574

mycorrhizae. We also observed significant positive correlations between bacterial abundance and C-acquiring enzymes (BG, CHB, and XYL) (Table 3), similar to previous reports (Brockett et al., 2012). Overall, the observed relationships between bacteria, fungi, and enzyme activities suggest functional differences between microbial communities with different F:B ratios. Prior studies report conflicting results regarding functional characteristics of communities with different F:B ratios, with some studies reporting functional differences (i.e. Blagodatskaya and Anderson, 1998; Malik et al., 2016), and others reporting no functional differences (i.e. Rousk and Frey, 2015; Thiet et al., 2006). Our results, which show clear associations between bacteria and fungi and specific extracellular enzymes, suggest that such functional differences may indeed exist.

The observed correlations between extracellular enzymes and bacterial vs fungal abundance (Table 3) resulted in C:N enzyme activity ratios that were negatively correlated with F:B ratios (Fig. 5D). Prior studies have shown low C- vs N-acquiring enzyme activity in arbuscular mycorrhizal (AM) fungi (Burke et al., 2011), potentially accounting for the negative F:B vs C:N enzyme relationship we observed if AM fungi are in fact abundant in our plots. Future studies should evaluate the hypothesis that abundance of mycorrhizal fungi can significantly affect patterns of extracellular enzyme activities measured in forest soils. In addition to relationships with F:B ratios, we observed treatment differences in C:N enzyme activity ratios that were dependent on sample date, with higher relative C-acquiring enzyme activity in canopy + O-horizon removal plots relative to O-horizon removal and reference plots only in the spring (Fig. 4A). These results suggest that extracellular enzyme responses to *R. maximum* removal are potentially dependent upon both season and the resident microbial community. Because microbial communities and associated extracellular enzyme activities differ among tree species in eastern US forests (Weand et al., 2010), we may also expect enzyme responses to *R. maximum* understory removal to depend upon the tree species composition of the remaining forest.

## 5. Conclusions

Overall, our results show that the combination of *R. maximum* canopy + Oi layer removal by burning increases soil C and N availability, resulting in increased microbial biomass and increased production of key microbial extracellular enzymes, while individual removal treatments had much smaller effects. Enzymes associated with C-acquisition show particularly strong responses, suggesting that soil C dynamics were altered with *R. maximum* removal. Further, responses to *R. maximum* removal were different between seasons, with a shift from relatively higher microbial C-acquiring enzyme activity in spring to relatively higher N-acquisition enzyme activity in summer, which was associated with increased F:B ratios. The observed increases in soil nutrients and microbial enzyme activity will potentially influence recovery rates of Appalachian forests, at least in the short term. Medium- and long-term microbial responses to *R. maximum* removal are difficult to predict; microbial activity may return to baseline levels after recovering from disturbances associated with removal treatments or may remain persistently higher due to continued absence of *R. maximum*. Regardless, these ecosystems should be continually monitored to

further inform the use of *R. maximum* removal to achieve forest management goals.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.soilbio.2018.09.008>.

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