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The influence of elevated CO_2 and soil depth on rhizosphere activity and nutrient availability in a mature *Eucalyptus* woodland

Johanna Pihlblad[1,2], Louise C. Andresen[3], Catriona A. Macdonald[1], David S. Ellsworth[1], Yolima Carrillo[1]

[1] Hawkesbury Institute of environment, Western Sydney University, Penrith Australia
[2] Birmingham Institute for Forest Research, University of Birmingham, Birmingham United Kingdom
[3] Department of Earth Sciences, University of Gothenburg, Gothenburg Sweden

Correspondence to: Johanna Pihlblad (m.pihlblad@bham.ac.uk)

Abstract. Elevated carbon dioxide (eCO_2) in the atmosphere increases forest biomass productivity, but only where soil nutrients, particularly nitrogen (N) and phosphorus (P) are not limiting growth. eCO_2, in turn, can impact rhizosphere nutrient availability. Our current understanding of nutrient cycling under eCO_2 is mainly derived from surface soil, leaving mechanisms of the impact of eCO_2 on rhizosphere nutrient availability at deeper depths unexplored. To investigate the influence of eCO_2 on nutrient availability in soil at depth, we studied various C, N and P pools (extractable, microbial biomass, total soil C and N, and mineral associated P) and nutrient cycling processes (enzyme activity and gross N mineralization) associated with C, N, and P cycling in both bulk and rhizosphere soil at different depths at the Free Air CO_2 enrichment facility in a native Australian mature *Eucalyptus* woodland (EucFACE) on a nutrient-poor soil. We found that the depth-induced decrease in nutrient availability, gross N mineralization was counteracted by the root influence and by eCO_2. Increases in available PO_4^{3-}, adsorbed P and the C:N and C:P ratio of enzyme activity with depth were observed. We conclude that the influences of roots and of eCO_2 can affect available-nutrient pools and processes well beyond the surface soil of a mature forest ecosystem. Our findings indicate a faster recycling of nutrients in the rhizosphere, rather than additional nutrients becoming available through SOM decomposition. If the plant growth response to eCO_2 is reduced by the constraints of nutrient limitations, then the current results would call to question the potential for mature tree ecosystems to fix more C as biomass in response to eCO_2. Future studies should address how accessible the available nutrients at depth are to deeply rooted plants, and if fast recycling of nutrients is a meaningful contribution to biomass production and the accumulation of soil C in response to eCO_2.

1 Introduction

With elevated carbon dioxide (eCO_2) in the atmosphere, higher photosynthesis rates can drive increases in forest biomass productivity (Ainsworth and Long, 2005; Norby and Zak, 2011). However, enhanced forest productivity in the long-term is not possible in areas where soil nutrients, particularly nitrogen (N) and phosphorus (P) (Fisher et al., 2012) limit growth (Ellsworth et al., 2017; Terrer et al., 2018, 2019). In contrast, plant-microbe interaction under eCO_2 might stimulate soil organic matter (SOM) decomposition and alleviate nutrient limitation (Luo et al., 2004; Drake et al., 2011; Wang and Wang, 2021). The mechanisms alleviating the nutrient limitation is that higher root exudation rates, stimulation of root growth and fine root production and turnover can potentially elicit SOM decomposition and subsequent nutrient release (Bernard et al., 2022). Root-mediated changes to SOM decomposition and nutrient cycling resulting from a changing climate may be especially important in forest

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systems where tree roots extend far below the soil surface, and where eCO_2 may also alter root distribution with depth (Iversen et al., 2008) (Iversen, 2010). However, current understanding of nutrient cycling under eCO_2 is mainly derived from surface soils, leaving mechanisms of the impact of eCO_2 on nutrient availability at deeper depths unexplored (Jackson et al., 1996).

In the organic rich surface layers of soil, where most fine roots are located, microbial activity is high (de Graaff et al., 2014). As SOM content, root density, and microbial biomass decline with depth, so does microbial activity and rate of processes in soil (Hobley and Wilson, 2016). Despite this, deeper SOM has been found to be more responsive to fresh C inputs (Fontaine et al., 2007) with the implication that the decomposition effect of fresh C from the rhizosphere is likely to increase with depth. With an extending root system, such as may occur under eCO_2 (Iversen, 2010), plants can introduce C where labile C may not have previously been abundant (Iversen et al., 2008; Kuzyakov and Blagodatskaya, 2015) thus promote microbial activity and accelerated C decomposition at depth, potentially releasing nutrients. Moreover, increased C to the rhizosphere can shift the stoichiometric balance of C relative to soil nutrients (de Graaff et al., 2006; Kuzyakov, 2010; Carrillo et al., 2014). With increased abundance of C, the microbial demand for N and P increases (Sistla and Schimel, 2012), in turn leading to an increase in microbial SOM decomposition (Bengtson et al., 2012; Carrillo et al., 2017). Further, microbes have been found to improve their nutrient use efficiency to compensate for the stoichiometric imbalance of decomposer and substrate (Mooshammer et al., 2014). This is manifested through accumulation of N and P in microbial biomass, faster gross mineralization rates, and smaller pools of available inorganic nutrients in the soil solution available for plant uptake. The phenomenon has been found for both N (Rütting et al., 2010) and P (Spohn, 2016; Spohn and Widdig, 2017). How these shifts in stoichiometry manifest in deeper soils is unclear but may have wide ranging implications for forest productivity in response to eCO_2.

Belowground allocation of plant-derived C has differential impacts on N and P owing to inherent differences in their cycling. Plant available N in inorganic form (ammonium and nitrate) is derived primarily through SOM decomposition involving the microbial processes of depolymerization and mineralization of organic compounds and through nitrification (Schimel et al., 2015). In contrast, plant available inorganic P (phosphate) can be sourced from both organic sources via microbial SOM decomposition, and inorganic sources via dissolution from primary minerals and desorption from secondary minerals (Adeleke et al., 2017) (Figure 1). Plant and microbial P limitation is often driven by the mechanism of transitioning P between inaccessible organically bound P to an available inorganic form via a dissolved phase, which renders it susceptible to sorption to secondary mineral surfaces like clays and metal hydroxides (Gérard, 2016). In older, highly weathered soils of higher clay content inorganic P availability can be more constraining for plant and microbial activity than N. In these soils, where the primary mineral P source has been depleted, most of the P left in the system is in organic form, either in biomass of plants and microbial cells, or in SOM (Walker and Syers, 1976; Lambers et al., 2008). Increased root exudation and microbial activity in the rhizosphere can increase decomposition of organic P in SOM through phosphatase enzyme production (Bünemann, 2015) and facilitate the release of mineral adsorbed P by releasing organic acids, competing for sorption sites and lowering soil pH. Therefore, the equilibrium of inorganic P between adsorbed and available forms is determined by root exudation, microbial enzyme production and soil mineralogy (Figure 1) all factors that are considered depth dependent properties.

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Given that N and P cycling in soil differs, and that the factors controlling those processes can vary with depth, soil nutrient stoichiometry also tends to vary with depth (Li et al., 2016). Soil C:N ratio tends to decrease with depth under increased microbial processing of C. Declining SOM content with depth will also lower the N content. In contrast, soil C:P can decrease, but more often remains unchanged as mineral adsorbed P remains in soil despite SOM content declining; the potential implication of which, is a reduction in soil N:P at depth (Li et al., 2016; Zhao et al., 2017). Therefore, many heavily weathered surface soils may be constrained in available PO_4^+, but at depth, some soils may be N limited. This is important in the context of eCO_2, because the response of SOM decomposition to increased labile C availability could be dependent on which nutrient is most limiting to microbes (Dijkstra et al., 2013), which in turn would be expected to depend on depth. Accordingly, extrapolations of nutrient limitation from surface soil processes to deeper soil layers become unreliable without accounting for mechanisms controlling nutrient processing as the stoichiometry changes with depth. The lack of experimental evidence concerning soil nutrient cycling processes in deeper soil render the assumption that native biomes will increase their productivity under eCO_2 contentious (Iversen et al., 2011; Rumpel and Kögel-Knabner, 2011).

The *Eucalyptus* Free Air CO_2 Enrichment (EucFACE) facility in eastern Australia has experimentally exposed a *Eucalyptus* woodland, on a low N and P soil, to eCO_2 concentration (+150 ppm) continuously since 2013 (Drake et al., 2016). To date the site has not seen any evidence of increase in aboveground biomass in the *Eucalyptus* trees under eCO_2 (Ellsworth et al., 2017) despite an increase in the photosynthetic rate of both the dominant tree species and the understory grasses in this ecosystem (Ellsworth et al., 2017; Pathare et al., 2017). The lack of plant biomass response to the CO_2 treatment is hypothesised to be caused by a severe P limitation of the soil, additions of which was shown to increase plant biomass in a tree stand close by not exposed to eCO_2 (Crous et al., 2015). In this system, mineralization and decomposition of SOM have only been investigated in the upper soil layers (Hasegawa et al., 2016; Castañeda-Gómez et al., 2020, 2021). The potential for the plants in this system to utilise nutrients in deeper soil layers is relevant because this highly weathered nutrient poor soil system may already have reached a maximum efficiency for nutrient cycling in the upper soil layer where SOM and microbial activity is greater. Despite the considerable number of P limited forests globally there are still large uncertainties surrounding rhizosphere activity and nutrient cycling in older, P-limited soils compared to younger soils in the northern hemisphere that are often N limited (Fisher et al., 2012; Terrer et al., 2019).

To investigate the influence of eCO_2 on nutrient availability in soil at depth, we studied various C, N and P pools (extractable, microbial biomass, total soil C and N, and mineral associated P) and nutrient cycling processes (enzyme activity and gross N mineralization) associated with C, N, and P cycling in both bulk and rhizosphere soil at different depths at the EucFACE facility. We asked: Q1. what is the difference between rhizosphere and bulk soil in terms of soil properties, and is this changed with soil depth? Q2. what is the effect of eCO_2 on nutrient availability and C:N:P stoichiometry in the rhizosphere, and does it change with soil depth? Given that increased root exudation will prime microbial nutrient mining, we hypothesize (1) nutrient availability (inorganic N and P) will be higher in the rhizosphere compared to bulk soil. We also hypothesize that (2) eCO_2 will increase availability of P to a greater extent than N in surface soil, but not at deeper layers; and that (3) eCO_2 will have less impact on N than P availability and increase the processes contributing to P release (P-targeting enzymes) more so than N release (N-targeting enzymes and gross N mineralization). This effect will be less

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important with depth because the overall N:P ratio declines with depth, alleviating the P limitation and thus shifting the demand from P to N.

2 Materials and methods

2.1 Experimental design

The study was performed at the *Eucalyptus* Free-Air CO_2 Enrichment (EucFACE) experiment located in a Cumberland Plain woodland with mature *Eucalyptus* trees in Sydney, Australia (33 37'S and 150 44'E, 23 m a.s.l.). The site has six experimental rings (n=3), each with a diameter of 25 m. The CO_2 treatment was implemented to three of the rings (eCO_2) since September 2012 and reached +150 ppm above ambient CO_2 (aCO_2) in February 2013 (Ellsworth et al., 2017). The remaining three rings are controls (aCO_2). The soil at the site is a developing red and/or yellow aeric podsol in weakly organised alluvial deposits (Ross et al., 2020) including iron-manganese nodules (Clarendon formation) with a metal oxide rich (field observation) transition to a hardpan clay layer (Londonderry clay; (Atkinson, 1988) found at a variable depth throughout the site (between 35-85 cm). The dominant tree species is *Eucalyptus tereticornis* and the dominant understory grass is *Microlaena stipoides*. The site has an average precipitation of 800 mm per year, with a total precipitation of 16.8 mm in the month leading up to the sampling campaign. The yearly mean temperature was 17 °C. For further detailed site description see Ellsworth et al., (2017).

2.2 Field sampling, soil preparation and root biomass determination

Soil cores (5 cm diameter) were collected from all rings in September 2017. Twelve cores were taken in each ring, spread as three in each of the four pre-established two by two-meter subplots designated for soil sampling (4 subplots per ring, total of 72 soil cores). Each core was sampled down to the clay layer which varied with depth across the site (35-85 cm). Each core was divided into the three depths for investigation: 0-10 cm, 10-30 cm, and transition (a 10 cm interval where sandy loam transitioned into clay). Samples were kept cool until further processing in the laboratory within one week of collection. Although the depth of the transition layer differed throughout the site the chemical properties are assumed to be similar within this zone across the site as the water here periodically builds up above the clay before it drains, creating conditions for podzolification. Soils were processed to separate bulk from rhizosphere soil. The rhizosphere soil was defined as any soil that was still attached to the fine roots when these were separated from soil and soil was collected by gently shaking roots. All other soil in the core was considered bulk soil. For both rhizosphere soil and bulk soil, subplots 1 and 2, and 3 and 4, were combined to two samples per ring and depth (n = 6 samples per ring). This was necessary to have sufficient rhizosphere soil sample for subsequent analysis. Samples were sieved to < 2 mm. Sub-samples for potential enzyme activity were frozen (-20 °C) immediately after sieving (see supplement for further methods). Soil samples to be analysed for nutrient availability and microbial biomass were stored field moist at 5 °C until processed. The roots already handpicked for rhizosphere soil were washed and dried within a week of sampling and later separated into larger and smaller than 3mm diameter fractions. Additionally, any remaining roots were hand-picked from a subsample (~50 g) of sieved soil and scaled to the total sample weight.

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2.3 Extractable carbon, nitrogen, and phosphorus and microbial biomass

Microbial biomass C, N, and P were determined on fresh soil following the fumigation extraction method of Vance et al. (1987). Briefly, fumigated samples were treated with ethanol free $CHCl_3$ under vacuum (fumigated for four days for C and N, and one day for P) and then extracted for C, N, and P using K_2SO_4 and Bray-P I. All extracts were filtered through Whatman 42 grade filter papers and frozen until analysis. Fumigated and unfumigated extracts of K_2SO_4 (0.5 M) were analysed for C and N on TOC-L (total organic carbon analyser, Shimadzu corporation, Japan). Fumigated and unfumigated extracts of Bray-P I were analysed for PO_4^{3-}, additionally unfumigated K_2SO_4 extracts were analysed for inorganic N (ammonium and nitrate), according to Rayment and Lyons (2011), by colorimetry (AQ2 Discrete Analyser, SEAL Analytical, Mequon, WI, USA). Soil was dried (70 °C) for determination of gravimetric soil moisture and air-dried soil was used for pH (1:5 s:w), (S20 SevenEasyTM pH, Mettler-Toledo International Inc., Columbus, OH, USA). Subsamples of the air-dried soil were cleared of visible root fragments and analysed for total soil C and N (LECO TruMac CN-analyser, Leco corporation, USA) and for mineral associated inorganic P.

2.4 Mineral adsorbed inorganic phosphorus

To quantify mineral associated inorganic P a one g air-dried subsample was extracted with NaOH-Na_2EDTA (0.25M NaOH and 0.05M Na_2EDTA) and horizontally shaken for 16 h at 80 rpm after which it was filtered (Rayment and Lyons, 2011). Extracts were diluted 1:10 with sterile water and analysed using the malachite green reagent (Ohno and Zibilske, 1991) in a clear 96 well plate. The plates were analysed by colorimetry on a CLARIOstar plate reader (BMG LABTECH GmbH, Germany) at 610 nm after one hour incubation at 25°C.

2.5 Pool dilution for gross N mineralization rates

To assess the gross N mineralization, rate an isotope pool dilution assay using ^{15}N enriched ammonium was made with a series of laboratory incubations following the method of Rütting et al. (2011). Ammonium concentration and ammonium-^{15}N excess from two time points was done on KCl extracts (Stange et al., 2007; Putz et al., 2018) with SpinMass (Sample Preparation of Inorganic Nitrogen MASSpectrometer) at ISOGOT (Dept of Earth Sciences, University of Gothenburg, Sweden). The^{15}N-label was added in duplicate fresh and sieved soil samples (5 g) with a label consisting of 10 µg $(^{15}NH_4)_2SO_4$ (^{15}N fraction of 99 %, Cambridge Isotope laboratory Inc.) in 0.25 mL milliQ water. After label addition, samples were incubated for 15 minutes and 24 hours under steady temperature (20 °C) and in darkness. The incubations were extracted with 1 M KCl (15 mL), shaken for one hour at 120 rpm and filtered through 42 grade ash-less Whatman filters and frozen until analysis. All gross mineralization rates were calculated using the equation in Kirkham and Bartholomew (1955).

2.6 Statistical analyses

The impact of CO_2 treatment, depth and their interaction were assessed separately for bulk and rhizosphere soil at three depth levels (0-10, 10-30, and transition). Two soil depths (0-10, 10-30) were used in the analysis of rhizosphere where insufficient amounts of rhizosphere soil were recovered during sampling. The subsequent pseudo-replication created with two samples per experimental unit (ring) were dealt with using a linear mixed effects model where CO_2 and depth and their interactions were fixed factors and ring a random factor with individual intersects (*lme4* package, Bates et al. 2015), corresponding to the EucFACE experimental design (Hasegawa et al., 2016). To assess the role of roots on CO_2 and depth effects, we used a linear mixed effects model

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with CO_2, depth (two depths: 0-10, 10-30) and soil type (bulk, rhizosphere) as fixed factors with all interactions and ring as a random factor with individual intersects (Bates et al. 2015). For gross N mineralization rate, the data was slightly skewed due to the ambient bulk 10-30 cm samples being below the SPINMAS detection limit so they could not be quantified.

Significance was determined with the *ANOVA* function (*car* package, Fox and Weisberg, 2019) with Kenward-Roger degrees of freedom estimation. Post-hoc analysis was performed with the *glht* function for multi-comparison (*multcomp* package, Hothorn et al. 2008). The post-hoc Tukey analysis all CO_2, depth, and soil factors were combined into their unique interactions and then processed in the linear mixed effects model as previously described. Normal distribution of residuals was assessed, and log transformations were performed where required to meet model assumptions.

3 Results

3.1 Fine root biomass

Fine root biomass density significantly decreased with depth and ranged from 0.12 $mg \cdot g^{-1}$ to 2.75 $mg \cdot g^{-1}$ with highest densities in the surface depth (0 to 10 cm) and the lowest density in the transition depth (Figure 2). There was a significant interaction between depth and CO_2 where in the topsoil (0 to 10 cm) elevated CO_2 (eCO_2) samples had a 28 % lower fine root density than ambient.

3.2 Carbon in total soil, dissolved and microbial biomass pools

Dissolved organic carbon (DOC) declined significantly with depth for both bulk and rhizosphere soil, and the decrease by depth was stronger for rhizosphere soil (25 %) than for bulk soil (11 %) (Figure 3A and C). The DOC was significantly higher (by 24 %) in rhizosphere soil than bulk soil (Table 1) (for 0-10 and 10-30 cm depths). Microbial C declined significantly with depth for both bulk soil and rhizosphere soil (Figure 3B and D) and was significantly higher in rhizosphere soil (Table 1, Figure 3) by 36 % (transition was excluded). Total soil C content had a significant effect of depth, and an interaction between CO_2 treatment and depth (Table 1); % soil C content was higher in the 0-10 cm depth under eCO_2 but was not different from ambient in the deeper depths (10-30 and transition) (Table 2).

3.3 Rate of gross N mineralization and N pools

Measured soil N content (including NH_4^+, NO_3^-, microbial N) declined significantly with depth for both bulk and rhizosphere soils (Figure 4). Ammonium, nitrate, microbial N, and gross N mineralization (Table 1) were significantly higher in rhizosphere soil than in the bulk soil at both 0 to 10 cm and 10 to 30 cm depths (Table 1). Total soil N content showed a significant interaction between CO_2 treatment and depth (Table 1) where % soil N content was higher in the 0-10 cm depth under eCO_2 but was the same as ambient in the deeper depths (10-30 and transition).

Gross N mineralization rate declined significantly with depth and was significantly higher in rhizosphere soil compared to bulk soil; furthermore, eCO_2 did not have a significant effect (Figure 5, Table 1). The multiple comparison showed the 0-10 cm bulk soil samples as being similar magnitude as the rhizosphere 10-30 cm

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samples. The 0-10 cm rhizosphere treatment were significantly higher than the ambient 10-30 cm rhizosphere, though it cannot be statistically separated from any other treatment group due to the high variability.

3.4 Soil Phosphorus

The three assessed P contents (extractable PO_4^{3-}, microbial P, and mineral associated inorganic P) significantly declined with increasing depth and were higher in the rhizosphere compared to bulk soil (Table 1 and Figure 6). For PO_4^{3-} there was a significant interaction between CO_2 and depth as the concentration of PO_4^{3-} did not decline with depth under eCO_2. Phosphate concentration in the 10-30 cm depth tended to be higher in eCO_2 soils compared to aCO_2 soils (Figure 6D). Microbial P in the bulk soil interacted with CO_2 treatment and depth, where microbial P was lower under eCO_2 compared to aCO_2 in the 0-10 cm depth only (Figure 6).

3.5 Stoichiometry of soil nutrient pools (C, N, P) and soil enzymes

The C:N and C:P of extractable nutrients in the bulk soil increased significantly with depth by 24.9 and 20.9 units of C per nutrient, respectively. However, under eCO_2 the C:N and C:P stoichiometry did not increase in bulk soil (Table 3 and 4). The rhizosphere soil N:P ratio significantly declined with depth. When soil was included as an interactive factor in the model (Table 4), C:N was significant by depth:soil. For extractable C:P ratio both the interaction between CO_2:depth and CO_2:soil was significant where C:P ratio declined with eCO_2 and depth but increased with depth when ambient. In the microbial biomass only C:P significantly increased with depth in bulk soil. The N:P of extractable N and P and microbial biomass stoichiometry significantly increased with depth. When both bulk and rhizosphere soil was considered (only 0-10 and 10-30 cm depth) soil and depth significantly affected extractable C:N and N:P, and the interaction of soil and depth was significant for soil C:N (Table 4). The bulk soil total C:N ratio decreased significantly with depth by 9 units. The rhizosphere soil C:N ratio increased slightly by only 1 unit, yet still significantly, with depth. There was also a significant interaction between CO_2 and depth in the C:N and C:P ratio of the enzymes (Table S1 and S2). The C:N and C:P ratios decreased 0.7 and 0.4 units with depth in ambient conditions but increased 0.4 and 0.3 with depth in eCO_2. The ratio between N and P targeting enzymes did not change with depth but was maintained in the range of 0.5-0.7 N enzymes per P enzyme. The pH showed a marginally significant effect from an interaction of depth and CO_2, where the pH increased slightly in the transition under eCO_2 (Table S1).

4 Discussion

We sampled rhizosphere soil and bulk soil in a depth profile in a *Eucalyptus* woodland experimentally exposed to eCO_2 for 5 years, with the goal to investigate how root activity influences nutrient availability and stoichiometry across depth and under eCO_2. Supporting our hypothesis (1), the nutrient availability increased in rhizosphere soil compared to bulk soil. However, we found no clear evidence to support the hypothesis that eCO_2 affected the rhizosphere soil to a greater extent than the bulk soil (Table 1). There was some evidence to support hypothesis (2), that eCO_2 affected the availability of P more than of N as available PO_4^{+} was more increased with depth in elevated compared to ambient CO_2 (Figure 6). Additionally, the low N:P ratio of enzymes supports hypothesis (3) that P was more limiting than N (Table S1).

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4.1 Depth effects on soil nutrients and microbial biomass

The effect of depth was overall significant and the microbial biomass C, N and P, DOC, inorganic N (NH_4^+ and NO_3^-), inorganic P (PO_4^+), and mineral adsorbed inorganic P all decreased in availability with depth (Table 1). However, when bulk and rhizosphere soil were analysed separately the availability of extractable P in the soil solution in the rhizosphere did not decline with depth under eCO_2 (Figure 6D). Increased P availability below surface soil in the rhizosphere has been found in previous studies at the site (Ochoa-Hueso et al., 2017), which measured nutrient availability down to 30 cm depth, and also in other forest sites investigating nutrient availability in deeper soil (Blume et al., 2002; Rumpel and Kögel-Knabner, 2011; de Graaff et al., 2014; Li et al., 2016). The microbial P concentration decreased under eCO_2 in the 0-10 cm depth in the bulk soil (Figure 6C), in line with the negative effect of CO_2 on fine roots (Figure 2), suggesting that root density and microbial P respond similarly to eCO_2.

Stoichiometry changed with depth differently for bulk and rhizosphere soil. The ratio of extractable C to N and to P in bulk soil increased with depth, as DOC decreased less with depth than inorganic N and P. However, contrary to our hypothesis the ratio between N and P was constant across depth in bulk soil. In the rhizosphere soil the ratio between DOC, and inorganic N and P remained constant with depth while the N:P ratio significantly decreased; hence, the rhizosphere inorganic P became relatively more available than N at deeper soil. We suggest there was more P available because there were fewer fine roots and lower microbial biomass to immobilise it. Furthermore, as P became limiting at depth more resources were used to increase its availability, supported by the consistently higher P targeting enzyme activity than N enzyme activity.

4.2 Rhizosphere effects on nutrient availability and mineralization across depths

It is a paradigm in rhizosphere research that microbial activity is high near the root because of the input of energy in the form of newly photosynthesised C (Kuzyakov et al., 2000; Kuzyakov and Cheng, 2001). Supporting this, we found that microbial biomass and nutrient availability was higher in the rhizosphere soil compared to bulk soil. Furthermore, the gross N mineralization rate increased in the rhizosphere compared to bulk soil. Given the positive links found between gross N mineralization and SOM decomposition (Bengtson et al., 2012; Zhu et al., 2014) these findings suggest that root-microbe interactions are facilitating decomposition and increasing nutrient availability (Andresen et al., 2020).

In contrast, the potential activities of enzymes responsible for depolymerizing and hydrolyzing N and P from SOM did not increase closer to the root (Table S1) supporting previous findings from the site that reported enzyme activities were not higher in the presence of roots (Ochoa-Hueso et al., 2017; Castañeda-Gómez et al., 2021). The lack of enzymatic activity response to roots in both surface and deeper soil depths could be due to the microbial community lacking access to energy and N to be able to synthesise enzymes (Olander and Vitousek, 2000). Alternatively, because of greater nutrient availability there is reduced need for enzyme production (Sinsabaugh et al., 2009). Finally, a shift in the microbial community composition favouring fungi over bacteria in the rhizosphere as has been observed at the site could lead to lower enzyme production per unit biomass (Castañeda-Gómez et al 2021).

The stoichiometry of enzymes targeting N and P is an indicator of microbial nutrient demand (Sinsabaugh et al., 2009). In this system, N does not appear to be the most limiting nutrient given the low ratio of N:P targeting

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enzymes. The low enzyme N:P ratio suggest that P is more highly sought by the microbes in this system (Allison and Vitousek, 2005; Sinsabaugh et al., 2008). We found this independent of soil depth, indicating that P is in higher demand than N in the entire soil profile. Interestingly, no difference in either enzyme amount or stoichiometry was found between bulk soil and rhizosphere soil which indicate that given a higher C availability in the rhizosphere, microbes did not increase their enzyme production to mine for organic P. However, P can also be sourced from non-organic sources (Gérard, 2016). This is supported by the high levels of mineral associated inorganic P in the rhizosphere at depth (Figure 5). We suggest that non-organic sources of P may be important to microbes in the rhizosphere as an alternative to high energy cost enzyme production.

The pattern of decline in nutrient concentrations in deeper soil profiles is well documented (Jobbágy and Jackson, 2001), though a decline in these concentrations still occurs in the rhizosphere soil with depth. Here we can show that root activity counteracts the decline associated with depth, maintaining a higher microbial biomass and nutrient availability in the rhizosphere soil compared to bulk soil (Finzi et al., 2015). Together with the evidence of higher gross N mineralization rate in the rhizosphere soil, we suggest that in this P limited mature forest, roots can drive the availability of both N and P even in deeper soil through microbial biomass turnover and a strong recycling of nutrients without large decomposition of SOM. Although we can show that deep rhizosphere has an impact on available nutrients our study cannot assess if plants are utilising the increased availability since we did not measure plant uptake. However, assuming at least part of plant nutrient immobilisation is via diffusion of concentration gradients (Gilroy and Jones, 2000), a higher nutrient concentration in the deeper rhizosphere soil is likely benefiting plants as well as microbes.

4.3 Elevated CO_2 and depth dependency of rhizosphere effects

Elevated CO_2 increases C availability and nutrients in the rhizosphere through increased rhizodeposition and nutrient mobilisation (Phillips et al., 2011; Kuzyakov et al., 2019). Because root density declines with increasing depth, we hypothesised that the effects of eCO_2 on C and nutrient availability will be less important with depth. Contrary to that hypothesis we found that eCO_2 interacted with depth by increasing the inorganic P availability at depth under eCO_2. Further, mineral associated inorganic P was constantly higher at depth in the bulk soil under eCO_2, though the trend is not significant. Metal hydroxide mineral rich clay is capable of strong adsorption of negative ions and organic complexes (Jilling et al., 2018; Rasmussen et al., 2018) which is present at EucFACE. Changes in pH can affect the equilibrium between mineral adsorption and solution concentration though the small increase in pH that was detected in the rhizosphere soil (less than 0.5 units compared to bulk soil, Table S1), it is not necessarily enough to change the sorption capacity. rather the higher PO_4^{3-} adsorption and concentration in solution indicates that higher rates of phosphate processes exist in that space. The different forms of soil P thus appears to respond to different drivers, while the microbial biomass did not immobilise the additionally available PO_4^{+} or access the mineral associated P. This supports that the microbes are not limited by P at depth. The question remains if plants can access the increased P availability at deeper soils.

The relative content and activity of C-degrading compared to N and P degrading enzymes was higher in the deeper soil under eCO_2 for both rhizosphere soil and bulk soil. These trends with depth suggest that the surface soil is more limited by nutrients (i.e. N and P poor) compared to deeper layers where C is a limiting factor for activity. Thus, eCO_2 may cause increased microbial activity and enzyme synthesis at depth rather than in the surface soil. The relative content of enzymes for N to P release ranged 0.5 to 0.8, and this indicated biological P

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limitation rather than N limitation and that ratio was consistent through the depth profile, though the total enzyme activity declined with depth. Only cellulase activity (CB, Table S1) was constant in all layers possibly indicating that plant matter is being decomposed throughout the soil profile. It was demonstrated by Castaneda-Gomez et al (2020) that root litter decomposition is increased under eCO_2 at the site and contributes to C loss from the system. Root litter decomposition can thus be an important source of nutrient release at depth. Further, eCO_2 has been found to increase the rate of root turnover in this system (Piñeiro et al., 2020), which is one of the main sources of C supply to the deeper soil, other than increased root exudation.

In this study the observed lack of influence of eCO_2 on nutrient availability and N mineralization at the surface is likely due to the topsoil being less limited by C than deeper soils (depth and CO_2 interaction). Though enzyme activities decrease with depth, they are more abundant per unit soil C deeper in the profile. Given the rather low eCO_2 fertilisation effect found on photosynthetic rate (Ellsworth et al., 2017; Jiang et al., 2020) and root production in this system (Piñeiro et al., 2020) the presumed limited increase in C release belowground is likely turned over without affecting the SOM decomposition. Mineral adsorbed P forms however, are sensitive to root derived changes in pH (Jones and Darrah, 1994), representing a different mechanism for affecting the P cycle separate from SOM decomposition. In the scenario where nutrients mostly become available through recycling, rather than SOM decomposition, it is unlikely that plant nutritional requirements under eCO_2 will be satisfied and support continued biomass growth even where roots are known to grow deeper (Iversen et al., 2011). This 'fast-in, fast-out' C cycle in this mature nutrient limited ecosystem under eCO_2 will not necessarily release long stored soil C to the atmosphere, but it is not likely to increase C sequestration by gaining additional plant biomass or soil C either.

There are several consistent trends of an increase in nutrient availability with eCO_2 in this study, but they were not statistically significant. These variables include available inorganic N, gross N mineralization rate, inorganic P, and mineral associated P. These trends in pools and processes may indicate an increase in both nutrient availability and up-regulation, if mild, of processes responsible for increased nutrient availability. Though the mature *Eucalyptus* trees have not responded to eCO_2 with aboveground biomass growth (Ellsworth et al., 2017) the understory species composition has shifted to include more nutrient-demanding grasses with eCO_2 (Hasegawa et al., 2018). Higher quality understory litter may in turn drive increased nutrient availability in the soil (Berg and McClaugherty, 1989). Given the necessarily low replication, common to many FACE experiments (Filion et al., 2000), and the lower-than-expected enhancement of photosynthesis in this FACE system (Ellsworth et al., 2017; Pathare et al., 2017; Jiang et al., 2020), an eCO_2 effect was expected to be statistically elusive, but here we do show that it can be discerned.

4.4 Conclusion

We found that nutrient availability and gross N mineralization were always higher in rhizosphere soil compared to bulk soils, but enzymatic activity was not. The effect of depth, generally, caused a decrease of available nutrients and process rates feeding into the available pools. However, the impact of roots and eCO_2 counteracted the decrease found with depth when interactions between soil depth and CO_2 or soil depth and soil type (bulk or rhizosphere) occurred. This response of lower concentrations found with increasing depth particularly affected

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available PO_4^{3-}, adsorbed P and the C:N and C:P enzyme activity. We can conclude that roots and eCO_2 can affect available nutrient pools and processes well below the surface soil of a forest ecosystem, though it is not clear if the plants can benefit and take up nutrients from deeper parts of the soil profile. Our findings indicate a faster recycling of nutrients in the rhizosphere, rather than additional nutrients becoming available through SOM decomposition. If the tree response to eCO_2 is hindered or prevented by nutrient limitations, then the current results would question the potential for mature tree ecosystems to fix more C as biomass in response to eCO_2. Future studies are suggested to focus on how accessible the available nutrients at depth are to deeper rooted plants, and if this fast recycling of nutrients is meaningful in production of plant biomass and accumulation of soil C response to eCO_2.

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Author contribution

The initial idea and experimental design were done by Johanna Pihlblad (JP) and Yolima Carrillo (YC) with support by Catriona A. Macdonald (CAM). The data was gathered by JP and with support by YC, CM, and Louise C. Andresen (LCA). JP did the data management, statistical analysis and wrote the first draft. All other authors contributed to writing of the final paper.

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Tables

Table 1. The effect of the factors CO_2 (eCO_2 and eCO_2), soil depth (*0 to 10 cm, 10 to 30 cm, transition*) and soil type (bulk and rhizosphere soil) and their interactions, shown as model F statistic output. *Asterisks and bold indicate the level of significance of P values: *** for P < 0.001; ** for P < 0.01 and * for P < 0.05.* The extractable nutrients NH_4^+, NO_3^- and PO_4^{3-}, DOC, and microbial biomass C, N and P are modelled on a $mg \cdot kg^{-1}$ basis, gross N mineralisation rate on a $mg \cdot kg^{-1} \cdot day^{-1}$ *basis, and soil C and N in %.*

	CO_2	depth	soil	CO_2:depth	CO_2:soil	depth:soil	CO_2:depth:soil
Df	1	1	1	1	1	1	1
Carbon							
DOC	0.29	**30.35** ***	**27.8** ***	0.01	0.05	1.46	0.94
Microbial C	0.08	**141.1** ***	**15.9** ***	1.92	0.6	2.34	0.01
Soil C	0.2	**236.89** ***	1.21	0.1	**7.94** **	0.69	1.69
Nitrogen							
NH_4^+	0.09	**24.08** ***	**25.96** ***	0.27	0.2	0.03	0.16
NO_3^-	0.46	**8.96** **	**16.36** ***	0.3	0.0	0.11	1.3
Microbial N	0.16	**122.42** ***	**18.32** ***	0.02	0.52	0.0	0.22
gross N min	2.04	**13.08** **	**8.81** **	0.37	0.05	0.92	NA
Soil N	0.0	**194.1** ***	0.19	0.01	**11.04** **	2.68	2.42
Phosphorus							
PO_4^{3-}	0.37	**32.63** ***	**33.18** ***	**8.6** **	2.21	0.17	0.06
Microbial P	0.48	**126.46** ***	**6.38** *	2.53	0.0	0.18	0.11
Mineral Pi *a*	0.03	**68.31** ***	**5.77** **	0.19	0.58	2.34	0.73

Table 2. Total soil C and N (%) and the C to N ratio for ambient aCO_2 and elevated eCO_2 in bulk soil at the three depths. Standard error is given in parenthesis. Results from statistical analysis are provided in Table 1.

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	Soil C %		Soil N %		C:N	
Depth	**Ambient**	**Elevated**	**Ambient**	**Elevated**	**Ambient**	**Elevated**
0-10	1.46 (0.2)	1.83 (0.2)	0.09 (0.0)	0.11 (0.0)	15.86 (0.6)	16.05 (0.4)
10-30	0.52 (0.1)	0.59 (0.1)	0.04 (0.0)	0.05 (0.0)	12 (1.1)	12.37 (0.9)
transition	0.15 (0.0)	0.17 (0.0)	0.02 (0.0)	0.02 (0.0)	6.59 (1.1)	7.34 (1.0)

Table 3. Extractable and microbial C, N and P stoichiometry (mg kg^{-1}/mg kg^{-1}) and soil C:N ratio for bulk soil (B) on the left of each column and rhizosphere soil (R) on the right for of a mature Eucalyptus forest soil exposed to ambient and elevated CO_2 for three depths (0 to 10 cm, 10 to 30 cm, transition). Stoichiometry was calculated on a mg kg^{-1} mass basis with standard error below in parenthesis.

	Extractable						Microbial						Soil	
	B	*R*	*B*	*R*	*B*	*R*	*B*	*R*	*B*	*R*	*B*	*R*	*B*	*R*
	C:N		**C:P**		**N:P**		**C:N**		**C:P**		**N:P**		**C:N**	
Ambient														
0-10	17.9 (3.5)	15.0 (3.5)	24.2 (1.6)	29.9 (3.1)	1.6 (0.2)	2.5 (0.5)	5.6 (0.6)	5.1 (0.4)	9.8 (0.6)	11.0 (0.9)	2.0 (0.2)	2.2 (0.1)	15.9 (0.6)	14.6 (0.5)
10-30	36.3 (10.4)	19.2 (5)	30.5 (3.2)	31.3 (6.4)	1.5 (0.5)	1.9 (0.3)	4.3 (0.6)	4.4 (0.8)	12.3 (2)	13.4 (3.5)	2.7 (0.2)	2.9 (0.3)	12.0 (1.1)	15.6 (0.8)
transition	56.8 (22.2)	NA	65 (18.7)	NA	1.9 (0.7)	NA	NA	NA	NA	NA	9.5 (3)	NA	6.6 (1.1)	NA
Elevated														
0-10	14 (2.6)	12.6 (2.4)	26.1 (2.4)	25.3 (0.2)	2 (0.1)	2.3 (0.4)	5.8 (0.3)	6.2 (1.1)	12.5 (1.2)	20.1 (7.3)	2.2 (0.2)	3.3 (1.3)	16.0 (0.4)	16.0 (0.5)
10-30	20.5 (6.3)	12.9 (2.5)	23.4 (1.4)	22.1 (1.8)	1.5 (0.3)	2.0 (0.3)	7.5 (1)	5.5 (0.9)	14.9 (2.8)	16.8 (3.1)	2.3 (0.2)	2.7 (0.3)	12.4 (0.9)	16.9 (0.9)
transition	24.3 (8.5)	NA	24.8 (7)	NA	1.2 (0.3)	NA	8.3 (5)	NA	NA	NA	17.8 (13.6)	NA	7.3 (1)	NA

Table 4. Model F statistic and significance of extractable and microbial C, N and P, and soil C:N. Where bulk and rhizosphere are shown separate, bulk was modelled with 3 depth levels whereas rhizosphere soil was modelled with only 2. Where bulk soil and rhizosphere soil are shown together (†) only the 0-10 and 10-30 cm depths are included in the model. Significance of P values are as indicated: *** indicate P < 0.001; ** indicate P < 0.01 and * indicates P < 0.05.

	Extractable			Microbial			Soil
	C:N	**C:P**	**N:P**	**C:N**	**C:P**	**N:P**	**C:N**
Bulk							
CO_2	0.32	0.62	0.04	0.45	0.16	0.3	0.16

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	Extractable			Microbial			Soil
	C:N	**C:P**	**N:P**	**C:N**	**C:P**	**N:P**	**C:N**
depth	**4.8 ***	0.51	1.7	0.67	0.78	**11 *****	**62.4 *****
CO_2:depth	0.34	2.48	0.84	0.62	0.12	1.27	0.06
Rhizosphere							
CO_2	0.14	0.77	0.01	0.62	0.54	0.23	3.9
depth	0.46	1.6	2.01	1.97	0.02	0.8	1.91
CO_2:depth	0.36	0.6	0.04	0.45	0	0.42	0
Bulk and Rhizosphere†							
CO_2	0.21	0.55	0.07	0.84	0.3	0.08	2.02
depth	**6.93 ***	0	**7.91 ****	1.16	0.27	2.5	**9.27 ****
soil	**11.8 ****	0.06	**13.58*****	1.73	1.28	1.53	**7.4 ***
CO_2:depth	0.52	3.23	0.06	1.57	0.02	1.54	0.01
CO_2:soil	0.2	1.78	1.35	0.04	1.47	0.29	0.96
depth:soil	3.04	3.01	0.84	0.94	0.13	0.04	**19.12 *****
CO_2:depth:soil	0.01	0.58	0.27	0.2	0	0.03	0.01

Figures

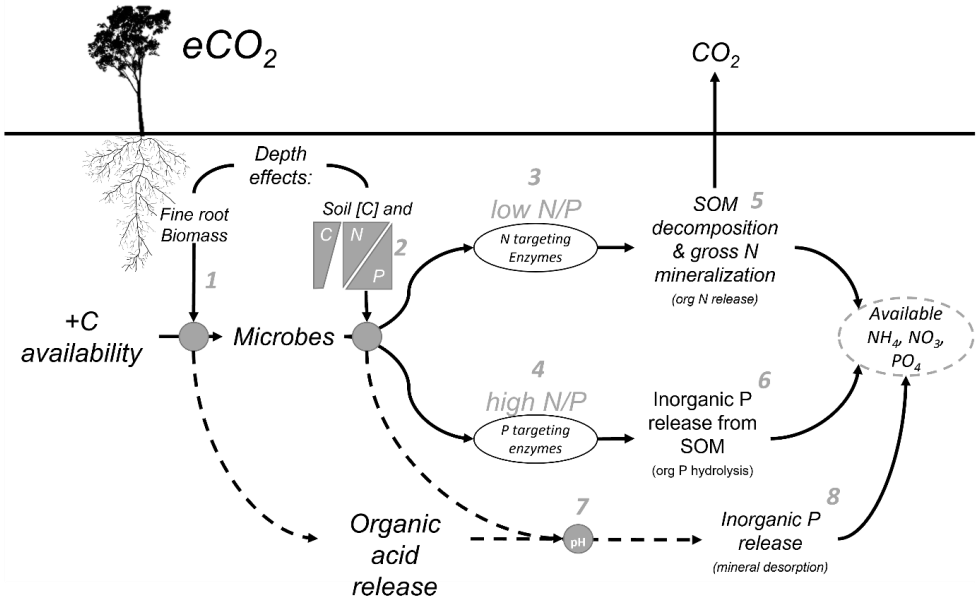

Figure 1. Conceptual diagram of the mechanisms affecting nutrient availability as influenced by soil depth. Elevated CO_2 increases C availability belowground, but the effect of that extra C is moderated by depth dependent mechanisms. (1) Root exudation in the rhizosphere soil is proportional to fine root biomass which decreases with

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depth. (2) The microbial strategy to release nutrients is a function of soil C content and N to P ratio, which also can change with depth. (3) The microbial strategy is a response to the N to P ratio either producing N targeting enzymes in low N to P conditions or (4) P targeting enzymes in high N to P conditions. (5) Nitrogen targeting enzymes act to decompose SOM and increase gross N mineralization, transforming N into NH_4^+ and ultimately NO_3^- which are available for plant uptake. (6) P targeting enzymes cut phosphates from organic molecules by hydrolysis. (7) One further mechanism behind nutrients release affected by eCO_2, is that soil pH is changed, impacting the soil sorption capacity, by the organic acid exudates from roots and microbial mineralization thereof. (8) The decreased acidity tips the balance of phosphates in solid and in solution, to increase soil solution content and P availability by mineral desorption.

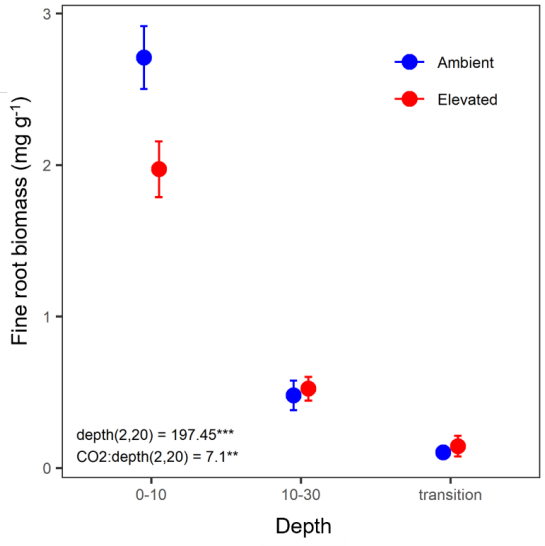

Figure 2. Biomass of fine roots of less than 3 mm thickness ($mg \cdot g^{-1}$) in the mature *Eucalyptus* forest soil exposed to ambient (blue) and elevated (red) CO_2 for three depths (0-10 cm, 10-30 cm, transition). Error bars indicate standard error. Mixed effects model output stated with (degrees of freedom, Df residuals) F statistic presented and asterisks for the P values for significance are as indicated: *** indicate $P < 0.001$ and ** indicate $P < 0.01$.

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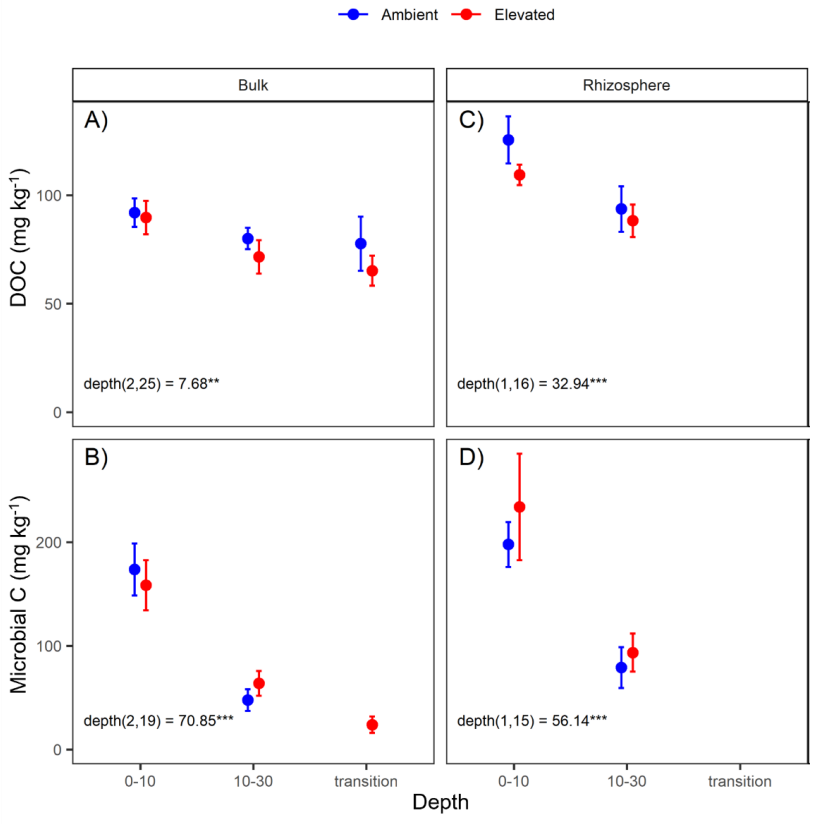

661

662 **Figure 3.** Dissolved organic carbon (DOC) and microbial biomass carbon (C) content for bulk and rhizosphere
663 soil of the mature *Eucalyptus* forest soil exposed to ambient (blue) and elevated (red) CO_2 for three depths (0 to
664 10 cm, 10 to 30 cm, transition). Error bars indicate standard error. Mixed effects model output stated with (degrees
665 of freedom, Df residuals) and F statistic presented and asterisks for the P values for significance are as indicated:
666 *** indicate $P < 0.001$ and ** indicate $P < 0.01$. Results from statistical analysis of comparison of soil types (bulk
667 and rhizosphere) are presented in Table 1.

668

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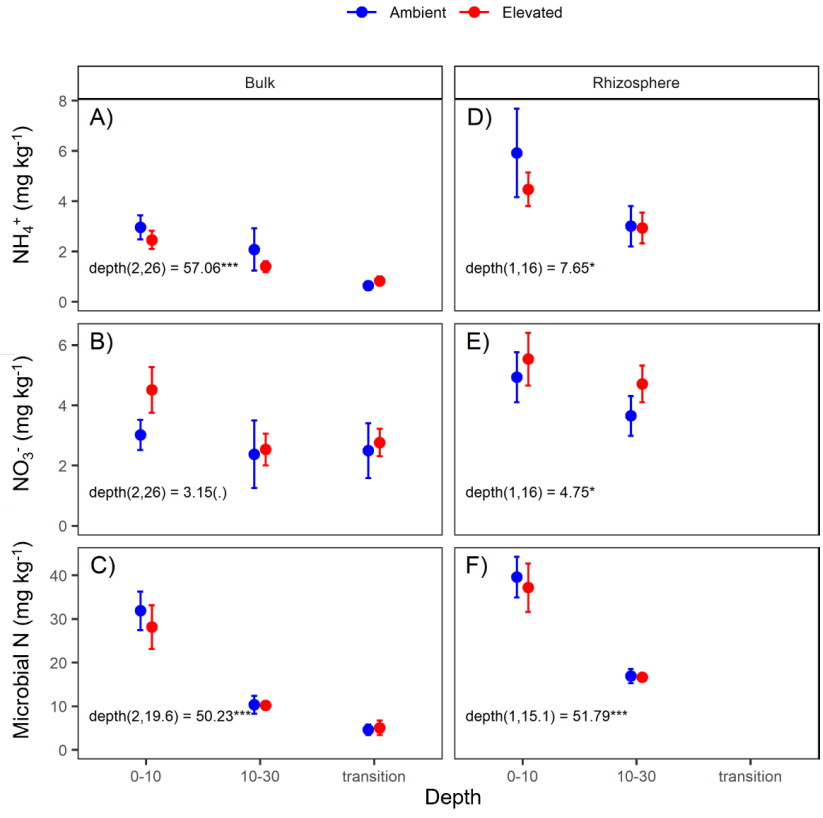

669

670 **Figure 4.** Nitrogen (N) pools in the forms of ammonium (NH_4^+), nitrate (NO_3^-) and microbial biomass N for bulk
671 and rhizosphere soil of the mature *Eucalyptus* forest soil exposed to ambient (blue) and elevated (red) CO_2 for
672 three depths (0 to 10 cm, 10 to 30 cm, transition). Error bars indicate standard error. Mixed effects model output
673 stated with (degrees of freedom, Df residuals) and F statistic presented and asterisks for the P values for
674 significance are as indicated: *** indicate $P < 0.001$, ** indicate $P < 0.01$, * indicates $P < 0.05$ and (.) indicates a
675 tendency to a significance $P < 0.1$. Results from statistical analysis of comparison of soil types (bulk and
676 rhizosphere) are presented in Table 1.

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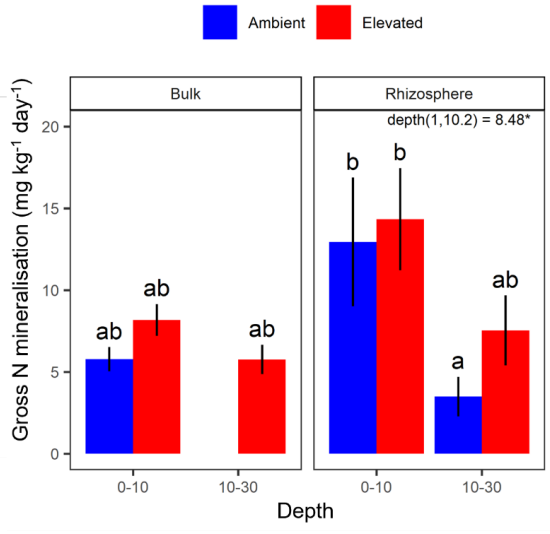

678

679 **Figure 5.** Gross N mineralization for bulk and rhizosphere soil of the mature *Eucalyptus* forest soil exposed to
680 ambient (blue) and elevated (red) CO_2 for two depths (0 to 10 cm, 10 to 30 cm. Error bars indicate standard error.
681 Mixed effects model output stated with (degrees of freedom, Df residuals) and F statistic presented and asterisks
682 for the P value for significance, * indicates $P < 0.05$. Results from statistical analysis of comparison of soil types
683 (bulk and rhizosphere) are presented in Table 1.

684

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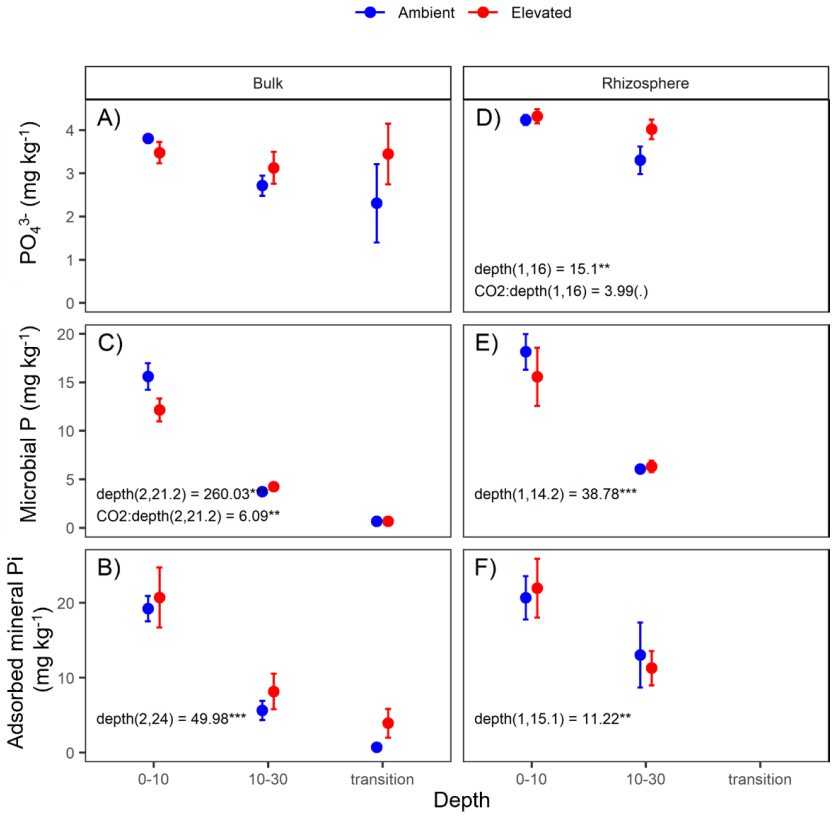

Figure 6. Measured soil P pools in the in forms of inorganic P (PO_4^{3-}), microbial biomass P, and mineral associated phosphate through adsorption for bulk and rhizosphere soil of the mature *Eucalyptus* forest soil exposed to ambient (blue) and elevated (red) CO_2 for three depths (0 to 10 cm, 10 to 30 cm, transition). Error bars indicate standard error. Mixed effects model output stated with (degrees of freedom, Df residuals) and F statistic presented and asterisks for the P values for significance are as indicated: *** indicate $P < 0.001$, ** indicate $P < 0.01$, * indicates $P < 0.05$ and (.) indicates a tendency to a significance $P < 0.1$. Results from statistical analysis of comparison of soil types (bulk and rhizosphere) are presented in Table 1.