

The stoichiometric legacy of fire regime regulates the roles of micro-organisms and invertebrates in decomposition

ORPHEUS M. BUTLER,¹ TOM LEWIS,² MEHRAN REZAEI RASHTI,¹ SARAH C. MAUNSELL,³ JAMES J. ELSER,⁴ AND CHENGRONG CHEN^{1,5}

¹Griffith School of Environment and Science and the Australian Rivers Institute, Griffith University, Nathan, Queensland, Australia

²Department of Agriculture and Fisheries and University of the Sunshine Coast, Sippy Downs, Queensland, Australia

³Department of Organismic and Evolutionary Biology, Harvard University, Boston, Massachusetts, USA

⁴Flathead Lake Biological Station, University of Montana, Polson, Montana, USA

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Abstract. Decadal-scale increases in fire frequency have the potential to deplete ecosystems of essential nutrients and consequently impede nutrient-limited biological processes via stoichiometric imbalance. Decomposition, a fundamental ecosystem function and strong driver of future fire occurrence, is highly sensitive to nutrient availability and is, therefore, particularly important in this context. Here we show that 40 yr of quadrennial (4yB) and biennial (2yB) prescribed burning result in severely P- and N-depleted litter stoichiometry, respectively, relative to fire exclusion. These effects exacerbated the nutrient limitation of microbial activities, constraining litter decomposition by 42.1% (4yB) and 23.6% (2yB) relative to unburned areas. However, invertebrate-driven decomposition largely compensated for the diminished capacity of micro-organisms under 4yB, suggesting that invertebrates could have an important stabilizing influence in fire-affected ecosystems. This effect was strongly positively coupled with the strength of microbial P-limitation and was not obviously or directly driven by fire regime-induced changes in invertebrate community assemblage. Together, our results reveal that high-frequency fire regimes promote nutrient-poor, carbon-rich ecosystem stoichiometry and, in doing so, disrupt ecosystem processes and modify the relative functionality of micro-organisms and invertebrates.

Key words: decay; eco-enzymes; invertebrates; litter; N : P ratio; phosphorus limitation; prescribed burning; stoichiometry.

INTRODUCTION

The increasing frequency, severity, and extent of vegetation fires associated with climate change will have significant and widespread ecological consequences (Noble and Slatyer 1981, Weber and Flannigan 1997, Westerling et al. 2011), many of which are poorly understood. In particular, changes in fire regime can trigger profound shifts in the balance or “stoichiometry” of carbon (C), nitrogen (N), and phosphorus (P) in ecosystems (Lagerström et al. 2009, Toberman et al. 2014, Butler et al. 2018), but the implications of such shifts are unclear. Recent or repeated fire events are often associated with N-depleted and P-enriched stoichiometry (i.e., lower N : P and C : P) in soil and plant material (Toberman et al. 2014, Muqaddas et al. 2015, Butler et al. 2018). Moreover, decadal-scale increases in fire frequency can lead to ecosystem P depletion through postfire leaching

and erosion (Pellegrini et al. 2018). Thus, protracted changes in fire regime could bring about severe stoichiometric imbalances between resources and consumers and, in doing so, alter the function of ecosystems by exacerbating the nutrient limitation of biological processes (Sterner and Elser 2002).

The process of plant litter decomposition is highly sensitive to stoichiometric imbalances on local scales (Enríquez et al. 1993, Güsewell and Gessner 2009, Mooshammer et al. 2012) and is particularly important in the context of climate change–altered fire regimes because of its roles in sequestering C and shaping fire fuel loads (Birk and Bridges 1989, Chapin et al. 2002, Brennan et al. 2006). Rates of microbially driven decomposition tend to be highest under C-limited (i.e., nutrient sufficient) conditions, under which mass loss through C hydrolyzation progresses rapidly (Enríquez et al. 1993, Güsewell and Gessner 2009). However, mass loss becomes constrained under N limitation (at high litter C : N or low litter N : P) and P limitation (at high litter C : P or N : P), because micro-organisms prioritize acquisition of the limiting nutrient over that of C (Sinsabaugh et al. 2009). Thus, the tendency for

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⁵Corresponding Author. E-mail: c.chen@griffith.edu.au

high-frequency fire regimes to deplete an ecosystem's nutrient stocks is likely to constrain microbially driven decomposition by inducing or exacerbating microbial N or P limitation.

However, during decomposition micro-organisms work in conjunction with litter invertebrate fauna, which influence decomposition via the regulation of micro-organism populations, and through the displacement, fragmentation, consumption, and conversion to frass of the litter material itself (Hättenschwiler et al. 2005). Prior research indicates that the contribution of invertebrates to decomposition can be enhanced under high-frequency fire regimes despite significant decreases in litter stoichiometric quality (e.g., increased litter C : N; Brennan et al. 2009). This suggests that invertebrate-driven decomposition is more resilient to stoichiometric imbalance than that of micro-organisms and might, therefore, be particularly important for the function and stability of fire-prone ecosystems. Such resilience could arise through various means. For instance, the greater mobility of invertebrates should enable preferential grazing of more-palatable litter components (Grime et al. 1996, Kagata and Ohgushi 2011, Dray et al. 2014), as well as recolonization and community restructuring in response to changing nutrient availability (Kaspari et al. 2017). Moreover, microbial decomposition is driven by the activities of extracellular coenzymes that are resource specific, and thus responsive to environmental cues and microbial demand for specific resources (Burns and Dick 2002). Invertebrates, on the other hand, typically ingest multiple elemental resources simultaneously, in composite form, and regulate resource assimilation internally (Behmer 2009, Halvorson et al. 2018). Thus, invertebrates may consume greater amounts of a low-quality resource to obtain the required amount of a limiting nutrient (Suzuki-Ohno et al. 2012, Jochum et al. 2017). Overall, it seems plausible that the contribution of invertebrates to decomposition will be enhanced under stoichiometric conditions where microbially driven decomposition is inhibited. However, we are aware of no studies that have explicitly investigated the relationships between diminished litter quality under high-frequency fire regimes and changes in the type and degree of nutrient limitation and relative functional contributions of micro-organisms and invertebrates.

We hypothesized that fire regimes strongly affect the C : N : P stoichiometry of litter and consequently the rates and relative importance of microbially- and invertebrate-driven decomposition, respectively. Thus, we ran a 277-d litter decomposition experiment across an experimental fire regime gradient in eastern Australia to evaluate the following predictions: (1) long-term, high-frequency fire will be associated with nutrient-limiting stoichiometric conditions for litter micro-organisms, (2) microbially driven decomposition will be constrained under fire regimes that produce strongly nutrient-limiting conditions, and (3) the positive effects of invertebrates on decomposition will be greatest under fire

regimes that induce or exacerbate microbial nutrient limitation.

MATERIALS AND METHODS

Study site and field methods

The study was carried out in a wet sclerophyll forest in southeast Queensland, Australia (Peachester State Forest; 26°52' S, 152°51' E). The forest is dominated by *Eucalyptus pilularis* (Blackbutt), with *Corymbia intermedia*, *E. microcorys*, and *Lophostemon confertus* (all Myrtaceae) also present in the canopy. The long-term average daily temperature at the study site is 23.3°C, and the mean annual precipitation is 1,684 mm (Bureau of Meteorology, 2018). Since 1969 the forest has been subjected to a long-term prescribed burning experiment that consists of three fire frequency treatments that have been maintained since the experiment was established in 1969: burned every 2 years on average since 1972 (2yB), burned every 4 years on average since 1972 (4yB), and unburned since 1969 (NB). Each treatment consisted of 4 randomized burning plots (as described by Toberman et al. 2014), with 12 burning plots in total. Each of these burning plots was ca. 27 × 30 m in area (Toberman et al. 2014). The main shortcoming of this design is that the treatments differ in time elapsed since the last fire as well as fire frequency, because the NB has remained unburned for over 40 years, whereas the 2yB and 4yB treatments were both burned 28 months prior to our study. Time since fire and fire frequency are, therefore, confounded for comparisons of NB against the 2yB and 4yB treatments, so treatment effects are most accurately attributed to fire “regime” rather than fire frequency per se.

In November 2015, 28 months after the most recent fires in the 2yB and 4yB treatments, samples of mixed-age *E. pilularis* leaf litter with minimal visual signs of herbivory and microbial activity were obtained from each burning plot. We ensured that only *E. pilularis* litter was collected and subsequently used in the decomposition experiment via visual assessment of leaf morphology. Within 1 week of sampling, individually labeled litter bags containing approximately 10 g (dry-weight equivalent) leaf litter that had been kept “fresh” (i.e., stored at 4°C and not dried) were installed in the field in 5 × 5-m experimental plots that were situated within the same larger burning plots from which the litter originated. Litter bags were 18 × 18 cm and were constructed with either (1) fine nylon mesh (aperture = 0.3 mm) on both sides or (2) fine nylon mesh on the downward-facing side and coarse mesh (aperture = 20 mm) on the upward-facing side, with a loose covering of 1-mm aperture mesh on top to prevent through-fall of litter or other material from above. The fine mesh-only bags were designed to exclude all but the smallest microinvertebrates (i.e., those with diameter <0.3 mm) and thus represent the effect of microbial community on litter decomposition (ML_{microbial}), whereas the bags with coarse mesh allowed

entry by surface-active invertebrates up to 2 cm in diameter and thus represent the combined effect of microbes and invertebrates (ML_{total}). Twelve litter bags of both types were installed in each of the 12 experimental plots (288 litter bags in total). Three bags of each type per experimental plot were then recollected on four dates over 277 d (days 24, 95, 188, and 277). There were no fires during the decomposition period.

Five days prior to each of the final three litter re-collection dates (days 95, 188, and 277), pitfall traps were installed in each experimental plot to assess the abundances of surface-active litter invertebrates. Pitfall trap arrays consisted of five 120-mL jars containing 50 mL of 70% ethanol distributed within a 1-m² area inside the 5 × 5-m litter bag area. Each jar was individually covered with a small plastic square “roof” that was elevated 3–5 cm above the litter surface using steel pegs. Pitfall traps were retrieved after ca. 120 h at the same time as litter bag retrieval. Invertebrates in the pitfall traps were sorted to Order.

Laboratory methods

Litter material from the three litter bags of each type per experimental plot was bulked together, weighed, and cut into small pieces (ca. 1 cm²) prior to analysis. Litter moisture was determined after drying at 65°C for 3 d. Oven-dried samples were finely ground prior to measurement of total C and N via dry-combustion (LECO CN analyzer; TruMac No. 830-300-400) and total P using molybdenum-blue spectrophotometry after digestion in nitric and perchloric acids (Jackson 1958, Murphy and Riley 1962). A separate portion of each litter sample was kept fresh (stored at 4°C) and used for analyses of litter pH and electrical conductivity (EC; litter:water ratio = 1 : 5), microbial biomass C (MBC), N (MBN), and P (MBP), and the potential activities of three extracellular enzymes involved in the acquisition of C, N, and P respectively, β -glucosidase (BG), *N*-acetyl- β -D-glucosaminidase (i.e., “chitinase”; CHN) and acid phosphatase (AP).

Litter MBC and MBN were measured via the chloroform fumigation method with 0.5-mol/L K₂SO₄ used as an extractant (Brookes et al. 1985, Vance et al. 1987). Concentrations of soluble organic C and total soluble N in K₂SO₄ extracts were quantified by high-temperature catalytic oxidation using a Shimadzu TOC-VCPH/CPN analyzer fitted with a TN unit (Shimadzu Scientific Instruments, Sydney, Australia). Litter MBP was also measured using chloroform fumigation, but with 0.5 mol/L NaHCO₃ as an extractant (Brookes et al. 1982). Concentrations of PO₄³⁻ in NaHCO₃ extracts were quantified with molybdenum-blue spectrophotometry. Concentrations of soluble organic C, total soluble N and PO₄³⁻ in extracts of nonfumigated samples were used to represent litter soluble C, N, and P (Toberman et al. 2014). The potential activities of BG, CHN, and AP were determined using *p*-nitrophenol spectrophotometric

methods (Tabatabai and Bremner 1972, Eivazi and Tabatabai 1977, 1988). All microbial biomass C, N, and P extractions occurred within 28 d of sampling, and enzyme activities were assayed within 15 d of sampling.

Statistical analyses

All analyses were conducted in R (R Core Team 2014). One-way analysis of variance was used to determine the effects of fire regime on the properties of the initial litter samples. Tukey’s honestly significant difference (HSD) test was used to make post hoc comparisons of fire regime means where required. Further, we used a one-tailed Student’s paired *t*-test to establish whether invertebrates had a detectable positive effect on litter mass loss. Paired differences ($ML_{total} - ML_{microbial}$) were not normally distributed (Shapiro-Wilk test *P* values were <0.001); therefore, mass loss data were log-transformed prior to *t*-testing (Shapiro-Wilk test *P* values for paired differences after log-transformation = 0.059). Additionally, we calculated decomposition rate constants *k*, using the following formula: $M_t = M_0 e^{-kt}$, where M_t = litter mass at a given collection time, *t* (measured in days), and M_0 = initial litter mass. Values of *k* are provided in Appendix S1: Table S1.

To evaluate the importance of the fire regime variable as a predictor of numerous biological and stoichiometric properties of litter throughout the experiment, we used a linear mixed-effects (LME) model comparison approach (with models fitted by restricted estimate maximum likelihood) using the lme4 package in R (Bates et al. 2015). Each litter property was analyzed separately. In some cases, litter property data were log-transformed prior to analysis to meet the assumption of homoscedasticity better. For each litter property, a “full” model consisting of “days of decomposition” (as a fixed effect, coded as a continuous variable), bag type (as a fixed effect), fire regime (as a fixed effect), and a random intercept effect for experimental plot was compared to a null model that consisted of the full model but without the fire regime term (*n* = 96). By including “days of decomposition” as a fixed effect in null models, we were able to establish the effect of fire regime while controlling for the temporal variation in litter properties throughout decomposition. Comparisons of full and null models (i.e., models with and without the fixed effect of fire regime) were made using *F*-tests based on the Kenward-Roger approximation for degrees of freedom (Luke 2017), using the “pbkrtest” package (Halekoh and Højsgaard 2014).

We used a similar approach to evaluate the effects of fire regime (as a fixed effect), bag type (as a fixed effect) and the fire regime × bag type interaction on litter mass loss. Mass loss data were natural log-transformed prior to LME model analyses to meet the assumption of homoscedasticity. First, we analyzed the bag types separately (*n* = 48), with full models consisting of “days of decomposition” (as a fixed effect), fire regime (as a fixed effect), and experimental plot as a random intercept

effect. As for other litter properties, null models were equivalent to full models but did not include a term for the fixed effect of fire regime (Appendix S1: Table S2). We then added the fixed effect of bag type to the full and null models and performed the model comparisons across both bag types so that fire regime was tested across both bag types ($n = 96$; Appendix S1: Table S2). We evaluated the effect of bag type (as a fixed effect), and the interaction between fire regime and bag type, on litter mass loss in the same manner (Appendix S1: Table S2). For bag type, the full model consisted of “days of decomposition” (as a fixed effect), bag type (as a fixed effect), and a random intercept term for experimental plot, whereas the null model was equivalent to the full model but did not contain a term for bag type. The effect of the interaction between bag type and fire regime was tested by comparing a full model consisting of “days of decomposition” (as a fixed effect), fire regime (as a fixed effect), bag type (as a fixed effect), the interaction between fire regime and bag type, and a random intercept term for experimental plot, to a null model that was equivalent to the full model but only included main effects (i.e., the interaction between fire regime and bag type was not included in the null model; Appendix S1: Table S2).

We then assessed the role of nutritional constraints (i.e., N and P limitation) in driving the potential differences in litter mass loss between fire regime treatments using a similar technique. Here, we used enzymatic stoichiometric ratios as indicators of proportional resource demand, or the tendency towards limitation by a particular elemental resource (i.e., C, N, or P; Sinsabaugh et al. 2008). Specifically, we evaluated the importance of the interaction between fire regime and each ecoenzymatic stoichiometric ratio for predicting litter mass loss. We interpreted significant interactions between fire regime and enzyme activity ratios as fire regime changing the manner in which the ratio was coupled with, or influenced, decomposition. To complement these analyses we used Pearson’s correlation to analyze relationships between overall enzyme potential activities and litter total N, total P, and total N : P, which were used to represent the overall and proportional potential supply of these nutrients.

The proportional effect of invertebrates on litter mass loss ($ML_{\text{invertebrates}} \% = [ML_{\text{total}} - ML_{\text{microbial}}]/ML_{\text{total}} \times 100$) was analyzed using the same LME modeling approach as overall mass loss. We also tested the main effects of, and interactions of fire regime with, the following characteristics of the surface-active forest floor invertebrate community on $ML_{\text{invertebrates}}$: ordinal richness and diversity (Shannon diversity, H'), total invertebrate abundance, and the abundance and relative abundance of Formicidae (ants; order: Hymenoptera), Coleoptera, and Collembola, which were the three most abundant taxa in pitfall traps. Finally, we used non-metric multidimensional scaling analyses (NMDS), permutational analysis of variance (PERMANOVA), and a

surface-fitting ordination method based on generalized additive models (GAMs; “ordisurf” in “vegan”) to analyze relationships between fire regime, invertebrate community composition, and the effect of invertebrates on decomposition. The NMDS and PERMANOVA analyses were performed separately for each invertebrate sampling date (i.e., days 95, 188, and 277) and raw abundance data were log transformed ($\log_e[x + 1]$) before calculation of Bray-Curtis dissimilarity. The “ordisurf” method uses generalized additive models to predict $ML_{\text{invertebrates}}$ based on a two-dimensional smooth of NMDS site scores on the first and second NMDS axes.

RESULTS

Effects of fire regime on litter chemical and biological properties

Initial one-way ANOVAs indicated that the concentrations of total, soluble, and microbial biomass C, N, and P in the initial litter samples were largely unaffected by fire regime treatment, with the exception of soluble P, which was significantly lower in the 4yB treatment than in the NB treatment (Table 1). Litter total N : P was reduced significantly in the 2yB treatment relative to other treatments, whereas litter soluble C : P was significantly higher in the 4yB treatment than the NB and 2yB treatments. Litter soluble N : P was highest in the NB treatment and lowest in the 2yB treatment ($P = 0.03$), as was litter moisture ($P = 0.008$). Litter pH and EC were not affected by fire regime.

Linear mixed-effects models revealed additional effects of fire regime and further indicated that many fire-induced changes to litter properties were maintained throughout decomposition (Appendix S1: Table S2). Given our core hypothesis, here we focus on stoichiometric properties rather than overall concentrations or activities. According to LMEs, litter total C : N : P ratios varied significantly among fire regime treatments throughout the entire decomposition period, with total C : N ratios significantly lower in the NB treatment compared to 2yB and 4yB (Fig. 1a), and total N : P ratios significantly lower in the 2yB treatment compared to NB and 4yB (Fig. 1c). Further, litter total C : P ratios were significantly higher in the 4yB treatment throughout decomposition relative to the 2yB treatment (Fig. 1b). The same pattern occurred for litter soluble C : P and N : P ratios (Fig. 1e, f), whereas soluble C : N ratios were unaffected by fire regime (Fig. 1d). The stoichiometry of microbial biomass throughout the decomposition period was affected by fire regime in a similar, but comparatively modest manner, with microbial C : N ratios higher in the 2yB than in the NB treatment, and C : P and N : P ratios lower in the 2yB treatment than in the 4yB treatment (Appendix S1: Table S2). Finally, patterns of eco-enzymatic stoichiometry throughout decomposition revealed that the relative

TABLE 1. Initial chemical properties (means \pm standard error; $n = 4$) of fresh *Eucalyptus pilularis* leaf litter collected from the Peachester State Forest prescribed burning experiment (Queensland, Australia) in November 2015; NB = unburned treatment, 4yB = quadrennially burned treatment; 2yB = biennially burned treatment.

Litter property†	NB	4yB	2yB	One-way ANOVA $F_{2,9}$ statistic (P value)
Moisture %	20.3 ^A (± 0.9)	17.5 ^{AB} (± 1.7)	13.8 ^B (± 0.2)	8.75 (0.008)
pH	3.72 (± 0.09)	3.54 (± 0.03)	3.56 (± 0.05)	2.45 (0.142)
EC ($\mu\text{S}/\text{cm}$)	444 (± 55.2)	365 (± 28.4)	299 (± 20.3)	3.69 (0.067)
Total C (%)	51.9 (± 0.3)	51.7 (± 0.6)	52.2 (± 0.3)	0.43 (0.663)
Total N (%)	0.60 (± 0.06)	0.52 (± 0.03)	0.45 (± 0.03)	3.75 (0.065)
Total P (%)	0.017 (± 0.002)	0.012 (± 0.001)	0.017 (± 0.001)	3.80 (0.064)
SOC (mg/kg)	3,169 (± 738)	2849 (± 225)	2,589 (± 233)	0.39 (0.689)
Soluble N (mg/kg)	242 (± 117)	46.0 (± 12.1)	19.1 (± 6.0)	3.18 (0.090)
Soluble P (mg/kg)	11.4 (± 2.0) ^A	6.3 (± 0.6) ^B	10.4 (± 0.7) ^{AB}	4.51 (0.044)
MB C (mg/kg)	14,415 (± 1655)	13,405 (± 2177)	13,368 (± 3511)	0.05 (0.948)
MB N (mg/kg)	805 (± 241)	369 (± 147)	522 (± 185)	1.29 (0.322)
MB P (mg/kg)	74.3 (± 19.3)	33.3 (± 3.5)	40.3 (± 7.5)	3.26 (0.086)
Total C : N	88.6 (± 9.5)	100.1 (± 6.7)	117.7 (± 7.3)	3.41 (0.079)
Total C : P	3,293 (± 652)	4595 (± 483)	3,048 (± 222)	2.93 (0.105)
Total N : P	36.4 ^A (± 3.3)	45.8 ^A (± 2.6)	25.9 ^B (± 0.8)	16.3 (0.001)
Soluble C : N	19.6 (± 5.0)	95.4 (± 45.5)	185.4 (± 61.6)	3.51 (0.075)
Soluble C : P	276 ^A (± 40.6)	462 ^B (± 58.9)	252 ^A (± 26.5)	6.86 (0.016)
Soluble N : P	18.6 ^A (± 6.1)	7.3 ^{AB} (± 2.1)	1.8 ^B (± 0.6)	5.34 (0.030)
MB C : N	21.7 (± 4.5)	60.1 (± 22.5)	30.9 (± 10.9)	1.87 (0.209)
MB C : P	244 (± 74.0)	400 (± 32.9)	400 (± 148)	0.85 (0.461)
MB N : P	14.1 (± 5.5)	12.0 (± 5.7)	14.2 (± 5.6)	0.05 (0.953)

† EC = electrical conductivity; soluble C and N = total 0.5 mol/L K_2SO_4 extractable organic C and N; soluble P = 0.5 mol/L NaHCO_3 extractable PO_4^{3-} ; MB = microbial biomass; SOC = soluble organic carbon; Tukey's HSD post hoc test letters provided where one-way ANOVA P values were < 0.05 (different letters indicate significantly different means).

nutrient demands of litter micro-organisms varied significantly between the fire regimes. Microbial demand for P relative to C in the 2yB treatment was 52.9 and 65.8% lower than in the NB and 4yB treatments, respectively (Fig. 1h). Moreover, microbial demand for P relative to N in the 2yB treatment was 93.5% lower than in the NB treatment and 66.4% lower than in the 4yB treatment (Fig. 1i).

Effects of fire regime on overall decomposition processes

When we controlled for litter bag retrieval time, LME model comparisons confirmed that microbially driven decomposition (i.e., decomposition in fine-mesh litter bags; $\text{ML}_{\text{microbial}}$) progressed more rapidly in the NB than the 4yB treatment (Fig. 1j; $P = 0.004$; Tukey's HSD P value = 0.003). On the other hand, fire regime had no strong effects on litter mass loss when micro-organisms and invertebrates were both involved in decomposition (i.e., in coarse-mesh litter bags; ML_{total} ; $P = 0.157$; Fig. 1k). However, bag type was not significant in LME models and when the effect of fire regime was tested across all bag types mass loss was again greater in the NB treatment than the 4yB treatment (Tukey's HSD P value = 0.008; Fig. 1l). Litter mass loss tended to be lower in the 2yB than the NB treatment for most sampling dates, but this difference was not statistically significant (Tukey's HSD P value for $\text{ML}_{\text{microbial}} = 0.055$;

Tukey's HSD P value for all bags together = 0.066). The interaction between fire regime and bag type was not significant according to LME models ($P = 0.157$).

Fire regime clearly modified the relationships between enzymatic stoichiometry and litter mass loss (Fig. 2a; Appendix S1: Table S2 and Fig. S1). Specifically, interactive effects of fire regime and enzymatic stoichiometry were highly significant for BG : CHN (C-acquiring; N-acquiring enzymes) and BG : AP (C-acquiring; P-acquiring enzymes; P values < 0.001), and marginally significant for CHN : AP ($P = 0.04$). The effect was most strongly pronounced for the fire regime \times BG : AP interaction (Fig. 2a; $P < 0.0001$), and was characterized by a strong, positive relationship between mass loss and BG : AP in the 4yB treatment. The potential activities of BG, CHN, and AP were strongly and positively associated with litter total N content (Pearson's correlation P values < 0.001 ; Fig. 3; Appendix S1: Table S3).

Effects of fire regime on invertebrates and their role in decomposition

Surface-active invertebrates had both positive and negative effects on decomposition, depending on fire regime and litter bag retrieval date (Appendix S1: Fig. S2), with values of $\text{ML}_{\text{invertebrates}}$ (i.e., the proportional effect of invertebrates on litter mass loss) ranging from -92.5 to 52.7% (overall mean = 4.4% ; standard

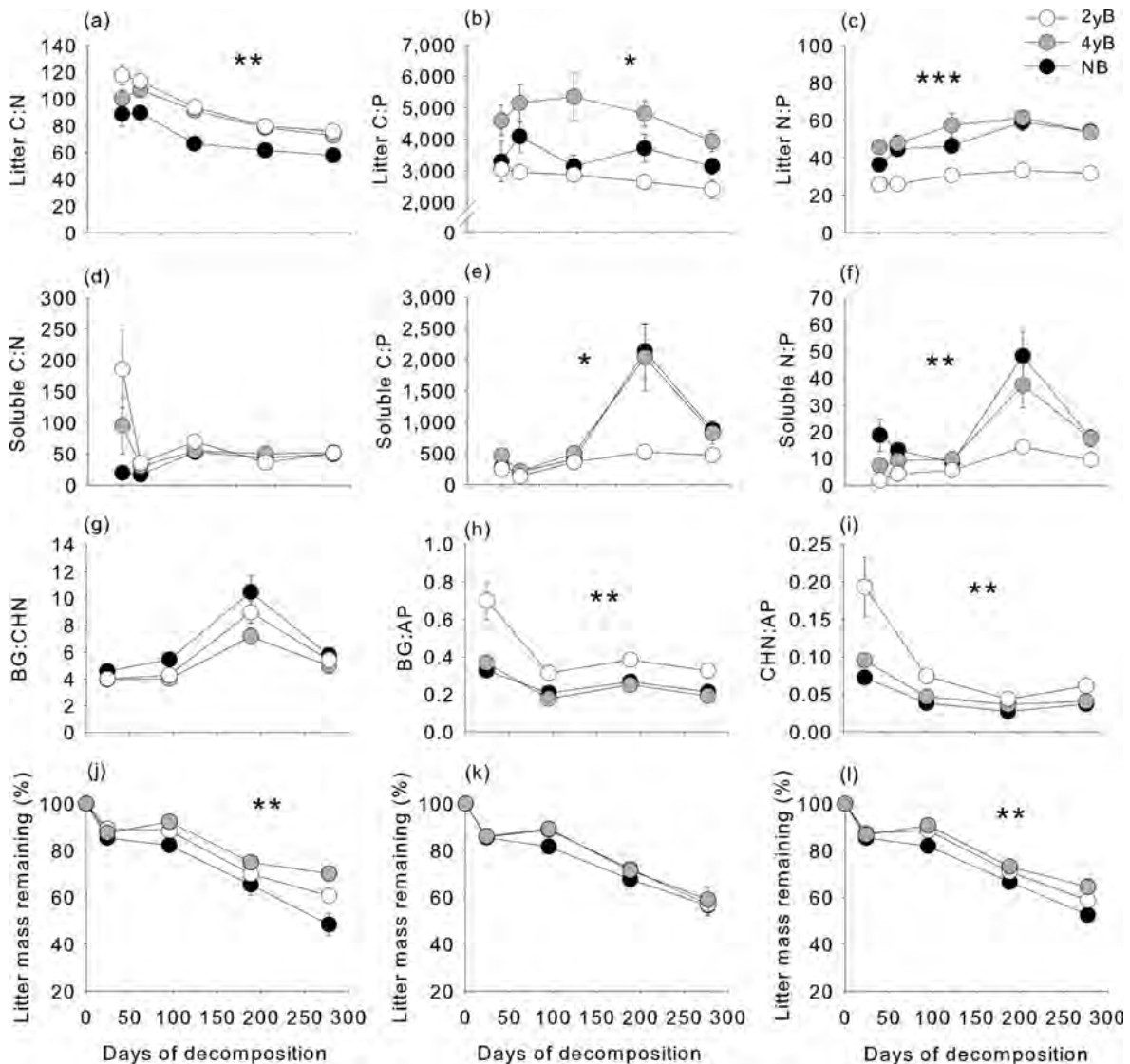


FIG. 1. Litter stoichiometric properties and mass loss throughout decomposition (means \pm standard error; $n = 8$), compared among the unburned (NB; black circles), quadrennially burned (4yB; grey circles), and biennially burned (2yB; white circles) treatments. Panels (a)–(c) show litter total carbon (C) : nitrogen (N), C : phosphorus (P), and N : P ratios, respectively; panels (d)–(f) show litter soluble C : N, C : P, and N : P ratios, respectively; panels (g)–(i) show litter β -glucosidase (BG):chitinase (CHN), BG : acid phosphatase (AP), and CHN : AP ratios, respectively (PNP = *p*-nitrophenol); and panels (j)–(l), respectively show the remaining percentage of litter mass in fine-meshed bags only ($ML_{\text{microbial}}$), in coarse-meshed bags only (ML_{total}), and across all bags; *P* values indicate significance of fire regime based on linear mixed-effect model comparisons: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

error = 3.5%; Student's one-tailed paired *t*-test *P* value = 0.02 for $ML_{\text{microbial}}$ vs. ML_{total}). Thus, litter bag type did not have a significant overall effect on mass loss when litter bag retrieval date and the random effect of experimental plot were accounted for in LME models (Appendix S1: Table S2). However, $ML_{\text{invertebrates}}$ varied significantly among the fire regimes when we controlled for the effects of litter bag retrieval date and experimental plot ($P = 0.03$; Appendix S1: Table S2). Specifically, the effect of invertebrates on decomposition was greater in the 4yB treatment than in the NB treatment (Tukey's

HSD test $P = 0.02$; Appendix S1: Fig. S2). In addition, the same LME model comparison approach indicated that $ML_{\text{invertebrates}}$ was strongly coupled with BG : AP, but that the nature of this coupling differed among fire regime treatments (fire regime \times BG : AP interaction P value = 0.027). Specifically, $ML_{\text{invertebrates}}$ increased with BG : AP in the 2yB treatment, but declined with BG : AP in the 4yB and NB treatments (Fig. 2b). Because of the presence of a single outlier in the BG : AP data (BG : AP = 1.36 in the 2yB treatment), we opted to perform the same LME analysis using log-

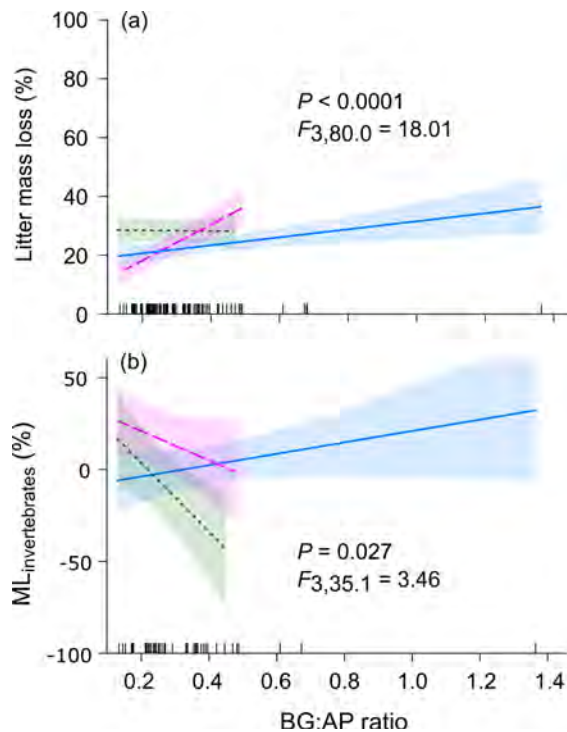


Fig. 2. Interaction plots from linear mixed-effect models showing the effect of fire regime treatment on the relationships between (a) litter β -glucosidase (BG):acid phosphatase (AP) ratios and litter mass loss (%; $n = 32$ for each fire regime treatment) and (b) litter BG : AP and the proportional effect of invertebrates on litter mass loss ($ML_{\text{invertebrates}}$; %; $n = 16$ for each fire regime treatment) when the effects of time, experimental plot, and fire regime (as a main effect) have been accounted for; for both panels, unburned treatment (NB) = green lines with short dashes, quadrennially burned treatment (4yB) = red lines with long dashes, and biennially burned treatment (2yB) = blue, solid lines; colored bands around lines indicate regions of 95% confidence. Tick marks on the x-axes represent observations of BG : AP, which in panel (a) ranged from 0.15 to 1.36 in 2yB, 0.13 to 0.48 in 4yB, and 0.14 to 0.46 in NB (overall mean = 0.34) and in panel (b) ranged from 0.15 to 1.36 in 2yB, 0.13 to 0.48 in 4yB, and 0.14 to 0.42 in NB (overall mean = 0.31); P values and F scores indicate significance of the BG : AP \times fire regime interactive effect on litter mass loss (panel a) and $ML_{\text{invertebrates}}$ (panel b).

transformed BG : AP data. We found that the relationships between $ML_{\text{invertebrates}}$ and BG : AP were similar (Appendix S1: Fig. S3; $P = 0.056$); thus, we focus on the results for untransformed BG : AP data hereafter.

We recorded a total of 15,322 invertebrate specimens in our pitfall traps, representing six taxonomic classes and 20 orders (Appendix S1: Table S4). All specimens were small enough that they could have passed through the 20-mm aperture mesh of the ML_{total} litter bags. Our NMDS and PERMANOVA analyses indicated that differences in community composition among fire regimes were significant on day 188 (PERMANOVA P value = 0.021) but not on days 95 or 277 (Fig. 4). The NB and 4yB

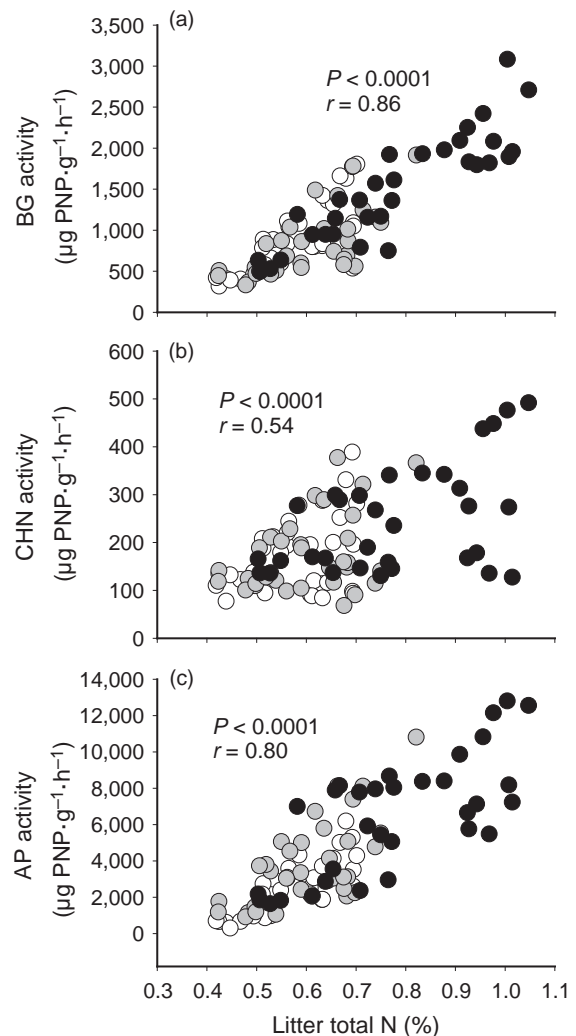


Fig. 3. Pearson's correlations between litter total nitrogen (N) and the potential activities of (a) β -glucosidase (BG), (b) chitinase, (c) acid phosphatase (AP); black circles = unburned treatment (NB) observations ($n = 32$), gray circles = quadrennially burned treatment (4yB) observations ($n = 32$), white circles = biennially burned treatment (2yB) observations ($n = 32$); Pearson's correlation coefficients (r) and P values are shown.

treatments were clearly differentiated in NMDS ordinations for day 188, and this effect was largely due to differences in the abundances of Araneae, Coleoptera, Psocoptera, and Thysanoptera (Appendix S1: Table S4).

According to LME models, $ML_{\text{invertebrates}}$ was not clearly related to total abundance, ordinal richness, ordinal diversity, the total or relative abundances of ants, Coleoptera or Collembola, or to the interaction of these variables with fire regime (P values > 0.05 in all cases; Appendix S1: Table S2). We did find some evidence, based on NMDS surface fitting, that $ML_{\text{invertebrates}}$ was related to invertebrate community composition on the level of order (Fig. 4), but this effect was modest and inconsistent between invertebrate sampling dates.

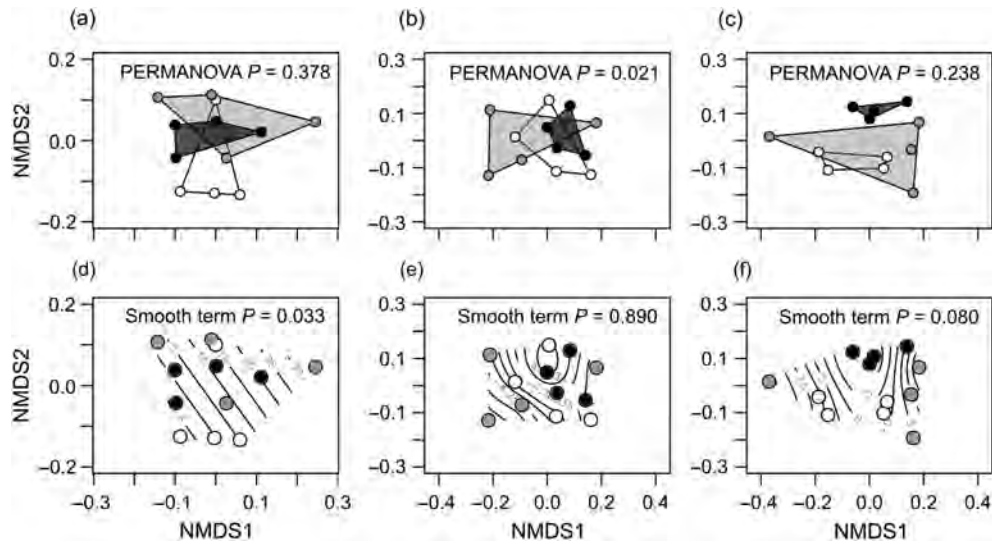


FIG. 4. Invertebrate communities represented using nonmetric multidimensional scaling (NMDS) ordinations (number of dimensions, $k = 2$) for (a) day 95 (stress = 0.15), (b) day 188 (stress = 0.17), and (c) day 277 (stress = 0.11; black hulls = unburned treatment [NB], gray hulls = quadrennially burned treatment [4yB], and white hulls = biennially burned treatment [2yB]) with P values indicating significance of fire regime based on permutational analysis of variance (PERMANOVA), and 'ordisurf' analyses of relationships between invertebrate community assemblage and proportional effect of invertebrates on litter mass loss ($ML_{\text{invertebrates}}\%$) on (d) day 95, (e) day 188, and (f) day 277, with two-dimensionally smoothed NMDS site scores used to predict $ML_{\text{invertebrates}}$ with generalized additive models (black circles = NB, grey circles = 4yB, and white circles = 2yB).

Specifically, there was a significant relationship between NMDS site scores and $ML_{\text{invertebrates}}$ on day 95 that was approximately linear on the first NMDS axis, no significant relationship on day 188, and a marginally significant nonlinear relationship on day 277 that was characterized by lower values of $ML_{\text{invertebrates}}$ around the center of the ordination space.

DISCUSSION

Ours is the first study to show that the stoichiometric imbalances that result from increasing fire frequency can impede microbially-driven decomposition and alter the relative functional contributions of micro-organisms and surface-active invertebrates. This effect was somewhat more robust and consistent for P than N (Fig. 1). Our results also indicate that fire regime-induced stoichiometric imbalances depend strongly on fire frequency, given that the 2yB and 4yB treatments were associated with N and P depletion, respectively. This is consistent with the findings of Toberman et al. (2014), who reported that litter in the biennially burned areas at Peacheseter had a lower N : P (N : P range: 31–36) than litter in unburned areas (N : P range: 51–56), but that the stoichiometry of litter in quadrennially burned areas did not differ from that of litter in unburned areas. The low N content of 2yB litter was most probably caused by repeated losses of ecosystem N through volatilization during combustion (Raison et al. 1984). Thus, the N-rich stoichiometry of 4yB litter, relative to 2yB litter, can be attributed to the greater fire return interval,

within which ecosystem N stocks might recover from losses of volatilized N (Toberman et al. 2014, Muqaddas et al. 2015). In contrast, the P-rich stoichiometry of 2yB litter suggests that *E. pilularis* trees in the 2yB treatment were using P in a manner that was not as conservative as their 4yB and NB counterparts. We suggest that this “leaky” P cycling was caused by the transient pulses in soil P availability that typically follow fires (Butler et al. 2018), and has the potential to accelerate ecosystem P depletion through runoff and leaching over the longer term (Peltzer et al. 2010, Pellegrini et al. 2018). Thus, the different P states in 2yB and 4yB litter are likely the result of interactions between fire regime and the temporal dynamics of rainfall events that drive runoff and leaching.

The depletion of litter nutrients under the high-frequency fire regimes had clear implications for decomposition. Litter mass loss was driven largely by loss of C (as opposed to N or P; Fig. 1a, b), much of which occurs through hydrolysis of organic C via BG activity (Burns and Dick 2002). In the 4yB treatment, BG activity was likely suppressed in favor of AP due to the strongly P-limited stoichiometric conditions (Mooshammer et al. 2012) and this had a clearly constraining influence on decomposition (Fig. 2a). However, C demand was not similarly suppressed by the N-depleted stoichiometry characteristic of 2yB litter. Given that enzymes are N-rich molecules (Sternner and Elser 2002), it might have instead been the synthesis of enzymes themselves that was N-limited and thus impeding decomposition in the N-depleted 2yB treatment. The strong couplings of

potential BG, CHN, and AP activities with litter total N support this view (Fig. 3; P values < 0.001), as do the low overall potential BG and AP activities in the 2yB treatment (LME model P values < 0.01) along with prior reports of increased litter BG and AP activities following N fertilization (e.g., Papanikolaou et al. 2010). On the other hand, microbially-driven decomposition of NB litter was most likely energy or C-limited and, therefore, progressed rapidly relative to that of 2yB and 4yB litter.

We acknowledge that much of fire regime's influence on decomposition is likely driven by other factors in addition to litter stoichiometry, particularly the consistently higher levels of moisture in the NB than in the 2yB ($P = 0.006$) and 4yB ($P = 0.056$) treatments (Table 1 and Appendix S1: Table S1). Disentangling the respective roles of, and interactions between, moisture availability, temperature, and litter stoichiometry in driving the effects of fire regime on decomposition will be an important focus for future work. However, our LME modeling approach allowed us to examine the effects of fire regime \times resource limitation interactions on decomposition while accounting for the more general effects of fire regime. Moreover, reciprocal litter transplant studies have shown litter chemistry to be a significant driver of decomposition in fire-affected ecosystems (Ficken and Wright 2017). Thus, without discounting the critical roles of moisture and other environmental factors, our study provides robust evidence that the stoichiometric imbalances associated with increasingly frequent fire can exert strong, constraining influences on decomposition.

Our results also hint at a potentially important relationship between the microbe- and invertebrate-driven aspects of decomposition in fire-affected systems, wherein the contribution of surface-active invertebrates to decomposition increases with the strength of microbial nutrient limitation (as indicated by eco-enzymatic stoichiometry; Fig. 2b). This relationship was only evident in the 4yB treatment and was only present between $ML_{\text{invertebrates}}$ and BG : AP ratios (i.e., BG : CHN and CHN : AP were not significant covariates in LME models; Appendix S1: Table S2). These findings are partially consistent with our prediction that invertebrate-driven decomposition would be more resilient to elemental imbalance than microbially-driven decomposition. However, they also reveal that invertebrate-driven decomposition at Peachester is more resilient to litter P depletion than to litter N depletion. We suggest that this resilience is driven by the tendency of invertebrates to ingest multiple elemental resources in composite form and regulate resource assimilation internally (Behmer 2009, Halvorson et al. 2018), such that the invertebrates in 4yB experimental plots might have consumed larger amounts of low-quality litter to obtain the required amount of P (Suzuki-Ohno et al. 2012, Jochum et al. 2017). The prospect of such a mechanism is strengthened by the seemingly limited role of invertebrate community characteristics in determining $ML_{\text{invertebrates}}$ in our study (Appendix S1: Table S2,

Fig. 4). We also note that the presence of invertebrates and their nutrient-rich frass might have stimulated microbially driven decomposition where it was otherwise impaired by P-limitation (Zimmer and Topp 2002, Kagata and Ohgushi 2011). We are not certain about the role of frass in our study, however, because frass can inhibit litter decomposition in some circumstances (Schowalter et al. 2011).

Previous studies have found that fire's effects on decomposition are mediated to at least some extent by invertebrate community assemblage (Brennan et al. 2009). The coarse level of taxonomic resolution in our study might have affected our ability to identify relationships between invertebrate community characteristics and decomposition. In particular, the Coleoptera and Formicidae present at Peachester likely include predatory and nonpredatory taxa that influence decomposition in different ways, and we were unable to account for this in our analyses. We note, however, that in the NMDS ordinations (Fig. 4) the NB experimental plots tended to cluster together whereas, overall, the 2yB and 4yB treatments tended to have more heterogeneous invertebrate assemblages, as suggested by the larger polygons for those treatments. Such effects are not unexpected, given that fire intensity and severity vary spatially on local scales because of variation in fuel load, fuel moisture and other factors, leading to spatial heterogeneity in the environmental conditions that shape biological communities (e.g., Rice 1993, Bowman et al. 2016). It seems possible that within-treatment community heterogeneity could correspond to invertebrate-driven decomposition that is, on average, somewhat resilient to the nutrient depletion in the 2yB and 4yB treatments, particularly if communities have been shaped by their local stoichiometric context.

Recycling of litter nutrients via decomposition is essential for plant growth and ecosystem functioning (Chapin et al. 2002), particularly where soils have inherently low levels of mineral nutrients. Our study reveals that frequent fire and fire exclusion have stoichiometric signatures in the decompositional environment that are conducive to slower and faster microbially-driven litter decay, respectively. Thus, the decadal-scale increases in fire frequency expected under climate change (Liu et al. 2010, Westerling et al. 2011) could result in ecosystems that are increasingly dependent on fire, which is limited only by energy (i.e., C; Bond and Keeley 2005), to remobilize litter-bound nutrients. At the same time, low rates of decomposition in fire-affected ecosystems promote fuel accumulation and thus reoccurrence of fire (Bradstock 2010, Bradstock et al. 2010).

Together, these effects are consistent with a positive feedback between fire, nutrient dynamics, and ecosystem flammability that underpins the ubiquity of pyrophilic vegetation on the Australian continent (Orians and Milewski 2007, Odion et al. 2010, Wood and Bowman 2012). In Australia, fires tend to promote a grassy understory and a high proportion of sclerophyllous taxa (e.g.,

Eucalyptus spp.; Noble and Slatyer 1981, Lewis et al. 2012), whereas long-term changes in fire regime can drive the shift between rainforest, wet and dry eucalypt forest, and grassland (Bowman 2000, Wood and Bowman 2012). Many of the plant taxa that have a positive association with fire have foliage with low nutrient levels and high proportions of woody, C-rich and, importantly, energy-dense and flammable tissue (Orians and Milewski 2007). Thus, Orians and Milewski (2007) argued that fire leads to C- (i.e., energy-) rich, nutrient-poor ecosystems that are highly flammable. It seems reasonable, then, that the patterns and processes observed in our study, which was focused on the level of species (i.e., *E. pilularis*), should also occur on a community or ecosystem level. This suggests that the stoichiometric legacies of high-frequency fire regimes have the potential to advance the transition toward increasingly fire-prone or pyrophilic ecosystems.

However, our results also suggest that surface-active invertebrates might delay such transitions by compensating, at least to some extent, for the reduced capacity of micro-organisms to process litter in frequently burned areas (Fig. 2 and Appendix S1: Fig. S2). Thus, invertebrates might make hitherto unrealized contributions to ecosystem function and stability under the potentially destabilizing influence of high-frequency fire. Further research will be necessary before strong conclusions can be drawn regarding this hypothetical aspect of invertebrate function. Nevertheless, our results highlight the need to consider invertebrate conservation in fire management, given that burning has varied but at times negative consequences for invertebrate abundance and diversity (Springett 1979, York 2000).

Taken together, our results reveal that long-term, high-frequency fire regimes promote nutrient-poor, carbon-rich ecosystem stoichiometry and, by doing so, can disrupt fundamental ecosystem processes and modify the relative functional contributions of litter-dwelling micro-organisms and surface-active invertebrates during decomposition. Such effects have important implications for fire behavior and biogeochemical cycling in fire-prone environments in Australia and globally. Thus, our findings provide important insights into the likely trajectories of ecosystem function, stability, and biogeochemical cycling in a future where human activities are disrupting natural fire regimes and distorting the cycles of C, N, and P (Vitousek et al. 1997, Bennet and Elser 2009, Westerling et al. 2011, Sardans et al. 2012).

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