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REGULAR ARTICLE

High-frequency fire alters soil and plant chemistry but does not lead to nitrogen-limited growth of *Eucalyptus pilularis* seedlings

**Orpheus M. Butler · Mehran Rezaei Rashti ·
Tom Lewis · James J. Elser · Chengrong Chen**

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Abstract

Background and aims Vegetation fire often raises levels of soil phosphorus (P) relative to those of nitrogen (N), while long-term fire exclusion is associated with accumulation of soil N and depletion of soil available P. Thus, high-frequency fire regimes might trigger N-limited plant growth in otherwise P-limited plant communities.

Methods We used soils from a long-term prescribed burning trial to grow *Eucalyptus pilularis* under several nutrient amendment conditions. We measured seedling growth, soil and plant chemistry, and root enzyme activities to assess nutrient status.

Results Biennially-burned (2yB) soils had higher labile P concentrations than long-unburned (NB) soils, and lower total and labile N:P ratios. This did not correspond to N-limited growth or stimulate seedling N demand. Seedlings grown with addition of N, P and micronutrients in combination (μMax treatment) attained 68% more biomass than unfertilised seedlings. Addition of P resulted in higher total biomass than addition of N, and similar biomass to the μMax treatment, suggesting partially P-limited growth. Plants grown in 2yB soils tended to be enriched with P, K, Ca and Mg compared to those grown in NB or 4yB soils.

Conclusions High-frequency prescribed burning depletes soil N relative to P, but this does not trigger a shift toward N-limited growth of *E. pilularis* seedlings. Instead, *E. pilularis* seedlings appear to grow under partial P-limitation which persists regardless of their soil's fire history.

Keywords Prescribed burning · Stoichiometry · Phosphatase · Chitinase · Ash deposition

Introduction

Many of fire's effects on the structure, function and stability of vegetation communities are driven, at least partly, by the changes in soil fertility associated with burning (Chambers and Attiwill 1994; Bowman 2000; Close et al. 2009). These changes can include increases in the availability of essential nutrients such as nitrogen (N) and phosphorus (P) in soil through ash-deposition, pyro-mineralisation and pH increase (Adams et al. 1994; Certini 2005; Schaller et al. 2015). Further, losses of soluble or particulate forms of nutrients can occur through post-fire run-off and leaching (Shakesby 2011) while carbon (C) and N can be lost as gases (i.e. volatilization) during combustion (Raison et al. 1984; Urbanski et al. 2008).

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O. M. Butler · M. Rezaei Rashti · C. Chen (✉)
Griffith School of Environment and Science and the Australian Rivers Institute, Griffith University, Brisbane, Australia
e-mail: c.chen@griffith.edu.au

T. Lewis
Department of Agriculture and Fisheries, University of the Sunshine Coast, Sunshine Coast, Australia

J. J. Elser
Flathead Lake Biological Station, University of Montana, Missoula, MT, USA

These effects are highly variable and context-dependent (Certini 2005). Further, they occur over a range of temporal scales, with volatilization occurring during burns (Urbanski et al. 2008), erosion mostly occuring within the first year after fire (Shakesby 2011), and ash-driven nutrient pulses being greatest immediately following fire and dissipating over a period of months to years (Certini 2005; Butler et al. 2018). In combination, however, these effects typically result in higher soil P availability, as well as lower total and labile N:P ratios, in soils that have been recently or frequently exposed to fire (i.e. 'fire-affected' soils; Butler et al. 2017a, 2018). On the other hand, fire exclusion is associated with a gradual accumulation of soil N and depletion of soil available P, consistent with normal patterns of soil development (Walker and Syers 1976; Muqaddas et al. 2015; Hume et al. 2016).

The effects of fire on soil chemistry and stoichiometry appear to be particularly strong when fire regimes are modified over long time-scales (Lagerström et al. 2009; Pellegrini et al. 2017). Thus, it has been argued that long-term absence of fire can lead to a severe imbalance between N and P in soil (i.e. increasing soil N:P ratios), which drives a strong state of P-limitation for biological processes and potentially leads to ecosystem decline in forests where fire is a common and normal agent of disturbance (i.e. eucalypt- or conifer-dominated forests; Wardle et al. 2004; Close et al. 2009; Peltzer et al. 2016). Conversely, long-term maintenance of high-frequency fire regimes is thought to have strong potential to relieve P-limitation where present or even induce N-limitation (e.g. Toberman et al. 2014).

Numerous field studies of mature plants indicate that changes in fire regime can alter the elemental content and stoichiometric ratios of plant material (Schafer and Mack 2010; Toberman et al. 2014; Zhang et al. 2015; Butler et al. 2017b). These effects have been linked to plant growth responses to fire and have been considered indicative of an 'easing' of (though not necessarily a transition between) N- and P-limitation following fires (Dijkstra and Adams 2015). However, while foliar stoichiometry is thought to be linked to soil chemistry and thus provides a useful diagnostic for assessing nutrient limitation status (Aerts and Chapin 1999; Güsewell et al. 2003; Güsewell 2004), the reliability of this metric in post-fire environments is unclear. It is not certain that the observed differences in foliar chemistry and stoichiometry between recently burned and unburned areas are driven directly or primarily by fire's effects on soil nutrient availability. This is because field studies have generally been unable to isolate the role of fire-altered soil chemistry from the effects of re-growth, age differences and other environmental conditions (i.e. increased light and reduced soil moisture) that might also influence plant elemental content in the post-fire environment (e.g. Butler et al. 2018).

Various species of *Eucalyptus*, the dominant genus in most fire-prone Australian forests, are highly sensitive to P nutrition and grow in a state of P-limitation in long-unburned soils (Kirschbaum et al. 1992; Guinto et al. 2002; Tng et al. 2014). However, it is not clear whether fire events or long-term increases in fire frequency can ameliorate this effect. Moreover, although sustained increases in prescribed fire frequency have been associated with potentially N-limiting ecosystem conditions (e.g. Toberman et al. 2014), to our knowledge few studies have tested whether the low soil N:P ratios in recently or frequently burned soils trigger N-limited growth in eucalypts. Guinto et al. (2002) tested the effects of N, P, and N and P together on the growth of several eucalypt species when grown in soils with various histories of fire exposure, and found that N tended to improve biomass yield for annually-burned soils after 120 days of growth, but not to the same extent as N and P together. However, the rates of relative growth and P-uptake of some eucalypts increase markedly after 120 days (Mulligan and Patrick 1985). Thus, longer growth trials are necessary to accurately characterise the type and degree of nutrient limitation for *Eucalyptus* seedlings in fire-affected soils.

We conducted a 219-day, laboratory-based growth trial of *Eucalyptus pilularis* (Smith) to investigate how a soil's long-term history of fire exposure affects *E. pilularis* growth, nutrient demand, elemental content, and the responses of *E. pilularis* growth and elemental content to nutrient additions. We also explored the potential implications of simulated ash deposition on the soil surface following fires in these contexts. We hypothesised that long-term maintenance of frequent prescribed fire and fire exclusion would bring about significant changes in soil properties, including lower N availability, higher P availability and, in particular, lower N:P stoichiometric ratios in recently, frequently burned soils. Thus, we predicted that plants grown in soils exposed to frequent fire would tend toward N-limitation, while plants grown in soils from long-unburned areas would tend toward P-limitation. We also expected that plants would be sensitive to soil moisture, such that dry conditions in the post-fire environment might exert more influence over plant growth than potential nutrient-limitation. Further, we predicted that the elemental composition of plants grown in soils with different fire histories would differ due to the varying soil nutrient levels associated with different fire frequencies, with plants grown in fire-affected soils having higher levels of non-volatile, biologically-essential elements (i.e. P, K, Ca, Mg) due to increased uptake or 'luxury' consumption of these potentially-limiting nutrients. Finally, we expected that variation in plant N:P ratios would be indicative of shifting strengths of plant nutrient demand in response to different fire regimes or soil nutrient addition.

Materials and methods

Study site and soil sampling

Soil samples (0–10 cm) were collected from the Peachester State Forest (hereafter 'Peachester'; 26°52′ S, 152°51′E) prescribed burning field trial in July 2011. This depth was considered reflective of overall soil nutrient availability for seedlings because soils at Peachester have a uniformly sandy texture until around 60 cm depth (Guinto et al. 2001). After sampling, soil samples were sieved at 4 mm, air dried and sealed in plastic bags at room temperature prior to their use in the plant growth experiment. The soils have been classified as Alfisols (USDA soil taxonomy; equivalent to red to yellow Kandosols in Australian soil taxonomy).

The vegetation at Peachester is a wet sclerophyll forest, and is dominated by *Eucalyptus pilularis.* Three fire frequency regimes have been maintained at Peachester since 1969: unburned (NB) since 1969, burned every four years (4yB) on average since 1972, and burned every two years (2yB) on average since 1972. The fire regime treatments are randomized and replicated in the field ($n = 4$), with each replicate plot having an area of ca. 0.08 ha. Burns have been conducted in winter or spring and were normally low intensity (<2500 kW m^{-1}), consistent with normal prescribed burning practises in the region and for this forest type (Lewis et al. 2012). Prior to sampling, the last burns had been conducted in 2007 for 2yB plots and in 2005 for 4yB (and 2yB) plots. As a result, fire frequency is confounded with time since fire; thus, fire treatments are most accurately considered in terms of fire 'regime' rather than fire frequency per se. The experimentally-imposed fire return intervals at Peachester are much shorter and more invariant than the 10—100 year fire return intervals estimated for wet sclerophyll forests under natural conditions (Enright and Thomas 2008).

Experimental design and setup

In March 2017, a laboratory-based, multi-factorial plant growth experiment was initiated. Here we note that this means there were six years between sampling and the growth experiment; however, we are confident that soils underwent minimal changes in edaphic properties during this time, as they were air-dried, sealed, and mixed well before and after storage. Fire regime was the first factor, with three levels corresponding to the fire frequency treatments at Peachester (NB, 4yB and 2yB). The second factor was resource amendment. We had six resource amendment treatments: +N; +P; +N, P and micronutrients (hereafter the 'µMax' treatment); ash addition; water restriction (hereafter 'drought'); and control. Each treatment combination had four replicates, corresponding to the fire regime replicate plots at Peachester. We used the field replicates of the fire regime treatments for the growth experiment in order to maintain the spatial variability among replicate plots.

Seventy-two plastic pots (width = 149 mm, height = 110 mm) with no drainage holes were filled with 1.1 kg of air-dry soil (weighed on an oven-dry soil basis) from Peachester. Deionized water was added to 75% of soil water holding capacity (WHC). Seeds of *E. pilularis* were then sown directly onto the soil surface on the 10th of March 2017, and WHC was maintained at 75% for an initial germination period of six weeks. After six weeks, seedlings were thinned to the two strongest individuals in each pot and soil moisture content was reduced to 50% of WHC during the following week. Plants were grown under laboratory conditions of 22 °C ambient air temperature and at a light intensity of 108–122 W m^{-2} provided by fluorescent bulbs (Sylvania Grolux). Plants were watered with deionised water to 50% of WHC twice per week throughout the course of the experiment.

On the 27th of April 2017 (two weeks after thinning), fertiliser solutions were added to pots, and this was treated as the first day of the experiment (i.e. day 0). The application rates for N (as NH_4NO_3) and P (as NaH_2PO_4) were 100 µg per gram of soil (oven-dry basis; equivalent to additions of 7.14 g N and P per m^2; consistent with Guinto et al. 2002). Application rates for other elements were as follows: 25 μg K g^{-1}, 12.5 μg Ca g^{-1}, 12.5 μg Mg g^{-1}, 5.45 μg Fe g^{-1}, 3.95 μg Mn g^{-1}, 2.0 μg Zn g^{-1}, 0.5 μg Cu g^{-1}, 0.45 μg Mo g^{-1}, 0.1 μg B g^{-1}, 37.3 μg Na g^{-1}, 81.9 μg Cl g^{-1} and 5.7 μg S g^{-1}. A second round of fertiliser solutions was applied twelve weeks later (day 96, 31st of July 2017). The water restriction treatment was implemented on the same day, and consisted of a reduction of moisture content to 35% WHC with no nutrient or ash amendments.

We used dried leaves of *I. cylindrica* collected from Peachester to produce ash for the simulated ash deposition treatments. *Imperata cylindrica* was selected because it is an abundant ground cover species in the 4yB and particularly 2yB treatments at Peachester (Lewis et al. 2012) and grows prolifically in 'over-burned' areas (de Groot et al. 2005). Thus, combusted *I. cylindrica* biomass is likely to comprise a significant portion of ash after prescribed burns at Peachester. Ash was prepared by combusting dried leaves of *I. cylindrica* in a furnace at 550 °C for 15 min. The combusted material was then roughly ground with a mortar and pestle. Previous work indicates that fuel loads for communities containing *I. cylindrica* are variable. Fuel loads ranging from 420 to 1860 g m^{-2} have been reported in *I. cylindrica* dominated grasslands (Pickford et al. 1992; Platt and Gottschalk 2001; de Groot et al. 2005), with the upper end of this range considered a 'high' fuel load. We aimed to approximate a moderate fuel load, adding 1.5 g of ash to each pot. This was equivalent to 97 g of ash per square meter, or 1150 g of dry *I. cylindrica* biomass per square meter, based on 8.4% of the grass biomass remaining as ash following combustion.

The growth trial was concluded, and plants harvested, on the 1st of December 2017, 219 days after initial amendments were added. During harvesting, plants were carefully clipped at the stem base and the above-ground biomass was put directly into paper bags for oven drying at 65 °C for five days. Two soil cores (5 cm diameter) were then taken from each pot, centred on the clipped plant stems. These cores were combined and then sieved at 2 mm to retrieve plant roots, and were subsequently used for the soil chemical analyses. The remainder of the soil was then sieved at 4 mm to retrieve the rest of the roots, which were added to the roots from the cores. Roots were then rinsed carefully with deionised water and a small sub-sample (ca. 0.3 g) of lateral root was taken, weighed fresh, and retained for enzyme activity measurements. The remaining root samples were oven-dried in the same manner as above-ground biomass.

Laboratory analyses

Initial soil properties

Soil pH and electrical conductivity (EC) were measured using a 1:10 soil:water ratio. Available N (NH_4^+ and NO_3^-) was measured using a Segmented Flow Analyser after extraction with 2 M KCl. Available PO_4^{3-} was determined using molybdenum-blue spectrophotometry after extraction with NH_4F (Bray and Kurtz 1945; Murphy and Riley 1962). Total labile fractions of C, N and P in soil (i.e. those forms of C, N and P that are readily accessible to micro-organisms) were estimated using a hot water extraction (incubation in water at 70 °C for 16 h; Sparling et al. 1998; Tutua et al. 2013). Hot water extracts were analysed for total organic C and total N using a Shimadzu TOCN analyser (Chen et al. 2005) and for total P using inductively-coupled plasma mass spectrometry (ICP-MS). Soil total C and N were determined via dry combustion (Leco TruMac TCN Determinator), and total P was determined using ICP-MS after samples were subjected to microwave digestion in 70% nitric acid. Samples for total C, N and P measurement were finely ground (< 15 μm) prior to analyses. All initial soil properties were measured in 2017, with the exception of water holding capacity (via the method described in Cassel and Nielsen 1986) which was determined soon after sampling in 2011.

Soil properties after growth trial

Labile C, N and P, and available N and P, were measured as above but without prior air-drying. In addition, concentrations of potassium (K), calcium (Ca), and magnesium (Mg) in hot water extracts were measured via ICP.

Plant properties

Oven-dried plant samples were finely-ground. The total P, K, Ca and Mg contents of above- and below-ground samples were then measured using ICP-MS after digestion in nitric-perchloric acid (Jackson 1958; Zasoski and Burau 2008). Total N content of above- and below-ground samples were measured using dry combustion (Leco TruMac TCN Determinator). Total (above- +

below-ground) biomass contents of all elements were subsequently calculated. In the week following harvest, the potential activities of chitinase (CHN) and acid phosphatase (AP) enzymes of root samples were determined following spectrophotometric methods (Tabatabai and Bremner 1969; Tabatabai and Dick 2002). Sigma-Aldrich codes for substrates were N9376 and P4744 for CHN and AP respectively. The enzymes CHN and AP contribute to the breakdown of N- and P-containing organic compounds, respectively, such that their activities on root surfaces are indicative of the demand of plants for N and P, respectively. Thus, we measured the activities of these enzymes to provide additional evidence of plant demand and limitation by N or P in response to fire regime and fertilisation treatments.

Calculations and statistical analyses

The total biomass of *E. pilularis* seedlings in each pot was divided by the number of plants, which was two in all but two cases, to give an average biomass per plant for each pot. The 'relative growth' (RG) for plants in the control, +N, +P, ash and drought treatments was then calculated as the proportion of the biomass attained by seedlings growing in the same soil (i.e. the same field replicate plot) but subjected to μMax treatment. Relative growth reflects the 'realised' growth as a proportion of maximum potential growth (i.e. μMax) for a given supply of energy, and thus provides a formal means of diagnosing nutrient limitation in autotrophs (Sterner and Elser 2002).

The effect of long-term fire regime on the chemical properties of initial soil samples was assessed using one-way analysis of variance. Factorial analysis of variance was used to analyse the effects of fire regime treatment and amendment category on the following properties of *E. pilularis* seedlings: total biomass, RG, root surface CHN and AP activities and CHN:AP activity ratios, N and P uptake (mg N and P per plant), and total biomass element (P, K, Ca and Mg) content and total biomass C:N, C:P and N:P ratios. The effect of simulated ash deposition on soil properties in NB, 4yB and 2yB soils was assessed against soils from control pots using factorial ANOVAs. Tukey's Honestly Significant Difference (HSD) was used as a post-hoc test for comparisons of treatment means where required. Data were tested for normality using the Shapiro-Wilk test and log transformed where necessary prior to ANOVAs. Relationships between plant N:P, as a common indicator of nutrient limitation status, and root surface chitinase and phosphatase activities were assessed with Pearson's correlation to determine whether variation in plant N:P due to fire or fertilisation is diagnostic of changes in N and P demand by seedlings. Statistix 8.0 analytical software was used for all analyses. Here we note that in one pot, a 2yB × +N treatment replicate, both seedlings died before the end of the experiment. This replicate was excluded from all statistical analyses.

Results

Initial soil properties

Soil properties varied significantly among the three fire regime treatments (Table 1). Soil water holding capacity was lower in the 2yB and NB treatment than in the 4yB treatment. Soil pH was lower in the 2yB treatment than the NB treatment, and EC was lower in the 2yB treatment compared to the NB and 4yB treatments. Total and HWE C were lower in the 2yB treatment than in the 4yB treatment, and so was total N. Soil HWE N, 2 M KCl extractable NH_4–N and 2 M KCl extractable mineral N were not affected by fire regime, while 2 M KCl extractable NO_3^- was lower in the 4yB treatment relative to the NB treatment. Total P, total organic P, total inorganic P and Bray extractable PO_4^{3-} were all unaffected by fire regime, whereas HWE P was significantly higher in the 2yB treatment relative to the NB and 4yB treatments. Total and HWE N:P ratios were significantly lower in the 2yB treatment compared to NB and 4yB treatments, while available N:P ratios were not significantly affected by fire regime.

Plant growth and nutrient demand

Amendment was a significant source of variation in the total biomass of *E. pilularis* seedlings at the end of the 219-day growth period (Fig. 1; Table 2). Seedlings subjected to the μMax treatment had higher biomass than those in the control, +N and drought treatments. However, plants in the μMax treatment did not have significantly more biomass than plants in the +P and ash treatments, and plants in the +P treatments had significantly more biomass than plants in the +N and drought treatments (Fig. 1). Fire regime did not have a significant effect on seedling biomass, and neither did the interaction between the fire regime and amendment (Table 2). There was no effect of amendment, fire

Table 1 Initial properties of soils† (0—10 cm) sampled from Peachester State Forest fire frequency plots in June 2011 ($n = 4$). Different letters indicate that means are significantly different according to Tukey's HSD, with significance determined at $p < 0.05$

Soil property	Fire frequency treatment			$F_{2,9}$ (p value)
	NB	4yB	2yB	
Water holding capacity (%)	47.7 (±4.0)^{A}	60.9 (±1.8)^{B}	38.6 (±3.6)^{A}	11.6 (0.003)
pH (1:10 soil to water)	3.77 (±0.03)^{A}	3.89 (±0.07)^{AB}	4.21 (±0.14)^{B}	6.31 (0.019)
Electrical conductivity (μS cm^{-2})	52.1 (±3.4)^{A}	52.4 (±3.3)^{A}	38.5 (±1.7)^{B}	7.66 (0.011)
Total C (%)	4.29 (±0.42)^{AB}	6.53 (±0.53)^{A}	3.07 (±1.06)^{B}	5.84 (0.024)
Total N (%)	0.18 (±0.02)^{AB}	0.26 (±0.03)^{A}	0.10 (±0.03)^{B}	10.4 (0.005)
Total P (%)	0.008 (±0.001)	0.012 (±0.002)	0.008 (±0.002)	1.96 (0.197)
Total organic P (mg kg^{-1})	58.9 (±5.2)	89.4 (±16.5)	61.2 (±15.9)	1.56 (0.262)
Total inorganic P (mg kg^{-1})	22.2 (±2.5)	33.6 (±3.8)	20.4 (±5.7)	2.92 (0.106)
HWE organic C (mg kg^{-1})	1133 (±129)^{AB}	1323(±140)^{A}	757 (±100)^{B}	5.39 (0.029)
HWE total N (mg kg^{-1})	97.6 (±11.8)	84.3 (±12.6)	62.7 (±8.4)	2.52 (0.135)
HWE total P (mg kg^{-1})	0.31 (±0.17)^{A}	0.20 (±0.06)^{A}	0.88 (±0.06)^{B}	5.77 (0.024)
2 M KCl extractable NH_4^+ (mg kg^{-1})	60.7 (±10.0)	57.7 (±7.0)	41.9 (±8.6)	1.36 (0.304)
2 M KCl extractable NO_3^- (mg kg^{-1})	14.0 (±2.4)^{A}	3.1 (±1.1)^{B}	7.2 (±3.0)^{AB}	5.54 (0.027)
Mineral–N (mg kg^{-1})	74.7 (±12.1)	60.8 (±8.1)	49.1 (±10.3)	1.55 (0.265)
Bray-1 extractable PO_4^{3-} (mg kg^{-1})	3.91 (±0.34)	4.51 (±0.32)	4.86 (0.61)	1.19 (0.349)
Total N:P	21.9 (±2.2)^{A}	21.6 (±1.2)^{A}	12.4 (±1.4)^{B}	10.8 (0.004)
HWE N:P	882 (±391)^{A}	507 (±133)^{A}	70.4 (±6.89)^{B}	6.65 (0.017)
Available N:P	18.9 (±1.9)	13.5 (±1.6)	10.5 (2.8)	3.91 (0.060)

† *C*, carbon; *N*, nitrogen; *P*, phosphorus; *HWE*, hot water-extractable; 'mineral' N = sum of NO3–N and NH4+–N; 'available' N:P = ratio of mineral-N to Bray-extractable PO43− –P

regime or their interaction on the relative growth rate of *E. pilularis* seedlings after 219 days of growth (Table 2).

Fire regime interacted with amendment to affect the CHN activity on *E. pilularis* root surfaces (Fig. 2a). Root CHN activities were lower in the 2yB soils compared to those in 4yB soils, but only in the +P treatment. On the other hand, root surface AP activities were only affected by amendment treatment (Fig. 2b), with μMax and + P treatments having significantly lower AP activities than the control, +N, and drought treatments. Root CHN:AP activity ratios were lower in the μMax treatment compared to control, ash and drought treatments, but not significantly different from +N or + P treatments (Fig. 2c).

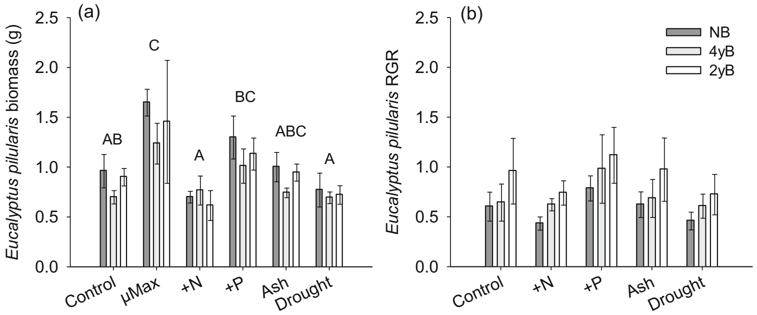

Fig. 1 Mean (± SE) (**a**) oven-dry biomass and (**b**) relative growth of *Eucalyptus pilularis* seedlings after 219 days of growth for each fire regime × amendment treatment combination (n = 4); Tukey's HSD test results for amendment shown (n = 12; different letters indicate significant differences at $P < 0.05$). Legend in panel 'b' applies to all panels in all figures

Table 2 Factorial analysis of variance of total *Eucalyptus pilularis* seedling biomass and relative growth rate[†] after 219 days of growth

	Total growth (biomass)					Relative growth rate				
Source	DF	SS	MS	*F*	*P*	DF	SS	MS	*F*	*P*
Amendment	5	0.728	0.146	6.47	<0.001	4	0.265	0.066	1.29	0.289
Fire frequency	2	0.078	0.039	1.73	0.187	2	0.233	0.116	2.26	0.117
Amendment × fire frequency	10	0.089	0.009	0.40	0.943	8	0.044	0.005	0.11	0.999
Error	53	1.193	0.023			44	2.268	0.052		
Total	70					58				

[†] Relative growth rate for a given seedling = total biomass as a proportion of the biomass attained by seedlings growing in the same soil (i.e. the same field replicate plot) but subjected to µMax treatment

Plant and soil chemical properties post-harvest

Fire regime and amendment both had significant effects on the uptake of N and P by *E. pilularis* seedlings. Seedlings grown in NB soils had accumulated 45.2% more N than those grown in 4yB soils, while plants grown in NB and 2yB soils accumulated 95.0 and 100.8% more P, respectively, than those grown in 4yB soils (Fig. 3). The µMax treatment resulted in significantly greater N and P uptake than all other treatments except the +P treatment, and the ash treatment was associated with greater P uptake than the +N treatment.

The N content of *E. pilularis* seedlings was significantly affected by the interaction between fire regime and amendment (Fig. 4a). Seedlings grown in NB and 2yB soils had higher N content under +N and µMax treatments than seedlings grown in 4yB soils. Moreover, seedlings grown in 2yB soils had higher N content when grown under the +N and µMax treatments compared to 2yB seedlings under other levels of amendment. On the other hand, *E. pilularis* P content was significantly affected by fire regime and amendment, but not their interaction (Fig. 4b). Total P content was lower in seedlings grown in 4yB soils, compared to those grown in NB and 2yB soils. At the same time, P content was higher in seedlings grown in the µMax and + P treatments relative to the control, +N, ash, and drought treatments.

Potassium content was significantly higher in plants grown in 2yB soils than plants grown in 4yB and NB soils (Fig. 4c). Further, plant K content was higher in the ash treatment than any other treatment, and lower in the +P treatment relative to control, +N, ash, and drought treatments (Fig. 4c). There was also more K in plants grown in the +N treatment than plants grown in the µMax treatment. The concentration of Ca in *E. pilularis* seedlings differed among all fire regimes (Fig. 4d), with plants grown in 2yB soils having the highest Ca content, and plants grown in NB soils having the lowest Ca content (Fig. 4d). Moreover, plant Ca content was higher in the +N treatment than all other treatments save the ash treatment. Seedling Mg content was higher in plants grown in 2yB soils than in NB soils (Fig. 4e), and was higher in plants subjected to the +N treatment than in plants subjected to the +P and drought treatments (Fig. 4e). Finally, S content was affected by the fire regime × interaction (Fig. 4f). Specifically, the +N treatment led to significant increases in seedling S content relative to the respective controls, but only for seedlings grown in the NB and 2yB soils (Fig. 4f). Seedling S content did not differ among control, +P, ash or drought treatments regardless of fire regime.

Eucalyptus pilularis seedlings grown in 2yB soils had significantly lower N:P ratios than those grown in 4yB soils but not NB soils (Fig. 5a). At the same time, addition of N only (i.e. the +N treatment) was associated with significantly higher seedling N:P ratios relative to the control treatment, while the addition of P only (i.e. the +P treatment), or N, P and micronutrients in combination (i.e. the µMax treatment), was associated with significantly lower seedling N:P ratios. Moreover, we found that seedling biomass N:P was significantly positively correlated with root surface AP activity (Fig. 5b), but not with root surface chitinase activity ($p = 0.088$, $r = 0.204$).

Soils in the ash treatment had higher P-availability, as indicated by Bray extractable P concentrations, which were 49% higher in ash-amended samples than in control samples (Table 3). The concentration of HWE K was also significantly higher in ash-amended soils,

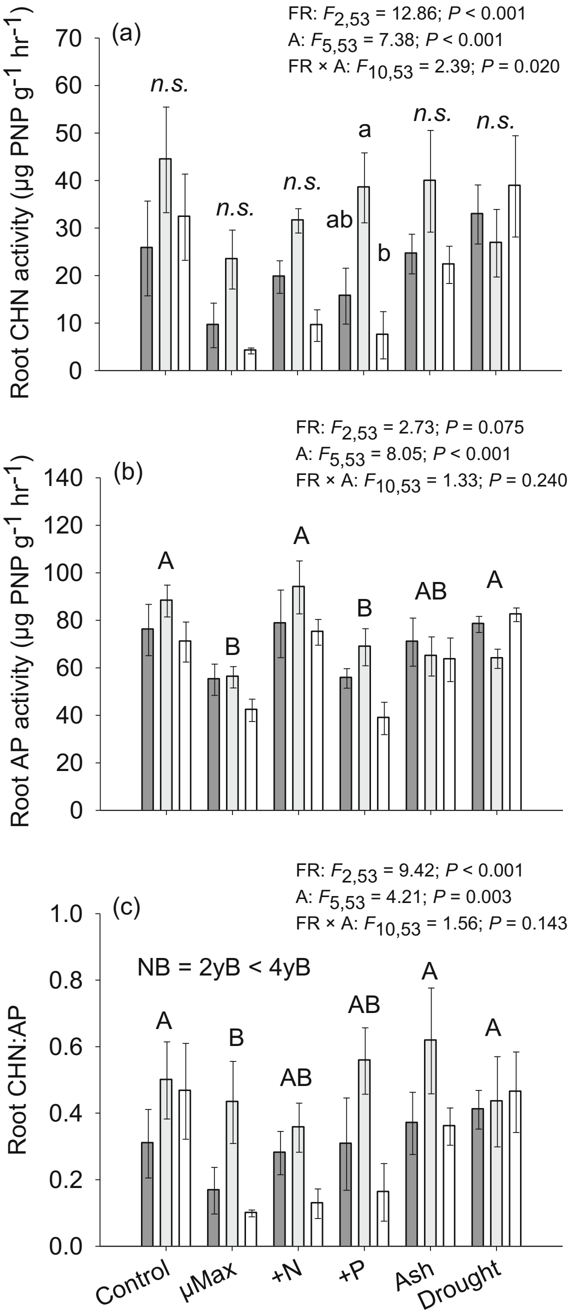

Fig. 2 Mean (± SE) root surface (**a**) chitinase (CHN) activity, (**b**) acid phosphatase (AP) activity and (**c**) CHN:AP activity ratio of *Eucalyptus pilularis* for each fire regime (FR) × amendment (A) treatment combination (n = 4); fire regime colours as defined in Fig. 1; *F*-statistics and *P* values provided; Tukey's HSD test results shown where $P < 0.05$, with uppercase letters indicating significant differences (at $P < 0.05$) among levels of amendment ($n = 12$); where the interaction between fire regime and amendment was significant, only comparisons of fire frequencies within levels of amendment are displayed, with lowercase letters indicating differences (at $P < 0.05$) among fire regimes for a given level of amendment; *n.s.* = differences among fire frequencies were not significant for the given level of amendment

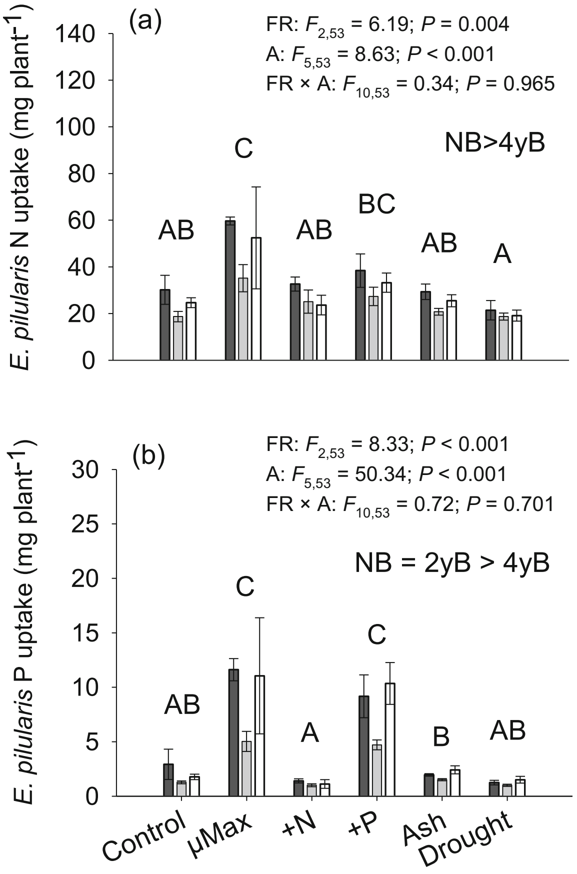

Fig. 3 Mean (±SE) uptake of (**a**) nitrogen (N) and (**b**) phosphorus (P) by *Eucalyptus pilularis* seedlings over 219 days of growth compared between each fire regime (FR) × amendment (A) treatment combination (n = 4); fire regime colours as defined in Fig. 1; *F*-statistics and *P* values provided; Tukey's HSD test results shown where $P < 0.05$, with uppercase letters indicating significant differences (at $P < 0.05$) among levels of amendment (n = 12)

increasing from 34.1 (±3.9) to 72.5 (±6.7) mg K kg soil^{-1}. On the other hand, HWE P was 90% lower in ash amended soils relative to control soils. Soil available N, HWE C, N, Ca and Mg concentrations were not affected by ash-amendment by the end of the 219-day growth period.

Discussion

Growth and nutrient demand of E. pilularis seedlings

The high biomass of seedlings in the μMax treatment indicated that the growth of *E. pilularis* was limited by nutrient availability under the conditions of light and

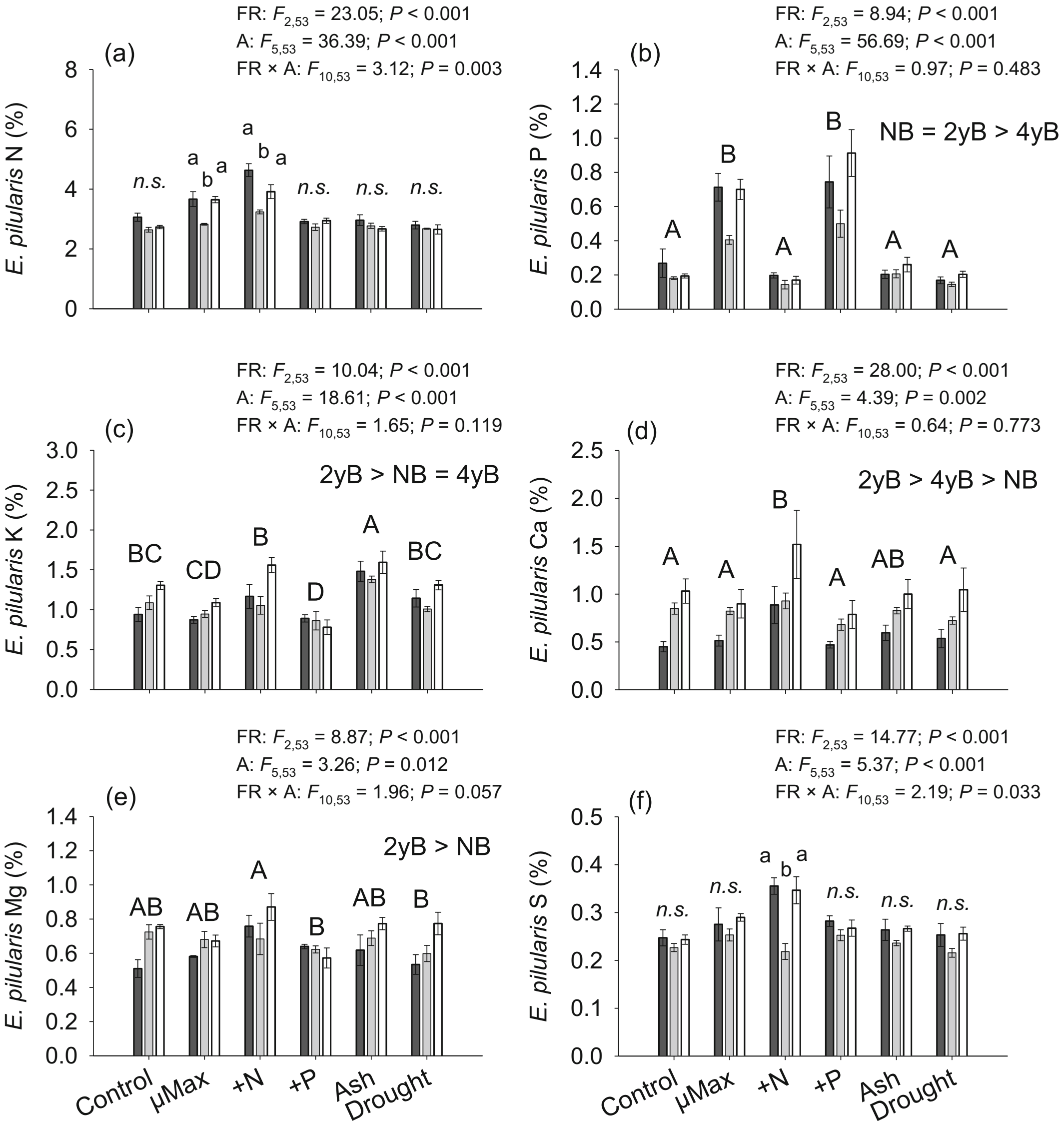

Fig. 4 Mean (±SE) concentrations of (**a**) nitrogen (N), (**b**) phosphorus (P), (**c**) potassium (K), (**d**) calcium (Ca), (**e**) magnesium (Mg), and (**f**) sulfur (S) in the biomass of *Eucalyptus pilularis* seedlings after 219 days of growth, compared between each fire regime × amendment treatment combination (n = 4); fire regime colours as defined in Fig. 1; Tukey's HSD test results shown where $P < 0.05$, with uppercase letters indicating significant differences among levels of amendment (n = 12); where the interaction between fire regime and amendment was significant, only comparisons of fire frequencies within levels of amendment are displayed, with lowercase letters indicating differences (at $P < 0.05$) among fire regimes for a given level of amendment; *n.s.* = differences among fire frequencies were not significant for the given level of amendment

moisture imposed in our experiment (Fig. 1). This was the case for all soils, regardless of fire history. However, we found no evidence that a soil's long-term history of fire exposure led to a shift from P- to N-limitation (Fig. 1a,b), or vice versa, contrary to our expectations and despite the lower total and HWE N:P ratios and higher labile P concentrations in 2yB relative to NB soils (Table 1). Moreover, our results clearly indicate that the growth of *E. pilularis* was not limited by N, even in the 2yB treatment where total N:P, HWE N:P and available N:P ratios were 43.4%, 92.0 and 44.4% lower, respectively, than in the NB treatment. This is consistent with previous studies, which reported that biennial burning, and associated soil N-depletion, did not have any impacts on the basal area growth of mature *E. pilularis* at Peachester (Guinto et al. 1999).

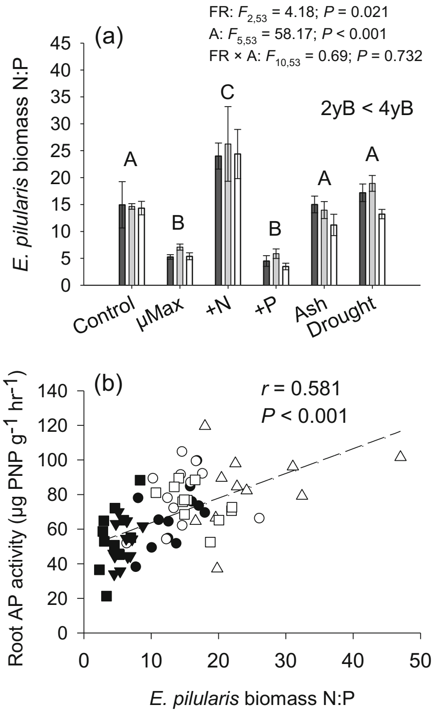

Fig. 5 **a** Mean biomass N:P of *Eucalyptus pilularis* seedlings after 219 days of growth for each fire regime × amendment treatment combination (n = 4); fire regime colours as defined in Fig. 1); *F*-statistics and *P* values provided; Tukey's HSD test results shown where $P < 0.05$, with uppercase letters indicating significant differences (at $P < 0.05$) among levels of amendment (n = 12); **b** correlation between the biomass N:P ratios and root surface acid phosphatase (AP) activities *E. pilularis* seedlings after 219 days of growth, with Pearson's correlation coefficient (*r*) and *P* value shown ($n = 71$); white circles = control (no amendment) treatment, black triangles = µMax treatment, white triangles = +N treatment, black squares = +P treatment, black circles = ash treatment, white squares = drought treatment

On the other hand, while not clearly evident based on the response of growth or RG to P addition (Fig. 1, Table 1), P-limitation cannot be so easily ruled out given the low seedling biomass N:P ratios (Güsewell et al. 2003; Güsewell 2004), the similar growth between µMax and + P treatments (Fig. 1), the clear difference in the response of seedlings to N and P fertilisation (Fig. 1), and the large increase in plant P uptake in response to P and N + P + micronutrient (i.e. µMax) fertilisation (Fig. 3b). Given these results, we suggest that P availability tended to constrain the growth of *E. pilularis* in soils from Peachester, but that a substantial part of this effect was driven by synergistic relationships or trade-offs between P and one or more additional nutrients (i.e. N, K, Ca, Mg, Fe, Mn, Zn, Cu, Mo, B, Na, Cl and S) in the soil–plant–microbial system. For instance, the diffusion and uptake of PO_4^{3-}, as well as rates of microbial P mobilisation, may have been enhanced or facilitated by other nutrients (Richardson et al. 2009; Tsai and Schmidt 2017). It is not clear whether this nutrient was K, Ca, Mg, S, or some other nutrient, given that the µMax treatment did not affect the concentrations of these elements in seedlings relative to the control treatment (Fig. 4c–f).

Alternatively, nutrient limitation may have shifted from P to other nutrients throughout the growth period, given the variation in P-demand and rates of P-uptake of *E. pilularis* seedlings that can occur during the first year of growth (Mulligan and Patrick 1985). Moreover, *E. pilularis* seedlings may have been limited by P and other nutrients simultaneously (Gleeson and Tilman 1992; Bracken et al. 2015). The lower root surface AP activity in µMax and + P treatments relative to controls indicates lower demand for P, which suggests that *E. pilularis* seedlings were dedicating more energy to acquisition of nutrients other than P in response to P fertilisation (Fig. 2b). This seems consistent with either shifting or multiple nutrient limitation. Interestingly, plant N demand (as indicated by root surface CHN activities) tended to be lower in P fertilised treatments, particularly in the N-depleted 2yB soils (Fig. 2a). Thus, it seems highly unlikely that N was one of the nutrients other than P that limited *E. pilularis* growth in our experiment.

Guinto et al. (2002) and Tng et al. (2014) both observed positive growth responses of eucalypts to P fertilisation when grown in long-unburned soils. Our results are consistent with this, but also show that the P-limitation of *E. pilularis* endures despite significant reductions in soil N:P ratios triggered by more than forty years of biennial burning. Guinto et al. (2002) noted a positive growth response of *Corymbia variegata* to N fertilisation when grown in annually-burned soils, although responses to simultaneous addition of N and P were greater. These findings contrast with our results that showed N fertilisation had no impact on *E. pilularis* growth in the 4yB and particularly 2yB soils in our experiment, but this difference might be due to differences in the plant species studied, the fire regime, or the length of the growing period. Our finding that P-limitation is highly persistent for *E. pilularis*, regardless of fire regime, supports the idea that long-term fire exclusion could result in severe P deficiency which, when coupled with soil N accumulation and soil P occlusion in the absence of burning (e.g. Jones and

Table 3 Chemical properties (means ± SE; n = 12) of soils in control and ash-amended pots at the end of the 219-day growth period; *F*-statistics and *P*-values are for the effect of amendment factor (control and ash amendment) based on factorial ANOVA†

Soil property	Control	Ash	Amendment factor $F_{1,17}$ (*P*-value)
NH_4^+ (mg kg^{-1})	35.35 (±11.9)	26.02 (±7.15)	0.53 (0.475)
NO_3^- (mg kg^{-1})	41.69 (±6.40)	60.00 (±10.69)	3.45 (0.080)
Mineral N (mg kg^{-1})	77.04 (±11.97)	86.02 (±13.63)	0.38 (0.543)
Bray P (mg kg^{-1})	3.96 (±0.34)	5.90 (±0.42)	11.25 (0.004)
HWE C (mg kg^{-1})	642.7 (±63.7)	582.7 (±48.2)	0.78 (0.390)
HWE N (mg kg^{-1})	155.2 (±18.4)	151.2 (±21.2)	0.03 (0.869)
HWE P (mg kg^{-1})	1.67 (±0.37)	0.16 (±0.03)	15.70 (<0.001)
HWE K (mg kg^{-1})	34.05 (±3.91)	72.54 (±6.71)	37.98 (<0.001)
HWE Ca (mg kg^{-1})	32.34 (±5.04)	33.71 (±4.75)	0.04 (0.839)
HWE Mg (mg kg^{-1})	33.48 (±5.44)	35.03 (±5.20)	0.05 (0.825)

N, nitrogen; *P*, phosphorus; *C*, carbon; *K*, potassium; *Ca*, calcium; *Mg*, magnesium; Mineral N = sum of NH_4–N and NO_3–N; HWE = hot water extractable forms of elements. † Fire frequency was a factor in these ANOVAs, but results for fire frequency are not reported because only samples from +Ash and Control treatments were included in the ANOVAs

Davidson 2014; Muqaddas et al. 2015), could contribute to the premature decline of eucalypt-dominated vegetation communities (Close et al. 2009; Jones and Davidson 2014). On the other hand, our results challenge the view that fire regime-induced soil N:P ratio shifts or changes in P-availability will have significant effects on the growth of dominant, P-limited tree species. However, time since fire may be critical in regulating the strength of this effect, given that our soil samples were taken 4 and 6 years after the most recent fires in 2yB and 4yB treatments, respectively, while post-fire pulses in P-availability are generally greatest immediately following burns and decrease with time (Adams and Attiwill 1991; Butler et al. 2018). Further study will be required to understand how time since fire might affect plant growth in fire-affected soils.

Another critical difference between our study's findings and those of previous studies was the overall amount and rate of biomass production. In our study, the total growth rate of *E. pilularis* control seedlings averaged 3.93 mg day^{-1} (860 mg per plant after 219 days), which was similar to the rate of (ca.) 3.6 mg day^{-1} observed by Mulligan and Patrick (1985), but was much less than the (ca.) 13.4 mg day^{-1} reported by Guinto et al. (2002; 1612 mg per plant after 120 days). This difference might be attributed to light and temperature, as Guinto et al. (2002) used a glass house, while we used artificial lighting in a laboratory. However, Mulligan and Patrick (1985) also used a glass house, and while soil soluble P levels were slightly lower in Mulligan and Patrick (1985) than those in Guinto et al. (2002; 2.29 mg P kg soil^{-1} versus 4.13 mg P kg soil^{-1}, based on 0.1 M H_2SO_4 soluble PO_4^{3-}; Heinrich and Patrick 1985; Guinto et al. 2001), this difference does not seem enough to explain the large differences in reported *E. pilularis* growth and growth rate between the studies. In any case, diagnosis of nutrient limitation in autotrophs always occurs in the context of a given light intensity (Sterner and Elser 2002), and the responses of *E. pilularis* growth to N, P and N and P together in Guinto et al. (2002) were reasonably consistent with the responses observed in our study. Thus, this aspect of our results essentially extends the findings of Guinto et al. (2002) to a wider range of environmental and experimental conditions.

Given the low-P status of many Australian soils, including those at Peachester, and the importance of P to eucalypt nutrition (Dell et al. 1987; Kirschbaum et al. 1992), it is perhaps not surprising that *E. pilularis* seems to grow under a persistent state of co-limitation by P and some other nutrient, regardless of fire history. Butler et al. (2018) argued that a tendency toward P-limitation in eucalypts might be a by-product of the eco-evolutionary dynamics of fire, wherein the rapid growth rates required for seedlings to reach maturity in fire-prone environments engenders a high P-demand (Elser et al. 2003; Bond 2008), thereby inducing P-limitation. Moreover, according to resource-ratio theory (Tilman 1982), a state of P co-limitation should confer a competitive advantage in fire-prone and post-fire environments, given that soil N can be depleted under high fire frequencies (Nave et al. 2011; Pellegrini et al. 2017), particularly relative to P (Table 1), while soil P availability can often be enhanced after fires (Schaller et al. 2015; Butler et al. 2018). Thus, transient increases in soil P availability following fires might play a key role in governing post-fire successional patterns in low-P environments, and might even contribute to the dominance of eucalypt species in many fire-prone ecosystems.

Effects of simulated ash deposition

Higher levels of soil available P, K and other nutrients following fire are thought to be driven in part by deposition of nutrient-rich, alkaline ash (Certini 2005), along with pyro-mineralisation of organic nutrient (i.e. P) forms (Cade-Menun et al. 2000; Hartshorn et al. 2009). In our study, simulated ash deposition was associated with concentrations of available P and HWE K that were 49 and 113.0% higher, respectively, than those in control soils (Table 3). Although this did not translate directly to greater yield or RGR of the seemingly P-limited *E. pilularis* seedlings in our study, enhanced growth of *Eucalyptus grandis* has been observed in studies of simulated ash deposition and soil heating in the past, and at least part of this effect is thought to be driven by increased P availability (Chambers and Attiwill 1994). Importantly, the concentration of highly 'labile' P (HWE P, which includes PO_4^{3-} and some simple, soluble organic P forms) was 90.4% lower in ash amended soils than in control soils. Although the reasons for this effect are unclear, this reduction in P lability could have limited the growth response of *E. pilularis* despite the significant increases in 'available' P as quantified based on the Bray extraction method.

At the same time, it may be relevant that microbial biomass can rapidly absorb increases in soluble PO_4^{3-} following fire (Huang et al. 2013). Under natural burning conditions a large portion of the surface soil is sterilized (González-Pérez et al. 2004; Wang et al. 2012), possibly limiting the extent of this effect. However, in our study ash was applied in the absence of heating, leaving an intact microbial biomass free to compete with *E. pilularis* seedlings for available P (Chen et al. 2004). Thus, it may be necessary for ash deposition to occur in conjunction with soil heating for P-limited plants to realize the full benefit of fire-enhanced P availability. We also note that intra-specific variation in plant biomass nutrient content in fire-prone forests (e.g. Butler et al. 2017b) means that species identity of the combusted material could influence ash nutrient content and subsequent changes in soil nutrient availabilities. Thus, our results for the effects of ash might only be applicable for ecosystems with a high proportion of *I. cylindrica* biomass in the fire fuel load.

Stoichiometry of E. pilularis seedlings

Several studies have argued that changes in plant biomass stoichiometry following fire are linked to, or indicative of, altered states or degrees of nutrient limitation (Schafer and Mack 2010; Toberman et al. 2014; Dijkstra and Adams 2015; Zhang et al. 2015). In particular, foliar N:P has often been cited as an index of N-versus P-limitation (Güsewell et al. 2003; Güsewell 2004), such that lower N:P ratios in post-fire environments have been taken as evidence of an 'easing' (but not necessarily a complete alleviation) of P-limitation in response to increased soil P availability (Dijkstra and Adams 2015; Butler et al. 2017b). Our study makes several important contributions in this context. First, the high P content (and low N:P ratios) of seedlings grown in μMax and + P treatments provides additional evidence that *E. pilularis* was at least partially P-limited in our study, given that sub-optimal cellular P quotas characteristic of P-limitation would have increased to meet or even exceed optimal levels following P fertilisation (Sterner and Elser 2002). Second, we clearly demonstrate that fire's effect on soil chemical properties (i.e. N:P ratios and P levels) is an important driver of fire's effect on plant elemental content and stoichiometry (Figs. 4 and 5). In particular, the low soil total and labile N:P ratios and high labile P concentration in the 2yB treatment soils (Table 1) are reflected in the low N:P of seedlings grown in 2yB soils, when compared to those grown in 4yB soils (Fig. 5a). Moreover, the high levels of K, Ca and Mg in plants grown in the 2yB soils, compared to the NB or 4yB soils (Fig. 4), are consistent with these elements being enriched in recently-burned soils (Certini 2005; Zhang et al. 2015), and with this enrichment being subsequently reflected in plant tissue chemistry (e.g. Zhang et al. 2015).

Thus, although the different growth stages achieved by seedlings under different amendments might have influenced inter-amendment comparisons of biomass N:P, our results support the view that changes in plant biomass N:P can indicate changes in the strength of N or

P limitation in response to fire-altered soil chemistry (Dijkstra and Adams 2015; Butler et al. 2017b). Seedling biomass N:P ratios were generally lower than twenty in all but the +N treatment, consistent with P-limitation (Güsewell et al. 2003), and seedling N:P ratios were positively related to root AP activity (Fig. 5b), meaning that seedlings demand more P at high seedling N:P. It follows that the strength of P-limitation for plants grown in 2yB soils was lower than that of plants grown in 4yB soils, but similar to plants grown in NB soils (Fig. 5a). However, this is only partially consistent with our expectations, given that soils from the NB and 4yB treatments had similar total and labile N:P ratios that were also higher than those in soils from 2yB treatments (Table 1), and it is unclear why biomass N:P ratios do not differ between plants grown in NB and 2yB soils.

Previous research indicates that fire's effects on foliar stoichiometry and soil—plant stoichiometric interactions are far from straightforward and clearly our results are no exception. Pellegrini et al. (2015) noted severe depletion of soil N relative to P after 58 years of increased fire frequency in savanna, but this did not correspond to altered foliar stoichiometry of the savanna vegetation. Zhang et al. (2015) observed similar effects in an alpine meadow on the Qinghai-Tibet plateau, while Cui et al. (2010) found that fire led to higher soil N and P availabilities and higher foliar N:P ratios in a temperate steppe ecosystem. Each of these studies was carried out under reportedly N-limiting conditions (Cui et al. 2010; Pellegrini et al. 2015; Zhang et al. 2015), and we suggest that this contributes greatly to the qualitatively and quantitatively different responses observed in our study. On the other hand, Butler et al. (2017b) found that recent, frequent fire was associated with lower foliar N:P ratios in a dry eucalypt forest on severely P-deficient soil. This response was absent for some species, indicating that taxa vary in their stoichiometric flexibility and their nutritional responses to fire. However, all of these field studies were subject to the potential confounding influence of post-fire changes in environmental conditions (e.g. higher light levels) and plant physiology and ontogeny that could affect plant tissue chemistry (e.g. P-enriched re-sprouts; Van de Vijver et al. 1999). Further research is warranted to better understand how soil chemistry interacts with these factors to influence plant biomass stoichiometry and, in doing so, continue to unravel fire's complex role in the shaping of ecosystems.

Conclusions

Our results show that long-term fire regimes can have marked effects on soil chemical properties and on N:P ratios in particular. However, while these effects are reflected in plant tissue stoichiometry to some extent, they do not necessarily correspond to altered growth rates or nutrient limitation of *Eucalyptus pilularis* seedlings. The severe depletion of soil N relative to soil P associated with high-frequency fire did not inhibit the growth, increase the N demand, or affect the growth response to N fertilisation of *E. pilularis* seedlings. Instead, *E. pilularis* grew under a persistent state of co-limitation by P and at least one other nutrient in all soils, regardless of their histories of fire exposure. This P-limitation could confer a competitive advantage on *E. pilularis* seedlings where large post-fire P flushes occur, potentially contributing to the tendency for this species to dominate in certain fire-prone environments within its climatic range.

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