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# A decade of free-air CO<sub>2</sub> enrichment increased the carbon throughput in a grass-clover ecosystem but did not drastically change carbon allocation patterns

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## Summary

1. The response of the soil carbon cycle to increasing atmospheric CO<sub>2</sub> concentration has far reaching consequences for the ecosystem carbon balance under future climatic conditions. We report on work carried out in the Swiss free-air CO<sub>2</sub> enrichment (FACE) experiment, where we used *in situ* <sup>13</sup>CO<sub>2</sub> labelling to determine whether elevated CO<sub>2</sub> (+230 µL L<sup>-1</sup>) concentration changes the fate of recently assimilated carbon in the soil microbial community.
2. Elevated CO<sub>2</sub> (eCO<sub>2</sub>) concentration had an overall positive effect on microbial abundance ( $P < 0.001$ ) with the gram-negative bacteria showing significantly increased quantities.
3. Gram-negative bacteria and saprotrophic fungi tended to utilize a higher amount of recently assimilated carbon under eCO<sub>2</sub>. Arbuscular mycorrhizal fungi (AMF) utilized plant-assimilated carbon within 1 day after the <sup>13</sup>CO<sub>2</sub> pulse and <sup>13</sup>C uptake patterns in AMF suggest that carbon transfer is faster under eCO<sub>2</sub> concentration than under ambient CO<sub>2</sub> (aCO<sub>2</sub>). Additionally, the respiration of recently assimilated carbon was significantly higher under eCO<sub>2</sub> than aCO<sub>2</sub> concentration.
4. Our data suggest that elevated atmospheric CO<sub>2</sub> concentration accelerated and increased the utilization of recently assimilated carbon by the microbial community without changing the microbial community composition drastically.
5. We conclude that a higher standing soil microbial biomass under eCO<sub>2</sub> concentration was the key cause for the higher carbon flow through the plant–soil system. Carbon utilization by microbial functional groups was only little affected by a decade of CO<sub>2</sub> enrichment.

**Key-words:** <sup>13</sup>C labelling, arbuscular mycorrhizal fungi, C flux, compound-specific <sup>13</sup>C analysis, elevated atmospheric CO<sub>2</sub>, free-air CO<sub>2</sub> enrichment, neutral lipid fatty acids, phospholipid fatty acids, soil carbon

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## Introduction

Human-induced environmental change is a major societal concern (IPCC 2007). Predicting and mitigating the impacts of human activity on the Earth's climate is therefore receiving considerable attention. In particular, understanding how terrestrial ecosystems will respond to elevated atmospheric CO<sub>2</sub> (eCO<sub>2</sub>) concentration is a priority research area in view of the existing feedbacks between the global carbon cycle and climate change (Luo *et al.* 1996; Carney *et al.* 2007; Selsted *et al.* 2012). The impact of eCO<sub>2</sub> concentration on carbon cycling has been investigated on small-scale mesocosms (Sowerby *et al.* 2000; Carney *et al.* 2007; Drigo *et al.* 2013), but also in large-scale field experiments (Van Kessel *et al.* 2000a; Denef *et al.* 2007; Hill *et al.* 2007; Selsted *et al.* 2012). One of the first free-air CO<sub>2</sub> enrichment (FACE) experiments was the Swiss FACE that aimed to understand the impact of eCO<sub>2</sub> concentration in combination with nitrogen (N) fertilization and management practice on grass-clover vegetation using *Lolium perenne* L. and *Trifolium repens* L. (Hebeisen *et al.* 1997). It has been reported that after more than 8 years, photosynthesis was increased in both species (Ainsworth *et al.* 2003a,b), which is in accordance with responses in other grasses (Albert *et al.* 2011) and forbs (Ainsworth & Long 2005). Increased uptake of atmospheric CO<sub>2</sub> has frequently led to the hypothesis that the increased carbon flow into the ecosystem could increase soil carbon sinks, but results are ambiguous.

Extensive research in the Swiss FACE showed that increased photosynthesis did not necessarily lead to a higher carbon storage potential of the soil (Sowerby *et al.* 2000; Van Kessel *et al.* 2000a; Drissner *et al.* 2007) and this has also been reported by others (Fitter *et al.* 1996; Carney *et al.* 2007; Selsted *et al.* 2012). This result is not surprising because increased carbon availability in the ecosystem does not only affect the carbon accumulated in plant biomass (shoots and roots; Hebeisen *et al.* 1997; Suter *et al.* 2002) but also assimilated carbon released into the soil matrix, which stimulates microbial activity and maintains plant available nutrient levels in soil (Fontaine, Mariotti & Abbadie 2003; Paterson *et al.* 2007; Talbot, Allison & Treseder 2008). A stimulation of the microbial biomass results in carbon being bound in the microbial community, and at the same time, these microbes mineralize soil organic matter (SOM) and release carbon by respiratory processes (Carney *et al.* 2007).

In the Swiss FACE, the microbial community has been intensively studied (Hartwig *et al.* 2000; Lüscher *et al.* 2000; Staddon, Jakobsen & Blum 2004; Drissner *et al.* 2007). The microbial biomass was stimulated under eCO<sub>2</sub> concentration (Sowerby *et al.* 2000; Drissner *et al.* 2007), but increased microbial activity led to an enhanced soil CO<sub>2</sub> efflux counterbalancing the carbon input (Sowerby *et al.* 2000). Increased soil CO<sub>2</sub> efflux has also been observed for a temperate heathland where eCO<sub>2</sub> in combination with warming and periodic summer drought induced soil carbon loss by 21% (Selsted *et al.* 2012).

One major microbial group associated with clover is the nitrogen-fixing gram-negative rhizobia bacteria that were found to balance soil nutrient levels under eCO<sub>2</sub> concentration in the Swiss FACE (Lüscher *et al.* 2000; Hartwig *et al.* 2002). In the grass-clover mixture, 59% of the nitrogen was supplied from atmospheric nitrogen fixation under ambient CO<sub>2</sub> (aCO<sub>2</sub>) concentration, and this amount was increased by 12% when plants were exposed to 600 ppm CO<sub>2</sub> (Zanetti *et al.* 1996).

Fungi were another important microbial group in the Swiss FACE: fungal enzyme production was increased under eCO<sub>2</sub> concentration and fungal abundance rose under low nitrogen fertilization (Drissner *et al.* 2007). Fungi have been shown to be a relevant driver of soil CO<sub>2</sub> efflux under eCO<sub>2</sub> concentrations (Carney *et al.* 2007) due to their ability to increase nutrient availability in the soil by mineralizing SOM (Carney *et al.* 2007; Drissner *et al.* 2007). Another important microbial group in terms of nutrient balance of an ecosystem is the group of mycorrhizal fungi. Mycorrhizal fungi provide nutrients, especially phosphorus, to plants in exchange for plant carbon. This is especially important in the

rhizosphere where phosphorous can be depleted and mycorrhizal hyphae can reach beyond the zone of nutrient depletion. In the Swiss FACE, arbuscular mycorrhizal fungi (AMF) were positively affected by eCO<sub>2</sub> conditions combined with low nitrogen fertilization (Staddon, Jakobsen & Blum 2004).

Here, we used an *in situ* <sup>13</sup>CO<sub>2</sub> pulse labelling approach (Ostle *et al.* 2000; Leake *et al.* 2006) to track and quantify the carbon flow through the ecosystem under ambient and elevated atmospheric CO<sub>2</sub> concentrations. We measured the <sup>13</sup>C enrichment of microbial fatty acid biomarkers to determine the effect of eCO<sub>2</sub> on the relative activity of major soil microbial functional groups. In particular, the <sup>13</sup>C label content of phospholipid fatty acids (PLFA) and neutral lipid fatty acids (NLFA) can be used to compare the relative activity of different groups of soil microorganisms, for example, gram-negative and gram-positive bacteria, saprotrophic and AMF (Treonis *et al.* 2004; Olsson & Johnson 2005). Elevated CO<sub>2</sub> is known to affect soil microbial communities, principally via altered carbon input quality, and clearly affects the relative activity of different soil microorganisms (Montealegre *et al.* 2002). We hypothesize that elevated CO<sub>2</sub> concentration changes the fate of carbon in the soil microbial community towards a higher carbon recovery in gram-negative bacteria, saprotrophic fungi and AMF.

## Materials and methods

### THE SWISS FACE EXPERIMENT

The research was carried out at the Swiss FACE site, Lindau, Switzerland (Hebeisen *et al.* 1997), where elevated atmospheric CO<sub>2</sub> concentration (+230 µL L<sup>-1</sup>) was imposed for 10 years from 1993. The lowland grassland site and treatments are fully described elsewhere (Schortemeyer *et al.* 1996). There were three replicate FACE rings and three replicate control (ambient) rings yielding a total of six labelling chambers. The labelling work reported here was entirely carried out in the mixed *L. perenne* L. and *T. repens* plots under low N addition (14 g m<sup>-2</sup> year<sup>-1</sup>).

### PULSE LABELLING WITH <sup>13</sup>CO<sub>2</sub>

The <sup>13</sup>C label was delivered to the vegetation as <sup>13</sup>CO<sub>2</sub> at 99 atom% <sup>13</sup>C (Staddon *et al.* 2003) at two CO<sub>2</sub> concentrations, that is, ambient (aCO<sub>2</sub>, 370 µL L<sup>-1</sup>) and elevated (eCO<sub>2</sub>, 600 µL L<sup>-1</sup>). This was achieved using an inexpensive and highly flexible method developed for labelling in the field, which, importantly, allowed the delivery of the <sup>13</sup>C label simultaneously at different CO<sub>2</sub> concentrations. Briefly, this method involved six individual labelling units consisting of 250 L capacity gas impermeable Tedlar bags, which were filled with air containing <sup>13</sup>CO<sub>2</sub> at 370 or 600 µL L<sup>-1</sup>. The 99 atom% <sup>13</sup>CO<sub>2</sub> was mixed into bottled CO<sub>2</sub>-free air to achieve the required concentration. The labelled air was then pumped at a flowmeter controlled rate of 1.0 L min<sup>-1</sup> to purpose built 20 cm diameter, 15 cm high, transparent acrylic labelling chambers, placed flush with the soil surface. The labelling areas were selected randomly within the low N mixed vegetation plots. Exhaust air from the labelling chambers was expelled 10 m outside the FACE and control rings. Labelling was carried out on 13 September 2002 in the afternoon and lasted for 3-5 h under sunny conditions.

We tested whether <sup>13</sup>C from the <sup>13</sup>CO<sub>2</sub> labelling pulse entered the soil directly in significant quantity by applying a <sup>13</sup>CO<sub>2</sub> pulse to vegetation that was kept dark (labelling chambers covered in opaque plastic; Staddon *et al.* 2003). Labelling in darkened chambers was carried out outside the FACE rings. Gas samples in unlabeled area were taken at least 2 m away from the labelled area. Soil-respired CO<sub>2</sub> was collected in the same manner as for the actual labelling experiment (detailed below). We found that the <sup>13</sup>C content of soil-respired CO<sub>2</sub> in unlabeled area and the labelled darkened area was similar at the relevant times after labelling.

## GAS, PLANT AND SOIL SAMPLE COLLECTION

Gas sampling was carried out to investigate the  $^{13}\text{C}$  content in soil  $\text{CO}_2$  efflux 2 h and 1, 2 and 4 days after  $^{13}\text{CO}_2$  labelling. Samples were taken within the labelled area and at 5 and 15 cm away from the outer edge of the labelled area. Soil-respired  $\text{CO}_2$  was collected using one 10 mL Exetainer vial per sampling point (Labco Ltd., High Wycombe, UK) that was placed upside down on the soil surface (Staddon *et al.* 2003). Vials were flushed with ambient air before the incubation so that, within treatments, they all had the same starting point. Care was taken not to include shoot respiration within the trapped soil-respired  $\text{CO}_2$ . The tubes were left in place for 4 h, after which, each tube was carefully lifted from the soil surface and capped. The soil-respired  $\text{CO}_2$  samples were stored at ambient temperature.

Samples of above-ground biomass were taken in all labelled areas to investigate the  $^{13}\text{C}$  content 2 h, 1 and 2 days after  $^{13}\text{CO}_2$  labelling. Samples were taken within the labelled area and were obtained as close as possible to where the Exetainer vials were located.

Soil cores (2 cm diameter) were taken in all labelled areas to examine the fate of recently assimilated carbon ( $^{13}\text{C}$ ) into the soil microbial community and to assess the microbial biomass 2 h and 1, 2, 3, 4, 6 and 14 days after the  $^{13}\text{CO}_2$  pulse. Cores were taken to a depth of 10 cm at the same location as the gas samples. Above-ground biomass samples and soil cores were frozen at  $-18\text{ }^\circ\text{C}$  until analysis.

## CARBON-13 ANALYSES OF GAS AND PLANT SAMPLES

The  $^{13}\text{C}$  content of  $\text{CO}_2$  (soil respiration) was analysed in subsamples transferred to evacuated 2 mL crimp seal vials for immediate processing. The vial headspace was purged into the He carrier stream of a PreCon trace gas preparation-concentration unit (Thermo Electron, Bremen, Germany) interfaced with a HP 6890 gas chromatograph coupled in continuous flow mode to a Finnigan MAT Delta PLUS isotope ratio mass spectrometer (Thermo Electron). As working standard, we used commercial  $\text{CO}_2$  calibrated against certified  $^{13}\text{CO}_2$  standards (Messer Griesheim, Krefeld, Germany).

Total carbon and the  $^{13}\text{C}/^{12}\text{C}$  ratio in plant material was measured by Dumas combustion on an elemental analyser (CE 1110; Thermo Electron, Milan, Italy) coupled in continuous flow mode to the Delta PLUS. Analyses were performed on dried, finely ground 2–3 mg samples weighed out into tin combustion capsules. As working standard for  $^{13}\text{C}$  determinations, commercial  $\text{CO}_2$  calibrated against certified  $^{13}\text{C}$ -sucrose material (IAEA, Vienna, Austria) was used.

## EXTRACTION, IDENTIFICATION AND $^{13}\text{C}$ ANALYSES OF FATTY ACIDS

The extracted PLFAs were selected as being specific to various groups of soil micro-organisms: general biota [16:0, 18:0 (both PLFAs synthesized by microbes and plants)], gram-negative bacteria (cy17:0, cy19:0, 18:1x7), gram-positive bacteria (i15:0, a15:0, i16:0, i17:0, a17:0, 10Me17), actinomycetes (10Me16, 10Me18) and saprotrophic fungi (18:1x9, 18:2x6,9). The NLFA 16:1x5 as specific to AMF was chosen as it has been shown to be a more sensitive indicator of AMF hyphae in soil than the PLFA 16:1x5 (Olsson 1999).

Lipids within soil (roots removed) were extracted in a one-phase mixture of citrate buffer, methanol and chloroform (0.8:2:1, v/v/v, pH 4.0). The lipids were fractionated into neutral lipids, glycolipids and phospholipids on pre-packed silica columns (100 mg sorbent mass, Varian Medical Systems, Palo Alto, CA, USA) as described by van Aarle & Olsson (2003). The fatty acid residues in neutral lipids and phospholipids were transformed into free fatty acid methyl esters and identified and quantified by gas

chromatography.

The  $^{13}\text{C}$  enrichment in fatty acid methyl esters was determined in a 20-20 IRMS (PDZ Europa Scientific Instruments, Crewe, UK) interfaced with a Hewlett Packard 6890 gas chromatograph (Hewlett Packard, Palo Alto, CA, USA). The chromatographic conditions were as described by Olsson *et al.* (2005). The  $^{13}\text{C}$  values were calculated based on atom%  $^{13}\text{C}$  of the reference  $\text{CO}_2$  gas, injected three times at the beginning and end of a chromatographic run. The reference  $\text{CO}_2$  was standardized with the Pee Dee Belemnite (PDB) standard. The precision of the reference gas  $^{13}\text{C}$  was 0.2‰. Integration for each peak was checked and corrected manually.

Carbon isotopic enrichment was highest in above-ground vegetation and was expressed as atom%  $^{13}\text{C}$  excess (APE) and calculated as follows:

$$\text{APE}(\%) = ^{13}\text{C content}_{\text{sample}} - ^{13}\text{C content}_{\text{control}} \quad \text{Eqn 1}$$

where the  $^{13}\text{C content}_{\text{sample}}$  is the measured  $^{13}\text{C}$  content in above- $^{13}\text{CO}_2$  pulse, and  $^{13}\text{C content}_{\text{control}}$  is the  $^{13}\text{C}$  content in above-ground vegetation before the experiment.

Measured  $^{13}\text{C}$  enrichments ( $\Delta^{13}\text{C}$ ) in fatty acids and soil respiration are calculated as:

$$\Delta^{13}\text{C}(\text{‰}) = \delta^{13}\text{C}_{\text{sample}} - \delta^{13}\text{C}_{\text{control}} \quad \text{Eqn 2}$$

where  $\delta^{13}\text{C}_{\text{sample}}$  is the  $^{13}\text{C}/^{12}\text{C}$  ratio of individual PLFAs and soil respiration after the  $^{13}\text{CO}_2$  pulse and  $\delta^{13}\text{C}_{\text{control}}$  before the  $^{13}\text{CO}_2$  pulse.

## DATA ANALYSES

Statistical analyses were performed using R version 2.12.1 (www.R-project.org). All data were checked for normality and where necessary log-transformed. Analysis of variance ( $\text{ANOVA}$ ) was performed to test for effects of  $\text{CO}_2$  concentration on  $^{13}\text{C}$  content in above-ground plant biomass. Sampling time was included as fixed variable to test for the effect of time.

Fatty acid quantities are presented as averages of sampling times and replicates (FACE rings) because fatty acid abundances are not assumed to change over the investigated time period. Standard errors were calculated using error propagation. The overall effect of  $\text{eCO}_2$  on fatty acid abundances was tested using a  $\text{MANOVA}$ , and the effect of  $\text{eCO}_2$  on individual fatty acids was analysed with a linear mixed-effect model (lme) using 'FACE ring' as a random variable because fatty acid quantities were consistently lower in one of the FACE rings.

The effect of  $\text{eCO}_2$  on  $\Delta^{13}\text{C}$  in microbial functional groups (averages of  $^{13}\text{C}$  values of functional group specific fatty acids) was analysed using the 'lme' function with 'FACE ring/time' as random variable. The model was extended for a 'varIdent' structure that accounts for inhomogeneous variances if the data did not show homogeneous variances. The same model was used to test for the effect of  $\text{CO}_2$  concentration on soil-respired  $\Delta^{13}\text{CO}_2$ .

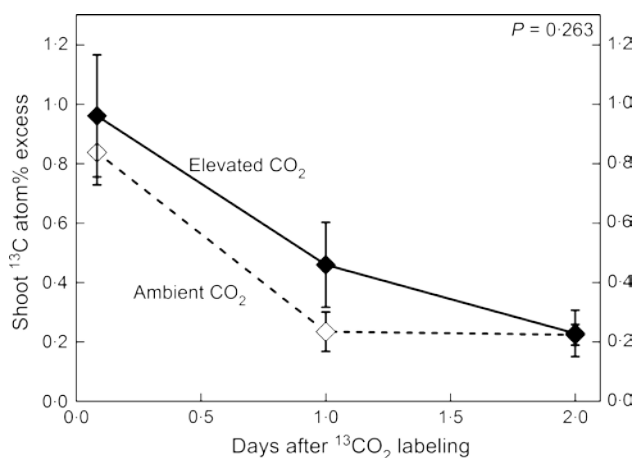
## Results

Plant  $^{13}\text{C}$  content decreased with time but plant  $^{13}\text{C}$  enrichment was not different between  $\text{CO}_2$  treatments (Fig. 1). Microbial fatty acid abundances were significantly higher under  $\text{eCO}_2$  compared to  $\text{aCO}_2$  concentration ( $\text{ANOVA}$ :  $P < 0.001$ ). This  $\text{CO}_2$ -induced increase was lowest for actinomycetes ( $9 \pm 4\%$ ) and highest for AMF (40%; Fig. 2). Gram-negative-specific  $\text{cy17:0}$  and  $\text{cy19:0}$  PLFAs were significantly more abundant in the  $\text{eCO}_2$  treatment ( $P < 0.05$ ). The same trend was observed for the gram-positive-specific biomarkers  $\text{i15:0}$  ( $P = 0.098$ ) and  $\text{i16:0}$  ( $P = 0.065$ ) and the actinomycetes PLFA  $\text{10Me16:0}$  ( $P = 0.057$ ). The AMF-specific NLFA  $\text{16:1x5}$  also tended to be more abundant under  $\text{eCO}_2$  ( $P = 0.061$ ).

The  $\Delta^{13}\text{C}$  in fatty acids was affected by functional group ( $P = 0.046$ ) and  $\text{CO}_2$  concentration ( $P = 0$ -

025) when considering all time points. There was no significant effect of eCO<sub>2</sub> on  $\Delta^{13}\text{C}$  in microbial functional groups. However, eCO<sub>2</sub> had a tendency to increase the  $\Delta^{13}\text{C}$  in gram-negative bacteria and saprotrophic fungi, whereas AMF were more enriched in <sup>13</sup>C when exposed to aCO<sub>2</sub> concentration (Fig. 3). The  $\Delta^{13}\text{C}$  in gram-positive bacteria ( $P = 0.40$ ) and actinomycetes ( $P = 0.50$ ) were unaffected by eCO<sub>2</sub> (Fig. S1, Supporting information).

There was a substantial difference in  $\Delta^{13}\text{C}$  of soil-respired CO<sub>2</sub> between aCO<sub>2</sub> and eCO<sub>2</sub> within the labelling plots (Fig. 4). The <sup>13</sup>C enrichment of soil-respired CO<sub>2</sub> was considerably higher at eCO<sub>2</sub> than aCO<sub>2</sub>. At 5 cm distance from the labelled plots, the <sup>13</sup>CO<sub>2</sub> efflux pattern was similar at aCO<sub>2</sub> and eCO<sub>2</sub> over the 4-day sampling period (data not shown).  $\Delta^{13}\text{C}$  was on average 4‰ in aCO<sub>2</sub> and was increased by ~2-5‰ under eCO<sub>2</sub> concentration. At 15 cm distance from the labelled plots, aCO<sub>2</sub> and eCO<sub>2</sub> plots showed a ~5‰  $\Delta^{13}\text{C}$  in soil CO<sub>2</sub> efflux. In aCO<sub>2</sub> plots, the measured  $\Delta^{13}\text{C}$  increased to ~8‰ and approached zero thereafter. In eCO<sub>2</sub> plots, measured  $\Delta^{13}\text{C}$  approached zero already 1 day after the labelling pulse.



**Fig. 1.** Above-ground <sup>13</sup>C enrichment at ambient and elevated CO<sub>2</sub> concentration over time. <sup>13</sup>C enrichment is expressed as <sup>13</sup>C atom% excess. Effect of the CO<sub>2</sub> treatment was statistically analysed with an analysis of variance (ANOVA) and is given in the upper right corner. Enrichments are presented as means  $\pm$  SE.

## Discussion

### PLANT CARBON UPTAKE

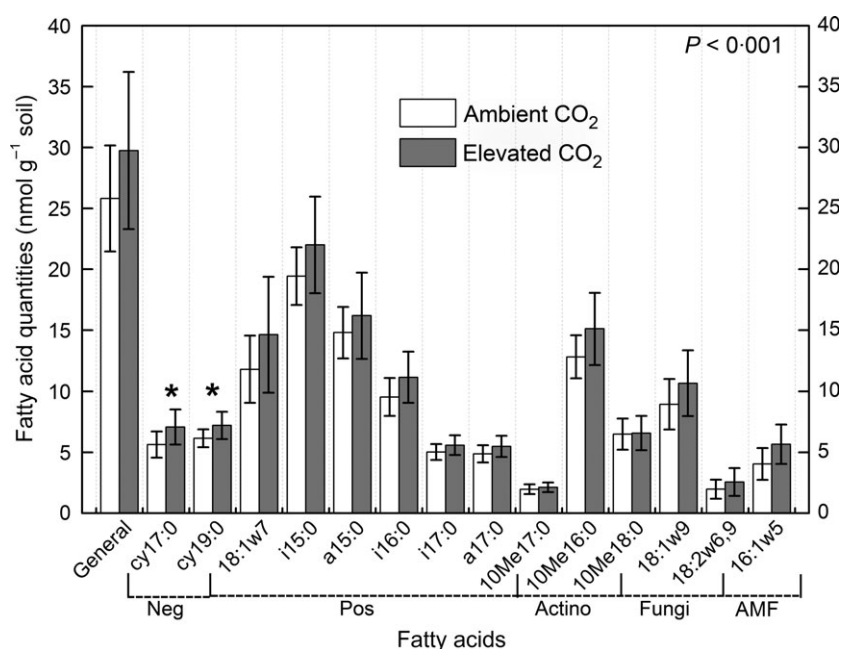
Our results show the same <sup>13</sup>C enrichment of above-ground biomass under aCO<sub>2</sub> and eCO<sub>2</sub> concentrations (Fig. 1). However, we can assume that the uptake of <sup>13</sup>CO<sub>2</sub> was higher under eCO<sub>2</sub> conditions because we know from earlier studies at the Swiss FACE that eCO<sub>2</sub> concentration affects the photosynthetic capacity of plants and results in increased carbon uptake (Ainsworth *et al.* 2003a, b). Seen in this context, our results suggest that photo-assimilated carbon is not stored in above-ground biomass but transported below-ground under the assumption that plant biomass is similar or higher under eCO<sub>2</sub> compared to aCO<sub>2</sub> (Ainsworth & Long 2005; Kongstad *et al.* 2012). This is a reasonable assumption because the labelling pulse was deployed in late summer when temperate plants increase the allocation of carbon to below-ground parts to prepare for the winter season. Increased fixation of CO<sub>2</sub> and a more rapid transfer of photo-assimilates below-ground under eCO<sub>2</sub> concentration (Hebeisen *et al.* 1997; Daepp, Nösberger & Lüscher 2001) create a bigger carbon pool for plant and below-ground activity but, at the same time, increase the nutrient demand for plants and microbes.

## MICROBIAL COMMUNITY RESPONSE TO ELEVATED CO<sub>2</sub>

Elevated CO<sub>2</sub> significantly increased the soil microbial PLFA abundance by ~16% compared to aCO<sub>2</sub> concentration (Fig. 2). This finding is in accordance with previous results from the same experimental site where total PLFA abundances were increased by about 25% (Drissner *et al.* 2007). Microbial PLFA abundance and thus biomass (Frostegård & Bååth 1996) likely increased due to the higher carbon input to the soil that stimulated microbial growth and activity due to, for example, rhizodeposition.

In particular, the abundance of gram-negative bacteria was significantly increased under eCO<sub>2</sub> concentration. Gram-negative bacteria are fast growing and highly dependent on plant photo-assimilates (Paterson *et al.* 2007; Jin & Evans 2010; Garcia-Pausas & Paterson 2011) and the increase of root growth (Jongen *et al.* 1995; Arndal *et al.* 2013) and connected rhizodeposition under eCO<sub>2</sub> can therefore have a facilitating effect on gram-negative bacteria.

In soil, the free-living gram-negative bacteria *Azotobacter spp.* (Strandberg & Wilson 1968) and the symbiotic (gram-negative) rhizobia are able to fix atmospheric nitrogen, and thus, they are a key component in the nitrogen balance of the soil. However, in the present study, it was not possible to



**Fig. 2.** Quantities of microbial phospholipid fatty acid (nmol g<sup>-1</sup> dry soil) and the neutral lipid fatty acid 16:1w5 under ambient and elevated CO<sub>2</sub> concentrations. The effect of the eCO<sub>2</sub> treatment is given in the upper right corner. Bars represent means ± SE. \*indicates significant differences on a level of  $P \leq 0.05$ .

determine whether these specific groups were part of the general increase in gram-negative bacteria biomass. In general, in other studies from the Swiss FACE, nodule formation was increased by 17–50% due to the symbiosis between plants and rhizobia under eCO<sub>2</sub> concentration (Schortemeyer *et al.* 1996; Montealegre *et al.* 2000). Nitrogen-fixing bacteria counterbalanced the expected negative effect of decreased above-ground biomass and lower nitrogen yield and maintained a similar C/N balance under aCO<sub>2</sub> and eCO<sub>2</sub> concentrations (Hartwig *et al.* 2000; Lüscher *et al.* 2000). Additionally, the increased nitrogen demand arising from increased carbon input under eCO<sub>2</sub> concentration was entirely supplied by nitrogen-fixing symbionts in the grass-clover mixture (Zanetti *et al.* 1996). However, the utilization of recently assimilated carbon by free-living nitrogen-fixing bacteria should be investigated in greater detail to confirm their dependence on rhizodeposits.

The AMF biomarker (NLFA 16:1w5) tended to be positively affected by eCO<sub>2</sub> concentration. Even

though this result was not significant, this trend is not a random effect because the extraradical mycorrhizal hyphae density was increased from 0.3 to 0.8 m g<sup>-1</sup> soil in aCO<sub>2</sub> to 1.0–1.7 m g<sup>-1</sup> soil in eCO<sub>2</sub> by October 2002, (Staddon, Jakobsen & Blum 2004) that is, 1 month after the pulse labelling. AMF are known to act as a carbon sink (Jakobsen & Rosendahl 1990; Olsson & Johnson 2005), and due to their symbiotic connection with the host plant, AMF are the first group to receive recently plant-assimilated carbon (Olsson & Johnson 2005).

## CHANGED MICROBIAL ACTIVITY

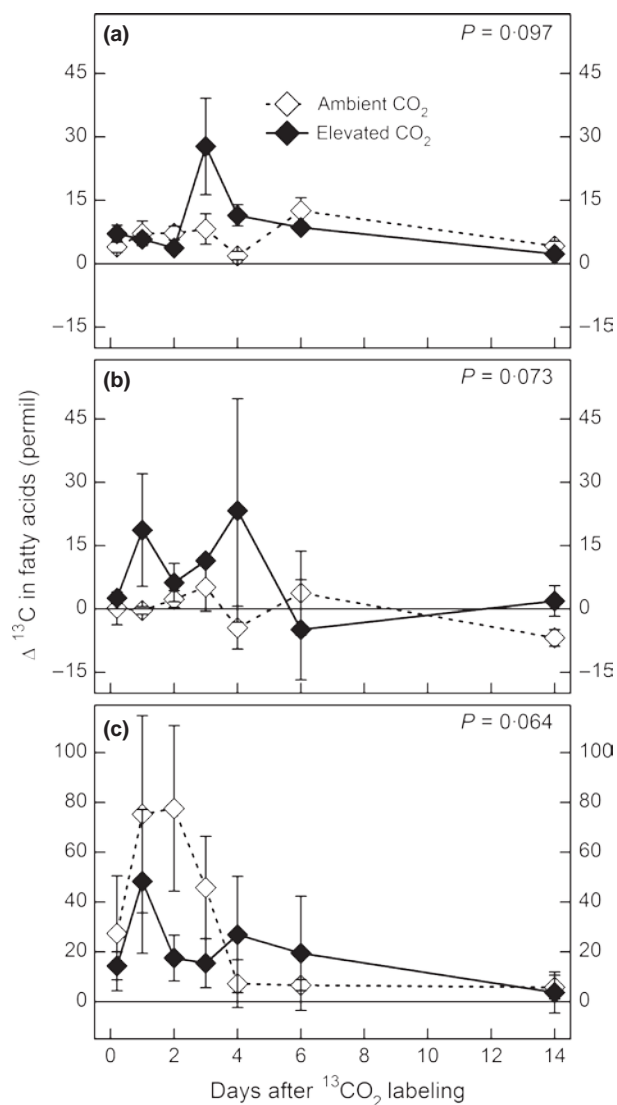
Arbuscular mycorrhizal fungi displayed distinct <sup>13</sup>C enrichment in the biomarker NLFA 16:1x5 under labelling at the two CO<sub>2</sub> concentrations (Fig. 3c). Under aCO<sub>2</sub> concentration, uptake of recently assimilated CO<sub>2</sub> peaked at 80‰ d<sup>13</sup>C between 2 and 3 days after labelling and decreased thereafter. In contrast, under eCO<sub>2</sub> concentration, the maximum d<sup>13</sup>C was measured to be ~44‰ 1 day after labelling. However, the lower d<sup>13</sup>C in the eCO<sub>2</sub> treatment is a result of pool dilution because extraradial mycorrhizal hyphae density was ~50% increased (Staddon, Jakobsen & Blum 2004) in soils with eCO<sub>2</sub> concentration. The measured isotopic value must therefore be diluted by 50% when rhizodeposition was similar in eCO<sub>2</sub> and aCO<sub>2</sub> treatments. When considering the isotopic dilution effect, the level of NLFA would be similar.

However, carbon transport has been shown to be faster under eCO<sub>2</sub> than under aCO<sub>2</sub> concentration (Selsted *et al.* 2012). Furthermore, the symbiosis between AMF and plant roots facilitate a very fast transfer of recently assimilated carbon between plants and AMF (Moyano, Kutsch & Schulze 2007) in exchange for nutrients (Jakobsen & Rosendahl 1990; Johnson, Leake & Read 2002; Olsson & Johnson 2005). In a <sup>14</sup>C labelling experiment, <sup>14</sup>C-labelled carbon that was taken up by plants was re-allocated below-ground and peaked in associated AMF within 16 h after the labelling pulse (Jakobsen & Rosendahl 1990). Our second sampling took place 1 day after the labelling implying that our measuring window was too wide to detect the early peak of enrichment in the eCO<sub>2</sub> treatment under fast carbon transfer conditions. Under aCO<sub>2</sub> concentration, carbon transfer is slower than under eCO<sub>2</sub> because the labelling peak seems to occur between 1 and 2 days after the labelling pulse. Thus, the symbiotic lifestyle of AMF results in a rapid transfer of carbon from the plant, and this carbon transfer is faster under eCO<sub>2</sub> than under aCO<sub>2</sub> concentrations. A more detailed evaluation of the impact of AMF on the net carbon balance of the system could be achieved with more frequent samplings.

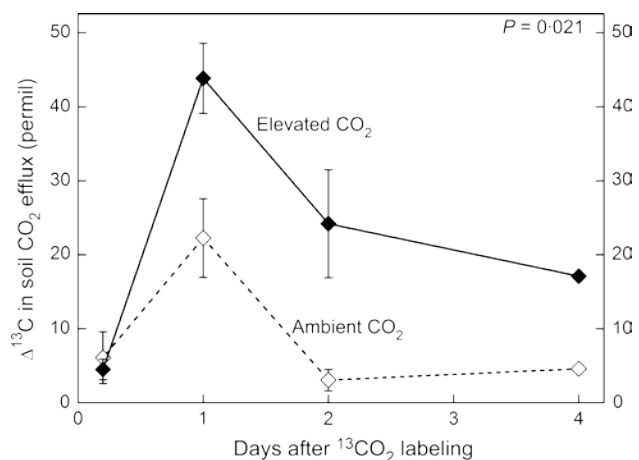
Fungi did not take up much of the recent plant-assimilated carbon under aCO<sub>2</sub> concentration (Fig. 3b). In contrast, fungi grown under eCO<sub>2</sub> concentration showed a dynamic <sup>13</sup>C uptake. At the Swiss FACE experiment, it has been reported that fungal and gram-negative-specific PLFA abundance were reduced under high nitrogen fertilization compared to the low fertilization treatment (Drissner *et al.* 2007). Thus, under low nitrogen fertilization, the abundance of fungi and gram-negative bacteria is important for, for example, maintaining the nutrient balance under induced nutrient stress in the eCO<sub>2</sub> treatment. In addition, soil enzyme activities were increased by 17–42% under eCO<sub>2</sub> concentration in October 2002 (Drissner *et al.* 2007), suggesting a crucial role of the fungal group in the maintenance of available nutrients.

Gram-negative bacteria under eCO<sub>2</sub> concentration received recently assimilated <sup>13</sup>C after a time delay of about 3 days (Fig. 3a). The observed peak enrichment at day three fits to the observation that carbon is first utilized by AMF and becomes available from rhizodeposits for bacteria and other soil organisms later (Olsson & Johnson 2005). The significant increase in gram-negative biomarker quantities coupled with a higher Δ<sup>13</sup>C indicates a high carbon demand in that microbial group which could be related to the effect of eCO<sub>2</sub> on rhizodeposition and the facilitation of gram-negative bacteria or to nitrogen-fixing

bacteria that have been shown to be abundant in this ecosystem (Lüscher *et al.* 2000; Drissner *et al.* 2007).



**Fig. 3.**  $\Delta^{13}\text{C}$  (%) in microbial functional groups at ambient and elevated  $\text{CO}_2$  concentrations over time: (a) gram-negative bacteria (average of cy17:0, cy19:0 and 18:1 $\omega$ 7), (b) saprophytic fungi (average of 18:1 $\omega$ 9 and 18:2 $\omega$ 9) and (c) arbuscular mycorrhizal fungi neutral lipid fatty acids (NLFA 16:1 $\times$ 5). Effects of the  $\text{CO}_2$  treatment are given in the upper right corners. Data are presented as means  $\pm$  SE.



**Fig. 4.** Soil-respired  $\Delta^{13}\text{C}$  (‰) at ambient and elevated  $\text{CO}_2$  concentration. Effect of the  $\text{CO}_2$  treatment is given in the upper right corner. Data are represented as means  $\pm$  SE.

## SOIL CO<sub>2</sub> EFFLUX

Soil CO<sub>2</sub> efflux showed a significantly higher  $\Delta^{13}\text{C}$  under eCO<sub>2</sub> concentration (Fig. 4). To evaluate the actual amount of respired <sup>13</sup>C under aCO<sub>2</sub> and eCO<sub>2</sub> concentrations, the isotopic values need to be weighted with the soil CO<sub>2</sub> efflux. However, soil CO<sub>2</sub> efflux was not assessed in this experiment, but it has been convincingly shown that eCO<sub>2</sub> concentration increases soil CO<sub>2</sub> efflux in grasslands (Luo *et al.* 1996; Selsted *et al.* 2012) and other ecosystems (Carney *et al.* 2007; Hyvönen *et al.* 2007). Therefore, the increased  $\Delta^{13}\text{C}$  of soil-respired CO<sub>2</sub> under eCO<sub>2</sub> conditions is probably also associated with a higher soil CO<sub>2</sub> efflux and the respiration of recently assimilated <sup>13</sup>C would therefore be higher under eCO<sub>2</sub> concentration.

Soil respiration is mainly composed of root and microbial respiration. Increased root biomass in *L. perenne* (Hebeisen *et al.* 1997; Van Kessel *et al.* 2000a; Daepf, Nösberger & Lüscher 2001) under eCO<sub>2</sub> concentration is a potential source of recently assimilated <sup>13</sup>C to the soil CO<sub>2</sub> efflux. Furthermore, increased symbiosis of N-fixing bacteria with *T. repens* (Schortemeyer *et al.* 1996; Zanetti *et al.* 1996; Montealegre *et al.* 2000) is another potential source of <sup>13</sup>CO<sub>2</sub> production. Also, a higher fungal importance under low nitrogen fertilization (Drissner *et al.* 2007) and an increased utilization of <sup>13</sup>C compared at aCO<sub>2</sub> can add a substantial amount of <sup>13</sup>C to the soil CO<sub>2</sub> efflux. CO<sub>2</sub> originating from these sources together can result in the observed higher  $\Delta^{13}\text{C}$  in soil respiration.

The main contribution of AMF to the measured  $\Delta^{13}\text{C}$  in soil CO<sub>2</sub> efflux under eCO<sub>2</sub> probably appeared within 16 h after the pulse labelling (Jakobsen & Rosendahl 1990) and was not captured. This assumption is in agreement with the finding that mycorrhizal fungi are strongly dependent on photosynthetic activity (Moyano, Kutsch & Schulze 2007) and a faster carbon turnover under eCO<sub>2</sub> concentration would therefore result in an earlier <sup>13</sup>C enrichment peak. This is supported by the observed lateral <sup>13</sup>C transport in 15 cm distance from the labelled plots where the <sup>13</sup>C enrichment peak in soil respiration occurred 1 day after labelling. This peak was delayed in the aCO<sub>2</sub> compared to eCO<sub>2</sub> treatment and can be explained by carbon transport within the mycorrhizal network that can cover relatively large distances (Johnson, Leake & Read 2002).

## SOIL CARBON STORAGE POTENTIAL

Plant-assimilated carbon can be distributed to different carbon pools, but will eventually be released back to the atmosphere. The increased carbon input to the soil under eCO<sub>2</sub> led to increased soil microbial biomass (Sowerby *et al.* 2000; Drissner *et al.* 2007) but the soil-respired CO<sub>2</sub> per unit microbial biomass stayed unchanged (Sowerby *et al.* 2000) likely due to higher rates of SOM mineralization (Drissner *et al.* 2007). After 4 years of eCO<sub>2</sub> fumigation in the Swiss FACE, the soil carbon pool remained unchanged under eCO<sub>2</sub> concentration as shown in previous studies (Van Kessel *et al.* 2000a,b). Our observations agree with those studies that reported a stimulated microbial community under eCO<sub>2</sub> and high impact of the fungal group in the studied ecosystem. Our results suggest that eCO<sub>2</sub> concentration increases the carbon throughput of the ecosystem (Figs 3 and 4) due to a higher soil carbon input and an increased soil microbial biomass. A higher carbon throughput but unchanged carbon allocation patterns under aCO<sub>2</sub> and eCO<sub>2</sub> concentrations point towards an unchanged net ecosystem carbon balance which is in agreement with previous observations (Van Kessel *et al.* 2000a,b).

## Conclusions

We presented the fate of carbon into above-ground and below-ground carbon pools in mixed grass-clover vegetation in a <sup>13</sup>CO<sub>2</sub> pulse labelling experiment after a decade of CO<sub>2</sub> fumigation. In this agricultural

relevant vegetation mixture, gram-negative bacteria and fungi have been shown to be important for the nutrient balance under eCO<sub>2</sub> especially. Our data suggest that the increase in microbial biomass is counterbalancing the increased plant nutrient demand under eCO<sub>2</sub> concentration. Furthermore, the increase in below-ground carbon allocation is balanced by a higher microbial activity, and thus, the release of CO<sub>2</sub> back to the atmosphere is increased. We conclude that, due to these balancing mechanisms, a decade of eCO<sub>2</sub> concentration did not drastically change carbon allocation in the studied grass-clover ecosystem.

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