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Community shifts and carbon translocation within metabolically-active rhizosphere microorganisms in grasslands under elevated CO$_2$

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BGD

4, 1437–1460, 2007

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Abstract

The aim of this study was to identify the microbial communities that are actively involved in the assimilation of rhizosphere-C and are most sensitive in their activity to elevated atmospheric CO_2 in grassland ecosystems. For this, we analyzed ^{13}C signatures in microbial biomarker phospholipid fatty acids (PLFA) from an in situ $^{13}CO_2$ pulse-labeling experiment in the Gießen Free-Air Carbon dioxide Enrichment grasslands (GiFACE, Germany) exposed to ambient and elevated (i.e. 50% above ambient) CO_2 concentrations. Carbon-13 PLFA measurements at 3 h, 10 h and 11 months after the pulse-labeling indicated a much faster transfer of newly produced rhizosphere-C to fungal compared to bacterial PLFA. After 11 months, the proportion of ^{13}C had decreased in fungal PLFA but had increased in bacterial PLFA compared to a few hours after the pulse-labeling. Nevertheless, a significant proportion of the rapidly assimilated rhizosphere-C was still present in fungal PLFA after 11 months. These results demonstrate the dominant role of fungi in the immediate assimilation of rhizodeposits in grassland ecosystems, while also suggesting a long-term retention of rhizosphere-C in the fungal mycelium as well as a possible translocation of the rhizosphere-C from the fungal to bacterial biomass. Elevated CO_2 caused an increase in the relative abundance of root-derived PLFA-C in the saprotrophic fungal PLFA 18:2ω6,9 as well as arbuscular mycorrhizal fungal PLFA 16:1ω5, but a decrease in the saprotrophic fungal biomarker PLFA 18:1ω9. This suggests enhanced rhizodeposit-C assimilation only by selected fungal communities under elevated CO_2.

1 Introduction

In the last ~150 years, the atmospheric CO_2 concentration has increased by ~33% due to human activity, and is predicted to continue to rise by ~0.4% per year (Alley et al., 2007). A continued rise in CO_2 may stimulate plant biomass production as well as root growth when sufficient mineral nutrients are available (Curtis and Wang, 1998; Ghan-

noum et al., 2000). This could result in greater carbon inputs into the soil due to higher rates of plant litterfall, root turnover and rhizodeposition (Rogers et al., 1994; Cotrufo and Gorissen, 1997; Sadowsky and Schortemeyer, 1997; DeLucia et al., 1999) as well as alterations in the chemical composition of plant tissues (e.g. higher C/N ratio) and

5 root exudates (Cotrufo et al., 1994; Jongen et al., 1995; Schortemeyer et al., 1996). Soil microorganisms are the key processors of soil organic matter and heavily rely on organic C supply for their growth. Any change in the amount and/or composition of plant material input into the soil in response to elevated CO_2 is therefore likely to affect soil microbial growth and metabolism of plant-derived substrates, and consequently

10 C and N cycling in soils (Zak et al., 1993). In N limited systems, an enhanced C input under increased CO_2 could also alter microbial community composition in favor of fungi. Fungi are capable of colonizing nutrient-poor and recalcitrant substrates due to their greater and more variable C:N ratio, their wide-ranging enzymatic capabilities and their ability to translocate essential nutrients through their hyphae over considerable

15 distances (Frankland et al., 1990; Hu et al., 2001). Alterations in soil microbial community composition could have significant consequences for C and N transformations. For instance, it has been postulated that bacteria-dominated food webs lead to greater short-term mineralization rates of organic C and N (Wardle et al., 2004), while fungal stimulation, in particular of arbuscular mycorrhizal fungi, may enhance C sequestration

20 (Treseder and Allen, 2000; Bailey et al., 2002) and N immobilization through hyphal translocation (Beare, 1997; Frey et al., 2000).

Microbial community composition is frequently assessed through in situ analyses of phospholipid fatty acids (PLFA), a diverse group of essential cell membrane lipids, several of which can be used as biomarkers for specific microbial groups (Vestal and White,

25 1989; Zelles, 1997). Phospholipids rapidly degrade following cell death and thus can be assumed to reflect the occurrence of living organisms. So far, contrasting results have been reported on the impacts of elevated CO_2 on the composition of the microbial community based on PLFA profiles, ranging from increased proportions of fungal (e.g. Klironomos et al., 1996; Rillig et al., 1999; Zak et al., 2000; Rønn et al., 2002) or

bacterial biomarker PLFAs (Montealegre et al., 2002; Sonnemann and Wolters, 2005; Drissner et al., 2007) to no effects at all on microbial community structure (Zak et al., 1996; Niklaus et al., 2003; Ebersberger et al., 2004). Possible causes for these discrepancies include the variety in analysis methods and the type of CO$_2$ fumigation systems used in different studies, as well as the presence of different plant species, diverse soil conditions, and variation in microbial communities colonizing the rhizosphere in different ecosystems. Moreover, most of these studies have assessed the effects of elevated CO$_2$ on the composition of the total microbial community, including both metabolically-active and inactive soil microbial communities. Specific information on the responses to elevated CO$_2$ of only those microbial community structures that are actively involved in organic matter transformations is crucial in order to better predict how key biogeochemical processes will function in an environment with increasing CO$_2$ concentrations.

The combination of ^{13}C stable isotope and PLFA analysis through gas chromatography – combustion – isotope ratio mass spectrometry (GC-C-IRMS) has made it possible to trace the flow of C from a ^{13}C-labeled substrate into the PLFA fraction of native microbial communities (Boschker et al., 1998), and to identify the microbial communities actively assimilating the labeled substrate-derived C. Stable isotope probing (SIP) of PLFA has been successfully attained through laboratory incubations with ^{13}C enriched substrate additions (e.g. Waldrop and Firestone, 2004; McMahon et al., 2005; Williams et al., 2006) as well as in situ through ^{13}C-CO$_2$ pulse-labeling of growing plants (Butler et al., 2003; Treonis et al., 2004; Prosser et al., 2006; Lu et al., 2007). By using in situ PLFA-based SIP analyses, several studies have demonstrated a dominant contribution of fungi in the immediate assimilation of rhizosphere-derived C (Butler et al., 2003; Treonis et al., 2004; Olsson and Johnson, 2005).

Phillips et al. (2002) was the first to introduce the PLFA-based SIP technique in elevated CO$_2$ research through a laboratory incubation with ^{13}C enriched substrates added to FACE soils. In their study, elevated CO$_2$ increased fungal metabolism of ^{13}C-labeled plant-derived cellobiose. Billings and Ziegler (2005) were the first to trace in situ

the ^{13}C depleted signature of supplemental CO_2 into PLFAs in the Duke-FACE experimental forest sites, excluding any potentially confounding effects of ^{13}C additions in the laboratory (cf. Phillips et al., 2002). Although the results from their study indicated clear differences in ^{13}C-depleted tracer incorporation among the different microbial groups in

5 the elevated CO_2 soils, the effect of elevated CO_2 on the metabolically-active microbial communities could not be assessed, as the control plots (the case in all FACE experiments) did not receive an equivalent ^{13}C depleted tracer. A more conclusive test of CO_2 effects on substrate utilization by specific microbial groups would require the use of an identical ^{13}C tracer incorporated in both elevated CO_2 and control plots.

10 In this study, we combined microbial community PLFA analyses with an in situ ^{13}C-CO_2 pulse-labeling approach in grassland sites from the long-term Gießen FACE experiment in order to (1) identify the microbial groups that are actively metabolizing recently produced rhizosphere-substrate in grassland ecosystems in a CO_2 enriched environment, and (2) elucidate which communities are most sensitive in their activity

15 to elevated CO_2. To our knowledge, this is the first study assessing in situ the effect of elevated CO_2 on active microbiota in grassland ecosystems through PLFA-SIP in long-term FACE experiments. The main hypothesis was that elevated CO_2 would have a larger impact on the activity of fungi, in particular mycorrhizal fungi, compared to bacterial communities due to the dominant role of fungi in rhizosphere carbon assimilation.

20 **2 Material and methods**

2.1 Research site description and ^{13}C-CO_2 pulse-labeling

The effect of elevated CO_2 concentration on active soil microbial communities was studied in permanent grassland soils from the University of Gießen long-term Free Air Carbon dioxide Enrichment experiment (GiFACE), which is located at the "Environmen-

25 tal Monitoring and Climate Impact Research Station Linden" near Gießen (Germany) at $50°32'$ N and $8°41'$ E and at an elevation of 172 m a.s.l. The GiFACE experiment was

established in 1998 to study the responses of a semi-natural grassland ecosystem to elevated CO_2. The extensively managed grassland (fertilization was $40 \, \text{kg N ha}^{-1} \, \text{yr}^{-1}$ since 1995) has not been ploughed for at least 100 years, but is mown twice a year since 1993. The mean annual precipitation and air temperature are 586 mm and 9.3°C,
5 respectively. The soil is a Fluvic Gleysol with a texture of sandy clay loam over a clay layer (FAO classification). The vegetation was dominated by an *Arrhenatheretum elatioris* (Br.-Bl.) *Filipendula ulmaria* sub-community vegetation. A detailed description of the experimental site can be found in Jäger et al. (2003).

   In September 2005, two $^{13}\text{C-CO}_2$ pulse-labeling events were conducted on plots E4
10 and K4 of the GiFACE experiment. These plots were selected because of their designation to soil process research in GiFACE. Plot K4 is naturally exposed to "ambient" CO_2 concentrations and E4 is experimentally exposed to "elevated" CO_2 concentrations at 50% above ambient levels. Since the start of the GiFACE experiment, the "elevated" E4 plot has been exposed to CO_2 with a more depleted $\delta^{13}\text{C}$ signature (i.e. –25‰
15 between 1998 and June 2004, and –48‰ after June 2004) compared to the CO_2 to which the "ambient" K4 plot was exposed (–8‰). The two pulse-labeling events were performed on two consecutive days under similar weather conditions, each for a period of 6 h. The pulse-labeling consisted of an automated supply of $^{13}\text{CO}_2$ through acidifying ^{13}C-labeled Na_2CO_3 (99 atom%) to photosynthesizing grasses inside a plexiglass
20 chamber (0.4×0.4×0.5 m) placed on top of a stainless-steel frame. This frame was inserted into the plots a few weeks prior to the start of pulse-labeling in order to minimize disturbance-induced soil respiration during the pulse-labeling. The CO_2 concentration inside the chamber was controlled and maintained at concentrations corresponding to those naturally occurring at the "ambient" K4 plot and those experimentally controlled
25 at the "elevated" E4 site.

2.2 Soil sampling

Soil samples were taken from three locations in each pulse-labeled plot at 3 h and 10 h after the start of the pulse-labeling, as well as 11 month later (i.e. August 2006) at

0–7.5 cm depth. Soil samples were also taken prior to the pulse-labeling (control samples). For the 3 h sampling time, the chambers were temporarily removed and, after soil sampling, re-installed for a second period of pulse-labeling. The three samples were mixed to one composite sample per plot. All samples were immediately stored on dry ice and transported to the laboratory. Subsamples used for PLFA-extraction were stored at –80°C. Prior to PLFA-extraction, the samples were thawed and sieved through a 2 mm sieve to remove all visible roots, macro fauna and fresh litter since plants contain large concentrations of the fungal biomarker PLFA "linoleic acid" (18:2w6,9c) (Zelles, 1997). The roots of each sample as well as 2 mm sieved soil subsamples were ball-milled to a fine powder and analyzed for total C and δ^{13}C analysis using an Elemental Analyzer (ANCA-SL, PDZ Europa, UK) connected to an Isotope Ratio Mass Spectrometer (Model 20-20, Sercon, UK) (EA-IRMS).

2.3 Phospholipid fatty acid extraction and quantification

The extraction and derivatization of PLFAs for compound-specific δ^{13}C analysis was adapted from Bossio and Scow (1995). Briefly, 6 g soil samples were extracted using chloroform/methanol/phosphate-buffer at a 1:2:1 ratio. Total lipids, retrieved in the chloroform phase, were partitioned on silica gel columns by sequential elution with chloroform, acetone, and methanol. The polar lipid fraction, eluting with methanol, was then subjected to mild alkaline transesterification (using methanolic KOH) to form fatty acid methyl esters (FAMEs) which were subsequently analyzed by capillary gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) (GC-C/TC Delta^{PLUS}XP Thermo Scientific) via a GC/C III interface. All samples were run in splitless mode and at an injector temperature of 250°C, using a CP-SIL88 column (100 m×0.25 mm i.d. ×0.2 µm film thickness; Varian Inc.) with a He flow rate of 1 ml min^{-1}. The oven temperature was programmed at 75°C for 2 min, followed by a ramp at 5°C min^{-1} to 180°C with a 20 min hold, and a final ramp at 2°C min^{-1} to 225°C with a 20 min hold.

Prior to GC-C-IRMS analysis, a mixture of two internal standards (12:0 and 19:0)

was added to the FAME extract. Individual fatty acids were identified based on relative retention times vs. the two internal standards and cross-referenced with several standards: a mixture of 37 FAMEs (37 Component FAME Mix, # 47885, Supelco Inc.), a mixture of 24 bacterial FAMEs (BAME mix, # 47080, Supelco Inc.) and several individual FAMEs (Larodan Inc.). On average, 25 PLFA peaks were detected and quantified, but only 16 were selected for ^{13}C analysis because of their use as biomarker fatty acids for different microbial communities (Zelles, 1997). The biomarker PLFAs analyzed within this dataset included: 18:1ω9c and 18:2ω6,9c (indicative of saprotrophic fungi), 16:1ω5 (indicative of arbuscular mycorrhizal fungi), i14:0, i15:0, a15:0, i16:0, i17:0, a17:0 (indicative of gram-positive bacteria), cy17:0, cy19:0, 16:1ω7c and 18:1ω7c (indicative of gram-negative bacteria) and 10Me PLFAs (indicative of actinomycetes) (cf. Fierer et al., 2003; Chung et al., 2007). These PLFAs comprised approximately 90% of the total PLFA-C concentration. We determined the ratios of the peak area of each individual PLFA to that of 16:0, a universal PLFA occurring in the membranes of all organisms. PLFA ratios less than 0.02 were excluded from the data set (cf. Drijber et al., 2000). The C concentrations of the individual biomarker PLFAs in the soil extracts were quantified using the quantitative "37 Component FAME Mix" which was run in a dilution series. For each individual standard FAME, a linear regression through the origin was performed between the chromatographic peak areas and known concentrations (ranging from 5 to 150 ng μl^{-1}). For each linear regression, a unique slope was obtained for each individual standard FAME, which decreased with increasing number of C atoms. The different slopes were plotted as a function of the number of C atoms of the PLFAs which gave a negative correlation with R^2=0.94 (Fig. 1). Using this correlation, the C concentration of each of the individual PLFAs in the soil extract could be quantified as follows:

$$[PLFA\text{-}C] = \frac{PA}{slope} \tag{1}$$

with [PLFA-C] = the C concentration of each PLFA (mmol PLFA-C l^{-1}), PA = the chromatographic peak area (Vs) of the individual PLFA, and slope = the slope of the linear

regression through the origin between the peak areas and concentrations of the individual PLFA. Quantification of chromatographic PLFA peak areas through GC-C-IRMS has also been done by others, but only by using one or two quantitative FAME standards, in most cases 12:0, 13:0 or 19:0 (e.g. Bouillon et al., 2004; Williams et al., 2006). Our quantitative analysis shows that PLFA concentrations could be greatly over- or underestimated when working with only one or two standards.

Finally, PLFA-C concentrations were calculated as relative proportions of the total PLFA-C (M_c) in order to correct for peak area changes due to reduced combustion efficiency with time.

$$M_{ci} = \frac{[\text{PLFA-C}]_i}{\sum [\text{PLFA-C}]_i} 100 \tag{2}$$

2.4 Carbon-13 phospholipid fatty acid analysis

The δ^{13}C values of the individual FAMEs obtained from the GC-C-IRMS were corrected for the addition of the methyl group during transesterification by simple mass balance:

$$\delta^{13}C_{\text{PLFA}} = \frac{\left[(N_{\text{PLFA}} + 1)\, \delta^{13}C_{\text{FAME}} - \delta^{13}C_{\text{MeOH}} \right]}{N_{\text{PLFA}}} \tag{3}$$

where N_{PLFA} refers to the number of C atoms of the PLFA component, $\delta^{13}C_{\text{FAME}}$ is the δ^{13}C value of the FAME after transesterification, and $\delta^{13}C_{\text{MeOH}}$ is the δ^{13}C value of the methanol used for transesterification (–36.7±0.4‰ vs. Pee Dee Belemnite by EA-IRMS).

Carbon-13 enrichment (expressed as $\Delta\delta^{13}$C) was calculated by subtracting the pre-labeling natural abundance PLFA δ^{13}C values from the post-labeling PLFA δ^{13}C values.

The proportion of rhizosphere-derived PLFA-C (F_i) was calculated for each individual PLFA by using the following equation (cf. Williams et al., 2006):

$$F_i = M_{ci} \frac{\delta_{li} - \delta_{ui}}{\delta_r - \delta_{ui}} \tag{4}$$

where M_{ci} is the relative percentage of C in each PLFA to that of the total PLFA, δ_{li} represents the δ^{13}C of the PLFA-C in the pulse-labeled plots, δ_{ui} represents the δ^{13}C of the PLFA-C in the unlabeled plots, and δ_r represents the δ^{13}C of the labeled roots (Table 1). The proportion (P_{Fi}) of rhizosphere-derived PLFA-C for each individual PLFA to that of the total rhizosphere-derived PLFA-C was then calculated for each fatty acid using the following equation (cf. Williams et al., 2006):

$$P_{Fi} = \frac{F_i}{\sum F_i} 100 \tag{5}$$

All data represent means and standard deviations of duplicate extracted samples from one composite soil sample of each pulse-labeled site (E4 and K4). Each soil extract was injected 2 times for GC-C-IRMS analysis, and the average chromatographic peak area and δ^{13}C value was used in all calculations.

3 Results and discussion

3.1 ^{13}C enrichment of root biomass and soil C

Both pulse-labeling events in plots K4 and E4 resulted in a rapid increase in the δ^{13}C signature of the root biomass and soil C (Table 1). Significant δ^{13}C enrichment was still visible in the root biomass as well as in the soil C 11 months post-labeling. Considerably lower δ^{13}C signatures were observed in soil and root biomass C in plot E4 compared to K4 in pre- as well as post-labeling samples, which reflected the incorporation of the depleted ^{13}C signature of the CO_2 used for the CO_2 fumigation in plot E4

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since 1998. Therefore, the data was presented as ^{13}C enrichment ($\Delta\delta^{13}$C) relative to pre-pulse labeling (control) samples to correct for these initial ^{13}C differences. However, even after this correction, δ^{13}C enrichment of soil and root biomass was generally greater in the K4 compared to the E4 plot. Possible soil CO$_2$ release due to physical disturbance at the 3 h soil sampling could have resulted in a greater isotopic dilution of the ^{13}CO$_2$ in the labeling chamber of plot E4 which had a more depleted δ^{13}C signature of soil gas CO$_2$ (~–27.8‰) compared to the K4 plot (~–24.2‰) (K. Lenhart, unpublished results). In addition, the greater leaf biomass collected from the pulse-labeled site in K4 (35.0 g) than in plot E4 (30.9 g) could have caused a greater ^{13}CO$_2$ uptake by the K4 grasses and a greater ^{13}C transport into the rhizosphere soil of K4. Because of these differences in ^{13}C incorporation into the rhizosphere of the two plots, ^{13}C-PLFA comparisons between K4 and E4 were done after expressing the data as proportions (P_{Fi}) of rhizosphere-derived PLFA-C of each individual fatty acid to that of the total rhizosphere-derived PLFA-C.

3.2 Rhizosphere-C uptake and translocation within microbial communities

Soil samples taken 3 h after the start of the pulse-labeling showed only very limited ^{13}C enrichment in the different biomarker PLFAs in the K4 and E4 plots (Fig. 2). After 10 h, a very large incorporation of ^{13}C was observed in the saprotrophic fungal PLFAs (18:1ω9, 18:2ω6,9) and arbuscular mycorrhizal fungal (AMF) PLFA (16:1ω5), while remaining low for the bacterial PLFAs. This suggested that both non-mycorrhizal fungi as well as AMF are closely associated with the root system in grassland soils (Butler et al., 2003) and actively utilize and incorporate newly produced rhizosphere-C into their biomass. The distribution of newly produced rhizosphere-C among individual PLFAs (P_{Fi}) also drastically differed from the relative contributions of individual PLFA-C to total PLFA-C (M_{ci}) (Fig. 3). This is consistent with the results of Williams et al. (2006) suggesting that only a subset of the living soil microbial community, in our case the fungi, was primarily responsible for assimilating newly produced rhizosphere-

C, while other microbial communities were active in metabolizing other sources of C. The importance of fungi in assimilating fresh plant-derived C has been demonstrated in various ecosystems based on high amounts of ^{13}C incorporation from a variety of substrates into the saprotrophic fungal biomarker PLFA C18:2ω6,9 (Arao, 1999; Butler et al., 2003; Waldrop and Firestone, 2004; Lu et al., 2007). Recent in situ studies using PLFA-based SIP by ^{13}C-CO$_2$ pulse-labeling have also reported a much faster incorporation of rhizosphere-C into fungal biomass (Treonis et al., 2004) as well as AMF biomass (Johnson et al., 2002; Olsson and Johnson, 2005) compared to bacterial biomass (Treonis et al., 2004).

The δ^{13}C enrichment in the fungal PLFAs was still largely present after 11 months but had decreased relative to the enrichment observed after 10 h (Fig. 2). This decrease in fungal PLFA-^{13}C enrichment between 10 h and 11 months was probably caused by a dilution from unlabeled rhizosphere-C assimilation during continued photosynthesis in the period following the pulse-labeling. Interestingly, 11 months after pulse-labeling, significant amounts of ^{13}C had been incorporated in the bacterial PLFAs at both plots. These results are consistent with the results of Olsson and Johnson (2005) who found over a time period of 32 days a decrease in the ^{13}C enrichment of AMF PLFA 16:1ω5, extracted from roots, and a concomitant increase in ^{13}C enrichment of bacterial PLFAs extracted from soil. Our data indicates that both saprotrophic and mycorrhizal fungi play a major role in the C flux from roots into soil microbial biomass. It can be postulated that the rapidly assimilated rhizosphere-C by fungi is over time retracted from degenerating fungal hyphae and becomes incorporated into bacterial biomass. Moreover, the observed long-term retention of C in fungal biomass could be of importance to soil organic C sequestration in grassland ecosystems.

3.3 Elevated CO$_2$ effect on rhizosphere-C assimilating microbial communities

Fungal communities have been shown to be highly responsive to increases in rhizosphere-C supply. A study by Griffiths et al. (1999) showed an increase in fungal PLFA 18:2ω6,9 when amounts of synthetic root exudates added to soil were in-

creased. Along with the results of this study which indicate that fungi are most actively utilizing rhizodeposits in the GiFACE grasslands (Fig. 2), it can be expected that fungal communities are most sensitive in their activity to any responses of plant-C inputs likely to occur under rising atmospheric CO_2 concentrations (Rogers et al., 1994). The total mol% distributions of PLFA-C (M_{ci}) were similar among elevated and ambient CO_2 treatments with the sole exception of a greater proportion of PLFA-C present in the fungal 18:2ω6,9 under elevated CO_2 (Fig. 3). Stimulated saprotrophic fungal and AMF activities under elevated CO_2 have been reported by others (Rillig et al., 1999; Klironomos et al., 1996; Zak et al., 2000; Treseder, 2004) and have been attributed to the greater substrate use efficiency of fungi in N-limited ecosystems. Receiving only 40 kg N ha^{-1} yr^{-1}, the grassland sites at GiFACE are also considered N-limited. However, other studies were unable to detect changes in microbial biomass (Allen et al., 2000; Kandeler et al., 2006) nor shifts between bacterial and fungal communities under elevated CO_2 (Zak et al., 1996; Rønn et al., 2002; Niklaus et al. 2003; Ebersberger et al., 2004). Several studies have even showed a strong response of bacteria (Sonnemann and Wolters, 2005) and an enrichment of gram-negative bacteria in particular under elevated CO_2 (Montealegre et al., 2002; Drissner et al., 2007), while the fungal biomass did not change. Until now, in situ microbial community structural analyses in FACE studies have been limited to total PLFA-C distribution examinations which do not distinguish between the metabolically-active versus inactive rhizosphere microbial communities. The advantage of a pulse-labeling approach in combination with ^{13}C-PLFA analysis is the additional information obtained on the response of those microbial communities that are actively assimilating newly produced rhizosphere-C. The response to elevated CO_2 of metabolically-active microbial communities may be undetectable through conventional total PLFA-C analyses due to the large background concentration of the mostly inactive total soil microbial community, but is of importance to better understand C cycling in terrestrial ecosystems under increasing CO_2 concentrations. Our ^{13}C-PLFA results indicated a greater proportion of root-derived PLFA-C under elevated compared to ambient CO_2 in the saprotrophic fungal 18:2ω6,9

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and AMF 16:1ω5 PLFAs, while the opposite was observed for the saprotrophic fungal 18:1ω9 PLFA (Fig. 3). Root-derived C proportions in bacterial PLFAs remained unaffected by elevated CO_2. PLFAs 18:1ω9 and 18:2ω6,9 are generally used as biomarker PLFA indicators for the presence of saprotrophic fungi (Phillips et al., 2002; Rønn et al., 2002; Chung et al., 2007; Drissner et al., 2007). However, PLFA analysis does not allow for specific detection of individual species of the microbial communities. It is possible that different fungal species are differently affected by the CO_2 treatments. Stable isotope labeling techniques in combination with molecular tools such as RNA-SIP (Griffiths et al., 2004; Rangel-Castro et al., 2005; Lu et al., 2006) could provide a better resolution of the individual active microbial communities at the species-level and should be explored in FACE research in order to better understand shifts in microbial species composition due to elevated CO_2.

Several studies have analyzed the microbial community composition under elevated CO_2 using other experimental methods than the PLFA-based SIP approach used in this study, such as extracellular enzyme activity assays (Moscatelli et al., 2005; Chung et al., 2006), PCR-DGGE analyses (Chung et al., 2006), substrate-induced respiration measurements, and 16S rRNA clone libraries (Lipson et al., 2005). Corresponding to our findings, most of these studies also suggested stimulated fungal pathways under increased atmospheric CO_2 concentrations. This fungal stimulation could be beneficial for ecosystem functioning as fungi are believed to play a positive role in soil structural stabilization (Bossuyt et al., 2001; Rillig et al., 2002), C sequestration (Treseder and Allen, 2000; Bailey et al., 2002) and N immobilization through hyphal translocation (Beare, 1997; Frey et al., 2000).

4 Conclusions

Using PLFA-SIP, the present study showed a rapid transfer of newly produced rhizosphere-C to fungal biomass (noticeable in less than 10 h) and a significant retention of rhizosphere-C in fungal biomass after 11 months in the surface 0–7.5 cm

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soil layer of grassland sites at GiFACE. The much slower incorporation of rhizosphere-C into bacterial PLFAs, but their significant ^{13}C enrichment after 11 months further suggests a potential fungi-mediated transfer of rhizosphere-C to the bacteria from degenerating fungal hyphae. This in situ pulse-labeling experiment demonstrated for the first time in long-term FACE experimental grasslands a stimulated fungal rhizosphere-C metabolism under elevated CO_2 through PLFA-based SIP analyses. Elevated CO_2 caused an increase in the relative abundance of root-derived PLFA-C in saprotrophic fungal 18:2ω6,9 and AMF 16:1ω5, but a decrease in saprotrophic fungal 18:1ω9, suggesting enhanced rhizodeposit-C assimilation by only selected fungal communities under elevated CO_2. Further pulse-labeling studies in combination with microbial biomarker SIP analyses in different long-term FACE experiments are required to test if the results observed in this experiment are reproducible for other ecosystems with different plant species and soil types, and to investigate if these microbial changes persist over time.

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Table 1. δ^{13}C values and δ^{13}C enrichment ($\Delta\delta^{13}$C, i.e. net increase relative to natural abundance δ^{13}C values of soil and roots from non-labeled control plots) for soil and root C. δ^{13}C measurements were done on composite 0–7.5 cm soil samples from 3 sampling points per pulse-labeled plot.

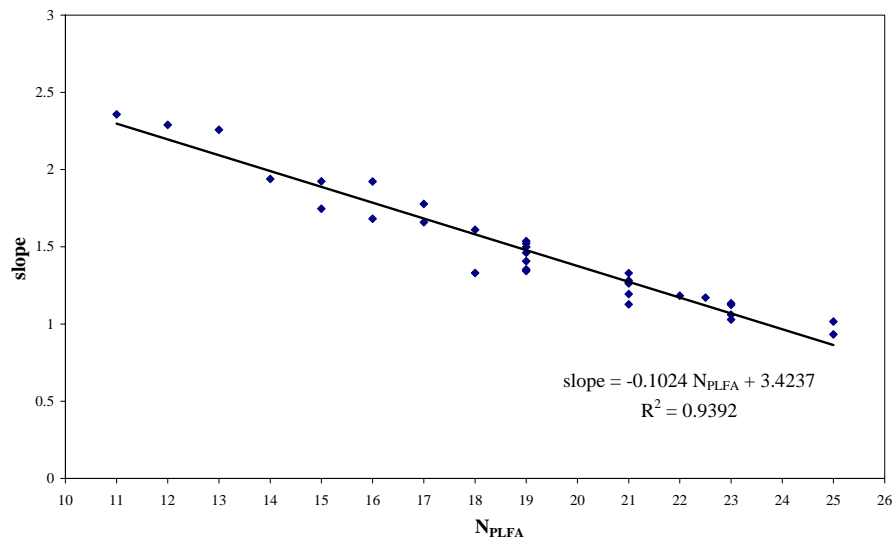| | Non-labeled | | Pulse-labeled | | | | | |
| | control | | 3 h | | 10 h | | 11 months | |
	K4	E4	K4	E4	K4	E4	K4	E4
				δ^{13}C (‰)				
Root C	−30.0	−35.2	13.2	3.3	31.0	−12.4	−9.1	−13.0
Soil C	−28.0	−30.4	−26.7	−27.9	−17.4	−26.5	−25.9	−27.8
				$\Delta\delta^{13}$C (‰)				
Root C			43.2	38.5	61.0	22.8	21.0	22.1
Soil C			1.3	2.5	10.6	3.2	2.1	1.9

Fig. 1. The relationship between the number of C atoms of PLFA components (N_{PLFA}) and their corresponding slope, obtained from linear regressions through the origin between the chromatographic peak areas and concentrations of individual standard FAMEs of the quantitative "37 Component FAME Mix" (# 47885, Supelco Inc.).

The chart shows data with the fitted equation:

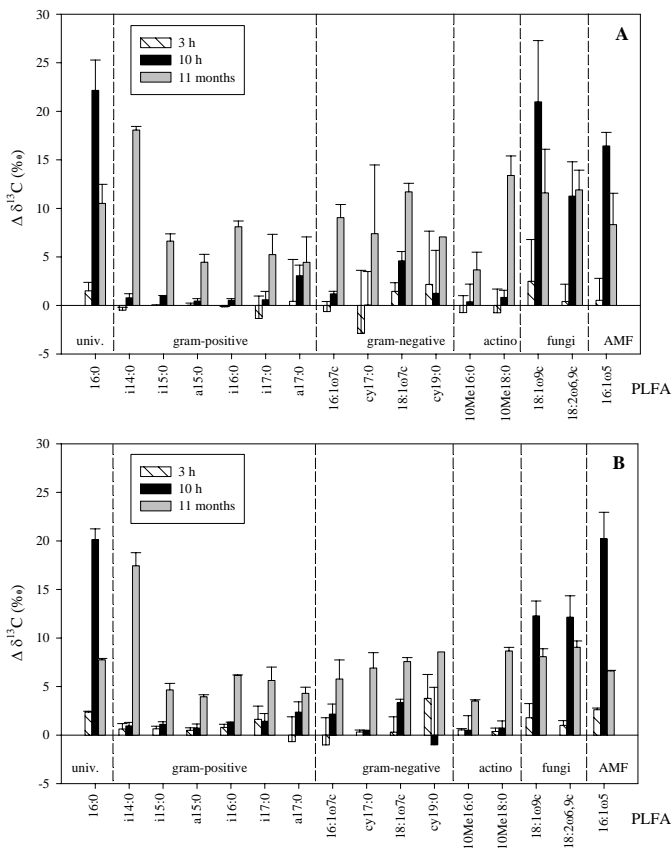$$\text{slope} = -0.1024\, N_{PLFA} + 3.4237$$
$$R^2 = 0.9392$$

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Fig. 2. Net increase of δ^{13}C values of individual biomarker PLFAs extracted from pulse-labeled 0–7.5 cm soil samples from plots K4 (A) and E4 (B) sampled 3 h, 10 h and 11 months post-labeling, in excess of those in the non-labeled control plots. Univ. = universal biomarker PLFA; gram-positive = gram-positive bacteria; gram-negative = gram-negative bacteria; actino = actinomycetes; AMF = arbuscular mycorrhizal fungi.
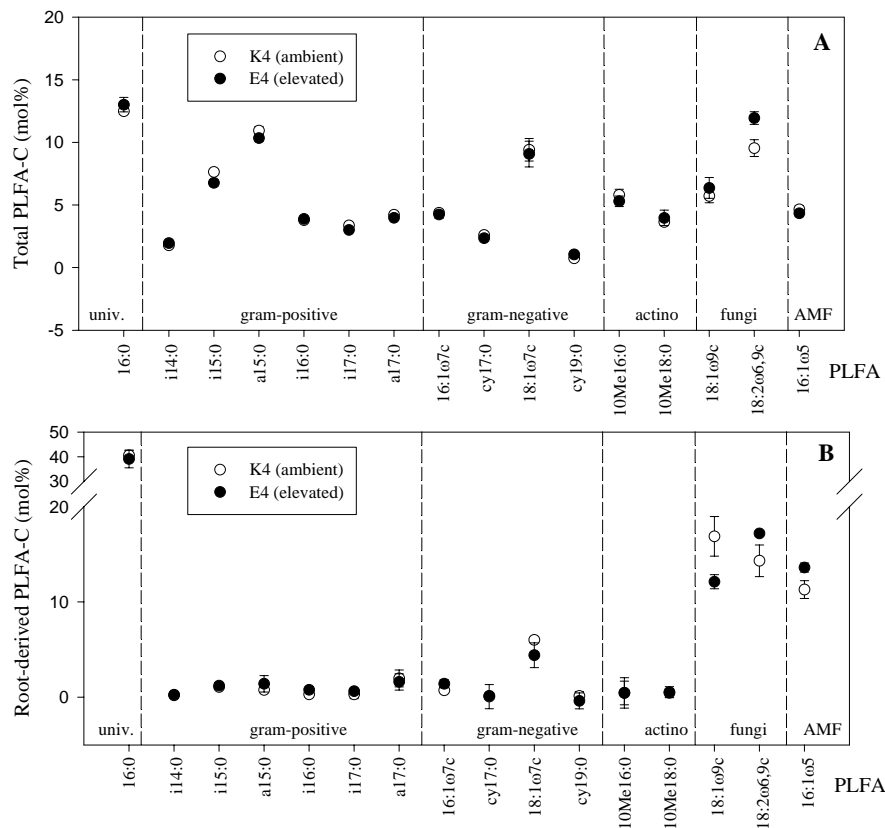
Fig. 3. The proportional distribution of total PLFA-C (M_{ci}) (A) and root-derived PLFA-C (P_{Fi}) (B) among individual biomarker PLFAs extracted from pulse-labeled 0–7.5 cm soil samples from plots K4 and E4 sampled 10 h post-labeling. Univ. = universal biomarker PLFA; gram-positive = gram-positive bacteria; gram-negative = gram-negative bacteria; actino = actinomycetes; AMF = arbuscular mycorrhizal fungi.