Reviewer 3 comments:

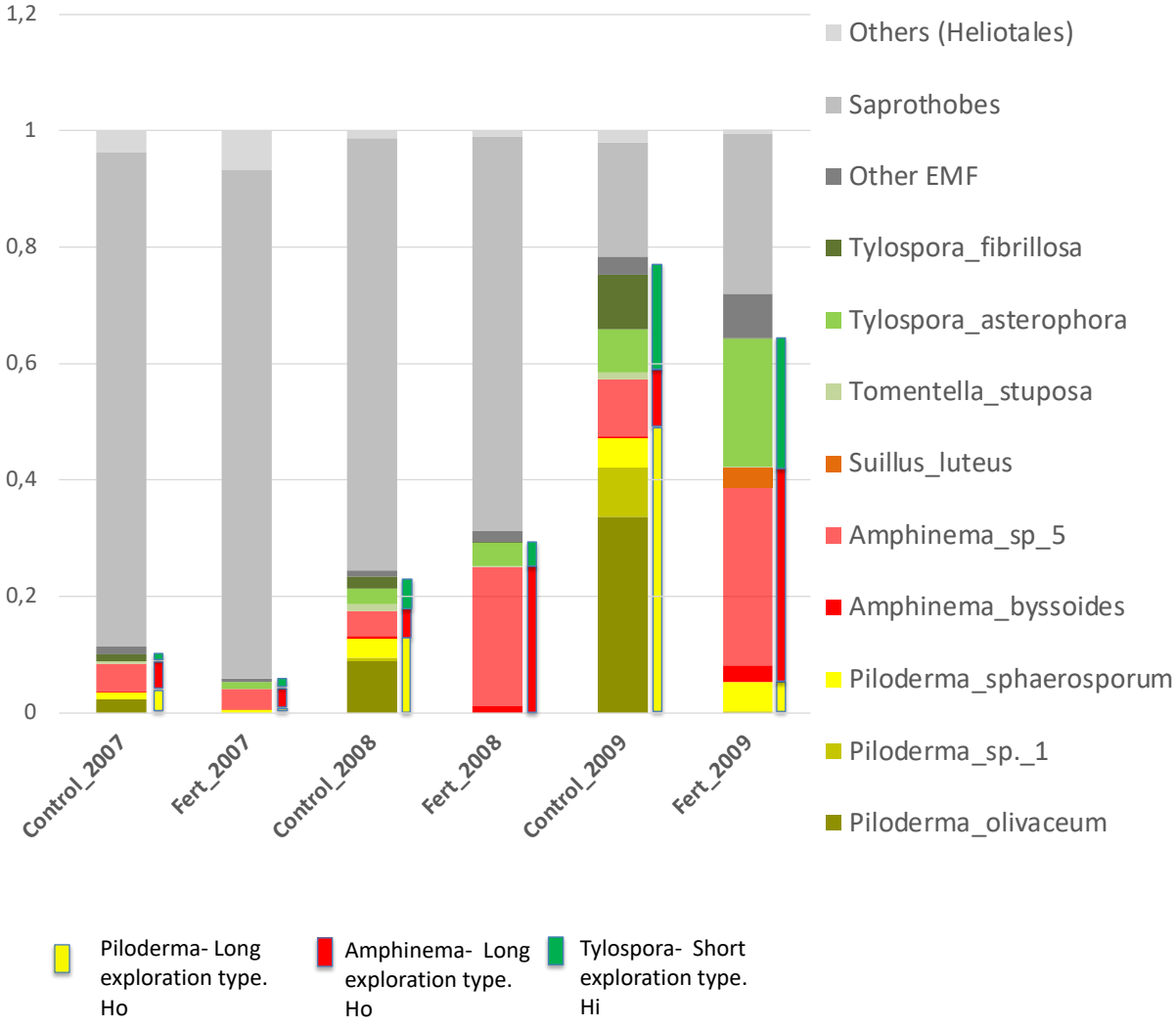
Thank you for the comments. The manuscript has improved a lot and with the suggested changes our findings are supported.

Comment:

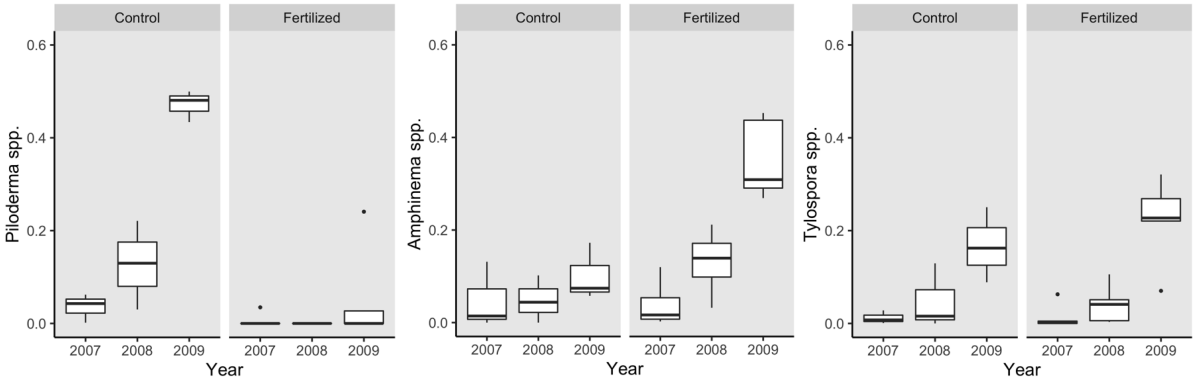
For instance, Almeida et al. could code for mycelial hydrophobicity and compare the relative sequence abundance of hydrophobic EMF taxa over time/across treatments. The effect of fertilization is particularly important given that the design of the study hinges on the idea that EMF with hydrophobic mycelia decline with fertilization

Answer:

A new Figure 3 where the most abundant species (at least 1% of the total fungal reads) are grouped by genus and by exploration type and hydrophobic properties of their mycelium will be presented:



Also, in the same figure a new panel with a boxplot of the most abundant genera will be shown:



Comment:

I would also like to see more information about the variability within the EMF community. For instance, instead of a stacked bar chart, Figure 3b could be broken out by treatment and each taxon could have an error bar.

Answer:

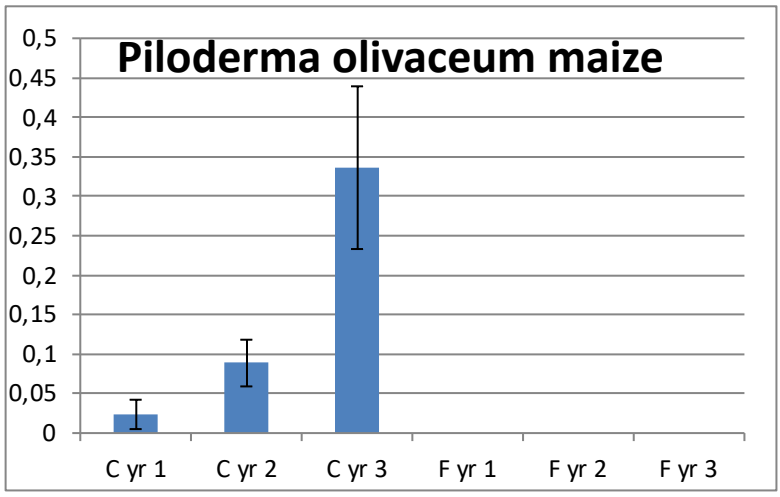
In the third panel in the new Figure 3 it can be seen how well represented the genera are across samples. Especially for the genus *Piloderma* there is low variability between replicates as it can be seen in the box plots especially in the third-year bags.

Comment:

Further, much of the discussion centers on the physiology and ecology of the most abundant species of EMF in the control plots (Piloderma oliviceum),but little information is available regarding how consistently this taxon shows up in the meshbags. If P. oliviceum is indeed abundant across most samples, this would strengthen the discussion of its role in potentially enhancing SOM hydrophobicity.

Answer:

P. olivaceum is quite well represented across the different replicates in the control plots as shown by the standard bar error:



However, now the results will center more around the **genus *Piloderma*** which is also well represented in the control plots as explained in my previous comment.

Comment:

Also, if possible, regressing the relative sequence abundance of hydrophobic EMF against the averaged contact angle of the substrate in an ANCOVA would strengthen the claim that EcMF mycelial hydrophobicity is imparting increased hydrophobicity to meshbag contents. If this emerges across N fertilization treatments, this would be particularly impactful.

Answer:

An ANCOVA was performed and the abundance of hydrophobic species were not significant while the effect of treatment was:

ANOVA Table (type II tests)

	Effect	DFn	DFd	F	p	p<.05	ges
1	Long.distance.exploration.type	1	15	0.538	0.475		0.035
2	Treatment.1	1	15	6.150	**0.025**	*	0.291

This means that fertilization does have an effect on the contact angle but this cannot be explained by the proportion of hydrophobic EMF species. This fits with the discussion where we argue that the hydrophobicity is conferred by species specific features of *Piloderma* which is very scares in the fertilized plot. I will explain more on the matter in my next

answer. However, I am not sure that a covariate analysis will be the best for our data and hypothesis.

The ANCOVA is used to test the effect of an independent categorical variable (in this case Fertilization) on a dependent continuous variable (Contact angle) controlling for the effect of a second continuous variable, the covariate (Hydrophobic EMF).

Generally, the covariate is not very relevant for the study question and the test is used to find if the differences between the treatments are due to initial selection differences (the covariate). So the test removes the variance between treatments caused by the covariate (Miller and Chapman, 2001 & Jamieson, 2004)

However, hydrophobic EMF should not be used as a mere covariate since it is the main dependent variable that we try to use to explain the changes in hydrophobicity in time and fertilization regime. In this case Hydrophobic EMF might be intimately associated with the treatments (as we hypothesized) and removing its effect in and ANCOVA would remove most of the variance between the treatments (Miller and Chapman, 2001).

Jamieson, J. (2004). Analysis of covariance (ANCOVA) with difference scores. International Journal of Psychophysiology, 52(3), 277-283.

Miller, G. A., & Chapman, J. P. (2001). Misunderstanding analysis of covariance. Journal of abnormal psychology, 110(1), 40.

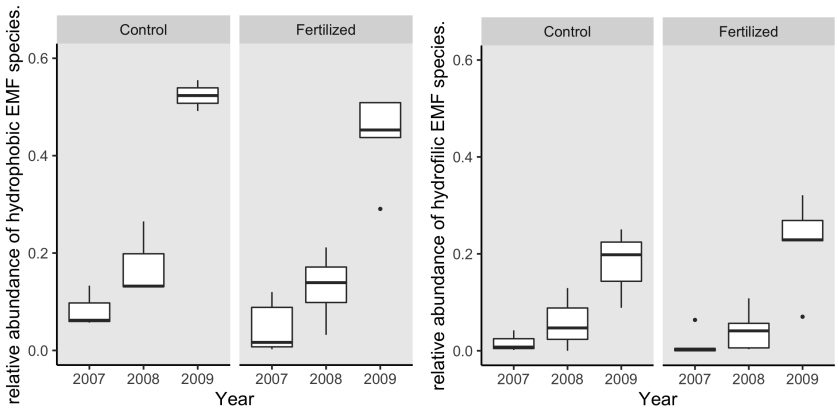
As suggested, regressions between the relative abundance of the **sum of reads from the hydrophobic EMF species** (those contributing at least with 1% of the total fungal reads) and the **averaged contact angle** has been performed and are explained in the next answer.

Comment:

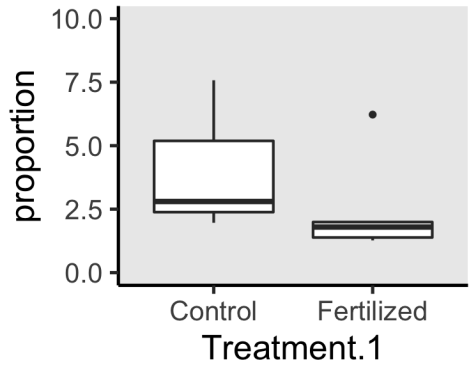
45-47: The hydrophobicity of living EMF mycelia is framed as a possible driver of SOM hydrophobicity here, but the subsequent analyses do not address how hydrophobic vs. hydrophilic EMF differ in abundance over the fertilization treatments. Is there a way to either change this framing, or address it with further analyses?

Answer:

Indeed, there is a trend for higher amount of hydrophobic types in the controls in comparison with the fertilized plots in the third year and a trend for lower amount of hydrophilic types in the fertilized plots in comparisons with the controls in the third year, but this trend was not significant:

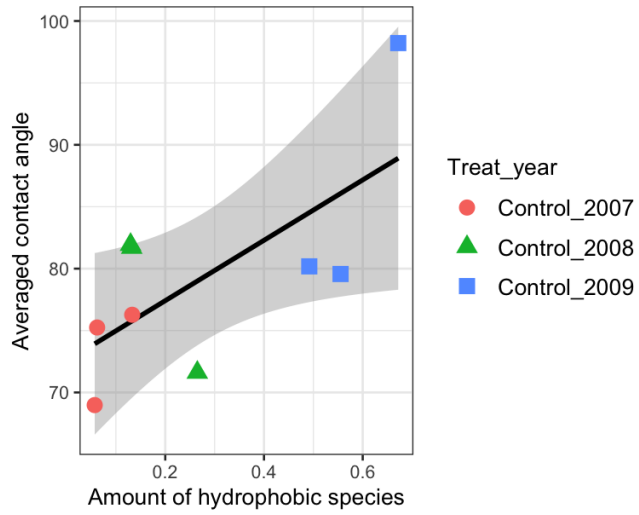


Also, the proportion of hydrophobic species in relation with hydrophilic species tended to be higher in the controls but this was not significant:



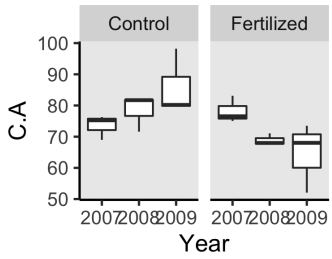
Moreover, if we make a correlation between the averaged contact angle (initial, 1s, 5s) and the abundance of hydrophobic types, there is no trend nor significance. This means that the combination of hydrophobic species might not explain **completely** the hydrophobic differences between the two treatments.

However, if we make a regression between the averaged contact angle and the abundance hydrophobic types using only the **control plots** the correlation is significant (**this figure will be added as an extra panel in Figure 3):**



This graph will be presented as another panel in figure 3 along with the other results from the community data.

This makes sense considering that both treatments have opposite trends regarding hydrophobicity (in the **fertilized plots** there was a decrease in hydrophobicity over time) while the relative abundance of hydrophobic EMF increased and at by the third year there were more abundant hydrophobic species than the hydrophilic ones even in the **fertilized plots**.



If the changes in hydrophobicity are explained by the fungal communities it is likely that the effect of fungi is related more with specific and unique differences in the community composition between **control and fertilization**.

The most clear and unique difference between both treatments is the presence of the *Piloderma* species which almost disappeared in the fertilized plots. It should be noted that by the third year the proportion of hydrophobic species in the control plots was up to 60% of the total fungal reads but the genera *Piloderma* alone contributed to up 50% of the total fungal reads in the three-years bags.

Therefore, this genus is a good candidate to explain the increase in hydrophobicity in the control plots.

This is already mentioned in the discussion; the changes in hydrophobicity cannot be explained only by the exploration type and the hydrophobic properties of the mycelium of the species. It depends on features specific of certain genera in this case *Piloderma*. This was tested before by Zheng et al. (2014) in an elegant experiment where they measure hydrophobicity of sandy soil. The fact that the long exploration types correlates with hydrophobicity only in the controls (where *Piloderma* makes up the majority of the reads) but not in the fertilized plots where *Piloderma* spp. is almost absent further supports the discussion of this paper.

I have added extra information concerning this in the results:

The more abundant hydrophobic EMF genera were Amphinema and Piloderma while the more abundant hydrophilic genera were composed of Tylospora and Tomentella (Fig 3b). The amount of combined hydrophobic species tended to be higher in the control plots (up to 60% of the total fungal reads) in comparison with the fertilized plots (up to 50% of the total fungal reads) in the three-years-incubation bags, but this increase was not significant. Additionally, the proportion of hydrophobic EMF species in relation to hydrophilic EMF species in the control plots tended to be higher than in the fertilized plots in the three-years bags but this was not significant. When both treatments (control and fertilization) where analyzed together, there was no correlation between the proportion of hydrophobic species and the contact angle. The proportion of hydrophobic species was positively correlated with the averaged contact angle (initial C.A, C.A at 1s and C.A at 5s) in the control plots (Pearson, T = 2.9, p<0.04) but not in the fertilized plots.

Piloderma increased in abundance over time in the control plots to become the dominating genus (up to 50 % of the relative abundance) after three years of incubation. The most dominant species in the control plots was Piloderma olivaceum which was reduced to 0% in the fertilized plots independent of incubation time (Fig 3b). Tylospora fibrillosa was also reduced in response to fertilization (Dunn test, χ^2 =13.4, p<0.0001), (Fig 3b), while T. asterophora showed an opposite trend (Dunn test, χ^2 =4.4, p<0.05). Amphinema sp 5. was the most abundant species in the fertilized plots and was enhanced by fertilization (Dunn test, χ^2 =3.8, p<0.05) (Fig 3b)

I have added extra information concerning this in the discussion:

Given the apparent association of EMF colonization with higher hydrophobicity over time, some EMF species may be expected to be more important than others for this process. We expected higher hydrophobicity in the control plots in response to a higher proportion of hydrophobic long distance exploration types species. ***Indeed, the proportion of hydrophobic EMF species in the control plots tended to be higher in comparison with the fertilized plots in the meshbags incubated for three years. From the hydrophobic species in the control plots, Piloderma* spp. *constituted the majority of fungal species with up to 50% of the total fungal reads****. The presence of Piloderma species like P.olivaceum, known to form hydrophobic mycelia, (Lilleskov et al., 2011, Agerer, 2001), and that was totally absent in the fertilized plots is likely to contribute significantly to hydrophobicity of SOM. **In the***

fertilized plots there was also an increase over time in the amount of hydrophobic EMF species (Amphinema being the most abundant hydrophobic genus) which was not accompanied by an increase in hydrophobicity. This may suggest that necromass from Amphinema do not accumulate to the same extent as for Piloderma and is probably not associated with the hydrophobicity in the meshbags. *These findings suggest that hydrophobicity of living mycelium might not necessary influence the water retention of the organic material to a large extent. This is consistent with the findings of Zheng et al. (2014) who found that the hydrophobicity of EMF mycelium does not necessary enhance soil water repellency.*

Comment:

48-50: I'm unclear about how by removing N and P from SOM, EMF activity may reduce soil C stocks. By inhibiting saprotrophic activity by outcompeting them for nutrients, wouldn't this enhance soil C stocks by reducing respiration by saprotrophic fungi (Gadgil effect)? Do you mean that EMF themselves are mineralizing C from SOM, decreasing soil C stocks?

Answer:

We agree that our wording here was not optimal. We have now reformulated the sentence to:"

In contrast to carbon accumulating activities by EMF, certain species may also reduce soil C stocks by oxidizing organic matter to release nitrogen and phosphorus. Some EMF species use 'brown-rot' Fenton chemistry and some use 'white-rot' peroxidases to do decompose SOM (Shah et al., 2016; Lindahl and Tunlid, 2015; Bödecker et al., 2014). This can result in 30% decrease in SOM according to Lindahl et al (2021).

Comment:

81-83: Here, you write that you would expect higher hydrophobicity in the control vs. fertilized plots due to the higher proportion of hydrophobic species – where is this tested? Genus-level assignments on mycelial hydrophobicity are available in the literature, as well as exploration type assignments that could offer further resolution on the effect of EMF mycelial traits on substrate hydrophobicity.

Answer:

See my previous answer from the comment for lines *45-47.*

Comment:

235: Table 1 would benefit from indicators of significance, either between treatments or over time. I found myself asking questions like “is the decline in the contact angle in the fertilized plots over time significant?” and struggling to locate the relevant information in the text. Alternatively, it could be visualized, which would also make it easier to interpret.

Answer:

Letters of significance were be added to the table:

Table 1:
Average and standard error of the ergosterol concentrations, total C%, C/N ratio, amount of new carbon (C3 mainly from EMF), % of EMF DNA reads, and contact angle determined 5 seconds after placement of water droplets placed on mesh bags material amended with maize compost (CA_{5S}; estimation of contact angle stability). **Low scores letters refer to statistical differences according to posthoc Tukey test and pairwise Dunn test. Asterisks correspond to statistic differences for the C.A after 5 (s) between the meshbag contents and the non-incubated reference material.**

258-260: Looks like you’re missing figure labels (a, b, c).

Answer:

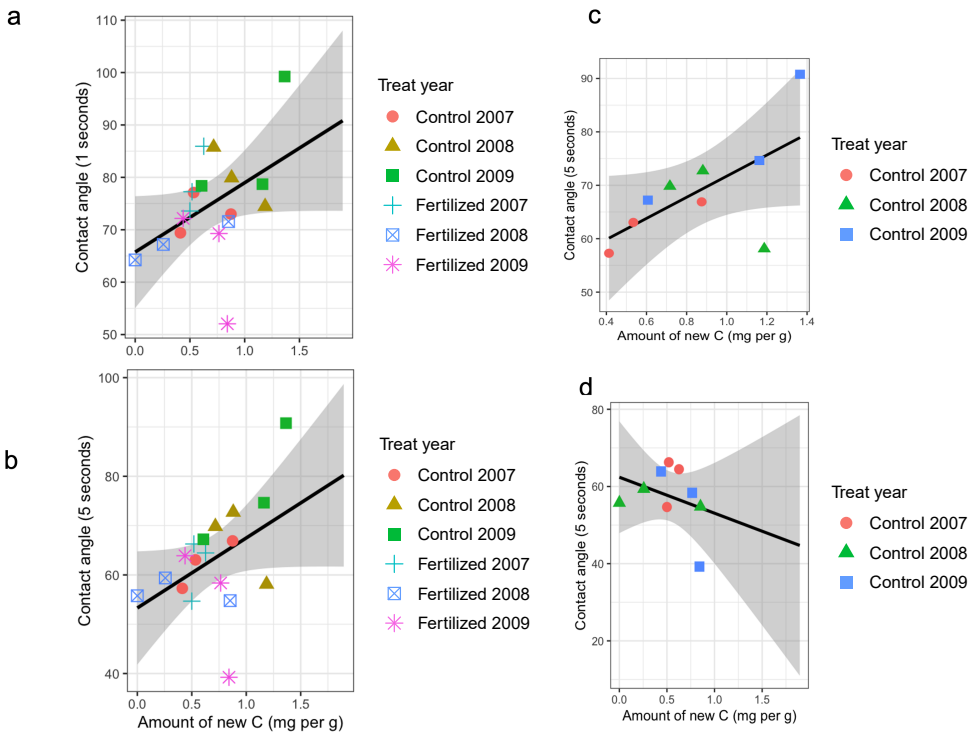
Fixed.

Comment:

272-273: Figure 2, would it be possible to make this a little cleaner (axis titles, the legend title)?

Answer:

Fixed. A better figure will be added. Note that new panels have been added after a comment of another reviewer:
When breaking down the data by fertilization regime, there was a positive correlation between amount of new C and the hydrophobicity for the CA5s in the control plots (Fig 2c) but the correlation tended to be negative in the fertilized plots (Fig 2d)



Comment:

Also, when the proportion of EMF reads is so low during the first two harvests, how can you attribute new C (C3 ingrowth into C4 substrate) to EMF alone? Table 1 indicates that roughly half of the "new" carbon enters the substrate by the end of the first incubation, although the proportion of EMF reads is only ~ 10%.

Answer:

That is a very good observation and indeed it looks peculiar that the amount of new C whose increase does not seem to match the increase in EMF sequences over time. The method used to measure the new carbon in the meshbag is not perfect it seems, and it comes with it´s flaws.

It seems to me that there is some extra carbon coming inside the meshbags that is not EMF. For example some soil solution might have come inside the bag during the first two years of incubation and contributed with some new C. Then the non-EMF new C might have built up so by the third year we do not see a clear difference between the 3 incubation periods.
So the method seems not to be perfect.

In that case it is hard to conclude that **all the new C** in the bags comes exclusively from EMF. However there is some indication that EMF might explain part of this new C since the number of EMF reads was significantly correlated with the new carbon in the meshbags (Pearson, $T = 2.4$, $p < 0.05$).

So, there is a trend for samples with high EMF sequences to have high new C. **I have added this result (not mentioned before) in the manuscript.**

An extra input of soil solution could be expected to affect both treatments, Fertilized and Control, and that could also explain why we do not see much difference between both treatments regarding new C.

Also, in Wallander 2011 (where part this data is published) a correlation between the new carbon and the ergosterol in the bags was found.

The positive correlation of EMF and the amount of new C have been added to the results and some extra information about other C than EMF coming inside the bags has been added to the discussion (as requested by referee number 2):

It should be also noted that we cannot rule out the possibility that soil solution entered the meshbags during the underground incubation. In soils, polymeric substances coming from SOM, root or microbial exudates can have hydrophobic properties (Vogelmann et al., 2013; Mataix et al., 2007). Hence, the hydrophobic changes in the material could be partly explained by other sources than EMF mycelium. However, the significant correlation between the new carbon in the bags and the EMF reads and the negative effect of fertilization on the C.A might suggest that hydrophobicity changes in the meshbag content are caused mainly by EMF.

Comment:

296: Figure 3, could you break panel b out to provide more information about how variable the EMF community is? Also, could you provide visual information about how the traits of the EMF community (hydrophobicity and/or exploration type) may be shifting according to sequencing results?

Answer:

Done.

Comment:

300: How do different kinds of EMF respond to the fertilization treatment over time? Hydrophobic vs. hydrophilic genera?

Answer:

See my previous answer from the comment for lines *45-47.*

Comment:

359-363: Here you argue that overall EMF abundance is linked with higher hydrophobicity. This is different from your original framing, where you implicate EMF producing hydrophobic mycelia.

Answer:

The main point of this sentence was to stress that an increase of **EMF over time** (while **non-EMF decreased**) was associated with the changes in the properties of the meshbag contents like C/N, new C and hydrophobicity.

However, since the increase in hydrophobicity and C/N ratios occurred in the control plots I have changed this sentence to be more precise:

*Thus, the EMF abundance was highest during the third year and this increase was associated **with higher C/N ratios and hydrophobicity in the control plots** and higher input of new C in the control and fertilized plots. This suggests a strong relation between EMF and the changes in the properties of the organic material in the meshbags.*

Later in the discussion (in the section: 4.2 Effect of incubation and fertilization on hydrophobicity) I go more in detail about why and how EMF colonization could have helped building up hydrophobicity).

Comment:

372-374: What is the mechanism of partner selection implied here? Reduced total C allocation to EMF fungi? How do you rule out environmental filtering and/or changes in EMF C sink strength? The Defrenne study cited here is correlative – how does it support your causal claim?

Answer:

We agree that our data does not support causal explanations here. We modified the text to:

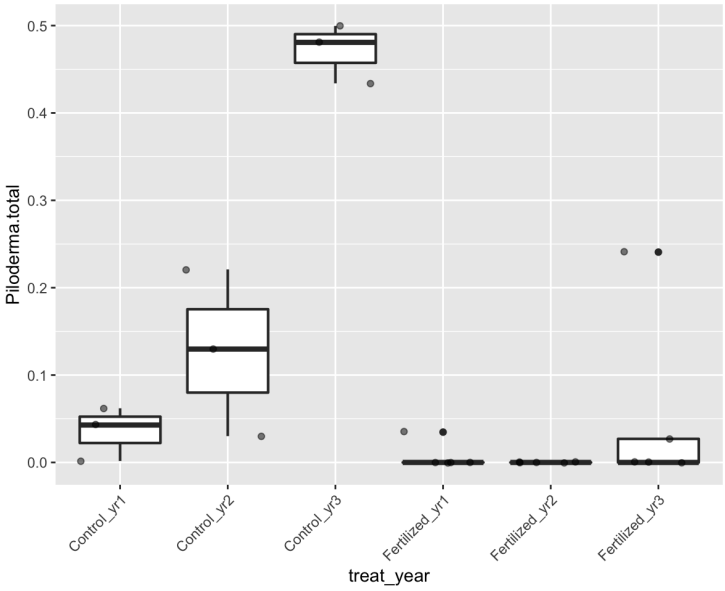
The decline of Piloderma in the fertilized plots may be a result abundant hydrophobic rhizomorphs that constitute a large C cost for the host (Defrenne et al., 2019), which is not economical for the symbiosis at high mineral N concentrations. Other more direct effects of the N fertilizer on the growth of Piloderma mycelium is however also possible.

Comment:

375-376: This is where I think more information about the homogeneity vs. patchiness of the EMF community would be helpful – are Piloderma spp. abundant across all samples?

Answer:

Yes. The genus *Piloderma* seems homogenously distributed in the Control plots replicates (in the fertilized plots this genus is extremely scarce) and the variability between samples in the control plots is not big (especially in the third year incubation) as you can see in this boxplot:



Comment:

415-419: Refer to comment from line 235. How can you attribute new C to EMF when their relative abundance after the first incubation was so low?

Answer:

See my previous comment on the matter.

Comment:

Also, the synthesis offered here (EMF necromass and biomass may contribute to SOM hydrophobicity) deviates from your original hypothesis, that EMF with hydrophobic mycelia in particular are contributing to SOM hydrophobicity.

Answer:

In this context I am discussing the correlation between the new carbon and the contact angle as a possible explanation for the building up of hydrophobicity in the meshbags.

However, since Hydrophobicity is enhanced over time only in the control plots, I added that specification in the discussion:

Therefore, these results suggest that the accumulation of biomass and necromass of EMF origin over time might contribute to the buildup of hydrophobicity in SOM ***in the control plots.***

Moreover, I added another specification in the results:

There was a positive correlation between the amount of new C and the hydrophobicity for both CA1s and CA5s (Pearson, T = 2 , p=0.06; T = 1.9, p=0.07 ; respectively). ***When breaking down the data by fertilization regime, there was a positive correlation between amount of new C and the hydrophobicity for the CA5s in the control plots (Fig 2c) but the correlation tended to be negative in the fertilized plots (Fig 2d)***

It is not surprising that the new C is correlated with the contact angle in the control plots. It is true that the new C might come from EMF in general but as it is mentioned later in the discussion (and earlier in my answers to the comments), the hydrophobic species in the controls contribute with the majority of fungal reads so they must be the ones contributing mostly to the new C and the changes in the water repellency of the material.

Later in this section I discuss the role of the individual species with hydrophobic mycelium in the control plots.

This is how the narrative and the flow of the discussion has been built. First, we see some indications of the effect of the new C on hydrophobicity and discus the role of EMF but at the end we argue for *Piloderma*. Is like a puzzle where the first pieces point out to an overall effect of EMF but ultimately is *Piloderma* that might explain the hydrophobicity changes in the material.

Comment:

437-439: This argument suggests that filamentous fungi contribute more than yeasts to SOM hydrophobicity – that is a much larger group than EMF fungi. How can you parse between the effects of filamentous fungi at large and EMF?

Answer:

Yes, filamentous fungi are not restricted to EMF only. However (as discussed in the first paragraph of the Discussion section 4.1) EMF became the most abundant fungal guild in the three-years-incubation bags with about 80% of the DNA reads in the Control plots. In the first years where non-EMF fungi were the most abundant fungal guild the new carbon, contact angles, and C/N ratios were the lowest which might suggest that the changes of the meshbag contents were more associated with EMF than with other filamentous fungi. That is why in this paragraph I argue that overall EMF abundance might be linked with higher hydrophobicity.

The reason we mentioned yeasts in this context was to attempt to explain the lack of an increase in **ergosterol** values over time while there **was** an increase in EMF reads and new carbon.

Comment:

445-449: This discussion would be strengthened by a more robust quantitative analysis of the relative abundance of hydrophobic/hydrophilic EMF across treatments. Where is your final hypothesis addressed?

Answer:

As mentioned in the comments above the regression between the averaged contact angle and the amount of combined hydrophobic species were performed and supported the hypothesis that hydrophobic EMF increased hydrophobicity in the control plots but not in the fertilized plots probably by the presence of the *Piloderma* genus which is absent from the fertilized plots.

Therefore, testing the correlation between CA and hydrophobic EMF with both treatments combined (control and fertilized) and then for each of them separately gives good support for the different hydrophobicity patterns between fertilized and control plots.

In addition, the multivariate analysis done as the PCA tested for all the different continuous variables measured in the organic material inside the meshbags and gave a good indication of how the **most abundant species** are associated with the different properties of the organic material.