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## **Title page**

**Title:** Elevated carbon dioxide increases soil nitrogen and phosphorus availability in a phosphorus-limited *Eucalyptus* woodland

**Running head:** Elevated CO<sub>2</sub> increases nutrient availability

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## Abstract

Free-Air CO<sub>2</sub> Enrichment (FACE) experiments have demonstrated increased plant productivity in response to elevated (e)CO<sub>2</sub>, with the magnitude of responses related to soil nutrient status. Whilst understanding nutrient constraints on productivity responses to eCO<sub>2</sub> is crucial to predicting carbon uptake and storage, very little is known about how eCO<sub>2</sub> affects nutrient cycling in phosphorus (P)-limited ecosystems. Our study investigates eCO<sub>2</sub> effects on soil N and P dynamics at the EucFACE experiment in Western Sydney over an 18 month period. Three ambient and three eCO<sub>2</sub> (+150 ppm) FACE rings were installed in a P-limited, mature Cumberland Plain *Eucalyptus* woodland. Levels of plant accessible nutrients, evaluated using ion exchange resins, were increased under eCO<sub>2</sub>, compared to ambient, for nitrate (+93%), ammonium (+12%) and phosphate (+54%). There was a strong seasonality to responses, particularly for phosphate, resulting in a relatively greater stimulation in available P, compared to N, under eCO<sub>2</sub> in spring and summer. eCO<sub>2</sub> was also associated with faster nutrient turnover rates in the first six months of the experiment, with higher N (+175%) and P (+211%) mineralisation rates compared to ambient rings, although this difference did not persist. Seasonally-dependant effects of eCO<sub>2</sub> were seen for concentrations of dissolved organic carbon in soil solution (+31%), and there was also a reduction in bulk-soil pH (-0.18 units) observed under eCO<sub>2</sub>. These results demonstrate that CO<sub>2</sub> fertilisation increases nutrient availability - particularly for phosphate - in P-limited soils, likely via increased plant belowground investment in labile carbon and associated enhancement of microbial turnover of organic matter and mobilisation of chemically-bound P. Early evidence suggests that there is the potential for the observed increases in P availability to support increased ecosystem C-accumulation under future predicted CO<sub>2</sub> concentrations.

## Introduction

Terrestrial biomes are responsible for sequestering a large proportion of global carbon (C) stocks ( $\approx 1\%$  of Earth's surface C and  $\approx 17\%$  of land C; Ciais *et al.*, 2013; Jobbágy & Jackson, 2000; Schlesinger & Bernhardt, 2013), with forest ecosystems accounting for more than half of global net primary productivity (NPP; Saugier *et al.*, 2001). Hence, understanding the impacts of climate change on these stocks and fluxes is central to predictions of terrestrial feedbacks on C cycling and thus future rates of atmospheric CO<sub>2</sub> increase. This is particularly so for forest and woodland ecosystems since these represent approximately 82% of total (above and below ground) C stocks in terrestrial biomes (Jobbágy & Jackson, 2000; Saugier *et al.*, 2001; Schlesinger & Bernhardt, 2013). Free-Air CO<sub>2</sub> Enrichment (FACE) experiments in terrestrial ecosystems have demonstrated a wide range of productivity responses to elevated CO<sub>2</sub> (eCO<sub>2</sub>), from sustained increases in plant biomass (e.g., Bader *et al.*, 2010; McCarthy *et al.*, 2010) to transient increases (e.g., Norby *et al.*, 2010; Reich *et al.*, 2006a) or even no response (e.g., Dukes *et al.*, 2005; Inauen *et al.*, 2012). It is now widely accepted that the size and persistence of CO<sub>2</sub>-fertilisation responses is likely to depend on soil nutrient status, with the biggest growth increases seen in experiments where nitrogen (N) availability is high (Reich & Hobbie, 2013; Reich *et al.*, 2006b). Indeed, soil N availability has been cited as the key determinant of productivity responses to eCO<sub>2</sub> in a wide range of studies (de Graaff *et al.*, 2006; Norby & Zak, 2011; Reich *et al.*, 2006b), with progressive N limitation (PNL) postulated to be a key mechanism reducing C fertilization responses over time (Luo *et al.*, 2004).

Although most large-scale and long-term eCO<sub>2</sub> studies have been carried out in N-limited systems and/or in young, rapidly growing stands (Reich *et al.*, 2006b), phosphorus (P) rather than N limits NPP in most of the ancient, highly weathered soils of the southern

Hemisphere and throughout the tropics (Vitousek *et al.*, 2010; Walker & Syers, 1976; Wang *et al.*, 2010). Furthermore, increasing rates of atmospheric N deposition is altering ecosystem nutrient stoichiometry (von Oheimb *et al.*, 2010) across more northerly latitudes, such that P limitation and NP co-limitation are now considered to be widespread across the globe (Cleveland & Townsend, 2006; Elser, 2012; Elser *et al.*, 2012; Menge & Field, 2007; Wang *et al.*, 2010). Whilst N constraints on productivity responses to eCO<sub>2</sub> have received considerable attention in the past decade, relatively few studies have looked at how low soil P availability affects plant response. Despite some evidence from glasshouse experiments that low soil P availability can constrain plant responses to eCO<sub>2</sub>, at least for young, pot-grown plants (e.g., Conroy *et al.*, 1990; Lewis *et al.*, 2010; Tobita *et al.*, 2010), there have been no such studies either under field conditions or at the ecosystem scale. In addition, recent studies by Wieder *et al.* (2015) and Zhang *et al.* (2014) using global C models predict serious constraints on terrestrial NPP and C storage in the future under NP co-limitation, highlighting the pressing need to include dynamic measures of soil P status in land-surface models. The issue of P constraints on CO<sub>2</sub> fertilisation - or even the concept of *progressive P limitation* (PPL) on the capacity of terrestrial ecosystems to sequester additional C - is, therefore, of global importance and one warranting urgent investigation.

Effects of eCO<sub>2</sub> on soil nutrient cycling vary considerably between experiments with, for example, a review of 47 studies by Zak *et al.* (2000) reporting both positive and negative impacts on soil N cycling, depending on ecosystem and/or dominant plant functional type. FACE experiments have generally shown decreases (Hovenden *et al.*, 2008; Lagomarsino *et al.*, 2008) or no change (Finzi *et al.*, 2002; Zak *et al.*, 2003) in soil N availability under eCO<sub>2</sub>. Relatively little attention has, however, been paid to the effects of eCO<sub>2</sub> on P cycling, or its impacts on both N and P availability in soils of very low nutrient status. Where P dynamics have been studied under field conditions, both increases (Khan *et al.*, 2010) and decreases

(Lagomarsino *et al.*, 2008) in P availability have been found under eCO<sub>2</sub>, depending on levels of N fertilisation. In addition to changing absolute levels of soil N and P availability, nutrient stoichiometry (i.e. N:P ratios) can also be altered by eCO<sub>2</sub> and climate change with, for example, soil, microbial and plant N:P ratios decreased under eCO<sub>2</sub> and increased under warming in a grassland FACE experiment (e.g., Dijkstra *et al.*, 2012).

Since nutrient availability is related to both uptake (i.e. plant and microbial immobilisation) and mineralisation processes, changes in plant or microbial traits associated with nutrient acquisition and/or community composition will influence nutrient dynamics. One of the key mechanisms behind eCO<sub>2</sub>-induced changes in soil nutrient dynamics is enhanced turnover of soil organic matter (SOM). This is typically associated with increased plant investment of C belowground - both in the form of increased root biomass and root exudates - when soil nutrient availability is low (Mason *et al.*, 2000; Treseder, 2004). Associated enhancement of plant- and microbial enzyme activities is also seen, with ratios of extracellular enzymes reflecting relative nutrient limitations. In other words, concentrations of enzymes associated with N mobilisation increase relative to those responsible for P mobilisation in N-limited sites, and *vice versa* in P-limited sites (Dijkstra *et al.*, 2012; Lagomarsino *et al.*, 2008; Treseder & Vitousek, 2001; Yin *et al.*, 2013). Increased belowground plant C investment has been reported in several forest FACE experiments to date with, for example, greater fine root production under eCO<sub>2</sub> contributing to sustained increases in NPP over six years in a sweetgum plantation (Norby & Iversen, 2006; Norby *et al.*, 2004). Moreover, there is now a growing body of evidence suggesting that enhanced root exudation of labile C under eCO<sub>2</sub> increases microbial decomposition of SOM, leading to increased nutrient turnover rates – via microbial priming, (Drake *et al.*, 2011; Finzi *et al.*, 2006; Lagomarsino *et al.*, 2008; Langley *et al.*, 2009; Larson *et al.*, 2002; Phillips *et al.*, 2011). Thus, increased root biomass (i.e. greater nutrient foraging capacity), priming of

microbial SOM decomposition and associated positive feedbacks to soil nutrient availability may be key mechanisms to offset PNL (and also PPL) and sustain the long-term enhancement of NPP under eCO<sub>2</sub>.

*Eucalyptus* species are planted widely, especially throughout the tropics and subtropics, playing an important role in regional economies as well as in global C sequestration (Varmola & Carle, 2002). In Australia, *Eucalyptus* woodlands account for 75% of native forest area, typically occurring on the very poor soils of the coastal fringes of the continent (Australian Government Department of Agriculture, 2012). Responses to eCO<sub>2</sub> have been studied for a small number of *Eucalyptus* species (e.g., Barton *et al.*, 2012; Conroy *et al.*, 1992; Crous *et al.*, 2011; Ghannoum *et al.*, 2010) and, where the role of P availability has been investigated, it has been found to severely constrain physiological responses to increased CO<sub>2</sub> concentrations (Conroy *et al.*, 1990, 1992; Lewis *et al.*, 2010; Thomas *et al.*, 2006; Tobita *et al.*, 2010). Whilst experimental (greenhouse) evidence implies that P-limited and/or N and P co-limited forests will not increase NPP under eCO<sub>2</sub>, at the field scale plant investment of additional C into nutrient mobilising strategies (e.g., increased phosphatase and/or organic acid exudation; Lambers *et al.*, 2008) may increase P availability and, potentially, facilitate CO<sub>2</sub> fertilisation of forest productivity. In this study, we investigate the effects of eCO<sub>2</sub> on soil nutrient dynamics in a novel field CO<sub>2</sub> exposure experiment in a mature, P-limited, native *Eucalyptus* woodland in Australia - EucFACE. We specifically test the hypothesis that increased plant and microbial nutrient demand under eCO<sub>2</sub> will result in faster turnover rates of soil organic N and P and drive an increase in availability of these nutrients. We further predict that, in this demonstrably P-limited ecosystem (Crous *et al.*, 2015), P availability will be increased to a greater extent than N, driving shifts in the stoichiometry of available nutrients, towards lower N:P ratios.

## Materials and methods

### Experimental site

The field site is a remnant Cumberland Plain Woodland, located at the Western Sydney University near Richmond, New South Wales, Australia (33°61' S, 150°74' E, 22 m altitude). Cumberland Plain Woodland is recognised nationally for its unique vegetation and declining, highly fragmented habitat and is listed as a critically endangered ecological community (Hancock *et al.*, 2013; N.S.W. Government Office of Environment & Heritage, 2011). Annual rainfall at the study site in 2012 and 2013 was 878 and 838 mm, respectively. Annual mean minimum and maximum temperatures were 10.5 and 24.0 °C in 2012 and 11.1 and 25.2 °C in 2013, respectively (Station 067105, Australian Government, Bureau of Meteorology, <http://www.bom.gov.au/>).

A detailed site description can be found in Crous *et al.* (2015). Briefly, the soil is a loamy sand Chromosol of the Clarendon Formation (Barton *et al.*, 2010; Isbell, 2002) with a sand/silt/clay content of 76%, 12% and 12% respectively (T. Gimeno, *pers. comm.*, July 1<sup>st</sup> 2014) and a mean soil pH of 5.45 (0-15 cm). Mean soil total N and P are 677 and 59 mg kg<sup>-1</sup>, respectively, and organic matter content is 1.8 % (0-15 cm). Annual mean soil temperature and moisture in the top 5-25 cm were 17.91 °C and 12.5%, respectively in 2013. Temporal patterns in soil temperature and moisture are shown in Fig. S1.

The dominant overstorey species at the study site is mature (>75-year old) *Eucalyptus tereticornis* (≈20 m canopy height), accompanied by a small proportion of *E. amplifolia*. Overstorey tree density is ≈600 trees ha<sup>-1</sup> and there is a diverse understorey of ≈70 species; understorey shrubs include *Breynia oblongifolia*, *Bursaria spinosa*, *Hakea sericea* and *Sida*

*rhombifolia*. *Microlaena stipoides* is the dominant grass, followed by *Cynodon dactylon* and *Paspalidium distans*.

### ***Eucalyptus* Free-Air CO<sub>2</sub> Enrichment (EucFACE)**

The EucFACE facility comprises six 25 m diameter arrays (FACE rings). Each FACE ring is made up of multiple vertical gas dispersal pipes (28 m height) that release CO<sub>2</sub> into the rings (Hendrey *et al.*, 1999). Elevated CO<sub>2</sub> treatment (ambient +150 ppm) was applied to three randomly selected rings, with the remaining three rings receiving ambient air. CO<sub>2</sub> treatment began on 18<sup>th</sup> September 2012, with an initial increment of +30 ppm; CO<sub>2</sub> concentrations in treated rings were increased by an additional 30 ppm every 4-5 weeks, reaching target concentrations of 540 ppm on 6<sup>th</sup> February 2013. Eight soil water content reflectometers (CS650-L, Campbell Scientific, Australia) were installed for each ring and soil temperature and moisture in the top 5-25 cm were recorded every 15 minutes.

### **Evaluation of N and P dynamics**

Multiple approaches were taken to assess pools and fluxes of inorganic N and P - soil extracts, soil solution and ion exchange resin membranes (IEMs). Nutrient concentrations in soil extracts reflect exchangeable pool sizes at a given point in time, indicating the amount of potentially available nutrients, the bioavailability of which highly depends on diffusion rates of nutrient ions. This is a simple, commonly used measure that allows comparison with other studies. Measurement of nutrient concentrations in soil solution reflects the pool of plant-available nutrients, but soil solution sampling using suction lysimeters is restricted to periods when soil moisture levels are high enough to allow a vacuum to be applied. Both extracts and soil solution measurements are instantaneous measures of nutrient availability, reflecting concentrations at the time of sampling (Abrams & Jarrell, 1992; Bowatte *et al.*, 2008). In



contrast, *in situ* incubation of IEMs provides an integrated measure of available nutrients over the whole incubation period (Abrams & Jarrell, 1992; Bowatte *et al.*, 2008; Qian & Schoenau, 2002). The three different metrics used in this study thus provide both instantaneous and integrating measures of nutrient availability over the study period. In addition, to evaluate treatment impacts on nutrient turnover rates and nutrient demand, we also measured *in situ* N and P mineralisation rates and the activities of extracellular enzymes responsible for decomposition of organic matter. Sampling schemes for each approach are shown in Fig. S1.

### Field sampling

Within each of the six FACE rings, four 2 m x 2 m plots were established for soil and soil solution sampling. Three soil cores (2 cm diameter, 0–10 cm depth) were sampled and pooled for each plot, every three months from June 2012. Sample locations were randomly located using a grid system to ensure previously sampled locations were avoided on subsequent sampling campaigns. On the same day of soil sampling, polyvinyl chloride (PVC) tubes (4 cm diameter, 15 cm length) were also installed within each plot to a depth of 10 cm to evaluate *in situ* net N and P mineralisation rates. Tubes were sealed with caps to prevent rainfall ingress and associated nutrient leaching; two small holes (4 mm diameter) were drilled in the side of each tube, to allow air-exchange. Tubes were incubated in the ground for three months. After this time, they were removed and the incubated soil was collected for analysis. New incubation cores were installed every three months. A total of 48 soil samples (i.e. 24 initial soil core samples ( $T_0$ ) and 24 incubated ( $T_{3\text{-months}}$ ) soil samples) were collected quarterly, from June 2012 to March 2014; this provided two pre-CO<sub>2</sub> sample points, and six following CO<sub>2</sub> treatment initiation in September 2012. All pooled soil samples were sieved (<2 mm) within one day of sampling, and plant roots and litter were removed. Soil moisture

content was determined on a 10 g subsample of fresh soil that was oven-dried at 105 °C for two days.

### **Plant accessible inorganic N and P - Ion exchange resin membranes (IEM)**

Ion exchange resin membranes (IEM; VWR International, Radnor, Pennsylvania, USA) were used to assess nitrate ( $\text{NO}_3^-$ ), ammonium ( $\text{NH}_4^+$ ) and phosphate ( $\text{PO}_4^{3-}$ ) availability. Two anion exchange membranes (AEM; 1 cm x 12.5 cm) and one cation exchange membrane (CEM) were inserted into the soil (0-10 cm deep) at eight locations within each ring: one set each in the four prescribed soil plots and a further four randomly located within each ring. Membranes were incubated *in situ* for a period of one month between July and December 2012, and for two months thereafter. IEMs were regenerated following standard protocols prior to each insertion (Snapp & Morrone, 2008). On collection, IEMs were well washed with deionised water to remove adhesive soil, and then extracted for  $\text{NO}_3^-$  and  $\text{NH}_4^+$  using the following modification of Bowatte *et al.* (2008)'s method. One of the AEM strips and the single CEM strip for each sample location were immersed in 90 ml of 2M KCl together, shaken at 100 rpm for an hour, and filtered (Whatman Grade 42). The second AEM strip in each sample location was used for  $\text{PO}_4^{3-}$  extraction using the Bray 1-P extraction with 0.03M  $\text{NH}_4\text{F}$ , according to Rayment and Lyons (2011). These AEM strips were immersed in 24.5 ml of 0.03M  $\text{NH}_4\text{F}$ , shaken at 100 rpm for 1 min and filtered immediately (Whatman Grade 42). Concentrations of  $\text{NO}_3^-$ -N,  $\text{NH}_4^+$ -N and  $\text{PO}_4^{3-}$ -P were analysed by automated colorimetry (AQ2 Discrete Analyzer, SEAL Analytical, USA). After extraction, IEM strips were scanned, and their surface areas were computed using ImageJ 1.46 (Wayne Rasband, National Institutes of Health, USA) in order to express results as concentrations per unit area ( $\text{ng cm}^{-2} \text{ day}^{-1}$ ). The ratios of plant available N and P were determined as ratios of total N ( $\text{NO}_3^-$ -N +  $\text{NH}_4^+$ -N) to  $\text{PO}_4^{3-}$ -P concentrations. Treatment

means and associated standard errors for N:P ratios were computed following log transformation, with results presented as geometric means.

### **Extractable soil inorganic N and P pools, and N and P turnover**

Extraction of soil inorganic N and P was conducted within two days of sampling.  $\text{NO}_3^-$  and  $\text{NH}_4^+$  were extracted using 2M KCl (Rayment & Lyons, 2011), while  $\text{PO}_4^{3-}$  was extracted by Bray 1-P extraction as described above with a 15 minute filtration time. Extracts were analysed using the SEAL AQ2 Discrete Analyzer as before, with results expressed on a mass of oven-dry soil basis. Net nitrification and N and P mineralisation rates were determined as the change in  $\text{NO}_3^-$ -N, total N ( $\text{NO}_3^-$ -N +  $\text{NH}_4^+$ -N) and  $\text{PO}_4^{3-}$ -P concentrations between core installation ( $T_0$ ) and removal three months later ( $T_{3\text{-months}}$ ).

### **Dissolved inorganic N, P and organic C in soil solution**

Dissolved organic C (DOC) and inorganic N and P in soil pore solution was collected using suction lysimeters (1900 Soil Water Sampler, Soil moisture Equipment Corp, USA) installed at two depths within each soil plot. The first lysimeter was installed with the ceramic cup located in the upper soil layer (10-15 cm), corresponding to the depth where the majority of fine root biomass is found (Jackson *et al.*, 1997), hereafter referred to as “shallow”. The second lysimeter was installed immediately above the impermeable layer, at a depth of 35-75 cm, hereafter “deep”.

Soil solution was sampled monthly when possible. However, in the very well drained, sandy soils of the study site, vacuum could only be maintained in suction lysimeters when soil moisture levels were high, so sampling was restricted to periods following significant rainfall episodes (>20 mm). After collection, lysimeter samples were immediately filtered (0.22  $\mu\text{m}$ , SLGP033RB, Merck Millipore, USA) and stored at  $\sim 20^\circ\text{C}$ . Concentrations of

NO<sub>3</sub><sup>-</sup>-N, NH<sub>4</sub><sup>+</sup>-N and PO<sub>4</sub><sup>3-</sup>-P were analysed using the AQ2 Discrete Analyzer (described above), and DOC was analysed using a total organic C analyzer (TOC-L CPH/CPN, Shimadzu Scientific Instruments, New South Wales, Australia) using the TOC-IC method.

### Soil extracellular enzyme activity

Effects of eCO<sub>2</sub> on potential activity of four commonly measured soil extracellular enzymes - acid phosphatase (AP), *N*-acetylglucosaminidase (NAG),  $\beta$ -glucosidase (BG) and  $\beta$ -cellobiohydrolase (CBH) - were examined using standard fluorescence techniques (Allison *et al.*, 2009; DeForest, 2009; German *et al.*, 2011; Saiya-Cork *et al.*, 2002), following the method outlined in DeForest (2009). Catalytic functions of these enzymes are described in detail in German *et al.* (2011). Briefly, AP and NAG are responsible for decomposition of organic P and N, respectively, while BG and CBH are involved in the decomposition of organic C. Incubation time, saturating concentrations of fluorescent marker (4-methylumbelliferone; MUB)-linked substrate (4-MUB-phosphate, 4-MUB-*N*-acetyl- $\beta$ -D-glucosaminide, 4-MUB- $\beta$ -D-glucopyranoside and 4-MUB- $\beta$ -D-cellobioside) and soil:buffer ratios were optimised for our soil samples according to German *et al.* (2011).

A subsample of frozen soil ( $\approx$ -20 °C) was mixed with 50mM of sodium acetate buffer (1:10 w/v), with pH adjusted to field soil (pH 5.5). The resulting soil homogenate was sonicated for 2 min (29 W, 3.2 kJ; VCX130, Sonics, Sonics & Material, Inc., Connecticut, USA) and stirred until assay. Shortly after (<5 min) soil homogenate (200  $\mu$ l) and 200 $\mu$ M of corresponding 4-MUB-linked substrates (50  $\mu$ l) were dispensed into a 96-well microplate (Nunc-Immuno™ MicroWell™ 96 well polystyrene plates, SIGMA-ALDRICH, Australia) along with reference standards and controls. The microplate was incubated at room temperature in the dark for 1 h, and then the reaction was stopped by adding 10  $\mu$ l of 1M NaOH to each well. After 1 min, fluorescence was measured at 369 nm (excitation) and 438

nm (emission) wave lengths using a fluorimetric microplate reader (2300, EnSpire® Multilabel Reader, PerkinElmer, Massachusetts, USA). Enzyme activity was expressed in units of  $\mu\text{mol MUB h}^{-1} \text{g}^{-1}$  (DeForest, 2009).

## Soil pH

Soil pH was measured in June 2012 and June 2013. Randomly located soil cores were sampled from depths of 0-15 and 15-30 cm in 2012 and 0-10, 10-20 and 20-30 cm in 2013, and refrigerated immediately after collection. Two cores per ring were sampled in 2012 and four per ring in 2013. Within two days of sampling, sieved soil samples ( $<2$  mm) were mixed with deionised water (1:2 w/v) and the pH of two analytical replicates was measured according to Rayment and Lyons (2011) using a pH meter (S20 SevenEasy™ pH, Mettler-Toledo International Inc., USA). Since different sampling depths were used in 2012 and 2013, a single, composite value was derived for a depth of 0-30 cm, using measured data, prior to analysis of treatment effects on soil pH.

## Statistical analysis

In this analysis, the unit of replication was the FACE ring ( $n = 3$ ). Although FACE rings were assigned treatments at random, adjacent ambient and  $\text{eCO}_2$  rings were paired for statistical analysis, based on similarities in soil properties as previously outlined in Drake *et al.* (2015). This resulted in three blocks, with one ambient and one elevated  $\text{CO}_2$  ring in each block (See Drake *et al.* (2015) supporting information for a detailed description of the blocking approach taken). The effects of  $\text{eCO}_2$  on nutrient concentrations, turnover rates and enzyme activities were assessed using linear mixed-effects models (LMM), for which block, ring and plot were random factors. Repeated-measures analysis of variances (ANOVA) were first performed with  $\text{CO}_2$ , Time and a  $\text{CO}_2 \times \text{Time}$  interaction as fixed factors to determine

the effects of CO<sub>2</sub> treatment, variation over time and interactions between the two. Data collected before and after CO<sub>2</sub> treatment began were analysed separately in order to assess initial pre-treatment differences. For soil pH, observations before and after CO<sub>2</sub> treatment commenced were analysed together in a single model as there was only one time point for each of pre- and post-CO<sub>2</sub> treatments. Where a CO<sub>2</sub> x Time interaction was indicated at  $P < 0.1$ , *a priori* contrasts were performed by single-degree-of-freedom comparisons using the ‘contrast’ package in R 3.1.2 (Crawley, 2012; Kuhn *et al.*, 2013; R Core Team, 2014) for each sampling event. Response variables were transformed (natural log, square root, cubic root or reciprocal) as required to ensure homogeneity of variances and normality of errors prior to analyses (Crawley, 2012; Fox & Weisberg, 2011).

After this, LMMs were undertaken with CO<sub>2</sub> and two covariates - soil temperature (Temp) and moisture (Moist) - and their interactions (CO<sub>2</sub> x Temp and CO<sub>2</sub> x Moist) as fixed factors in order to evaluate the role of soil conditions in responses to CO<sub>2</sub> treatment. Hereafter, this analysis is referred to as LMM<sub>cov</sub>. Mean values of soil temperature and moisture were calculated for the relevant incubation periods for mineralisation rates and IEM-adsorbed nutrients, and for the three months period immediately prior to sampling for extractable soil nutrients and extracellular enzyme activity analyses. Response and explanatory variables were again transformed as required to meet model assumptions. Model fit was evaluated based on variance explained by only fixed factors and both fixed and random factors according to Nakagawa and Schielzeth (2013). Confidence intervals (95%) on parameters predicted by LMM<sub>cov</sub> were computed by performing parametric bootstrap using the ‘lme4’ package in R 3.1.2 (Bates *et al.*, 2014).

$P$  values were obtained for minimal adequate models by deleting non-significant ( $P > 0.1$ ) factors from maximal models (Crawley, 2012), but given the small number of treatment replicates ( $n = 3$ ), marginally significant ( $P \leq 0.1$ ) factors were kept. Kenward-Roger Degrees

of Freedom Approximation was employed to estimate residual degrees of freedom for  $F$  tests in LMMs using the 'lmerTest' package in R 3.1.2 (Kenward & Roger, 1997; Kuznetsova *et al.*, 2014). All statistical analyses were performed using R 3.1.2.

## Results

### Plant accessible inorganic N and P - Ion exchange resin membrane (IEM)

Repeated-measures ANOVA showed a significant  $\text{CO}_2 \times \text{Time}$  interaction (Fig. 1a,  $F_{9, 413} = 2.16$ ,  $P < 0.05$ ) and revealed seasonally-dependent effects of  $\text{CO}_2$  on IEM-adsorbed  $\text{NO}_3^-$ -N. Over the 18 month study period,  $\text{NO}_3^-$ -N concentrations were 93% higher under  $\text{eCO}_2$  compared to ambient rings. Although concentrations were higher in  $\text{eCO}_2$  rings compared to ambient before  $\text{CO}_2$  treatment commenced, the difference was not statistically significant (+78%,  $P = 0.09$ ). Furthermore, once  $\text{CO}_2$  treatment began, differences were larger and statistically significant in December 2012 (+120%,  $P < 0.05$ ), June 2013 (+192%,  $P < 0.01$ ) and January 2014 (+216%,  $P < 0.05$ ).  $\text{LMM}_{\text{cov}}$  found that soil temperature ( $P < 0.001$ ) and moisture ( $P < 0.001$ ) were also strong drivers of  $\text{NO}_3^-$ -N concentrations, with greater availability at higher temperatures and soil moistures (Table 1, Fig. 2).

Elevated  $\text{CO}_2$  increased IEM-adsorbed  $\text{NH}_4^+$ -N concentrations by 12% over the study period. Although small, treatment differences were consistent across time, resulting in a marginally significant effect ( $F_{1, 2} = 15.3$ ,  $P = 0.06$ , Fig. 1b). IEM-adsorbed  $\text{NH}_4^+$ -N was also correlated positively with soil temperature ( $\text{LMM}_{\text{cov}}$   $P < 0.01$ ) and negatively with soil moisture ( $\text{LMM}_{\text{cov}}$   $P < 0.001$ , Table 1). The relationship with soil moisture was stronger in ambient than  $\text{eCO}_2$  treatments, with a significant  $\text{CO}_2 \times \text{Moist}$  interaction ( $\text{LMM}_{\text{cov}}$   $P < 0.05$ ) implying that  $\text{NH}_4^+$  availability was higher in  $\text{eCO}_2$  than ambient rings under wet conditions (Fig. 2).

Elevated CO<sub>2</sub> had a positive effect on IEM-adsorbed PO<sub>4</sub><sup>3-</sup>-P, with a more consistent pattern of significant monthly increases in concentrations under eCO<sub>2</sub> than those for NO<sub>3</sub><sup>-</sup>-N and NH<sub>4</sub><sup>+</sup>-N, and also a significant CO<sub>2</sub> x Time interaction (Fig. 1c,  $F_{9, 414} = 2.35$ ,  $P < 0.05$ ). Treatment effects varied between seasons with increases under eCO<sub>2</sub> seen predominantly in warmer months; IEM-adsorbed PO<sub>4</sub><sup>3-</sup>-P concentrations were 80% higher in eCO<sub>2</sub> rings between September 2012 and March 2013, and 69% between September 2013 and February 2014, with the largest increase (+124%) observed in December 2012. Treatment differences disappeared in the cooler part of the year (April-August 2013). LMM<sub>cov</sub> demonstrated treatment-specific correlations with soil temperature and moisture (Table 1, Fig. 2). While PO<sub>4</sub><sup>3-</sup>-P concentrations were scarcely correlated with soil moisture in eCO<sub>2</sub>, there was a positive relationship with moisture in ambient rings. In contrast, the positive relationship between PO<sub>4</sub><sup>3-</sup>-P concentrations and temperature was stronger in eCO<sub>2</sub> rings compared to ambient. Modelled relationships between PO<sub>4</sub><sup>3-</sup>-P concentrations, temperature and moisture highlight that the stimulation in PO<sub>4</sub><sup>3-</sup> availability in eCO<sub>2</sub> (compared to ambient rings) is greatest under low soil moisture, particularly under warmer conditions (Fig. 2).

Over the study period, there was no significant effect of eCO<sub>2</sub> on N:P ratios (Fig. 1d). Although not statistically significant, ratios were 69% higher in eCO<sub>2</sub> rings compared to ambient rings before CO<sub>2</sub> treatments commenced ( $P > 0.1$ ). Whilst this pre-CO<sub>2</sub> difference persisted during the colder months (+39%; April-August 2013), ratios were consistently lower in eCO<sub>2</sub> compared to ambient rings (-17%) during the warmer months where eCO<sub>2</sub>-associated increases in P availability were observed (Fig. 1c and d). LMM<sub>cov</sub> revealed treatment-specific correlations with soil temperature (Table 1, Fig 2; CO<sub>2</sub> x Temp  $P < 0.05$ ) and moisture (CO<sub>2</sub> x Moist  $P = 0.10$ ). N:P ratios from IEM samples were significantly, negatively correlated with soil temperature (LMM<sub>cov</sub>  $P < 0.001$ ), with a steeper slope for eCO<sub>2</sub> than for ambient treatments. This indicates that, during warmer periods, P availability



increased to a greater extent than N availability under eCO<sub>2</sub>, compared to ambient treatment (Fig. 1, Fig. 2).

### **Extractable soil inorganic N and P pools**

There was no evidence of an overall treatment effect on KCl-extractable NO<sub>3</sub><sup>-</sup>-N (Fig. 3a), although concentrations were negatively correlated with soil temperature (LMM<sub>cov</sub>  $P < 0.01$ ). KCl-extractable NH<sub>4</sub><sup>+</sup>-N concentrations were 23% higher under eCO<sub>2</sub> compared to ambient across the study period ( $F_{1,2} = 3.25$ ,  $P > 0.1$ , Fig. 3b), with a significant CO<sub>2</sub> x Time interaction ( $F_{5,110} = 2.90$ ,  $P < 0.05$ ); treatment differences were significant on only three occasions - December 2012 (+52%,  $P < 0.05$ ), June 2013 (+48%,  $P < 0.05$ ) and December 2013 (+35%,  $P < 0.05$ ). LMM<sub>cov</sub> also showed significant, positive associations between extractable NH<sub>4</sub><sup>+</sup>-N and both soil moisture ( $P < 0.001$ ) and temperature ( $P < 0.001$ , Table 1).

Repeated-measures ANOVA showed no evidence of a CO<sub>2</sub> effect on extractable PO<sub>4</sub><sup>3-</sup>-P (Fig. 3c). Pre-treatment values, on the other hand, showed a significant CO<sub>2</sub> x Time interaction ( $F_{1,22} = 7.40$ ,  $P < 0.05$ ), with concentrations 27% higher in June 2012 ( $P < 0.05$ ) and 16% lower in September 2012 ( $P > 0.1$ ) in designated eCO<sub>2</sub> rings compared to ambient. Once CO<sub>2</sub> treatments were running, concentrations were generally higher (+14%) in eCO<sub>2</sub> compared to ambient rings, with increases of 23% seen in March and 19% in December 2013. Of particular note was the relatively large (+27%) increase in eCO<sub>2</sub> rings over the first 3-months of CO<sub>2</sub> fumigation, compared to a lack of change in ambient rings over the same period (Fig. 3c). Extractable PO<sub>4</sub><sup>3-</sup>-P was also positively related to soil temperature (LMM<sub>cov</sub>  $P < 0.001$ , Table 1).

## Dissolved inorganic N, P and organic C in soil solution

Dissolved organic carbon (DOC) concentrations were generally higher in eCO<sub>2</sub> rings compared to ambient (+25% in the shallow layer and +36% in the deep layer over the 18 month study period). Although treatment effects were not statistically significant in the shallow layer (Fig. 4a), there was a significant CO<sub>2</sub> x Time interaction in the deep layer ( $F_{8, 106} = 2.90$ ,  $P < 0.01$ , Fig. 4b), with higher concentrations under eCO<sub>2</sub> in October 2012 (+158%,  $P < 0.01$ ), February 2013 (+33%,  $P = 0.08$ ), March 2013 (+37%,  $P < 0.05$ ) and November 2013 (+43%,  $P < 0.05$ ). For dissolved inorganic N or P in soil solution, on the other hand, there was limited evidence of eCO<sub>2</sub> effects on concentrations in either shallow or deep soil layers (Fig. S2).

## N and P turnover

Repeated-measures ANOVA demonstrated a significant CO<sub>2</sub> x Time interaction effect on net N mineralisation, with increases apparent in the early months after treatments commenced, and decreases seen later in the experiment ( $F_{5, 110} = 2.46$ ,  $P < 0.05$ , Fig. 5a). N mineralisation rates in eCO<sub>2</sub> rings were almost double those in ambient rings between October 2012 ( $P = 0.09$ ) and April 2013, but less than half those recorded between July and November ( $P < 0.05$ ) 2013. LMM<sub>cov</sub> suggests that soil temperature and moisture are also strong drivers of N mineralisation rates, with rates positively related to soil temperature ( $P < 0.05$ ) and negatively related to soil moisture ( $P < 0.05$ , Table 1).

Nitrification showed a similar temporal pattern to N mineralisation with a significant CO<sub>2</sub> x Time interaction ( $F_{5, 109} = 2.38$ ,  $P < 0.05$ , Fig. 5b). Nitrification was 163% higher in eCO<sub>2</sub> rings relative to ambient rings between January 2013 ( $P = 0.06$ ) and April 2013, but 24% lower in October 2013. LMM<sub>cov</sub> demonstrates a significant CO<sub>2</sub> x Moist interaction effect on nitrification rates ( $P < 0.05$ , Table 1), indicating that although rates appear to be

unrelated to soil moisture in eCO<sub>2</sub> rings they were negatively related to moisture in ambient rings.

Over the study period, CO<sub>2</sub> effects on net P mineralisation rates were not significant. Although P mineralisation rates were lower in eCO<sub>2</sub> rings compared to ambient during the pre-treatment period, these differences were not significant at the 5% level of probability ( $P = 0.09$ ). Of particular note, however, is the substantial increase in net P mineralisation rates during the first three months immediately after CO<sub>2</sub> treatments were initiated (Fig. 5c). The change from pre-treatment mineralisation rates to those measured three months later (post CO<sub>2</sub> switch on) was an order of magnitude higher in eCO<sub>2</sub> than in ambient rings. LMM<sub>cov</sub> highlights the importance of soil conditions as drivers of P mineralisation, with the latter correlated negatively with soil moisture ( $P < 0.01$ ) and positively with temperature ( $P = 0.06$ , Table 1).

### **Soil extracellular enzyme activity**

There was no evidence of an eCO<sub>2</sub> effect on the activities of four extracellular enzymes responsible for turnover of organic C, N and P (Fig. 6). A similar temporal pattern was seen for all enzymes, with greatest activities measured during the summer months. LMM<sub>cov</sub> demonstrated that the activities of all four enzymes were negatively correlated with soil moisture ( $P < 0.001$ ), while BG, NAG and AP were also positively correlated with soil temperature (Table 1). These soil variables, especially moisture, explained the substantial temporal variation in enzyme activity and accounted for between 70-90% of the model variance.

## Soil pH

Elevated CO<sub>2</sub> lowered bulk soil pH (0-30 cm) by 0.18 units in eCO<sub>2</sub> rings, compared to ambient, after 9-months of CO<sub>2</sub> fumigation, although even this fairly large difference was significant only at  $P = 0.09$  (Fig. 7).

## Discussion

During the first 18-months of CO<sub>2</sub> fumigation, we found seasonally-dependent positive effects of eCO<sub>2</sub> on soil P availability in this mature *Eucalyptus* woodland. Consequently, ratios of available N:P decreased in warmer months to a greater extent under eCO<sub>2</sub> than ambient treatment. Such a rapid and proportionally large increase in soil P availability as soon as CO<sub>2</sub> treatment began, and the seasonality of these responses, are particularly interesting in the context of a demonstrably P-limited ecosystem (Crous *et al.*, 2015).

Few studies have looked at how soil P availability is affected by eCO<sub>2</sub>, and those that have report conflicting findings. For instance, Johnson *et al.* (2004) found no significant effects of eCO<sub>2</sub> on P availability during the first two years of CO<sub>2</sub> fumigation in a (non P-limited) sweetgum plantation. In contrast, Huang *et al.* (2014) found increased available P under eCO<sub>2</sub> following five years' CO<sub>2</sub> fumigation in an open-top chamber study in a highly weathered, P-deficient subtropical forest in China. They suggested that this was primarily associated with higher soil moisture levels under eCO<sub>2</sub> and associated increases in turnover of organic matter. Dijkstra *et al.* (2012) found decreased N:P ratios of available soil nutrients under eCO<sub>2</sub> during a three year study in a northern mixed-grass prairie. They also attributed this to CO<sub>2</sub>-associated increases in soil moisture content. However, since soil moisture did not differ between CO<sub>2</sub> treatments at EucFACE during the study period ( $10.4\% \pm 1.7$  in

ambient and  $10.5\% \pm 1.6$  in eCO<sub>2</sub> rings, Mean  $\pm$  1.S.E,  $n = 3$ ) this is unlikely to have made a significant contribution to the observed early treatment effects on P mineralisation rates.

Greater proportional increases in P availability relative to N in our study likely reflect the larger plant and microbial demand for P, compared to N, given the known P limitation of plant growth at the site (Crous *et al.*, 2015). Positive effects of eCO<sub>2</sub> on soil P availability were confined to the warmer months of the growing season, resulting in a greater reduction in N:P ratios in eCO<sub>2</sub> compared to ambient rings. This, however, did not lead to significant treatment difference in available N:P ratios, probably owing to initially higher values in eCO<sub>2</sub> compared to ambient rings prior to CO<sub>2</sub> fumigation, driven by higher NO<sub>3</sub><sup>-</sup>-N concentrations. Dijkstra *et al.* (2012) showed that green plant N:P ratios had strong, positive correlations with those of IEM-adsorbed nutrients for five dominant species in a northern-mixed prairie. In their study, N:P ratios for green plant biomass (8-14) and IEM-adsorbed nutrients (2-16) were relatively low, with the former generally higher than the latter, indicating N limitation (Güsewell, 2004). At EucFACE, on the other hand, N:P ratios for tree leaves ( $\approx 20$ , Crous *et al.*, 2015) and IEM-adsorbed nutrients ( $\approx 92$ ), suggest a substantially larger soil pool size for N, compared to P, at our study site (Güsewell, 2004).

It is feasible that plants were adopting the strategy of investing a proportion of their photosynthate belowground to “mine” available P, potentially via exudation of carboxylates during periods of strong photosynthesis and active plant growth (Lambers *et al.*, 2008 and 2012). Indeed, Drake *et al.* (2015) found that eCO<sub>2</sub> stimulated light-saturated photosynthetic rates in canopy leaves (ca. +22 %) during the first five months of CO<sub>2</sub> fumigation at our site. This was also associated with a significant increase in soil CO<sub>2</sub> flux, indicating increased flow of labile C from plants to soil under eCO<sub>2</sub> (Drake *et al.*, 2015). Increased plant C allocation to root exudates under eCO<sub>2</sub> has been observed in studies elsewhere, particularly during warmer periods (Phillips *et al.*, 2011; van Hees *et al.*, 2005). Such an increase in

belowground C investment in our study is further suggested by the observed trend towards treatment-related increases in DOC in soil solution in both shallow (+25%) and deep (+36%) soil layers.

Increased exudation of labile C has been shown to enhance microbial decomposition of soil organic matter (SOM) – so called “priming” (Phillips *et al.*, 2011; Qiao *et al.*, 2014; van Hees *et al.*, 2005) - leading to increased nutrient availability, and is likely to have contributed to observed increases in both N and P mineralisation rates early in our study. Khan *et al.* (2008, 2010) also report increased soil P availability under eCO<sub>2</sub> in a poplar plantation following four years of CO<sub>2</sub> fumigation. They found that eCO<sub>2</sub> increased organic P but had no effect on total soil P content in a P-limited study site, proposing priming of organic matter decomposition as one of the sources of increased P.

Interestingly, the early eCO<sub>2</sub>-enhancement of N and P mineralisation did not persist beyond the first six months in the present study. Similar findings have been reported in FACE studies elsewhere with, for example, no effects of eCO<sub>2</sub>, or negative effects on nutrient turnover rates found for plantation forests in the USA (Finzi *et al.*, 2002; Zak *et al.*, 2003) and Italy (Lagomarsino *et al.*, 2008), and for a temperate Australian grassland (Hovenden *et al.*, 2008). The disappearance of early treatment effects on N and P mineralisation rates during the cooler, drier months of the first year of our study is perhaps not surprising, given the close relationship between soil temperature, moisture and microbial activity (Brockett *et al.*, 2012; Hackl *et al.*, 2005). However, treatment effects on mineralisation did not re-appear as conditions warmed later in the year (September 2013-March 2014) despite a strong re-occurrence of the stimulation in IEM-adsorbed PO<sub>4</sub><sup>3-</sup>-P concentrations during spring and summer of the second year; there was also a lack of treatment effects on extracellular enzymes over the study period. The disconnect between treatment effects on mineralisation rates/enzyme activity and associated increases in nutrient availability, and the relatively

greater increases in P availability compared to N, all suggest that factors other than priming of SOM decomposition are driving observed patterns in P availability.

Non-priming mechanisms that could contribute to observed patterns of soil P include exudation of carboxylates by plants and also soil acidification (Vance *et al.*, 2003). The latter could result from 1) proton ( $H^+$ ) release from plant roots (Hinsinger, 2001) and 2) carbonic acid due to the dissolution of  $CO_2$  in soil water under  $eCO_2$  (Andrews & Schlesinger, 2001). Protons are released from plant roots to maintain charge balance in association with cation uptake (Gahoonia & Nielsen, 1992; Shen *et al.*, 2004). Exudation of carboxylates – the anionic component of organic acids – and  $H^+$  release from plant roots have long been known for a wide range of plants, especially in soils of very low P availability – resulting in mobilisation of chemically-bound P and enhanced turnover of organic P pools (Gardner *et al.*, 1983; Hinsinger, 2001; Imas *et al.*, 1997; Lambers *et al.*, 2008, 2012; Shen *et al.*, 2004; Vance *et al.*, 2003). Although the relationship between soil pH and P availability depends strongly on soil types and associated plant communities (e.g., Betencourt *et al.*, 2012; Devau *et al.*, 2010; Staunton & Leprince, 1996), there is evidence of increased plant P uptake with lower rhizosphere soil pH in P-limited, neutral-moderately acidic soils/solution-culture (e.g., Gahoonia & Nielsen, 1992; Riley & Barber, 1971; Shahbaz *et al.*, 2006). Furthermore, given that bulk soil provides a relatively dilute signal for plant-driven changes in rhizosphere chemistry (Gahoonia & Nielsen, 1992; Hunter *et al.*, 2014; Nichol & Silk, 2001; Radersma & Grierson, 2004), the decrease of nearly 0.2 pH units in bulk soil pH observed under  $eCO_2$  in this study may well be indicative of ecologically significant levels of carboxylate exudation and/or  $H^+$  release. On a similar note, Delucia *et al.* (1997) demonstrated that an increase in pine seedling biomass after four months'  $CO_2$  fumigation was associated with decreased phosphatase activity but increased soil concentrations of a carboxylate (oxalate) under  $eCO_2$ , and increased plant P uptake. Taken together, the significantly higher concentrations of DOC

in soil solution and lower soil pH seen under eCO<sub>2</sub> in our study provide indirect support for an involvement of organic acids and/or H<sup>+</sup> release in observed patterns of nutrient availability, although direct measurements of exudate chemistry would be required to confirm this.

We employed multiple metrics for assessing pools and fluxes of soil nutrients that can be broadly classified as instantaneous and integrating measures, according to the timescale over which sampling is carried out. Instantaneous measures of nutrient concentrations in soil extracts and soil solution are commonly used, but give only a snapshot of the size of inorganic N or P pools (Bowatte *et al.*, 2008). The ability to detect treatment effects on soil nutrient availability using instantaneous measures depends on sample timing in relation to environmental constraints on potential responses. For example, snapshot sampling may fail to capture treatment signals during very dry periods where extracellular enzyme activity is constrained by low soil moisture (Baldrian *et al.*, 2010; Steinweg *et al.*, 2012) and where increased plant and/or microbial immobilisation masks increases in rates of nutrient cycling. On the other hand integrating measures such as the bi-monthly *in situ* IEM exposures used in this study reflect not only nutrient stocks but also associated fluxes, and thus provide a better representation of overall treatment effects on nutrient availability to plants and microbes (Bowatte *et al.*, 2008; Qian & Schoenau, 2002). Also, the less destructive sampling method using IEMs allowed more frequent sampling and a greater number of replicates, making statistical analysis more robust. Overall, effects of eCO<sub>2</sub> treatment were generally small or negligible for instantaneous measures relating to nutrient availability (i.e. extracts, enzyme activity and soil solution) compared to the more integrating measures (i.e. IEMs and *in situ* mineralisation rates) used in this study. Such differences between measurement approaches have not been reported in FACE or open-top chamber studies elsewhere, possibly because soil nutrient availability is generally higher and soil moisture levels less variable at other sites,



compared to EucFACE (e.g., Finzi *et al.*, 2001; Hungate *et al.*, 1999; Lagomarsino *et al.*, 2008; Schleppi *et al.*, 2012).

In this study, concentrations of IEM-adsorbed nutrients were relatively high before CO<sub>2</sub> treatment began (June-August 2012), particularly for NO<sub>3</sub><sup>-</sup>-N. This may reflect relatively high precipitation in the first half of 2012 and associated high levels of soil moisture. Indeed, precipitation in the first six months in 2012 (723 mm) was greater than that during the same period in any of the preceding 20 years, as well as being 30% and 175% higher than levels recorded for the same period in 2013 and 2014, respectively (Station 067105, Australian Government, Bureau of Meteorology, <http://www.bom.gov.au/>).

Both net N and P mineralisation rates were negatively associated with soil moisture in the present study. Denitrification might have contributed to lower rates of N mineralisation at high soil moistures, although this is unlikely to have been a major factor since N<sub>2</sub>O emissions at EucFACE were negligible (L. Nazaries, *pers. comm.* October 1<sup>st</sup> 2015). This is in line with findings from Martins *et al.* (2015) demonstrating negligible soil N<sub>2</sub>O emissions from a nearby paddock (<500 m away). Negative relationships between soil moisture and both N and P mineralisation rates may have resulted from higher plant and microbial nutrient immobilisation under wetter conditions as has been previously reported in Dijkstra *et al.* (2012).

Early results from this experiment suggest that increased plant investment in belowground C under eCO<sub>2</sub> - as evidenced by increased soil DOC concentrations and concurrent increases of ≈10% in soil CO<sub>2</sub> efflux (Drake *et al.*, 2015) - is driving the observed increases in nutrient availability, particularly for P. This is the first study to investigate ecosystem responses to eCO<sub>2</sub> in a mature, P-limited woodland and, as such, provides novel insight into soil nutrient dynamics in a higher CO<sub>2</sub> world. Initial results suggest that there is at least the potential for CO<sub>2</sub>-driven increases in soil P availability to support increased C-

accumulation in nutrient-poor, P limited ecosystems as CO<sub>2</sub> concentrations continue to rise. Further work will be undertaken to derive detailed carbon and nutrient budgets for EucFACE to determine whether observed increases in soil nutrient availability do indeed support increased NPP under eCO<sub>2</sub> at this site.

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## Supporting information captions

Figure S1. Sampling scheme for nutrient measures, soil moisture and temperature (5-25 cm), and timing of precipitation events during the study period. The points for ion exchange membrane (IEM) and mineralisation samples represent the mid-points of incubation periods. The vertical dotted line indicates when CO<sub>2</sub> treatments began (18<sup>th</sup> September 2012).

Figure S2. Temporal changes in concentrations of dissolved NO<sub>3</sub><sup>-</sup>-N, NH<sub>4</sub><sup>+</sup>-N and PO<sub>4</sub><sup>3-</sup>-P in soil solution in the shallow (a, b and c) and deep soil layers (d, e and f; Mean  $\pm$  1.S.E,  $n = 1$  to 3). The vertical dotted line indicates the point at which CO<sub>2</sub> treatments commenced (18<sup>th</sup> September 2012). Summaries of repeated-measures ANOVAs for data collected after this time are shown for each variable. Where there is a significant CO<sub>2</sub> x Time interaction ( $P < 0.05$ ), results of post-hoc comparisons between CO<sub>2</sub> treatments are shown above the relevant bars, with the following significance codes:  $P < 0.1$  ( $\dagger$ ),  $< 0.05$  (\*),  $< 0.01$  (\*\*) and  $< 0.001$  (\*\*\*).

**Table**

Table 1 Summary of linear-mixed effects models of CO<sub>2</sub> and soil variable effects (LMM<sub>cov</sub>) on ion exchange resin membrane (IEM)-adsorbed nutrients, soil extractable nutrients, turnover rates of inorganic N and P, and potential soil enzyme activity. Fixed factors are CO<sub>2</sub>, soil moisture (Moist) and temperature (Temp), and their interactions (CO<sub>2</sub> x Moist and CO<sub>2</sub> x Temp). Degrees of freedom for each explanatory variable is 1, with residual degrees of freedom referred to as  $Df_{Res}$ .  $P < 0.1$  is shown in bold, otherwise shown as ns (not significant).

Response variables			Predictors				
			CO <sub>2</sub>	Moist	Temp	CO <sub>2</sub> xMoist	CO <sub>2</sub> xTemp
IEM	NO <sub>3</sub> <sup>-</sup> -N	<i>F</i>	-	15.11	13.65	-	-
		<i>Df<sub>Res</sub></i>	-	446	429	-	-
		<i>P</i>	ns	<b>&lt;0.001</b>	<b>&lt;0.001</b>	ns	ns
	NH <sub>4</sub> <sup>+</sup> -N	<i>F</i>	8.07	290.19	8.03	4.60	-
		<i>Df<sub>Res</sub></i>	2	120	434	119	-
		<i>P</i>	0.119	<b>&lt;0.001</b>	<b>0.005</b>	<b>0.034</b>	ns
	PO <sub>4</sub> <sup>3-</sup> -P	<i>F</i>	6.59	3.31	76.15	4.31	8.24
		<i>Df<sub>Res</sub></i>	2	457	430	397	431
		<i>P</i>	0.128	<b>0.070</b>	<b>&lt;0.001</b>	<b>0.039</b>	<b>0.004</b>
	N:P ratios	<i>F</i>	0.03	2.20	15.06	2.79	6.51
		<i>Df<sub>Res</sub></i>	2	436	430	436	430
		<i>P</i>	0.880	0.139	<b>&lt;0.001</b>	<b>0.095</b>	<b>0.011</b>
Soil extractable	NO <sub>3</sub> <sup>-</sup> -N	<i>F</i>	-	-	9.16	-	-
		<i>Df<sub>Res</sub></i>	-	-	119	-	-
		<i>P</i>	ns	ns	<b>0.003</b>	ns	ns
	NH <sub>4</sub> <sup>+</sup> -N	<i>F</i>	-	15.63	13.79	-	-
		<i>Df<sub>Res</sub></i>	-	76	118	-	-
		<i>P</i>	ns	<b>&lt;0.001</b>	<b>&lt;0.001</b>	ns	ns
	PO <sub>4</sub> <sup>3-</sup> -P	<i>F</i>	-	-	20.16	-	-
		<i>Df<sub>Res</sub></i>	-	-	59	-	-
		<i>P</i>	ns	ns	<b>&lt;0.001</b>	ns	ns
Mineralisation	N mineralisation	<i>F</i>	-	5.55	5.65	-	-
		<i>Df<sub>Res</sub></i>	-	70	118	-	-
		<i>P</i>	ns	<b>0.021</b>	<b>0.019</b>	ns	ns
	Nitrification	<i>F</i>	0.88	2.83	-	4.66	-
		<i>Df<sub>Res</sub></i>	2	135	-	54	-
		<i>P</i>	0.462	<b>0.095</b>	ns	<b>0.035</b>	ns
	P mineralisation	<i>F</i>	-	10.71	3.53	-	-
		<i>Df<sub>Res</sub></i>	-	20	119	-	-
		<i>P</i>	ns	<b>0.004</b>	<b>0.063</b>	ns	ns
Enzyme	CBH <sup>I</sup>	<i>F</i>	0.13	283.20	-	3.21	-



	<i>Df<sub>Res</sub></i>	2	48	-	49	-
	<i>P</i>	0.758	<0.001	ns	0.079	ns
BG <sup>2</sup>	<i>F</i>	-	214.12	4.14	-	-
	<i>Df<sub>Res</sub></i>	-	47	49	-	-
	<i>P</i>	ns	<0.001	0.047	ns	ns
NAG <sup>3</sup>	<i>F</i>	-	213.80	5.65	-	-
	<i>Df<sub>Res</sub></i>	-	51	49	-	-
	<i>P</i>	ns	<0.001	0.021	ns	ns
AP <sup>4</sup>	<i>F</i>	-	489.98	20.40	-	-
	<i>Df<sub>Res</sub></i>	-	49	48	-	-
	<i>P</i>	ns	<0.001	<0.001	ns	ns

<sup>1</sup> $\beta$ -D-cellobiohydrolase. <sup>2</sup> $\beta$ -glucosidase. <sup>3</sup>N-acetylglucosaminidase. <sup>4</sup>Acid phosphatase.

### Figure captions

Figure 1. Temporal changes in ion exchange resin membrane (IEM)-adsorbed (a)  $\text{NO}_3^-$ -N, (b)  $\text{NH}_4^+$ -N, (c)  $\text{PO}_4^{3-}$ -P and (d) log of N:P ratios (Mean  $\pm$  1.S.E,  $n = 3$ ). Treatment means and associated standard errors of N:P ratios were computed following log transformation, with results presented as geometric means. The vertical dotted line indicates when  $\text{CO}_2$  treatments commenced (18<sup>th</sup> September 2012). Summaries of repeated-measures ANOVAs for data collected after this time are shown for each variable. Where there is a significant  $\text{CO}_2 \times \text{Time}$  interaction ( $P < 0.05$ ), results of post-hoc comparisons between  $\text{CO}_2$  treatments for each month are shown above the relevant bars with the following significance codes:  $P < 0.05$  (\*),  $< 0.01$  (\*\*) and  $< 0.001$  (\*\*\*)

Figure 2. Conditional scatter plot of ion exchange resin membrane (IEM)-adsorbed nutrient concentrations ( $\text{NO}_3^-$ -N,  $\text{NH}_4^+$ -N,  $\text{PO}_4^{3-}$ -P and N:P ratios) against soil moisture for a given soil temperature range: Cold (12 to 16 °C), Moderately warm (16 to 20 °C) and Hot (20 to 24 °C), plotted with predicted lines and associated 95% confidence intervals. Predicted values were estimated from linear-mixed effects models with two covariates (soil moisture

and temperature;  $LMM_{cov}$ ), demonstrating treatment-specific correlations with these soil variables (Table 1). Bootstrap analyses were employed to approximate 95% confidence intervals for coefficients estimated by  $LMM_{cov}$ .

Figure 3. Temporal changes in KCl-extractable (a)  $NO_3^-$ -N, (b)  $NH_4^+$ -N and (c) Bray-extractable  $PO_4^{3-}$ -P (Mean  $\pm$  1.S.E,  $n = 3$ ). The vertical dotted line depicts the point at which  $CO_2$  treatment commenced (18<sup>th</sup> September 2012). Summaries of repeated-measures ANOVAs for data collected after this time are shown for each variable. Where there is a significant  $CO_2 \times$  Time interaction effect on values before and/or after  $CO_2$  treatment commenced ( $P < 0.05$ ), results of post-hoc comparisons between  $CO_2$  treatments for each time period are shown above the relevant bars with the following significance codes:  $P < 0.1$  ( $\dagger$ ),  $< 0.05$  (\*),  $< 0.01$  (\*\*) and  $< 0.001$  (\*\*\*).

Figure 4. Temporal changes in concentrations of dissolved organic carbon (DOC) in soil solution in (a) shallow and (b) deep layers (Mean  $\pm$  1.S.E,  $n = 1$  to 3). The vertical dotted line indicates the point at which  $CO_2$  treatments commenced (18<sup>th</sup> September 2012). Summaries of repeated-measures ANOVAs for data collected after this time are shown for each variable. Where there is a significant  $CO_2 \times$  Time interaction ( $P < 0.05$ ), results of post-hoc comparisons between  $CO_2$  treatments for each time interval are shown above the relevant bars, with the following significance codes:  $P < 0.1$  ( $\dagger$ ),  $< 0.05$  (\*),  $< 0.01$  (\*\*) and  $< 0.001$  (\*\*\*).

Figure 5. Temporal change in (a) N mineralisation, (b) nitrification and (c) P mineralisation rates (Mean  $\pm$  1.S.E,  $n = 3$ ). The vertical dotted line indicates the point at which  $CO_2$  treatments began (18<sup>th</sup> September 2012). Summaries of repeated-measures

ANOVAs for data collected after this time are shown for each variable. Where there is a significant CO<sub>2</sub> x Time interaction ( $P < 0.05$ ), results of post-hoc comparisons between CO<sub>2</sub> treatments are shown above the relevant bars with the following significant codes:  $P < 0.1$  (†), 0.05 (\*),  $< 0.01$  (\*\*) and  $< 0.001$  (\*\*\*).

Figure 6. Temporal changes in potential enzyme activities (Mean  $\pm$  1.SE,  $n = 3$ ): (a)  $\beta$ -D-cellobiohydrolase, (b)  $\beta$ -glucosidase, (c) *N*-acetylglucosaminidase and (d) acid phosphatase. The vertical dotted line indicates the point at which CO<sub>2</sub> treatments commenced (18<sup>th</sup> September 2012). Summaries of repeated-measures ANOVAs for data collected after this time are shown for each variable.

Figure 7. Mean soil pH (0-30 cm depth) measured in June 2012 (pre-CO<sub>2</sub> treatment) and June 2013 (after 9-months of CO<sub>2</sub> fumigation; Mean  $\pm$  1.SE,  $n = 3$ ). Results of post-hoc comparisons between CO<sub>2</sub> treatments for each year are shown above the relevant bars.

## Figures

## Supporting information









