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## ***Interactive comment on “Community shifts and carbon translocation within metabolically-active rhizosphere microorganisms in grasslands under elevated CO<sub>2</sub>” by K. Denef et al.***

### **Anonymous Referee #2**

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Paper outlines results of a pulse-chase labeling study conducted in two plots at the GiFACE site which was designed to investigate the influence of elevated CO<sub>2</sub> on the use of rhizosphere-derived C by the soil microbial community. The focus of the paper is very topical given need to understand how elevated CO<sub>2</sub> will impact C-cycling in terrestrial ecosystems particularly within the soil. The work presented here provides a contribution to the current literature on this topic by affirming that substrate utilization by soil fungi can be enhanced under elevated CO<sub>2</sub>. Although other investigations have pointed to a stimulatory effect, inhibitory effect and no effect of elevated CO<sub>2</sub> on fungal activity in various ecosystems this work is new in its approach using a <sup>13</sup>C labeling approach in situ at a FACE site. Advantage of this approach is that it allows for the

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assessment of active microbial components in situ, unfortunately as designed it did not allow for quantification of C flow from the grasses into the soil microbial community. But alas we cannot have everything in every study! What the authors were able to point out in this study was the distribution of the of rhizosphere-derived C among the PLFA resolved, so it is a relative measure of the activity of the soil microbes supported by rhizosphere-derived C. With this we get a more refined picture of the alteration of microbial use of rhizosphere C under elevated CO<sub>2</sub> in a grassland ecosystem.

So overall I find this paper a worthy contribution. I do however have a couple questions/suggestions to consider in a revision of this manuscript. Some more serious than others, such as need to explain the remaining 13C label at 11 months in the context of fungal biomass turnover and /or remaining root materials detected as fungal PLFA and lack of statistical analyses to support the statements made in the results/discussion section.

1. There needs to be an explanation for the 3 h, 10 h, and 11 month sampling times.
2. Were there any big differences in the field conditions between the first two sampling events and 11 months later? I ask this because the fact that the 13C remained (and still quite elevated) in the fungal biomarkers suggest very little activity by the fungi in these soils over that 11 month period.
3. Could the authors specify whether they visually inspected the soil for fine roots and removed them or simply sieved (2mm). This is important considering, as the authors clearly point out, the overlap between plants and fungi containing 18:2w6 PLFA. I bring this up, again, because of the remaining enrichment of the fungal PLFA 11 months after the labeling was conducted. I wonder if that would simply have been fine roots that remained in the soil since the 13C of the root material collected at 11 months was 21 permil for K4 and 22 permil for E4 and the 18:2w6 PLFA for example remained at about 11 and 7 permil, respectively. This needs to be more thoroughly explained by providing (1) further evidence for the true identification of the PLFA as fungi, (2)

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explaining the results in the context of typical fungal biomass turnover in grassland soils (i.e. does it make sense that fungal biomass would turnover less than once a year...if so provide the context to explain that...it is important!), and/or (3) directly discussing the difference between the roots and fungal PLFA isotopic composition at 11 months.

4. In the methods there needs to be direct reference to the literature that specifically identifies the individual PLFA biomarkers as belonging to specific groups. All too often papers cite other papers that have “used” a particular biomarker to identify a particular group rather than providing the true evidence (specific study of PLFA in groups of microorganisms) for these biomarkers. For example Fierer et al. 2003 and Chung et al 2007 did NOT study the PLFA composition of individual microorganisms but applied the use of PLFA for a particular question, while Olsson (1999) for example specifically demonstrates the presences of 16:1w5 in AMF and 18:2w6 in fungi in general. This particular point is also relevant to the final discussion/conclusion of the data. How confident are you in saying that 18:1w9 is only derived from saprotrophic fungi?

5. There is some discussion of the standard curves used to quantify the FAMEs detected via IRMS, however, in the end the authors revert to using only the mol% of individual PLFA. So a few questions related to this: (1) There is some use of GC-C-IRMS to quantify PLFA and as the authors point out there are problems with that approach so why wasn't a FID used since it has a much wider linear dynamic range than combustion IRMS and since quantification requires multiple concentrations of standards particularly with combustion IRMS where linearity is a problem!?, (2) were individual FAMEs verified using GCMS (mass spectra) to compare to standards, I know from experience that the use of mixed standards like the one from Supelco is problematic if used alone in the identification of FAMEs from real samples (perhaps the authors could simply provide the information on the individual standards used to make these identifications), (3) was the mol% increase in 18:2w6,9 PLFA due to a true increase in the quantity of that PLFA or a decrease in other PLFA? One cannot tell from the data presented and it seems very relevant to the goals of the study to find out if ele-

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vated CO<sub>2</sub> stimulated a increase in the biomass of fungi or inhibited growth in bacterial biomass. So this means the direct quantification data on the individual PLFA would be very relevant and the precision and accuracy of this measure is more critical to the results/discussion than as currently written, (4) Could the authors provide the error (%CV or standard deviation) in their measurement of FAME quantities using this approach?

6. There needs to be a report of the precision of the d<sup>13</sup>C PLFA analysis such as %CV or mean deviation of the duplicate injections (average of that value across all samples would be sufficient). Also the variation in the correction measured and used for the methanol in derivatization of fatty acids to FAME. How was the methanol analyzed for d<sup>13</sup>C value via EA-IRMS without fractionation due to volatilization? It would be prudent for the authors to report the correction factor used and how it compares to those studies where the methyl group was measured via internal standard realizing that this could not be done in this case since mild alkaline transesterification was used (i.e. could not methylate free fatty acid isotopic standards).

7. Some attention to statistics are needed. Was this omitted because there were no replicates conducted within each of the two plots? I am sensitive to this sort of criticism recognizing the work involved in conducting these experiments and the subsequent analyses. Perhaps a statement explaining the reasons for not conducting the pulse chase experiments in more than one elevated CO<sub>2</sub> and one control plot would be sufficient. Not having any level of replication precludes use of any statistics to test the question posed regarding the impact of elevated CO<sub>2</sub> on rhizosphere-derived C, but I am not entirely sure this is even the case since it was not explicitly stated. Other details such as what the error bars on figures 2 and 3 represent. If these were duplicate injections then standard deviations are not appropriate, however, mean deviations would be and a simple t-test on those data could at least tell us whether within the error of the analysis itself there were significant differences realizing that this is not a true test of the treatment effect. Again, this draw back does need to be highlighted within the methods section but does not have to be a fatal flaw if limitations on replication are

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warranted in this case to provide an in situ measure of C-flow.

8. In the first paragraph of the results/discussion the authors describe the potential uneven labeling of the two plots and therefore difference in  $^{13}\text{C}$  incorporation into the rhizosphere between the two plots. This is a fine assessment except for two things, (1) the explanation that greater leaf biomass was collected from the control plot as one reason for the uneven labeling...is confusing. I am not sure I understand how that is related to the different in  $^{13}\text{C}$  incorporated into the two plots, (2) need to explicitly state that the actual flux of new C into PLFA could not be calculated but was used to determine the relative proportion of rhizosphere-derived C into individual PLFA to assess differences in the composition of microbes using rhizosphere-derived C. The authors might consider placing this discussion within the methods section to specify how the data were used.

9. On page 1448 in the full paragraph authors should consider discussing the context of the remaining  $^{13}\text{C}$  label in the fungal PLFA. Is this realistic in terms of what is known about fungal biomass turnover/growth rates. This is a very interesting aspect of the paper but remains underdeveloped. It is also a point that raises questions regarding the use of the 18:2w6,9 PLFA as a biomarker for fungi (i.e. were plant inputs a factor?).

10. Page 1449 lines 5-10. What was the change in the actual quantities of the 18:2w6,9 PLFA. It would be useful to indicate this to specify whether the change in mol% was due to an increase in fungal biomass or reduction of other microbial components under elevated  $\text{CO}_2$  (or both). Also were these differences also seen in the 3h and 10h measurements or was this only detected in the second year?

11. The difference in the effect on 18:1w9 and 18:2w6 is intriguing but I am not convinced of the authors conclusion regarding this difference. Could the authors consider how specific the 18:1w9 PLFA is as a biomarker for saprotrophic fungi?

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