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Biogeosciences Discussions



# *Interactive comment on* "Community shifts and carbon translocation within metabolically-active rhizosphere microorganisms in grasslands under elevated $CO_2$" *by* K. Denef et al.

**K. Denef et al.**

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We found the comments given by reviewer 2 very constructive and most of his/her suggestions were followed in the revised manuscript (see list below). Reviewer 2 is obviously familiar with this topic, and appears to have an in-depth understanding of the huge amount of practical work involved (pulse-labeling, compound-specific isotope measurements, correction factors, biomarker issues etc.). Both reviewers commented on the unfortunate lack of replication, but nevertheless, reviewer 2 did not seem to find this a reason to reject the paper. We agree that it is unfortunate that we were not able to do a pulse-labeling on the replicated treatment sites at GiFACE, but there were several reasons why this was the case. With respect to the suggestions of reviewer 2, we justified the reasons for the unreplicated nature of this experiment in a new section in

the materials and methods (pg. 5-6) (also listed below). In addition, as reviewer 2 also recognized, we would like to stress that the work involved in pulse-labeling experiments and isotope-biomarker analyses cannot be underestimated and limited us to just one treatment plot per pulse-labeling. Since this type of pulse-labeling has never been done in a FACE experiment, we believe our paper is still very innovative and unique and will attract much scientific interest. The main purpose of this paper was therefore to show some of the early findings to other researchers in this area, and encourage further research with these powerful techniques. We believe that it can have a significant contribution to future work in this exciting and rapidly growing research area.

We had some concerns about the comments given by reviewer 1 and did not agree with most of his/her remarks. We do not believe that he/she fully understands the advantages of the techniques applied in this experiment. We explain this in the order of his/her comments:

1) Untypical situation at GiFACE for future increased CO2 concentrations:

We agree with the reviewer that the grassland sites in Giessen respond differently to elevated CO2 when compared with other FACE experiments in grasslands, in terms of soil C changes (no differences observed in GiFACE). However, weak to no changes in soil C with elevated CO2 (despite substantial aboveground biomass changes) have been also found by others (Gill et al., 2002, reviews by van Groeningen et al., 2006 and Jastrow et al., 2005). There are many possible explanations for this, based on the type of ecosystem investigated (in our case N-limited, wet grassland, lower CO2 enrichment in the replicated design compared to most FACE studies etc.). When acknowledging the N-limitations in the Giessen FACE sites, also quite atypical responses in terms of aboveground productivity (increased in GiFACE with elevated CO2) were found (Kammann et al., 2005). In most nutrient-limited grasslands, elevated CO2 causes only a weak or no stimulation of plant productivity. When ecosystems are nutrient limited, immobilization of nutrients (N in particular) in plant biomass and SOM provides negative feedback to plant growth and this could lead to progressive N limitation of plant



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response to CO2 enrichment (see review by Hu et al., Plant and Soil, 2006). However, at GiFACE, quite large increases in the aboveground biomass with elevated CO2 have been documented (Kammann et al., 2005) and were attributed to possible stimulation of root growth and mycorrhiza resulting in better nutrient acquisition. This stimulation of the activity of the mycorrhiza was confirmed in our study. However, we do not agree with the reviewer that the CO2 effects observed in this study reflect an "atypical situation"; for future increased CO2 concentrations since it is not known yet what a "typical" future response will be. Can we talk about "typical" ecosystem behavior under elevated CO2 when we can only base this on observations from only a few ongoing FACE manipulation experiments in drastically different ecosystems around the world? The FACE experiment in Giessen is as valuable for elevated CO2 research as other FACE experiments since it is unique for its type of ecosystem, and would otherwise not continue to be maintained if it is non-representative of ecosystem changes in response to future CO2 increases.

2) "cuvette-effects" of the chamber system:

We believe that reviewer 1 does not fully understand the advantage/strength of the pulse-labeling system to trace the flow of plant-C through the microbial communities IN-SITU and to investigate the response of only those microbial communities that are "actively"; involved in assimilating plant-C in-situ. For this type of pulse-labeling, a chamber approach is the only technique that can be used. This, of course, has its disadvantages as well (temperature increases, moisture build-up from evapotranspiration), but some control was attempted by cooling the circulating air, and by having a water condensation trap in front of the IRGA. Even the CO2 concentration in the chamber was reasonably controlled. The problem with turbulence is not so clear to us. The frames were inserted in the soil some time before the actual labeling (indicated in the text, pg.5). This could have caused some disturbance, but again, not possible to be avoided. We think the advantages of the pulse-labeling technique weigh out these disadvantages, which seem to us to have mainly an effect on the plants and to a lesser



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degree on the microbial communities over only a 6 hour period.

3) Lack of field replication:

As stated before, this is indeed unfortunate, but cannot be changed. The experiment and subsequent analyses are quite complex and labor-intensive. Moreover, some limitations exist at GiFACE due to concerns about the preservation of the natural state of these grasslands. This is a long-term elevated CO2 experiment and one of the only ones that is in a temperate, (semi)-natural wet grassland ecosystem. It is understandable that they would like to avoid too much disturbance and isotope inputs from pulse-labeling experiments (plots are not that big either). Therefore, we were limited to do this pulse-labeling on only one elevated and one ambient site. It seems like reviewer 2 understands this and only asks for an explanation to be added in the text, which was done in the new manuscript (pg. 6). As we stated earlier, we agree that replication is necessary for justified statistical analyses and to draw conclusions. But as this is the first time a pulse-labeling was done at a FACE experiment, our study mainly serves to indicate the potential of this technique to investigate in-situ the microbial communities' activities in response to elevated CO2. However, acknowledging the need for a truly replicated study, we changed this part in the conclusions section (pg. 14-15) and indicated that additional pulse-labeling studies in combination with microbial biomarker SIP analyses are also needed in the fully-replicated FACE experiment at Giessen to test if the results observed in this trial experiment are reproducible at GiFACE.

4) PLFA quantification

Both reviewers commented on the fact that no attempts were made to quantify the absolute amount of fungal and bacterial PLFAs. We agree that relative amounts of fungal and bacterial biomarker PLFA-C (as a proportion of the total) does not reveal which of the communities change in absolute quantities. Therefore, we decided to show the absolute concentrations of the biomarker PLFAs in nmol PLFA-C/g soil instead of the proportional distribution of PLFA-C amongst the different biomarkers (see Fig. 3).



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After doing a simple t-test on the data (soils were extracted in duplicate, so n=2), this resulted in no effect of elevated CO2 on the concentration of any biomarker PLFA at any time (3h, 10h, 11 months), at least within the analytical error of the PLFA-analysis.

2. Reply to specific comments of reviewer 2:

1. We added an explanation for the choice of the 3 h, 10 h, and 11 month sampling times (pg. 6). Soil samples were taken at 3 h and 10 h after the start of the pulse-labeling to investigate the short-term incorporation of new rhizosphere-C into microbial communities. Soil samples were taken 11 months later to determine any long-term retention of 13C label in root, soil and microbial biomass and to assess possible rhizosphere-C transfer pathways through different microbial communities over time.

2. According to the reviewer, the high amount of 13C remaining in the fungal PLFAs after 11 months suggests very little activity by the fungi in the 11 month period. However, to our opinion, it is possible that the fungi that were living at the 11 month sampling time (most likely not the same ones as those present in the soil at the time of pulse-labeling) and that were actively assimilating root-derived C, were able to incorporate some of the 13C remaining in the roots after 11 months. Since the harvested roots (including both living and dead roots) were still substantially enriched after 11 months, fungal 13C enrichment might just be a result of continued root-C assimilation by new fungi, either through root decomposition, or by the use of living root products, which could still be 13C enriched. Perhaps it is also possible that there is some active recycling of plant-derived as well as microbial-derived 13C-containing products by the fungi. We discussed this in more detail on pg. 11-12.

3. More details were provided on the removal of roots from the soil samples (pg. 6). This was first done by sieving, followed by manual removal of roots by forceps. The reviewer also raised the question of possible contamination of the fungal PLFA by plants (remaining roots in the soil samples which contain similar PLFAs as the fungal biomarker 18:2w6,9). This was acknowledged in the text (pg. 11-12), but pointing out



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that other plant-specific PLFAs should be detected in the soil extracts, which was not the case in our study. We also referred to the study by Treonis et al. (2004) to indicate that root-derived C rather rapidly turns over in fungal PLFAs in grassland soils. This supports our statement that the remaining enrichment in fungal PLFA 13C is rather a result of continued assimilation or recycling of root-derived C.

4. We agree with the reviewer that we did not refer to the appropriate studies that have proved the unique presence of specific PLFAs in specific microbial groups. We added a list of new references to the text (pg. 8). Later in the text, we indicated the uncertainty about the use of the 18:1w9c PLFA as fungal biomarker (pg. 13-14).

5. (1) We now expressed the quantification data in absolute PLFA-C concentrations (nmol PLFA-C/g soil). The quantification was done by GC-C-IRMS and not by GC-FID. Quantification by GC-C-IRMS has been done by others as well (e.g. Williams et al., 2006; Bouillon et al., 2004), however these studies only used a few FAME standards to quantify all identified PLFAs (as mentioned on pg. 8). We believe that our method was much more precise. We do agree with the reviewer that this type of quantification is atypical, but this was done to avoid double analyses on one soil extract and lack of access to a GC-FID with the same GC column. Moreover, our quantification regression (Fig. 1) showed very promising for using GC-C-IRMS to quantify PLFAs. It still remains a complex analysis since an entire standard series of a quantitative standard mixture needs to be analyzed in triplicate prior to each batch of samples. This takes up almost a full day of analysis. But overall, we are happy with the results and the values of these PLFAs seem comparable to other studies. (2) A list was provided of the individual standards used to confirm the PLFA identification in addition to the 37 component FAME mix and the bacterial BAME mix from Supelco. The identification was only done based on retention times on the GC-C-IRMS and not confirmed by GC-MS. However, this type of identification (based on retention times) has been done by others as well (e.g. Quezada et al., 2006; Bouillon et al., 2004). (3) Instead of mol% data, we now presented the absolute concentrations of PLFA-C in nmol PLFA-C/g soil. (4)



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A separate section was included in the manuscript (pg. 9) reporting the error on the PLFA quantification and 13C analyses.

6. The precision of the GC-C-IRMS analysis is now indicated on pg. 9 (determined on duplicate injections of one soil extract). The methanol d13C was measured by elemental analyzer-IRMS (cfr. Treonis et al., 2004). This was done by adding a small aliquot of methanol on an absorbent (Chromosorb-W) inside a tin cup, which was sealed immediately after methanol addition. We made sure to analyze these samples immediately after filling the cups, to avoid volatilization. If volatilization occurs with concomitant fractionation, volatilization should also occur during the derivatization of the fatty acids, with possible fractionation. In this way, it is practically impossible to determine the true signature of the methanol-C that was added to the fatty acid during mild alkaline transesterification. However, slight changes in this signature (as a possible result of fractionation during volatilization) only have a minor effect on the overall enrichment signature of the PLFAs in this 13C enrichment study.

7. A section explaining the statistics was added to the manuscript (pg. 9). We agree with the reviewer that a simple t-test can already indicate whether, within the error of the analysis itself, there were significant differences between treatments, while acknowledging that this is not a true test of the treatment effect due to the lack of treatment replication.

8. (1) We agree with the reviewer that the greater biomass in K4 does not clearly explain the greater 13C enrichment of the grass, roots and soil in K4. Even if there was more net 13C uptake (in absolute amount) by the greater plant biomass in K4, there would have been also a greater d13C dilution effect caused by the larger background 12C of the greater biomass. We therefore left this out and focused on the possible greater dilution by soil CO2 (which was more 13C-depleted in E4) in the E4 plot upon soil disturbance caused by soil sampling after 3 h. (2) We decided to work with the proportional 13C enrichment of the PLFAs relative to a universal biomarker PLFA that is present in all organisms (i.e. 16:0). This has also been done by others (e.g. Butler



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et al., 2003; Lu et al., 2007) to indicate differences in the C-assimilating activity of microbial communities among soil types or over time. We decided to do this, since in pulse-labeling studies, it is difficult to determine the 13C signature of the true source of rhizosphere-C that the microbial communities are assimilating. We realized that using the 13C signature of the root biomass (as was done previously) is not really correct, since sometimes the PLFAs were more enriched in 13C than the root material itself. By expressing the 13C-PLFA data proportional to the 13C enrichment of the universal biomarker PLFA, one bacterial biomarker PLFA also showed an increase in relative 13C enrichment due to elevated CO2 in addition to the arbuscular mycorrhizal fungi, and a decrease was again found for the 18:1w9c PLFA with elevated CO2. This was explained in the method section on pg. 9.

9. See earlier response in 2. and 3.

10. Figure 3 now shows the quantity of the individual PLFAs in nmol PLFA-C/g soil for sampling times 3 h, 10 h and 11 months.

11. We discussed this uncertainty of the uniqueness of the 18:1w9c biomarker for fungal biomass on pg. 13-14. However, we also provided some arguments why we believed this biomarker has the same origin as the other fungal biomarkers, based on the rapid 13C enrichment only in these fungal biomarkers (as well as the universal PLFA 16:0).

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