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Short-term carbon cycling responses of a mature eucalypt woodland to gradual stepwise enrichment of atmospheric CO_2 concentration

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Abstract

Projections of future climate are highly sensitive to uncertainties regarding carbon (C) uptake and storage by terrestrial ecosystems. The *Eucalyptus* Free-Air CO_2 Enrichment (EucFACE) experiment was established to study the effects of elevated atmospheric CO_2 concentrations (eCO_2) on a native mature eucalypt woodland with low fertility soils in southeast Australia. In contrast to other FACE experiments, the concentration of CO_2 at EucFACE was increased gradually in steps above ambient (+0, 30, 60, 90, 120, and 150 ppm CO_2 above ambient of ~400 ppm), with each step lasting approximately 5 weeks. This provided a unique opportunity to study the short-term (weeks to months) response of C cycle flux components to eCO_2 across a range of CO_2 concentrations in an intact ecosystem. Soil CO_2 efflux (i.e., soil respiration or R_{soil}) increased in response to initial enrichment (e.g., +30 and +60 ppm CO_2) but did not continue to increase as the CO_2 enrichment was stepped up to higher concentrations. Light-saturated photosynthesis of canopy leaves (A_{sat}) also showed similar stimulation by elevated CO_2 at +60 ppm as at +150 ppm CO_2. The lack of significant effects of eCO_2 on soil moisture, microbial biomass, or activity suggests that the increase in R_{soil} likely reflected increased root and rhizosphere respiration rather than increased microbial decomposition of soil organic matter. This rapid increase in R_{soil} suggests that under eCO_2, additional photosynthate was produced, transported belowground, and respired. The consequences of this increased belowground activity and whether it is sustained through time in mature ecosystems under eCO_2 are a priority for future research.

Keywords: carbon cycling, climate change, elevated carbon dioxide, *Eucalyptus*, woodland

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Introduction

Projections of future climate are highly dependent on feedbacks between the terrestrial carbon (C) cycle and climate change, particularly the degree to which elevated concentrations of atmospheric CO_2 (eCO_2) will increase ecosystem C uptake and storage (Friedlingstein *et al.*, 2006, 2014). The response of forests and woodlands is particularly important, given that these ecosystems dominate the terrestrial C cycle (Melillo *et al.*, 1993; Dixon *et al.*, 1994; Pan *et al.*, 2011). There is a rich literature regarding the major patterns of plant physiological and structural responses to CO_2 enrichment (e.g., Ainsworth & Long, 2005; Ainsworth & Rogers, 2007; Leakey *et al.*, 2009). However, ecosystem-scale responses to CO_2 enrichment are less certain, given that ecosystem-scale effects depend on the allocation and fate of C under eCO_2, as well as feedbacks related to nutrient uptake and nutrient-use efficiency (Luo *et al.*, 2004; DeLucia *et al.*, 2005; McCarthy *et al.*, 2010; Norby *et al.*, 2010; Drake *et al.*, 2011; Reich *et al.*, 2014).

Atmospheric CO_2 enrichment is likely to increase ecosystem C inputs from photosynthesis, at least in the short term, but whether this leads to an increase in ecosystem C storage is dependent on many processes. In some temperate forest experiments, the additional C fixed under eCO_2 was used to stimulate additional soil exploration by fine roots and nutrient extraction from soil organic matter (SOM) decomposition, such that soil nitrogen (N) uptake increased under eCO_2, providing a positive feedback to growth (Norby *et al.*, 2004; Finzi *et al.*, 2007; Drake *et al.*, 2011; Talhelm *et al.*, 2014; Taylor *et al.*, 2014). In contrast, a whole-tree chamber experiment in a strongly N-limited boreal forest showed no response of tree growth to eCO_2 in the absence of N-fertilization despite sustained increases in

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canopy C uptake; this additional C was returned to the atmosphere through increased respiration, particularly in soils (Comstedt *et al.*, 2011; Hall *et al.*, 2013; Sigurdsson *et al.*, 2013). A similar response was noted in a central European hardwood forest where eCO_2 increased photosynthesis and soil C outputs without increasing growth in mature hardwood trees (Bader *et al.*, 2013). In addition, eCO_2 consistently increased photosynthesis and soil CO_2 efflux (R_{soil}) independent of N supply in a temperate grassland (Adair *et al.*, 2011; Lee *et al.*, 2011), but plant biomass responded significantly less to eCO_2 under low vs. higher experimental N inputs (Reich & Hobbie, 2013). Thus, it is possible for eCO_2 to increase C inputs and C outputs similarly, leading to an enhanced rate of C cycling through an ecosystem without stimulating net C storage. This possibility is supported by the growing understanding that canopy photosynthesis dynamically interacts with soil CO_2 efflux on a relatively short timescale of hours to days in forests and woodlands (Högberg *et al.*, 2001; Irvine *et al.*, 2005; Stoy *et al.*, 2007; Drake *et al.*, 2008; Mencuccini & Holtta, 2010; Vargas *et al.*, 2011; Han *et al.*, 2014). Thus, the belowground consequences of increased photosynthesis under eCO_2 are a key area for current and future research.

All previous Free-Air CO_2 Enrichment (FACE) studies have implemented the elevated CO_2 treatment with a single large step change in CO_2 concentration, or $[CO_2]$. This approach may introduce transient C uptake and transport behavior that is inconsistent with the ecosystem response to a gradual increase in $[CO_2]$ (Luo & Reynolds, 1999). In addition, experimental work with potted *Bromus inermis* has shown that a gradual increase in $[CO_2]$ over 6 years led to a different functional community of mycorrhizal fungi relative to a single-step change in $[CO_2]$, with strong effects on plant growth under eCO_2 (Klironomos *et al.*, 2005). In an attempt to minimize potential artifacts associated with a single large increase in $[CO_2]$, the *Eucalyptus* Free-Air CO_2 Enrichment experiment (EucFACE) implemented an elevated CO_2 treatment through a 6-month stepped 'ramp' consisting of +0, 30, 60, 90, 120, and 150 ppm CO_2 above ambient, with each step lasting approximately 5 weeks. This 6-month increase in $[CO_2]$ can still be considered a rapid step change relative to the gradual increase in atmospheric $[CO_2]$, but this approach may minimize unmeasured and unintended artifacts associated with a large pulse of C assimilation. Moreover, this stepped ramp provided an opportunity to study the short-term response of C cycle fluxes to CO_2 enrichment across a range of CO_2 concentrations in an intact ecosystem.

We examined the response of a eucalypt woodland to stepwise increases in CO_2 concentration with a focus on leaf-level photosynthesis, R_{soil}, and potential changes in soil microbial biomass or activity during the ramp period. We concentrated on these measures of the C cycle as they represent major components of C uptake and loss for this ecosystem and may change rapidly in response to eCO_2 relative to large pools of ecosystem C that are expected to change more slowly (e.g., wood biomass, soil C). We measured initial soil properties (pH, total C, N, and P concentrations) and then specifically addressed the following questions: (1) what is the nature of the photosynthetic and R_{soil} response to stepwise CO_2 enrichment and (2) does microbial biomass or activity respond in the short-term to stepwise CO_2 enrichment? Answering these questions during this early period of the EucFACE experiment may help to resolve mechanisms underpinning fast response components of the C cycle and define priorities for future research.

Materials and methods

Site description

The *Eucalyptus* Free-Air CO_2 Enrichment experiment (EucFACE) is located within a 35-ha remnant patch of Cumberland Plain woodland (Hancock *et al.*, 2013) at 23 m elevation above sea level, approximately 50 km northwest of Sydney, Australia (33°37′S, 150°44.3′E). Open *Eucalyptus* woodlands are a dominant cover type in Australia (Yates & Hobbs, 1997; Burrows *et al.*, 2002); similar woodlands, savannahs, and grasslands cover ~11% of the global land surface and account for ~30% of terrestrial net primary production (Field *et al.*, 1998; Burrows *et al.*, 2002). EucFACE has several unique characteristics relative to the previous forest FACE experiments: the presence of old trees, old and weathered soils with low nutrient availability, and phosphorus limitation of tree growth (Crous *et al.*, 2015). The site is 5 km from the Hawkesbury River on an ancient alluvial floodplain. The soil is an alluvial formation characterized by surface soils of slightly acidic loamy sand with low organic C, underlain by discontinuous layers of clay. The soil was originally reported as a grey chromosol but is better described as an aeric podosol (Isbell, 2002; Barton *et al.*, 2010).

Climate

The site has a humid temperate-subtropical transitional climate. Across the last 20 years, the site experienced a mean annual rainfall of 720 mm and a mean annual temperature of 17 °C (Australian Bureau of Meteorology, station 067105; http://www.bom.gov.au). The mean maximum temperature of the warmest month (January) was 30 °C and the mean minimum temperature of the coldest month (July) was 3.6 °C, with approximately 15 days per year with a minimum temperature below 0 °C. Precipitation was variable but occurred year-round with larger amounts during summer months

(258 mm during the summer months of December–February, 182 mm during the spring months of September–November, 107 mm during the winter months of June–August, and 173 mm during the autumn months of March–May).

Vegetation

The site is an open woodland with an overstory of a single dominant tree species (*Eucalyptus tereticornis* Sm.) at a density of ~600 trees ha^{-1} with a minor component of *E. amplifolia* Naudin. A minor component of noncanopy trees and shrubs is also present, including *Melaleuca decora* Salisb. and Britten, *Acacia parramattensis* Tindale, *Breynia oblongifolia* Mull. Arg., *Hakea sericea* Schrad. and J.C. Wendl., and *Bursaria spinosa* Cav. There is a diverse understory (~70 species) consisting primarily of grasses and forbs; common species include *Microlaena stipoides* Labill., *Commelina cyanea* R.Br., and *Pratia purpurascens* R.Br.

Free-air CO_2 enrichment

Six circular 25-m-diameter plots were established in 2010 in locations chosen to minimize pretreatment plot-to-plot variation in species composition, tree density, and tree size. The minimum distance between plots was 80 m. Plots were assigned to the ambient or elevated CO_2 treatments in a completely randomized design.

The FACE facility was based on the four previous forest FACE experiments (Hendrey *et al.*, 1999), including fully instrumented control plots. Each plot was surrounded by a large horizontal plenum suspended one meter above the forest floor that delivered gas to 32 vertical vent pipes in a cylindrical frame up to 28-m height. The vertical vent pipes release prediluted CO_2 through adjustable ports installed at 50-cm intervals along the vertical vent pipes. The release of CO_2 into the canopy air space was controlled using a proportional–integral–differential algorithm to achieve the desired CO_2 concentration in the three 'elevated' CO_2 plots (Lewin *et al.*, 2009). An identical system fumigates with ambient air only in the three 'ambient' plots to control for potential microclimate artifacts of the system.

In contrast to previous experiments that implemented a single-step change in CO_2 concentration upon commencing CO_2 enrichment (e.g., from ambient to +200 ppm, Luo & Reynolds, 1999), the concentration of CO_2 at EucFACE was increased in a 'ramp' of five 30-ppm steps; each step lasted approximately 5 weeks. Fumigation with CO_2 commenced on September 18, 2012, at +30 ppm CO_2, and the concentration set-points were ramped up to +60 ppm on October 25, 2012, +90 ppm on November 28, 2012, +120 ppm on January 2, 2013, and finally to the full-strength treatment of +150 ppm on Feb 6, 2013 (Fig. 1a). This study encompassed the pretreatment period through this series of [CO_2] steps through a 6-week period of the full treatment. The FACE system performance was satisfactory throughout the study period; 5-min averages of canopy CO_2 concentration measurements were within 25% of the target [CO_2] 84% of the time (81% at +30 ppm, 82% at +60 ppm, 84% at +90 ppm, 85% at +120 ppm, and 88% at +150 ppm CO_2).

Soil respiration (R_{soil})

The rate of CO_2 diffusion from the soil surface to the atmosphere (soil CO_2 efflux or soil respiration; 'R_{soil}' hereafter) was measured at eight random locations within each of the six FACE plots. A 20-cm-diameter PVC collar was inserted into the soil to 7 cm depth at each location; collar insertion to 7 cm was required to eliminate lateral diffusion of CO_2 beneath the collars in the sandy soil at the site. These collars were permanently left in place to reduce soil disturbance by repeated measurement and to ensure that the same soil was measured over time (King *et al.*, 2004). Collars were installed 2 weeks prior to the first measurement and 4 weeks prior to CO_2 enrichment. A small amount of understory plant biomass was removed from inside the collars by clipping such that measurements of CO_2 efflux could be attributed to R_{soil} alone, as is common (e.g., Bremer *et al.*, 1998; Craine *et al.*, 1999; Frank *et al.*, 2002). Complementary campaigns of manual surveys and automated chamber measurements were implemented to measure spatial and temporal variation of R_{soil}.

Manual survey measurements of R_{soil} were taken at all 48 collar locations (6 FACE plots × 8 collars per plot = 48 locations) approximately every 2 weeks from 10:00 hours to 13:00 hours beginning on September 2, 2012; the plot mean R_{soil} was estimated as the mean rate measured across the eight collars. R_{soil} was measured with two identical portable infrared gas analyzers (IRGAs; 20-cm-diameter chamber, model Li-8100-103; Licor Environmental, Lincoln, NE, USA) with a 2.5-min observation length and a 30-s deadband. The rate of CO_2 accumulation in the system during the measurement was used to estimate R_{soil} by an exponential curve-fit. One collar within each FACE ring was occupied by an automated chamber; this autochamber was temporarily removed for manual measurement of R_{soil} during each survey. In addition to three sets of pretreatment measures, two to three sets of manual survey measurements were recorded during each 5-week step of the CO_2 ramp.

Automated measurements of R_{soil} were taken at 30-min resolution at one of the eight collar locations within each FACE plot using six identical 20-cm-diameter chambers interfaced with IRGAs (model Li-8100-104 long-term chambers and Li-8100A IRGAs; Licor). One long-term chamber and one IRGA were deployed in each of the FACE plots at a randomly chosen collar location. The observation length was 4.5 min with a 30-s deadband and a 30 s prepurge and postpurge. These measurements began on September 10, 2012, 1 week prior to commencement of CO_2 fumigation, and we report data until March 21, 2013, 6 weeks after the CO_2 treatment reached the full +150 ppm. There was minimal data loss to poor-quality fits or equipment failures. We collected 53086 R_{soil} observations from September 10, 2012, to March 21, 2013 of 55704 potential measurements; a data retention rate of 95%. We reported daily averages of these data.

Soil volumetric water content (VWC) and soil temperature (T_{soil}) were measured one meter from each of the eight collars per plot with permanently installed time-domain reflectometry probes inserted into the soil at a 45° angle (eight per plot; CS650-L; Campbell Scientific, Logan, UT, USA); VWC was measured from 0 to 15 cm depth and T_{soil} was measured at

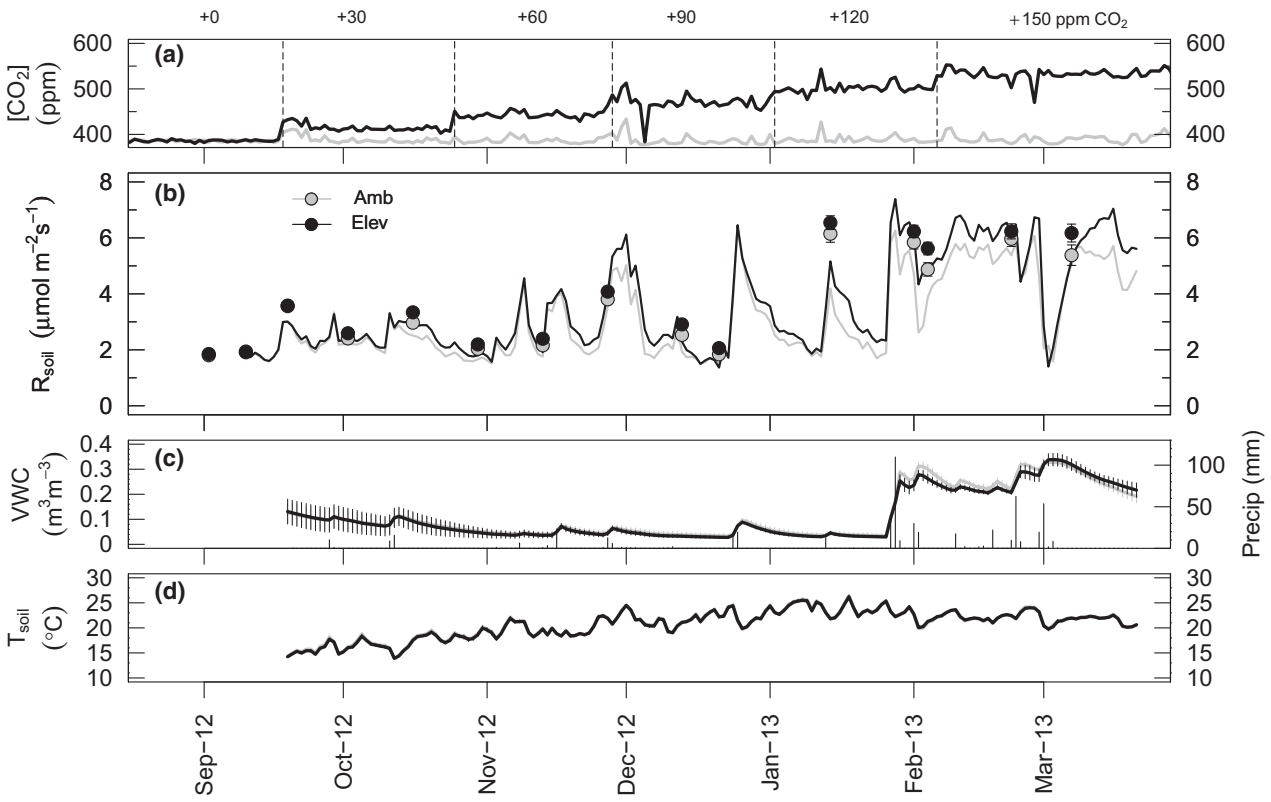

Fig. 1 The rate of CO_2 diffusion from the soil to the atmosphere (R_{soil}) as the concentration of atmospheric CO_2 was increased in five steps at EucFACE. Daytime means of the atmospheric CO_2 concentration (a) increased in the elevated CO_2 treatment as the setpoints were increased over time. The vertical dashed lines denote when the $[CO_2]$ setpoints were increased; the $[CO_2]$ of these setpoints are listed across the top. In (b), dots reflect the mean R_{soil} of survey measurements (±1SE) and lines reflect the daily mean R_{soil} of autochamber measurements. Precipitation resulted in variable soil volumetric water content from 0 to 15 cm depth (VWC; c). Soil temperatures at 5 cm depth (T_{soil}) increased and then decreased during this time period (d). Error bars reflect ±1SE (n = 3); note that error bars were too small to be visible in some cases.

5 cm depth. These data were recorded at 15-min intervals by a datalogger in each plot (C3000; Campbell Scientific); we report daily averages across plots for these measurements.

Photosynthesis

Photosynthesis in the upper tree canopy was measured in four campaigns: April, May, October 2012, and February 2013. The first two campaigns occurred during the pretreatment period, while subsequent campaigns were performed when the eCO_2 treatment levels were +60 and +150 ppm CO_2. Measurements for each campaign were taken across two to three sunny days using four open-flow portable photosynthesis instruments (Li-6400 with Li-6400-02B LED light source, Licor). We measured light-saturated photosynthetic rates of upper canopy leaves (A_{sat}) with a light intensity of 1800 μmol m^{-2} s^{-1} and a cuvette CO_2 concentration matching the $[CO_2]$ within the target atmosphere of each experimental plot (~390 ppm CO_2 in ambient plots and in elevated plots for pretreatment measurements, 450 ppm for the October measurements at +60 ppm and 540 ppm for the February measurements at +150 ppm). Measurements were made in the mid-morning (9:30-11:10 AEST) on a single cohort of fully expanded and mature leaves. Leaf temperatures were controlled at a constant value within each campaign at the prevailing seasonal air temperature (26, 22, 22, and 28 °C, for four campaigns, respectively). Upper canopy leaves at a mean height of 20 m were accessed from a gondola suspended from the jib of a freestanding tower crane permanently installed adjacent to each experimental plot (36-m-tall crane with 35-m-long jib; J4010, Jaso Cranes, Idiazábal, Guipuzkoa, Spain). We measured two leaves per tree in three dominant canopy trees per experimental plot, with whole-plot averages used for analyses. For further details, see Gimeno *et al.* (2015).

We used a leaf-scale coupled photosynthesis and stomatal conductance model based on the Farquhar–von Caemmerer–Berry model of photosynthesis (Farquhar *et al.*, 1980) and an optimal stomatal conductance model (Medlyn *et al.*, 2011). This model was developed by Duursma *et al.* (2014) and assumes no acclimation in response to eCO_2, which may be appropriate given the short time frame of these observations (weeks to months). We used this model to predict the direct effect of stepwise CO_2 enrichment on A_{sat} given the environmental conditions at the site. Constant values for the

maximum velocity of rubisco carboxylation ($V_{c,max}$) and RuBP regeneration (J_{max}) at 25 °C were used (80.0 and 139.7 μmol m^{-2} s^{-1}, respectively), based on initial measurements of CO_2 response curves at EucFACE (*D. Ellsworth, unpublished data*). The temperature sensitivities of photosynthetic parameters followed Medlyn *et al.* (2002). A constant g_1 stomatal parameter of 4.3 was used based on the average for trees in both treatments (Gimeno *et al.*, 2015). Mean air temperature, vapor pressure deficit (VPD), and canopy [CO_2] were calculated daily from morning canopy measurements at EucFACE (9:30-11:10 AEST); this time frame was used to match the measurements of A_{sat}. VPD was calculated from measurements of air temperature and relative humidity measured with shielded sensors (HMP115, Vaisala; Campbell Scientific) mounted at 23.5 m height on a central tower within each experimental plot.

Microbial biomass and activity

Microbial biomass carbon (C_{mic}) and nitrogen (N_{mic}) were determined on soils collected in September, December 2012 and March 2013 encompassing the pretreatment period, +90 and +150 ppm CO_2, respectively. Four soil cores (3 cm diameter by 10 cm depth) were taken from each of four randomly assigned 2 × 2 m locations designated for long-term soil sampling and monitoring within each FACE plot; these cores were bulked to provide four samples per FACE plot (four locations × six FACE plots = 24 samples). Samples were immediately returned to the laboratory, stored at 4 °C, and sieved (<2 mm) within 1 day of sampling. Soil gravimetric water content was determined in duplicate on 5 g of fresh soils following drying at 105 °C for 24 h. C_{mic} and N_{mic} were extracted using the fumigation extraction method as previously described (Brookes *et al.*, 1985). Total dissolved carbon and nitrogen in filtered soils extracts were determined on a total organic carbon analyzer fitted with a total nitrogen measurement unit (TOC-L TNM-L; Shimadzu, Sydney, Australia). C_{mic} and N_{mic} were calculated as the difference between the fumigated and nonfumigated samples, using a conversion factor of 0.45 for C (Beck *et al.*, 1997) and 0.54 for N (Brookes *et al.*, 1985). Data were expressed as μg C/N g^{-1} oven dry soil.

Laboratory incubations were carried out to determine basal microbial respiration rates on sieved soil (i.e., in the absence of plant roots) using the MicroResp™ system (Campbell *et al.*, 2003). Approximately 250 mg fresh soil of each sample and 40 μL water was added to each of four deep wells in a 96-deep-well plate (ThermoScientific, Australia). The 96-deep-well plates were immediately sealed with a two-way PTFE-coated rubber matt containing a single hole that allowed connection and CO_2 diffusion between the deep-well plate and the colorimetric detection plate (Campbell *et al.*, 2003). Plates were clamped and incubated at 25 °C for 4 h. Rates of basal respiration were determined by measuring the change of absorbance (570 nm) in the detection plate between 0 and 4 h using a predetermined calibration curve and conversion calculation as described by Campbell *et al.* (2003). Data were expressed as μg CO_2-C g^{-1} dw h^{-1}. To assess the metabolic rates of soil microbial communities, we calculated the microbial metabolic quotient (qCO_2), as basal respiration rate per unit microbial biomass (Anderson & Domsch, 1993).

Soil properties

Soil pH, total C, total N, and total P were measured across three depths (0–10, 10–20, and 20–30 cm) using the soils sampling procedure described above (four locations × six FACE plots = 24 samples per depth). Soil pH was determined in water (1 : 5 weight/volume) following shaking at 180 rpm for 1 h and settling for 20 min (SevenEasy pH Meter, Mettler-Toledo Ltd., Melbourne, VIC, Australia). Total C and N (%) were determined on finely milled soil (Retsch Mixer Mill MM 400; 2 min at 25 vibrations per second) via Dumas combustion and elemental analysis (TruMac C N, LECO, Castle Hill, NSW, Australia). Total P was determined following Aqua Regia digestion and inductively coupled plasma mass spectrometry (ICP-MS, Environmental Analysis Laboratory, Southern Cross University, Lismore, NSW, Australia). None of these variables differed between the ambient and elevated CO_2 plots (ANOVA, $P > 0.1$), so we report site averages and the range across plots (Table 1).

The soils at EucFACE were slightly acidic with low but variable concentrations of total C, N, and P (Table 1). Averaged across 0–30 cm depth, the C:N ratio was 12.2, the C:P ratio

Table 1 Mean soil properties for three depths at the EucFACE site. Numbers in parenthesis are ± one standard error, n = 6 plots, with four subreplicate measurements per plot. Range reflects variation across the six plots

	Depth		
Property	0–10 cm	10–20 cm	20–30 cm
Soil pH (H_20)	Mean: 5.29 (0.09)	Mean: 5.37 (0.09)	Mean: 5.28 (0.07)
	Range: 5.06–5.68	Range: 5.11–5.66	Range: 5.01–5.50
Total C (%)	Mean: 1.34 (0.15)	Mean: 0.74 (0.08)	Mean: 0.37 (0.07)
	Range: 1.00–2.00	Range: 0.52–1.06	Range: 0.25–0.66
Total N (%)	Mean: 0.11 (0.01)	Mean: 0.06 (0.006)	Mean: 0.03 (0.004)
	Range: 0.08–0.16	Range: 0.05–0.09	Range: 0.03–0.05
Total P (mg kg^{-1})	Mean: 76.28 (7.08)	Mean: 44.78 (2.60)	Mean: 51.82 (5.24)
	Range: 51.29–102.45	Range: 38.94–56.86	Range: 40.55–75.09

was 175.7, and the N:P ratio was 14.4; these values were quite similar to an analysis of grassland and forest soils, which reported global averages of 14.3, 186.0, and 13.1 for C:N, C:P, and N:P, respectively (Cleveland & Liptzin, 2007).

Statistical analysis

While the treatments were assigned completely randomly, subsequent measurements supported pairing the plots into three blocks for this analysis (Fig. S1). Plot pairs of 1–2, 3–4, and 5–6 were established based on spatial proximity, soil C and N content, soil moisture as measured by the time-domain reflectometry probes, and pretreatment tree basal area (Fig. S1). Each block contained a single replicate plot of each CO_2 treatment ($n = 3$). Thus, statistical analyses followed a randomized complete block design with three blocks. Given the modest statistical power of forest FACE experiments, we follow Oishi *et al.* (2014) and report results with strong ($P < 0.05$) and moderate ($0.05 \leq P \leq 0.10$) statistical significance. All analyses were performed using the 'lme' function within the 'nlme' package in R version 3.1.0 (R Development Core Team, 2012; Pinheiro *et al.*, 2013). All analyses were evaluated for the assumptions of residual normality and variance homoscedasticity; log-transformations were often necessary to stabilize the residual variance.

The survey R_{soil}, photosynthesis, and microbial data were analyzed in a mixed-model analysis of variance framework (ANOVA) treating block and CO_2 treatment as whole-plot factors and time as a split-plot factor. Pseudoreplication was avoided by including a random term of block by treatment to test the main effect of CO_2 treatment and a random term of block by time nested within treatment to test the time and treatment: time interaction. For the survey R_{soil} data, individual measurement locations exhibited consistent variation over time (e.g., spearman rank-order correlation between pretreatment data and measurements 4 months later, $\rho = 0.68$, $P < 0.001$), so the analysis considered pre- and post-treatment data separately with the average pretreatment R_{soil} included as a covariate in the post-treatment analysis.

Daily averages of the automated R_{soil} data were analyzed in a repeated-measures ANOVA framework treating block, time, and CO_2 treatment as fixed effects with a random term of block:treatment to test the main effect of CO_2 treatment. As there were no subplot measurements in this analysis, the time and time:treatment terms were tested with the residual error. Variation in VWC and T_{soil} was highly correlated with R_{soil}, so VWC and T_{soil} were combined into a covariate following Davidson *et al.* (2012) in an attempt to remove these environmental effects and clarify any CO_2 effect on R_{soil}. The covariate was calculated as:

$$\text{covariate} = R_{ref} \times D^{(\text{VWC}_{opt} - \text{VWC})^2} \times Q^{\frac{(T_{soil} - 10)}{10}} \quad (1)$$

where VWC and T_{soil} were measured variables (units of % and °C), VWC_{opt} was the optimal soil moisture at which maximum R_{soil} was observed (20%), while R_{ref}, D, and Q were assumed constant at 2.4, 0.995, and 2.5, respectively (R_{ref} reflects the reference rate of R_{soil} at 10 °C in μmol m^{-2} s^{-1}, D is a calibration parameter, and Q reflects the change in R_{soil} with a 10 °C change in temperature). We compared many repeated-measures variance–covariance structures using the Akaike information criteria (AIC) and found the autoregressive moving average with an autoregressive order of one and a moving average order of two to be most appropriate (lowest AIC score).

We also performed a power analysis by Monte Carlo simulation to assess the magnitude of pretreatment difference in R_{soil} we could expect to detect. We simulated 1000 sets of data while preserving our sampling intensity (three dates with eight collars in each of six plots) and the underlying variation across dates, plots, and collars.

Results

R_{soil}

There were no pretreatment differences in the rate of R_{soil} between ambient and elevated CO_2 plots (Fig. 1b; survey data, mixed-model ANOVA, $P = 0.77$). Given the observed level of spatial variation and the sampling intensity of the pretreatment survey measurements, the power analysis indicated an 18, 55, and 80% chance of detecting a pretreatment difference of 10, 20, and 30%, respectively ($P < 0.1$). Thus, the low replication of this experiment, like all FACE studies (Filion *et al.*, 2000), resulted in modest statistical power for the detection of small treatment effects.

Following atmospheric CO_2 enrichment to +30 ppm, R_{soil} increased by approximately 10% in the elevated CO_2 plots, as shown by the survey and autochamber data (Fig. 1b). As the level of CO_2 enrichment was stepped up to +60, 90, 120, and 150 ppm, the CO_2 induced increase in R_{soil} did not grow larger; the CO_2 treatment effect on R_{soil} was maintained at approximately 10% throughout the ramp (Figs 1b and S2). Herein, we focus on the survey data when making inference about eCO_2 effects, as these measurements

Table 2 Analysis of variance results for the survey and autochamber R_{soil} datasets during the CO_2 ramp at EucFACE

Term	DF_{num}	DF_{den}	*F*-value	*P*-value
Survey R_{soil} dataset				
Block	2	2	22.9	0.04
CO_2 treatment	1	2	9.0	0.09
Time	11	44	81.3	<0.0001
CO_2 treatment: Time	11	44	0.1	0.99
Pretreatment covariate	1	498	178.2	<0.0001
Daily autochamber R_{soil} dataset				
Block	2	2	4.3	0.18
CO_2 treatment	1	2	3.6	0.19
Time	175	645	18.1	<0.0001
CO_2 treatment: Time	175	645	0.7	0.99
VWC and T_{soil} covariate	1	645	42.1	<0.0001

have more extensive within-plot sampling (eight observations per plot) relative to the autochamber measurements (one observation per plot), but we also present the autochamber measurements given their rich temporal sampling.

The CO_2 effect on survey R_{soil} was modestly statistically significant (Table 2, $P = 0.09$), but the interaction between CO_2 treatment and time was not significant ($P = 0.99$), indicating that the effect of eCO_2 treatment was not variable in time. Thus, eCO_2 treatment increased R_{soil}, but this effect did not increase as the atmospheric concentration of CO_2 was increased beyond +30 ppm CO_2. We suggest that the main effect of eCO_2 was detectable despite the modest statistical power because a statistically significant amount of variation was accounted for by blocking and the average pretreatment R_{soil} covariate.

The daily autochamber dataset also indicated that R_{soil} increased by approximately 10% following CO_2 enrichment at +30 ppm CO_2 (Fig. 1b). Repeated-measures analysis suggested that this was a nonsignificant trend for the main effect of CO_2 treatment (Table 2, $P = 0.19$) and the CO_2 effect was not variable in time (CO_2 treatment: time interaction, Table 2, $P = 0.99$). While the CO_2 effect on R_{soil} was not statistically significant, the trends in the autochamber data support the inference from the survey data that the eCO_2 increased R_{soil} by ~10% (Figs 1b and S1), but the effect of eCO_2 on R_{soil} did not increase as eCO_2 rose beyond +30 ppm CO_2 enrichment.

Volumetric water content (VWC) from 0 to 15 cm depth was not altered by eCO_2 (ANOVA, $P > 0.8$; Fig. 1c). Similarly, T_{soil} at 5 cm depth was not affected by eCO_2 (ANOVA, $P > 0.75$; Fig. 1d). R_{soil} responded positively to rain events and the associated increase in VWC, with the exception of a large rain event (>70 mm in 24 h) in March 2013 that flooded the plots and was associated with temporarily reduced R_{soil} (Fig. 1).

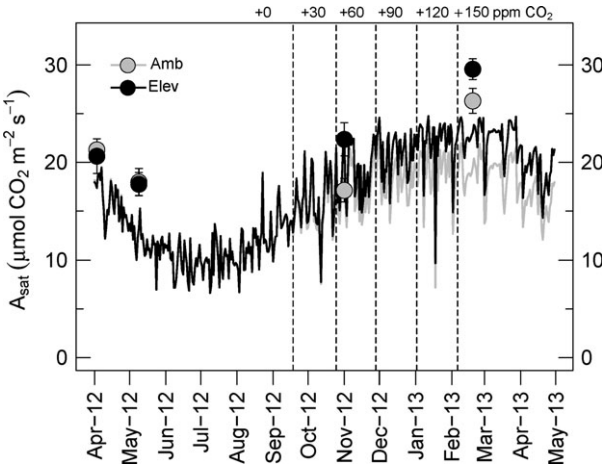

Fig. 2 Measured and modeled rates of light-saturated photosynthesis in upper canopy leaves during the mid-morning (A_{sat}) during the stepwise increase in [CO_2] at EucFACE. A_{sat} was measured on four campaigns (large circles) and modeled for each day (lines). Error bars reflect ±1SE ($n = 3$).

Photosynthesis

Prior to CO_2 fumigation, light-saturated photosynthesis (A_{sat}) of mature leaves was 20.8 ± 1.3 (mean ± SE of 6 replicate plots) and 17.9 ± 1.3 μmol CO_2 m^{-2} s^{-1} in April 2012 and May 2012, with no pretreatment differences between the plots assigned to the ambient or elevated CO_2 treatments ($P > 0.8$; Fig. 2). A_{sat} increased significantly in response to +60 ppm CO_2 enrichment in November 2012 from 17.1 ± 1.2 μmol CO_2 m^{-2} s^{-1} in ambient CO_2 to 22.4 ± 1.7 μmol CO_2 m^{-2} s^{-1} in the elevated CO_2 treatment, an increase of 31% ($P < 0.01$). However, the magnitude of the effect of elevated CO_2 was not greater at a higher enriched concentration of CO_2 later in the ramp; A_{sat} on February 19, 2013 was 12.5% higher in elevated than ambient CO_2 (29.6 ± 1.1 vs. 26.3 ± 1.7 μmol CO_2 m^{-2} s^{-1}) even though the elevated treatment was +150 ppm CO_2. Considering the

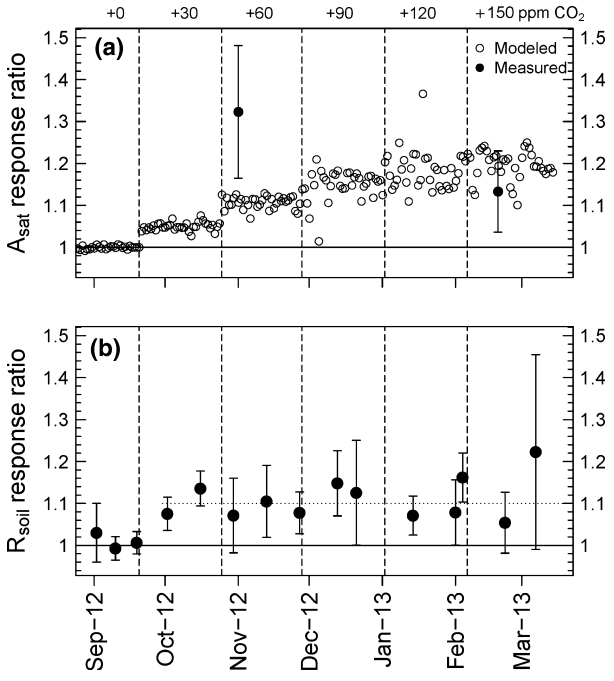

Fig. 3 Response ratios of light-saturated photosynthesis (A_{sat}; a) and soil CO_2 efflux (R_{soil}; b) during the stepwise increase in [CO_2] at EucFACE. Small open circles reflect modeled A_{sat} (a), while black-filled circles reflect direct measurements of two A_{sat} campaigns (a) and fifteen R_{soil} survey campaigns (b). Error bars reflect ±1SE of three blocks. The horizontal dotted line at 1.1 in (b) reflects the mean response ratio of R_{soil} to eCO_2.

postfumigation data only, the main effect of CO_2 treatment was significant ($P = 0.01$) while the date by CO_2 interaction was not ($P = 0.48$), indicating that the effect of elevated CO_2 on A_{sat} did not differ between +60 vs. +150 ppm CO_2 enrichment.

Response ratios

The model predictions of A_{sat} were largely consistent with the observed magnitude and seasonality in measured A_{sat} (Fig. 2). The model predicted a stimulation of A_{sat} in response to +30 ppm CO_2 enrichment with a response ratio of ~1.04 (Fig. 3a). This response ratio was predicted to increase to ~1.1 as the CO_2 was stepped up to +60 ppm and to plateau at ~1.2 at +120–150 ppm. The modeled response of A_{sat} to eCO_2 exhibited day-to-day temporal variation (Fig. 3a) because of variation in VPD and air temperature, which affected the [CO_2] within leaf airspaces. The observed response ratio of A_{sat} during the +60 ppm step was considerably higher than the model predictions, although the observation had a relatively large standard error. However, the observed and modeled A_{sat} response ratios were in agreement at +150 ppm CO_2 enrichment (Fig. 3a).

The response ratio of R_{soil} was ~1.0 prior to CO_2 fumigation, increased to ~1.1 following +30 ppm CO_2 and remained relatively constant thereafter. This pattern was observed in the response ratios of the survey R_{soil} data (Fig. 3b) and the autochamber R_{soil} data (Fig. S2). The autochamber response ratios were more variable than the survey R_{soil} data given that they reflect only one measurement location within each plot. Both datasets indicate that the response ratio of R_{soil} did not increase as the concentration of atmospheric CO_2 was increased.

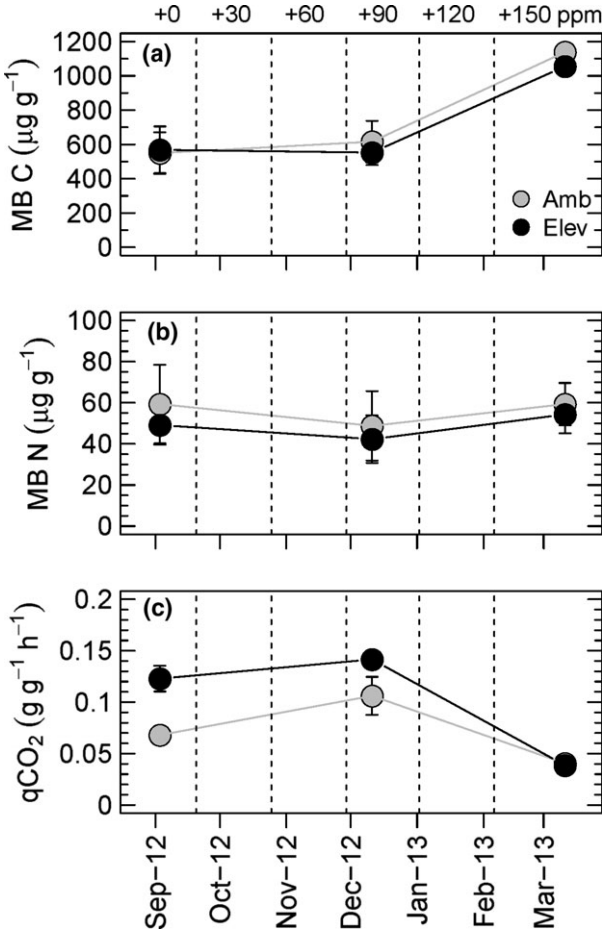

Fig. 4 Microbial biomass and activity across three measurement timepoints during the study period. Microbial biomass carbon (MB C; a), microbial biomass nitrogen (MB N; b), and the rate of microbial respiration per unit microbial biomass (respiratory quotient, or qCO_2; C).

Microbial biomass and activity

There were no differences between the ambient and elevated CO_2 plots for microbial biomass C, or N, either pre- ($P > 0.1$) or post-treatment (Fig. 4a,b; $P > 0.5$). There were strong pretreatment differences among the plots for microbial respiration per unit microbial biomass (qCO_2; Fig. 4c; $P < 0.01$). These pretreatment differences were maintained in the middle of the CO_2 ramp in December 2012. However, the treatment difference in qCO_2 was not observed in March 2013, as microbial biomass C was high during this wet time period (Fig. 4). The pretreatment difference in qCO_2 returned in subsequent measurements (Fig. S3). Collectively, these data indicate that microbial biomass or activity did not increase in response to CO_2 enrichment during this short-term study.

Discussion

Atmospheric CO_2 enrichment stimulated photosynthetic C uptake and soil CO_2 efflux in this mature eucalypt woodland; A_{sat} was increased by 15–30%, and R_{soil} increased by ~10% relative to the control plots. We observed a positive response of A_{sat} and R_{soil} to a small incremental increase in the concentration of CO_2 (+30 or 60 ppm CO_2), but no further enhancement of these rates with a greater increase in CO_2. The lack of any change in soil microbial biomass or activity suggests that the increase in R_{soil} with elevated CO_2 enrichment likely reflected increased root and rhizosphere respiration, rather than increased microbial decomposition of soil organic matter. The rapid increase in R_{soil} with eCO_2 implies the loss of C that might otherwise accumulate as organic material, reducing the potential C accumulation in this ecosystem with CO_2 enrichment.

The photosynthetic data suggest that eCO_2 increased the light-saturated photosynthetic rates of upper canopy leaves (A_{sat}) by 15–30% by eCO_2 during this early period of the experiment. While the observed data are sparse during the stepped ramp, the model predictions of the direct CO_2 effect without physiological adjustment suggested an asymptotic response to stepwise eCO_2, with a maximum response ratio of ~1.2 at +150 ppm CO_2. The model predicted that the +30 and +60 ppm CO_2 treatments would increase the CO_2 concentration inside leaf airspaces (C_i) during $V_{c,max}$ limited photosynthesis, resulting in stimulations of modeled A_{sat}. However, subsequent steps of CO_2 enrichment (i.e., +90, +120, and +150 ppm CO_2) resulted in J_{max} limited photosynthesis in the model and thus a comparatively modest additional eCO_2 effect on A_{sat} through well-known mechanisms related to rubisco kinetics and the suppression of photorespiration (Farquhar *et al*., 1980; Long & Bernacchi, 2003). It is clear that eCO_2 stimulated photosynthetic C uptake during this early period of the experiment, although physiological adjustment to eCO_2 over longer periods may modify this response, particularly acclimation that results in downregulation of photosynthetic capacity (e.g., Ellsworth *et al*., 2012).

The rapid increase in R_{soil} following the start of the eCO_2 treatment, and lack of evidence of other potential sources for that C, suggests that the flux of C belowground was increased by eCO_2. By mass balance, the observed increase in R_{soil} must be matched by additional soil C inputs (e.g., litterfall or belowground C flux) or the loss of C from soil pools (Giardina & Ryan, 2002). The short-term nature of the response indicates that the stimulation of R_{soil} cannot be explained by higher litterfall (which did not differ among treatments, R.A. Duursma, T.E. Gimeno, M.M. Boer, K.Y. Crous, M.G. Tjoelker and D.S. Ellsworth, submitted). The lack of any CO_2-induced increase in soil moisture, microbial biomass, or microbial activity suggests that increased microbial decomposition of bulk soil organic matter is also unlikely. Thus, we suggest that the observed increase in R_{soil} with eCO_2 (Fig. 1) most likely followed directly from eCO_2 stimulation of photosynthesis (Fig. 2) and increased C transport belowground, leading to increased root and rhizosphere C substrate availability. This is consistent with previous experiments that have measured an increase in root respiration with eCO_2 (Janssens *et al*., 1998; Drake *et al*., 2008) and the rapid (<2 weeks) belowground transport of the fumigation isotopic label at Duke FACE (Andrews *et al*., 1999). While water savings and increased soil moisture have been linked to increases in R_{soil} in other eCO_2 experiments (e.g., Pendall *et al*., 2003), this is not always the case (e.g., Adair *et al*., 2011). The apparent increase in belowground C flux with eCO_2 likely has implications for soil nutrient availability and C cycling at the site that merit further study.

We observed positive responses of A_{sat} and R_{soil} to a small incremental increase [CO_2] (e.g., +30, 60 ppm), but no further enhancement of those rates with greater increase in [CO_2]. While other variables such as temperature, soil moisture, and duration of exposure to eCO_2 varied during this experiment, the magnitude of observed responses did not change as the concentration of CO_2 was increased. Our results are consistent with tunnel chamber CO_2-gradient studies on grassland ecosystems, which found that biomass production and R_{soil} increased with [CO_2] in an asymptotic manner that saturated at approximately 500 ppm (Gill *et al*., 2006; Fay *et al*., 2009). The similar extent of stimulation of A_{sat} to the elevated [CO_2] treatment at ~520 ppm as at ~430 ppm is consistent with well-known rubisco kinetics and the shape of an A_{sat}:C_i curve (the direct response of assimilation to the concentration of carbon dioxide inside leaf airspaces; Farquhar *et al*., 1980; Long & Bernacchi, 2003). The asymptotic nature of R_{soil} is less understood, but we suggest that the lack of further increase in A_{sat} as the [CO_2] was increased throughout the ramp could limit the additional photosynthate available for C allocation belowground. If the short-term responses observed here reflect lasting responses, C sequestration in this system may not increase as strongly with rising [CO_2] as predicted by earth system models, which typically predict increases in primary production and C sequestration across a broad range of CO_2 concentrations up to at least 1000 ppm (Cramer *et al*., 2001; Friedlingstein *et al*., 2006; Arora *et al*., 2013).

While this study provided a unique investigation into ecosystem responses to eCO_2 across a range of CO_2 concentrations, it also had several limitations. First, the concentration of atmospheric CO_2 was confounded with time and other variables that varied with time, including VWC, T_{soil} (Fig. 1), and leaf area index (R.A. Duursma, T.E. Gimeno, M.M. Boer, K.Y. Crous, M.G. Tjoelker and D.S. Ellsworth, submitted). While we cannot think of a mechanism by which these variables would interact with [CO_2] in a manner that could lead to the responses observed here, we cannot discount this possibility. Secondly, this short-term study did not address any of the potential long-term effects of eCO_2, such as physiological acclimation, altered nutrient availability, or ecosystem C storage. Whether long-term adjustment to eCO_2 alters the short-term responses observed here is an important subject of future research. Finally, we did not measure photosynthesis or soil microbial processes during all steps of the CO_2

ramp, and thus, we may have missed intermediate responses.

Conclusions

We utilized a unique stepwise CO_2 enrichment experiment to study the short-term response of a mature eucalypt woodland to increasing atmospheric [CO_2]. We found that A_{sat} and R_{soil} responded positively to the initial increment in [CO_2] (e.g., +30, 60 ppm CO_2), but this response did not increase in magnitude as the concentration of CO_2 was raised. We found no measurable change in VWC, soil microbial activity, or soil microbial biomass with eCO_2, suggesting that the R_{soil} response was likely driven by a stimulation of rhizosphere respiration (roots and closely associated microbes), rather than an increase in microbial decomposition of SOM. As a consequence of the apparent increase in belowground C flux with eCO_2, altered root and microbial activities are possible and merit further study.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Data used to support establishing pairing plots into blocks at EucFACE.
Figure S2. Response ratio of soil CO_2 efflux (R_{soil}) as measured by autochambers during the stepwise increase in [CO_2] at EucFACE.
Figure S3. Microbial respiration per unit microbial biomass (qCO_2) over eighteen months.