Nitrogen dynamics after two years of elevated CO₂ in phosphorus limited *Eucalyptus* woodland

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**Abstract** It is uncertain how the predicted further rise of atmospheric carbon dioxide (CO₂) concentration will affect plant nutrient availability in the future through indirect effects on the gross rates of nitrogen (N) mineralization (production of ammonium) and depolymerization (production of free amino acids) in soil. The response of soil nutrient availability to increasing atmospheric CO₂ is particularly important for nutrient poor ecosystems. Within a FACE (Free-Air Carbon dioxide Enrichment) experiment in a native, nutrient poor *Eucalyptus* woodland (EucFACE) with low soil organic matter (≤ 3%), our results suggested there was no shortage of N. Despite this, microbial N use efficiency was high (c. 90%). The free amino acid (FAA) pool had a fast turnover time (4 h) compared to that of ammonium (NH₄⁺) which was 11 h. Both NH₄-N and FAA-N were important N pools; however, protein depolymerization rate was three times faster than gross N mineralization rates, indicating that organic N is directly important in the internal ecosystem N cycle. Hence, the depolymerization was the major provider of plant available N, while the gross N mineralization rate was the constraining factor for inorganic N. After two years of elevated CO₂, no major effects on the pools and rates of the soil N cycle were found in spring (November) or at the end of summer (March). The limited response of N pools or N transformation rates to elevated CO₂ suggest that N availability was not the limiting factor behind the lack of plant growth response to elevated CO₂, previously observed at the site.

**Keywords** Gross N mineralization rate · Depolymerization · Free amino acids · Phosphorus limitation

**Introduction**

Ecosystem effects of elevated atmospheric carbon dioxide (CO₂) concentration are a global concern, with the atmospheric CO₂ concentrations recently having
surpassed 415 ppm (McGee 2019). Worldwide, several experiments manipulate atmospheric CO₂ concentration at the field scale in open air by the FACE (Free Air Carbon dioxide Enrichment) technique (Miglietta 2001; Ellsworth et al. 2017) to investigate direct physiological responses of vegetation and effects on whole ecosystems. The question of whether plant growth is limited by carbon (C) or nutrient supply is relevant as both nutrient loading and elevated atmospheric CO₂ currently alter natural terrestrial environments (Steffen 2015; Stevens 2019). Recently reviewed evidence suggest that the CO₂ response in plant biomass is controlled by stocks of plant available nitrogen (N) and phosphorus (P) (Terrer 2019). Hence, C, N and P responses to CO₂ are likely to be intimately linked. However, if plant growth is limited by one or more nutrient (Chapin et al. 1987; Čapek 2018), an increased plant growth under elevated CO₂ is only possible if nutrient mobilization also increases. Eventually, a down-regulation of growth responses (progressive nutrient limitation, PNL) might occur through long-term changes in nutrient cycles. However, PNL under eCO₂ has only been confirmed in few cases (Norby 2011; Zähle 2014). Beginning with photosynthesis, CO₂ can stimulate a cascade of potential effects in an ecosystem, leading to an increase in plant growth and in belowground allocation (root growth) (Andresen et al. 2016b; Körner 2018). Subsequently, increased rhizodeposition could stimulate organic matter decomposition (rhizosphere priming) and nutrient mineralization, leading to increased nutrient availability, to meet the extra nutrient demand (Dijkstra et al. 2013; Kuzyakov 2015; Jilling et al. 2018; Moreau et al. 2019; Schleppi 2019). A meta-analysis found that gross N mineralization rate was stimulated by CO₂ across N limited ecosystems, but not in ecosystems limited by P (Rütting & Andresen 2015), potentially because the cascade response to labile C deposition switches from N mining to P mining (Dijkstra et al. 2013).

Organic nitrogen (Nₐₒᵣᵍ) plays a key role in soil N cycling and amino acids constitute an important direct resource of N for microbes and plants across ecosystems (Chapin III 1995; Kuzyakov 2013). It is known that many vascular species, including Eucalyptus species (Warren 2006, 2009), can take up intact amino acids (Andresen et al. 2011; Näsholm et al. 2009) as well as inorganic N (Nᵢₘᵢᵣᵩ) sources (ammonium and nitrate). It has recently been suggested that amino acids are as abundant as Nᵢₘᵢᵩ in a Eucalyptus woodland with Brown Sodosol soils in New South Wales (Australia) (Prendergast-Miller et al. 2015). Similarly, amino acids were reported as abundant as Nᵢₘᵢᵩ in Eucalyptus, Melaleuca and Banksia dry bush with Aridisol soils in Western Australia (Farrell 2013). Hence amino acids might be as relevant an N source as Nᵢₘᵢᵩ across Australia (Lee et al. 2018). The transformation of Nₐₒᵣᵍ via N-rich polymers to free amino acids (FAA) directly from readily available detritus, is driven by soil microbes. Additionally, Nᵢₘᵢᵩ in mineral associated organic matter (MAOM) can be destabilized for further microbial transformation by organic acids in plant exudates (Schimel & Bennett 2004; Kuzyakov 2015; Jilling et al. 2018). Traditionally, for ecosystems with N limited vegetation having typical high soil C to N ratio, the liberation of FAAs from proteins during depolymerization is seen as the rate-limiting step in the transformation pathway of N from Nₐₒᵣᵍ to Nᵢₘᵢᵩ. Contrastingly, in more fertile ecosystems, both gross N mineralization and depolymerization rates can constrain Nᵢₘᵢᵩ availability (Schimel & Bennett 2004; Jones et al. 2009). Recently it has been noted that even liberation of FAA from MAOM can be a limiting factor for gross N mineralization rate (Jilling et al. 2018). Hence, FAA and Nᵢₘᵢᵩ pools can have several sources and sinks and it is unclear if these pool sizes are good indicators of the gross rates (Gårdenäs et al. 2011; Hobbie & Hobbie 2012). Gross rates determined by the ¹⁵N pool dilution technique allow the estimation of instantaneous rates, accounting for all sources (aided by all types of functional enzymes) and sinks together. For Australian ecosystems, only limited knowledge about gross rates driving the soil N cycle exist, and their responses to future climate change such as elevated CO₂ are unknown.

Investigation of FAA pools and production rates under elevated CO₂ are rare (Chen et al. 2014; Wild et al. 2018). Hypothetically, the abundance of FAAs in soil can increase as rhizodeposition increases. Such an increase in FAAs can occur directly from an increase in the release of FAAs in root exudates (Xiong 2019), or indirectly from an increase in the depolymerization rate. To our knowledge, no theory is available to explain why some individual FAAs would be more abundant than others. Further, individual FAA abundance and production rates are unknown for most FACE sites. In fact, most conclusions about the responses of the N cycle in FACE studies are based on
N\textsubscript{in}, leaving a large knowledge gap on N\textsubscript{org} responses to CO\textsubscript{2}. Studies on rates of gross N mineralization and depolymerization are lacking in Australia, and as many soils in Australia are considered as nutrient poor, because of a long history of weathering (Turner & Condron 2013), further understanding the importance of N\textsubscript{org} in N cycling in such nutrient poor ecosystems is important.

At the Cumberland Woodland site central to this study, measurements have shown seasonal variations of the nutrient pools, but with minimal effects from FACE (ambient + 150 ppm CO\textsubscript{2} increase; Hasegawa et al. 2016; Ochoa-Hueso et al. 2017). The study site soil has a low P status (\textasciitilde 60 mg kg\textsuperscript{-1}; Crous et al. 2015) and is considered to have P-limited vegetation, as Eucalyptus stem diameter (Crous et al. 2015) and root biomass (Nielsen et al. 2015) increased significantly following 2 years of P fertilization (50 kg P ha\textsuperscript{-1} yr\textsuperscript{-1}). We hypothesised that (i) N\textsubscript{org} would be an important factor in N cycling in these nutrient poor soils, and based on the outcome of a meta analysis of gross N mineralization rates (Rüttig & Andresen 2015) that (ii) gross N mineralization and depolymerization rates would show minimal response to elevated CO\textsubscript{2}. We used $^{15}$N pool dilution to assess gross N mineralization and depolymerization rates, in combination with quantification of the N\textsubscript{in} and FAA pools, in order to determine the role of FAAs in the N cycle and its response to elevated CO\textsubscript{2} in a P limited Cumberland Plain woodland (EucFACE).

**Methods and materials**

**Field site**

The EucFACE experiment is located within a Cumberland Plain Woodland in Western Sydney, New South Wales (NSW), Australia. The site is characterized by a relatively open canopy of evergreen Eucalyptus tereticornis (Forest Redgum) trees of 17 to 23 m height, at a low density of 600 to 1000 trees ha\textsuperscript{-1} with a basal area of 27 m\textsuperscript{2} ha\textsuperscript{-1} (Crous et al. 2015; Duursma et al. 2016; Ellsworth et al. 2017). Cumberland Woodland is listed as an endangered ecological community in Commonwealth Legislation (under Sect. 181 of the Environment Protection and Biodiversity Conservation Act 1999; EPBC Act). The understory is a diverse mix of grasses and forbs with a total of 86 species present (Hasegawa et al. 2018). The climate is a humid temperate-subtropical transitional climate. In the southern hemisphere spring months are September to November, summer months are December to February, autumn months are March to May and winter months are June to August (Drake et al. 2016). Growing season can be defined as August to March but this has much variation and relies on rainfall patterns and soil moisture (Collins et al. 2018). Soil volumetric water content is highly variable at the site, ranging from (rare) flooded conditions of 35% to exceptionally dry soils of 5% (Drake et al. 2018; Gimeno et al. 2018). This variation in soil volumetric water content arises mostly from variable weather patterns at the site, but typically, the summer receives most rainfall. The soil is formed from weakly organised alluvial deposits and primarily an Aeric Podosol with areas of Densic Podosol (Australian soil classification) (Ross 2020). The soil is a slightly acidic loamy sand with low soil organic matter content and there is a hard impermeable clay layer that varies in depth across the site from 35 to 75 cm depth.

**FACE treatment**

The main EucFACE experiment consists of six instrumented circular rings of 25 m diameter, connected by a set of vertical pipes that release CO\textsubscript{2} into the ecosystem in a controlled manner using the FACE technique with CO\textsubscript{2} pre-dilution (Hendrey 2006). Carbon dioxide is injected in three randomly selected rings. Three other rings are instrumented controls where CO\textsubscript{2} is not added. The rings are separated by 80 to 100 m. The target of the FACE treatment is 150 ppm above ambient atmospheric CO\textsubscript{2} concentration. Injection of CO\textsubscript{2} began in September 2012 and gradually reached + 150 ppm in February 2013.

**Soil sampling**

Soil was sampled 26 and 30 months after CO\textsubscript{2} treatment began, on November 17, 2014 (late spring) and on March 9, 2015 (early autumn). Within each of four 2 m $\times$ 2 m sub-plots dedicated for soil sampling, located within each ring, four soil cores were sampled to a depth of 10 cm using a 5 cm diameter metal auger. In parallel, separate samples were collected from the top 2 cm. For each depth, replicates from within each sub-plot were mixed resulting in four replicate
samples per ring. Leaf and bark litter as well as roots and stones were removed by hand. Soil was then sieved through a 2 mm sieve and kept at 4 °C for up to 2 weeks until labelling or extraction took place. The average field soil temperature from November 1, 2014 until March 31, 2015 was 22.5 °C ± 0.1 with a minimum of 18.8 °C and a maximum of 25.8 °C. The incubating soils were kept at room temperature, varying between 22 and 25 °C, for acclimation prior to the labelling. During calculations (see later), an average of the four sub-plots was used to cover the spatial variation in one plot. Bulk density (g dry soil cm⁻³) of the top 10 cm was determined for each ring in August 2017.

Soil water sampling

To measure soil water amino acid content, we obtained soil water from each of the four sub-plots within each ring, using permanently installed lysimeters with ceramic suction cups at two different depths (1900 soil water sampler from Soil Moisture Equipment Corp., Santa Barbara, California). The first was installed to a depth of 10 to 15 cm (upper cup) and the second immediately above the clay layer (lower cup). The day before sampling (December 10, 2014) a vacuum was applied to the cup using a hand pump to pull soil water into the cup. The following day, the pumped soil water was collected using PVC piping and a hand pump. Samples were sterilized by filtering (0.22 μm SLGP033RB, Millipore, OH, USA) and stored at 4 °C until analysis. All water samples were analysed for individual amino acid concentration.

Soil properties

Gravimetric soil water was determined by drying 5 g fresh soil at 105 °C until weight constancy. Soil organic matter (SOM) content was determined by loss on ignition (550 °C, 5 h). Extractable inorganic N was determined from fresh soil (2 g) for each sub-plot following extraction with 2 Mol·L⁻¹ KCl. Nitrate (NO₃⁻) and ammonium (NH₄⁺) concentrations were determined by colorimetry (AQ2 Discrete Analyser, SEAL Analytical, Mequon, WI, USA). Nutrient content is presented as mg (NO₃⁻ or NH₄⁺) kg⁻¹ dry soil, and by use of bulk density, on a m² basis in the top 10 cm.

Air dried and ground soil (approximately 60 μg) was accurately weighed in triplicate into tin capsules and analysed by the IRMS (GSL elemental analyzer coupled to a 20–22 isotope ratio mass spectrometer Sercon Ltd., Crewe, UK) for total N and C and for ¹⁵N and ¹³C abundance. Uncertainties of δ¹⁵N and δ¹³C were lower than 0.3 ‰.

Free amino acids were extracted from fresh soil (10 g) from each sub-plot using 20 mL of a 10 mMol·L⁻¹ CaSO₄ (120 rpm, 1 h) and filtered (Whatman qualitative filter papers no. 1) after sedimentation for 30 min. An internal standard (200 μL) consisting of norvaline (0.072 mg mL⁻¹), norleucine (0.071 mg mL⁻¹) and 4-chlorophenylalanine (0.096 mg mL⁻¹) was added to the CaSO₄ extracts and the soil water, which were then transferred to solid phase extraction (SPE) columns ( Dionex OnGuard II H, 1 mL cartridge, ThermoScientific) by vacuum suction. The SPE columns were then frozen and shipped to Ghent, Belgium (ISOFYS laboratories; Andresen et al. 2016a) for analysis.

Nitrogen turnover rates by pool dilution technique

Two isotope labellings were performed in the laboratory to study gross soil N turnover rates: 1. gross depolymerization rate (amino acid production rate) by ¹⁵N amino acid pool dilution, and 2. gross N mineralization rate by ¹⁵N–NH₄ pool dilution. Gross N mineralization rates were assessed in November and March but depolymerization only in March. For each sub-plot (four sub-plots per treatment ring), sub-samples of fresh soil (10 g) were weighed into 6 cm diameter dark flasks, producing a layer of soil c. 5 mm thick. Samples were then labelled by adding the isotope enriched label solution to the soil. In November (but not March), the soil moisture was so low that applying the isotope label was difficult as the soil was hydrophobic. Therefore, 1 mL deionized water was slowly added dropwise to each soil one day before labelling. The two ¹⁵N labels were: 1. a 20 amino acids-mixture (AA; ‘cell free powder’ from Cambridge isotope laboratories; see (Andresen et al. 2015)) with 99% ¹⁵N, dissolved in 0.1 Mol·L⁻¹ HCl, containing 0.36 g AA L⁻¹ corresponding to 3 μg AA-N g⁻¹ soil.; and 2. ammonium sulphate with 99.8% ¹⁵N, amended at a rate of 2 μg N g⁻¹ soil. Label addition was done by dripping 1.5 mL of the ¹⁵N enriched label onto the soil surface in an even
spatial pattern (in November only 0.5 mL of label was added to account for the 1 mL of deionized water already added). After label addition, the flasks were incubated in a dark chamber at 25 °C. At ten minutes and at 7 h after label addition soils from sub-plots were extracted as follows. The incubations with the 15N-NH4 label with 20 mL of 1 Mol L−1 KCl (120 rpm, 60 min), and incubations with the 15N-amino acid label with 20 mL of 3.7% formaldehyde in 10 mMol L−1 CaSO4 solution (120 rpm, 60 min). After 30 min, the slurries were decanted and filtered (Whatmann 42 ashless, diam. 125 mm). The KCl extracts were frozen and transported to ISOGOT labs in Sweden for analysis. The CaSO4 extracts were transferred to SPE columns as above, then frozen and transported to ISOFYS for analysis.

Measurement of NH4+ and 15N-NH4 by SPINMASS

The KCl extracts were analysed for 15N-NH4 using the SpinMass (Sample Preparation of Inorganic Nitrogen MASSpectrometer) at ISOGOT labs by automated determination of NH4-N and its 15N abundance in liquid samples. The procedure is a reaction of the KCl extracted sample (c. 9 mL) containing NH4+ with NaOBr solution (c. 1 mL) by the Rittenberg reaction (Stange 2007) and then transfer of the produced N2 gas into the quadrupole mass spectrometer (QMS, GAM 400, InProcess Instruments GmbH, Bremen). The QMS determines 14N14N (mass 28), 14N15N (mass 29) and 15N15N (mass 30) in N2 gas from which the 15N enrichment and concentration of NH4+ was calculated. The minimum amount required (detection limit) at SpinMass for δ15N in ammonium is 1 μg N.

Abundance and isotopic composition of individual amino acid analysis by GC–MS

The method to assess the individual free amino acid (FAA) content and isotopic enrichment is described in detail elsewhere (Wanek et al. 2010; Andresen et al. 2015, 2016a). In short, at arrival at ISOFYS lab, the SPE columns were washed with 10 mL of ultrapure water after which the FAAs were eluted with 30 mL 3 Mol L−1 NH4OH. The NH4OH extraction solvent was removed by evaporation at reduced pressure (c. 30 mbar by rotovap), and amino acids were derivatized by ethyl chloroformate in ethanol/pyridine to block the polar groups of amino acid in order to make them volatile for GC separation. Finally, the individual FAAs were measured by gas chromatography–mass-spectrometry (GC–MS, Trace GC–DSQ, Thermo Fisher). Detection limit for individual amino acids is lower than 0.02 mg·kg⁻¹ soil.

Calculations

Depolymerization rate (DSON, amino acid production rate), gross N mineralization rate (M, NH4⁺ production rate) and the consumption rates of amino acids (CFAA) and ammonium (CNH4) were calculated using the analytical equations (Kirkham 1954) for gross mineralization or depolymerization (Wanek et al. 2011). In most cases the formula for decreasing concentration between time steps was used as the difference of concentration was greater than the detection limit. The rates are expressed as mg N·kg⁻¹ dry soil·h⁻¹; or on a soil carbon (C) basis as mg N·kgC⁻¹·h⁻¹; and on m² basis by using the bulk density.

The turnover time was calculated by dividing the pool size (NH4-N or FAA-N) by the production rate (depolymerization rate or gross N mineralization rate). Half-life (t1/2) of a pool is the time when half of a pool size would be produced (t1/2 = turnover/2). Half-life is a measure that compares to results from studies of amino acids with 14C methods.

The microbial N use efficiency (NUE) is the efficiency of N immobilization and is calculated as NUE = (CFAA + CNH4-M)/(CFAA + CNH4); consumption of FAA and NH4+ express the microbial N uptake and gross N mineralization (M) express the microbial N loss (Wild et al. 2018).

Statistical testing

The effect of the factors (CO2 treatment, time and depth when available) on the variables was assessed using “R” software, by fitting linear mixed effect models with the function “lmer” from package “lm4” (Bates 2017). When only one time point was available, ring was included as random effect. For variables with repeated measures, the fit of a model with sub-plot within ring as a random effect was tested against a model with only ring as random effect. AIC (Akaike Information Criterion) of the latter was always lower and thus, only ring was retained as a random effect in
Results

Soil properties

The soil C to N ratio was unaffected by CO₂ treatments (aCO₂: 13.4 ± 3.0 mean and standard error; n = 12 and eCO₂: 14.1 ± 3.6; n = 12). Total soil N (0.02%) and C (0.2%) was higher in March than in November (p = 0.0015 and p = 0.0007 respectively; Table 1). Soil NO₃⁻ and NH₄⁺ content and gravimetric soil moisture, at either depth or date, did not significantly respond to CO₂ treatment (Fig. 1; Table 1).

Free amino acids

The total FAAs content from the soil extracts (on dry soil base) was 2 to 3 times higher in March compared to November (not significant; Table 1). Individual FAAs in soil extracts were more variable in March than in November (Table S1) and were dominated by the presence of aspartic acid, glutamic acid, serine and threonine on both dates (Fig. 2a). Ten out of 14 FAAs were either significantly, or by tendency, different at the two dates, but with no significant effect of CO₂ treatment (Table S2). Soil water total FAA concentration was c. 30% higher in the lower than in the upper lysimeter (Table S1). Soil water was dominated by the amino acids serine, glycine and lysine (Fig. 2b and Table S1), with significant effect of time only for glycine and lysine (Table S2).

Gross rates

Both gross N mineralization, depolymerization and the amino acid consumption rates (M, DSON and CFAA; Fig. 3) were highly variable but were not significantly affected by elevated CO₂ treatment or time (month), either on the dry soil weight or the soil carbon base (Table 2). Only the ammonium consumption rate (CNH₄) significantly differed between the two dates (0.007) while the interaction of sampling date and treatment had marginal difference (p = 0.058) (Table 2). In the aCO₂ treatment, CNH₄ was higher in November compared to March (time p = 0.007 and time × treatment; Table 2). The soil nitrogen cycle

Based on our observations we can characterize the main pools and fluxes of N in this ecosystem (Fig. 4 and Tables 3 and 4). The pool size (g N m⁻²) of Nṁ was two to five times larger than the FAA pool (F(1,41) = 415.7, p < 0.001; linear mixed model), especially in November, but was not significantly different between the two dates or CO₂ treatments. Depolymerization rate DSON was a factor three to four greater than M (F(1,41) = 79.3, p < 0.001; linear mixed model), and the CFAA was a factor 8 to 12 greater than CNH₄ (F(1,40) = 340, p < 0.001; linear mixed model). Consequently, the turnover of the FAA amino acid pool was faster (4 h) than the turnover of the NH₄⁺ pool (11 h) (Fig. 4 and Table 4). The half-life of the NH₄⁺ pool was on average 5 h and was around 1.5 h for the FAA pool (98 min; Table 4). In March, NUE was 84% in elevated CO₂ and 90% in control treatments.

Discussion

This study is one of the first relating gross N mineralization and depolymerization rates and N pools in Australian soils using ¹⁵N techniques. Using a ¹⁴C approach, Farrell et al. (2013) determined amino acid and peptide half-life (alanine, dialanine and trialanine) across grassland, bush and forest in Western Australia. With ¹⁵N techniques, Rutting & Hovenden (2020) determined gross N mineralization rate in a Tasmanian grassland (TasFACE). Our study is however, the first conducted in Australian woodland within an eCO₂ field facility (EucFACE).

Our results serve to enhance the understanding of soil N cycling and ecosystem response to elevated CO₂. By determining available soil N stocks and their turnover rate, we showed that both inorganic and
organic sources of N are available in soil for plant uptake. Even though our $^{15}$N labelling experiment did not directly involve plants, results from other experiments have demonstrated *Eucalyptus* plant uptake of intact FAAs (Warren 2006, 2009). Other experiments using $^{15}$N tracing suggest that the competition between plants and microbes for N sources is dominated by microbial uptake (Warren 2009; Kuzyakov 2013). We found that the microbial nutrient use efficiency NUE was very high (84% in eCO$_2$ to 90% in aCO$_2$), thus leaving a 10–16% margin of nutrients potentially available for plants. High NUE is usually related to N limitation of microbes, but a wide range in NUE has been observed for soils with C to N ratios below 20 (Mooshammer et al. 2014). The EucFACE soil C to N ratio of 13 to 15 suggest this is not an ecosystem with N limited plant growth. Thus according to the meta-study by Terrer et al. (2019), a positive biomass

### Table 1  Soil properties in 0 to 10 cm depth

|                      | aCO$_2$   | eCO$_2$   | time    | treatment | time*treatment |
|----------------------|-----------|-----------|---------|-----------|----------------|
| **Soil moisture (%)**|           |           |         |           |                |
| November             | 2.0 (0.3) | 1.9 (0.2) | n.d     | n.d       | n.d            |
| March                | 3.6 (0.5) | 4.1 (0.6) | n.d     | n.d       | n.d            |
| **Soil organic matter (%)**|          |           |         |           |                |
| November             | n.d       | n.d       | n.d     | n.d       | n.d            |
| March                | 2.7 (0.2) | 2.9 (0.3) |         |           |                |
| **Soil nitrogen (%)**|           |           |         |           |                |
| November             | 0.10 (0.01)| 0.10 (0.01)| 11.591 (0.0015) | ns | ns            |
| March                | 0.12 (0.01)| 0.11 (0.01)|          | ns | ns            |
| **Soil carbon (%)**  |           |           |         |           |                |
| November             | 1.3 (0.1) | 1.4 (0.1) | 13.67 (0.0007) | ns | ns            |
| March                | 1.5 (0.1) | 1.6 (0.2) |          | ns | ns            |
| **Soil $\delta^{15}$N (%)**|         |           |         |           |                |
| November             | 2.4 (0.2) | 2.7 (0.2) | ns      | ns        | ns             |
| March                | 2.3 (0.2) | 2.7 (0.3) |          | ns | ns            |
| **Soil $\delta^{13}$C (%)**|         |           |         |           |                |
| November             | 25.6 (0.2) | 26.1 (0.2) | ns      | ns        | ns             |
| March                | 25.8 (0.2) | 26.4 (0.2)|          | ns | ns            |
| **Soil NO$_3$ (mg$\cdot$kg$^{-1}$)**|       |           |         |           |                |
| November             | 1.71 (0.15)| 1.92 (0.19)| ns     | ns        | ns             |
| March                | 2.04 (0.33)| 1.68 (0.35)|        | ns | ns            |
| **Soil NH$_4$ (mg$\cdot$kg$^{-1}$)**|        |           |         |           |                |
| November             | 1.29 (0.45)| 1.74 (0.61)| ns    | ns        | ns             |
| March                | 1.56 (0.55)| 1.40 (0.17)|        | ns | ns            |
| **FAA (mg$\cdot$kg$^{-1}$)**|         |           |         |           |                |
| November             | 0.6 (0.2) | 0.3 (0.1) | ns      | ns        | ns             |
| March                | 1.2 (0.5) | 1.0 (0.6) |          | ns | ns            |

Gravimetric soil moisture (%), by weight loss at 100 °C, soil organic matter content (%), by loss on ignition at 550 °C, total soil nitrogen and carbon (%), by IR mass spectrometry, total soil $\delta^{15}$N (%) and $\delta^{13}$C (%o, by isotope ratio mass spectrometry), soil NO$_3$, NH$_4$, PO$_4$ and total free amino acids (FAA, mg kg$^{-1}$ dry soil). The average (n = 3) with standard error in parenthesis for ambient (aCO$_2$) and elevated (eCO$_2$) CO$_2$ treatments (EucFACE) sampled on November 17, 2014 and March 10, 2015

Statistical significance was tested by linear mixed effect model and ANOVA with treatment, time and the interaction of time and treatment as factors. $F$ values are presented with $P$ value in parenthesis, when a significant effect ($< 0.05$) was observed. Non-significant effects are indicated as ns. Soil moisture and soil organic matter measures were made only at one time point and thus time and interactive effects were not determined $n.d$. Data was log transformed when necessary.
response to eCO₂ is likely, based on this soil’s C to N ratio.

The EucFACE soil pool of FAA-N was smaller than the N\textsubscript{in} pool. Only a few studies of natural (unfertilized) Mediterranean or Australian soils provide parallel N content on FAA and N\textsubscript{in} pools. Soil content of FAAs and N\textsubscript{in} across three woodlands on Brown Sodosoil (with soil C to N ratios of 19 to 30) in New South Wales had smaller or similar content of FAA as N\textsubscript{in} (Prendergast-Miller et al. 2015). Smaller or similar FAA content as N\textsubscript{in} was found across natural soils in Western Australia (Farrell 2013), and a trend with less FAA-N than N\textsubscript{in} was found in a grassy woodland ecosystem dominated by Eucalyptus melliodora, and Blakely’s Red Gum (Eucalyptus blakelyi) near Canberra (with soil C to N ratio of 12 to 17) (Macdonald et al. 2014).

Gross N mineralization rates in the EucFACE soils were within the same order of magnitude as rates measured in an Eucalyptus forest on Durustalf soil with a clayey texture in Western Australia (Mediter- ranean climate) (Banning et al. 2008). In addition, gross N mineralization and ammonium consumption rates in our study were within one order of magnitude as measured in a native forest in Queensland (Australia), that had a subtropical climate and higher soil moisture (Burton et al. 2007) compared to our study. Furthermore, the EucFACE gross N mineralization rate fell within the high-end range of gross N mineralization rate measured in five Eucalyptus plantations in Portugal on Cambisols (Mediterranean climate) (Gómez-Rey et al. 2010). Finally, the half-life of FAA in EucFACE soils were within the same range as those observed in ¹⁴C-labelled amino acids experiments in Australian woodland soils (Farrell 2013; Prendergast-Miller et al. 2015). Hence, we consider the N dynamics of this Cumberland Plain woodland to be typical of woodlands in similar climatic regions or similar soils. In the lab incubations, the amount of plant accessible N made available through depolymerization (e.g. FAA-N release) was three to four times the amount made available by gross N mineralization (e.g. NH₄-N release). Hence, the amino acid pathway is more efficient than the gross N mineralization in making N available for plant uptake (Warren 2006, 2009). This plant driven short circuit- ing of the N-cycle (Chapin III 1995), is thus an important part of the N cycle as we hypothesized. With the gross N mineralization rate being slower than depolymerisation in our soils, gross mineralization, rather than depolymerisation, is the limiting step for N\textsubscript{in} availability. Hence, according to the paradigm suggested by Schimel and Bennett (2004), and mod- ified by Jilling et al. (2018), this Cumberland Wood- land has significant NH₄⁺ and monomer N sources, either from readily available detritus or from MAOM. Further, both gross N mineralization and depolymerization rates are potential controlling factors for plant N availability (Schimel & Bennett 2004; Jilling et al. 2018).
Fig. 2 Free a amino acid nitrogen (µg amino acid N g⁻¹ dry soil) in soil extracts (10 mMol L⁻¹ CaSO₄) in November and March (0 to 10 cm depth). Data is presented as average of ambient (aCO₂) and elevated (eCO₂) treatments within each time point; b amino acid nitrogen (µg N amino acid L⁻¹) in soil water in November from upper (15 to 20 cm) and lower (30 to 60 cm) lysimeters. Data is presented as average of ambient (aCO₂) and elevated (eCO₂) treatments. Error bars are standard error. Data is presented in Table S1 and significant effects in Table S2.
Individual amino acids from the two pools (CaSO$_4$ extracts and soil lysimeter water) represent the pool of available FAAs to plants and are made available by depolymerization and rhizodeposition. In addition, cell destruction may potentially bias the content as an artefact (Hobbie & Hobbie 2012). Soil FAA extracts were dominated at both sampling times by aspartic acid, glutamic acid, serine and threonine. These are acidic (aspartic and glutamic acid) and polar (serine and threonine) amino acids which, due to their electronegativity (soil pH 5.5), are mobilized by extraction (Warren & Taranto 2010). The soil water did have a different set of dominant FAAs compared to the soil CaSO$_4$ extracts. The basic amino acid lysine, which is a positively charged compound, dominated in the soil water along with serine and glycine. These are both low molecular weight amino acids and will likely emerge from degradation and destabilization of larger amino acid molecules prior to leaching (glutamine, asparagine and threonine have serine as basic structure; likewise, glycine is the smallest fundamental structure of all amino acids). The larger FAA concentration in the lower than upper lysimeter water suggest either a leaking of FAAs down in the soil or a less intense FAA consumption and gross N mineralization in the lower soil layer.

From the spring (November) to the late summer (March) sampling time, soil properties did change slightly, whereby higher total C, total N and FAA content were observed in late summer, which corresponded to a smaller ammonium consumption in late summer. However, the lack of strong responses in N transformation rates to eCO$_2$ at EucFACE were in accordance with our hypothesis and with the observed lack of increase in plant growth, litter inputs (Ellsworth et al. 2017; Jiang 2020) and changes in senesced...
litter chemistry (Crous et al. 2019). Water availability, which was low during both samplings, controls degradation and biological activity, along with root exudation (Bengtson et al. 2012). These factors are both theoretically enhanced under elevated CO₂ (Gimeno et al. 2018). Even though observations at the EucFACE indicated no CO₂ stimulation of plant growth, there were fluctuating stimulations of photosynthesis and belowground C allocation. Therefore, potential stimulation of priming of SOM and MAOM degradation followed by mineralization would theoretically have been possible (Duursma et al. 2016; Ellsworth et al. 2017; Collins et al. 2018; Drake et al. 2018; Jilling et al. 2018). As a parallel, a lack of response in depolymerization and gross N mineralization rates to CO₂ was also found in a nutrient poor heathland (Larsen et al. 2011; Holmstrup et al. 2017; Wild et al. 2018).

Our study spanned surface soils (0–10 cm), where most of the root influence occurs in this system (Ochoa-Hueso et al. 2017). However, as N supplies and root density (and thus activity) changes with depth, we cannot rule out that a response of soil N cycling to CO₂ occurs in deeper soil layers (Zähle 2014). Although root C and N dynamics (Castañeda-Gómez et al. 2020) were not part of our study, the behaviour of the soil microbes in the short-term incubation is driven by impacts from long-term exposure to experimental treatments which includes

| Month    | CO₂ treatment | M gross N mineralization rate | C₉NH₄ gross N consumption rate | DSON depolymerization rate | CFAA amino acid consumption rate |
|----------|---------------|-----------------------------|-------------------------------|---------------------------|----------------------------------|
|          |               | mg N kg⁻¹ dry soil h⁻¹       | mg N kg⁻¹ dry soil h⁻¹         | mg N kg⁻¹ dry soil h⁻¹    | mg N kg⁻¹ dry soil h⁻¹            |
| November | aCO₂          | 0.098 (0.01) 7.721 (0.79)    | 0.234 (0.06) 16.531 (3.27)    | n.d  n.d                  | n.d  n.d                          |
| November | eCO₂          | 0.081 (0.02) 6.407 (1.74)    | 0.108 (0.02) 7.789 (1.09)     | n.d  n.d                  | n.d  n.d                          |
| March    | aCO₂          | 0.111 (0.02) 7.429 (1.16)    | 0.115 (0.02) 7.683 (0.92)     | 0.460 (0.04) 30.174 (1.81) | 0.954 (0.05) 64.047 (3.19)       |
| March    | eCO₂          | 0.159 (0.07) 10.563 (4.08)   | 0.083 (0.01) 5.908 (1.13)     | 0.419 (0.04) 30.554 (4.62) | 0.894 (0.08) 67.247 (9.12)       |
| Linear mixed model |               | time: ns                               | time: 8.38 (0.007)                         | treatment: ns                        | treatment: ns                        |
|          |               | time × treatment: ns                             | time × treatment: 3.83 (0.058)                    | treatment: ns                        | treatment: ns                        |
|          |               | time: ns                               | time: 10.17 (0.003)                         | treatment: ns                        | treatment: ns                        |
|          |               | time × treatment: ns                             | treatment: ns                        | treatment: ns                        | treatment: ns                        |

Data is presented as mean values with standard errors in parenthesis. Gross nitrogen mineralization rate (M) and consumption rate (CNH₄) are expressed as mg NH₄-N kg⁻¹ dry soil h⁻¹ and in italics mg NH₄-N kg⁻¹ h⁻¹. Gross depolymerization rate DSON (amino acid production) and amino acid consumption rate CFAA are expressed as mg AA-N kg⁻¹ dry soil h⁻¹ and in italics as mg AA-N kg⁻¹ h⁻¹. M and CNH₄ were obtained by the pool dilution assay using ¹⁵N-labelled NH₄ in November and March. DSON and CFAA were obtained by the pool dilution assay using ¹⁵N-labelled amino acids only in March. The time steps 10 min to 7 h were used for calculating all rates by the analytical equations developed by Kirkham and Bartholomew (1954).

Statistical significance was tested by linear mixed effect model and ANOVA with treatment (for DSON and CFAA with only one time point) and time, and the interaction of time and treatment, were factors. F values are presented with P-value in parenthesis, when a significant effect (< 0.05) or a tendency (< 0.1). Non-significant effects are indicated as ns and n.d. means not determined. Data was log transformed when necessary.
Fig. 4 Conceptual diagram for an area of one (m$^2$) in the top 10 cm soil layer of average soil nitrogen (N) pools and fluxes, for the aCO$_2$ (a) and eCO$_2$ (b) treatments in March 2014. Pools (total soil N, amino acid N (AA-N), ammonium-N (NH$_4^+$) and nitrate–N (NO$_3^-$)) are presented in gN m$^{-2}$. Fluxes (gross N mineralization rate (M), NH$_4^+$ consumption rate (C$_{NH4^+}$), depolymerization rate (D$_{SON}$) and amino acid consumption rate (C$_{EA}$)) are presented in gN m$^{-2}$.day$^{-1}$. Turnover time, indicated by circular arrows for AA-N and NH$_4^+$ pools, is the time needed to produce the entire pool. Data and standard errors are presented in Table 3.
the direct and indirect root influence (Bengtson et al. 2012; Ochoa-Hueso et al. 2017).

The weak or missing N cycle response under FACE treatment in some ecosystems – grassland, heathland or forest observed here and elsewhere (Wild et al. 2018; Schleppi 2019; Rütting 2020), could be due to limitation of another nutrient, such as P (Dijkstra et al. 2013; Rütting & Andresen 2015). At EucFACE, evidence derived from Eucalyptus leaf C:N:P stoichiometry revealed strong P re-translocation compared to N in both ambient and CO2 treated trees (Crous et al. 2019), emphasising that P is highly limiting for the trees in this ecosystem. Phosphorus limitation within this ecosystem was further supported by evidence from a P-addition experiment near the experimental rings that demonstrated a significant increase in both above ground and below ground plant growth following P additions (Crous et al. 2015; Nielsen et al. 2015). When P (and not N) is limiting, increased rhizodeposition may not result in enhanced soil organic matter decomposition. Thus a stimulation of N mineralization is prevented because microbial P limitation forces microorganisms to target P rather than N (Dijkstra et al. 2013). Therefore future studies should target the P dynamics in the EucFACE experiment to better understand ecosystem response to eCO2 in nutrient poor systems.

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Table 3  Mean soil nitrogen (N) pools and rates on area (m²) basis in the 0 to 10 cm soil depth of the ambient (aCO2) and elevated CO2 (eCO2) treatments

| Nutrient pool or flux              | Treatment | November 2014 | March 2015 |
|-----------------------------------|-----------|---------------|------------|
| Total soil N g N m⁻²              | aCO2      | 133.8 (20.7)  | 160.3 (29.4)|
| Total soil N g N m⁻²              | eCO2      | 130.8 (14.7)  | 146.2 (26.1)|
| Inorganic N (NO₃ + NH₄) g N m⁻²    | aCO2      | 0.20 (0.04)   | 0.24 (0.05)|
| Inorganic N (NO₃ + NH₄) g N m⁻²    | eCO2      | 0.25 (0.08)   | 0.20 (0.01)|
| Amino acid N g N m⁻²               | aCO2      | 0.115 (0.04)  | 0.219 (0.10)|
| Amino acid N g N m⁻²               | eCO2      | 0.050 (0.02)  | 0.175 (0.10)|
| NH₄ consumption rate g AA-N m⁻² day⁻¹ | aCO2    | 0.671 (0.382) | 0.388 (0.109)|
| NH₄ consumption rate g AA-N m⁻² day⁻¹ | eCO2    | 0.342 (0.009) | 0.269 (0.071)|
| Gross N-mineralization rate g AA-N m⁻² day⁻¹ | aCO2    | 0.314 (0.061) | 0.376 (0.067)|
| Gross N-mineralization rate g AA-N m⁻² day⁻¹ | eCO2    | 0.281 (0.045) | 0.535 (0.201)|
| Depolymerization rate g AA-N m⁻² day⁻¹ | aCO2    | n.d           | 1.477 (0.145)|
| Depolymerization rate g AA-N m⁻² day⁻¹ | eCO2    | n.d           | 1.428 (0.146)|
| AA-N consumption rate g AA-N m⁻² day⁻¹ | aCO2    | n.d           | 3.197 (0.281)|
| AA-N consumption rate g AA-N m⁻² day⁻¹ | eCO2    | n.d           | 3.168 (0.351)|

Numbers in parenthesis are standard error (n = 3)

Table 4  Nitrogen (N) turnover time (hours) for the ammonium (NH₄-N) and the free amino acid (FAA-N) pools based on gross N mineralization and depolymerization rate respectively, for the ambient (aCO₂) and elevated CO₂ (eCO₂) treatments

| Turnover | Treatment | November 2014 | March 2015 | t₁/₂ |
|----------|-----------|---------------|------------|------|
| NH₄-N    | aCO₂      | 11            | 11         | 5    |
| NH₄-N    | eCO₂      | 16            | 7          | 3    |
| FAA-N    | aCO₂      | n.d           | 4          | 107 (min) |
| FAA-N    | eCO₂      | n.d           | 3          | 88 (min) |

Data is presented in Table 3. Half-life (t₁/₂) of the pool in March in hours for NH₄ and in minutes for AAs.
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**Author contribution** All authors planned or carried out the 15N-labelling experiments at the HIE with soils from the EucFACE experiment in Richmond, NSW Australia. Analysis of NH₄, NO₃ and PO₄₃ was carried out at HIE by CM. Analysis of amino acids was carried out at ISOFYS labs in Ghent, Belgium by LCA and SB. Analysis of 15N-NH₄ extracts was carried out at ISOGOT in Gothenburg, Sweden by LCA and TR. Calculations and statistical analysis done by LCA, TR and LCG. LCA wrote the first draft and all authors contributed to the development of the current paper.

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