Mark Anthony´s comments (Reviewer 1)

Thanks Mark for your positive comments! It is very nice to know that there are fellow young researchers interested in EMF. Organisms so interesting and so important for the biogeochemical cycles!

Even if minor, your comments improved the manuscript a lot.

Comment:

Line 56: change ‘to’ to ‘for’

Answer:

Done

Comment:

Line 57: I have been curious of this framing of these results because ericoid mycorrhizal fungi include many very strong decomposers (e.g. Burke and Cairney 2002, Mycorrhiza; Kohler et al. 2015, Nature Genetics)

Answer:

That is true.

This statement is based on findings from Clemmensen et al. (2015) from boreal forests. As you mentioned Ericoid mycorrhiza could have decomposing abilities also and it would be interesting to mention more about this fungal guild in this introduction. However, the purpose of this sentence was to explain that fungal community composition can affect carbon stocks. Our paper explores EMF mostly so going more in detail into the ericoid mycorrhiza would feel a bit out of our scope.

Comment:

Line 61: Though a great study, I would not say that Lindahl et al. (2021) could conclude causality in their work, and thus I would not say that C. acutes ‘resulted in...’. Rather, I would say ‘was linked to’.

Answer:

Agree and done

Comment:

Line 65-68: These results by Lilleskov et al. (2011) are very important, but some of the summaries at the genus level need to be reconsidered. For example, increasing evidence suggests that members of the Russula and Lactarius genera include species that respond both negatively and positively to N additions (e.g. Morrison et al. 2016, Fungal Ecology; Van der Linde et al. 2018, Nature; Moore et al. 2021, GCB; Anthony et al. 2021, ELEMENTA).

Answer:

Russula and Lactarius were omitted

Comment:

Line 94-95: Each fertilisation treatments includes a 50 kg N ha-1 yr-1 range, why is this? Maybe adding one additional clause to clarify. Because you ultimately consider fertilisation a single treatment, it is easily defendable but it would be good to stand lone in the paper versus needing to read Bergh et al. (2008) first. Can you also describe how long the N addition treatment was fertilized prior to installing the mesh bags?

Answer:

The aim of the additions was to optimize growth without inducing N leaching, as explained in Bergh et al (2008). The following sentence was added to the text:

In the fertilization treatments specific amounts of N (ammonium and nitrate) were applied to optimize plant growth without inducing leaching. The amount of N additions were based on needle N determinations and monitoring of N in soil water (Bergh et al 2008). Thus, the fertilization was applied by hand as 50-100 kg N ha^{-1} *every year for the first fertilization regime and as 100-150 kg N* ha^{-1} *every second year in the second fertilization regime* ***(fertilization begun in 2002****).*

Comment:

Line 95-96: Can you provide more information on the fertilisation of other macro- and micronutrients? Because micronutrient loss is also hypothesised to influence how fungi respond to N additions (e.g. Whalen et al. 2018, GCB).

Answer: The following text was added:

To avoid nutrient imbalance caused by fertilization, the amount of micronutrients was adjusted to optimum nutrient proportions for Picea abies (as calculated by Ingestad 1978).

Comment:

Line 116: It would be interesting to know why in November versus a time when tree growth and belowground C allocation is presumably higher.

Answer:

Different seasonal peaks for EMF biomass have been reported. Most commonly, EMF growth peaks in early Autumn probably due to more C allocated by the trees after the resource has been given to the leaves in Spring and Summer (This coincides with the production of fruiting bodies). However, it has also been reported that during warmer months EMF growth can peak. By collecting the samples in November, we can be sure that most of the productive season was captured. The one-year bags were incubated for 8 months so the bags were buried from Spring until late autumn. Therefore, independent of the seasonal peak the bags were belowground when more growth can be expected.

Comment:

Line 154: Please add one sentence about how sequences were denoised, given this is 454 data it is especially important bioinformatic detail.

Answer:

The following sentence was added:

The trim seqs operation was run with the following exclusion parameters: all sequences that mismatched the sample ID barcode at more than one position, mismatched the primers at more than 2 positions, had homopolymers longer than 10 bp, were shorter than 150 bp, or had an average base call quality score below 20 over a moveable window of 40 bases.

Line 169-172: Was this part of the work done manually?

Answer:

Yes

Lien 174: I realise the bioinformatics was done many years ago, but because the submission is current, I encourage updating language around the Zygomycota to be consistent with current taxonomic consensus (see Spatafora et al. 2016, Mycologia).

Answer:

Fixed

Comment:

Line 175: Does 'unknown ectomycorrhizal status' also refer to non-ectomycorrhizal taxa or just taxa thought to be ecto but not confirmed?

Answer:

It refers to taxa that are found in genera of ambiguous ectomycorrhizal status, either because their mycorrhizal status is unknown, or because a few members of the genus have been shown to be ectomycorrhizal under certain circumstances but the genus is generally not considered obligately ectomycorrhizal. This includes many ericoid and saprotrophic as well as ectomycorrhizal fungi. In our study this group was primarily populated by OTU's within the Heliotales.

Comment:

Line 180-182: Maybe just say 'relative abundance'?

Answer:

Done

Comment:

Line 215: Can you also provide technical details on the C/N measurements and the ergosterol analysis?

Answer:

The C/N ratios were calculated just by dividing total C by total N. Those were extracted using an elemental analyzer connected to an Isoprime isotope-ratio mass spectrometer (Isoprime, Manchester, UK).

Extra information of the basics of ergosterol extraction is added:

To estimate ectomycorrhizal growth, the fungal cell membrane compound ergosterol was measured as a biomarker of fungal biomass. Ergosterol was extracted from 5 g of the pooled sand-maize mixture from the meshbag. Briefly the sample was subjected to saponification using a solution of 10 % KOH in methanol and the non-polar phase (where the ergosterol is present) was separated using cyclohexane. The ergosterol was quantified by high-performance liquid chromatograph (Hitachi model L2130), a UV detector (Hitachi model L2400). For more detailed regarding the protocol see Wallander et al. (2011)

Comment:

Line 221: Was this a PCA of all these variables together or was some type of vector fitting used to fit the non-fungal values (e.g. envfit function in vegan)? I am also a bit concerned of using PCA on relative abundance data given how many zeros are probably in the data and co-linearity among some of the taxa. Is there a reason why PCoA was not used with Bray-Curtis distance or a distance-based redundancy analysis also using Bray-Curtis dissimilarity? Both of these would be more suitable non-parametric alternatives. I am also guessing from Figure 4 that this is a PCA of both fungal relative abundances and organic matter properties together. Thus, was some type of transformation used to put everything on the same scale? Additionally, can you define what was the criteria for being the most abundant fungal OTU. Was there a cutoff based on sequence proportion and/or occurrence across the sampling units? I am also guessing this is at the OTU level?

Answer:

No vector fitting was used in this PCA. This graph is not a plot from Vegan and it is not based on distance matrixes obtained from the relative abundance of the fungal community data. In this PCA I plotted the measurements of the organic matter properties of the meshbag contents and the Relative abundance of the most abundant EMF species. Therefore, the data was transformed to be in the same scale. The function PCA in R scales the vectors as a default function:

For this graph the criteria was the more abundant EMF species relative to the total fungal reads. The most abundant genera in the control plots was *Piloderma* with up to 50% of the total fungal reads and *Piloderma olivaceum* alone contributed with about 30% of the reads by the third year. The same goes for the fertilized plots where the genus *Amphinema* contributed with up to 40% of the total fungal reads while *Amphinema* sp 5. contributed with up to 30% of the total fungal reads. The other species that do not belong to the genera *Piloderma, Amphinema* or *Tylopora* contributed with less than 5% of the total reads.

For the bar graph presented in figure 3 the species that contribute with at least 1% of the total fungal reds are presented individually. The others are grouped in the bar called “Other EMF”.

Comment:

Line 257: I think the alphabetic labels are missing on the figure (e.g., a, b, & c).

Answer:
Fixed.

Line 268: I would also be interesting to have the Pearson correlation coefficient here.

Answer:

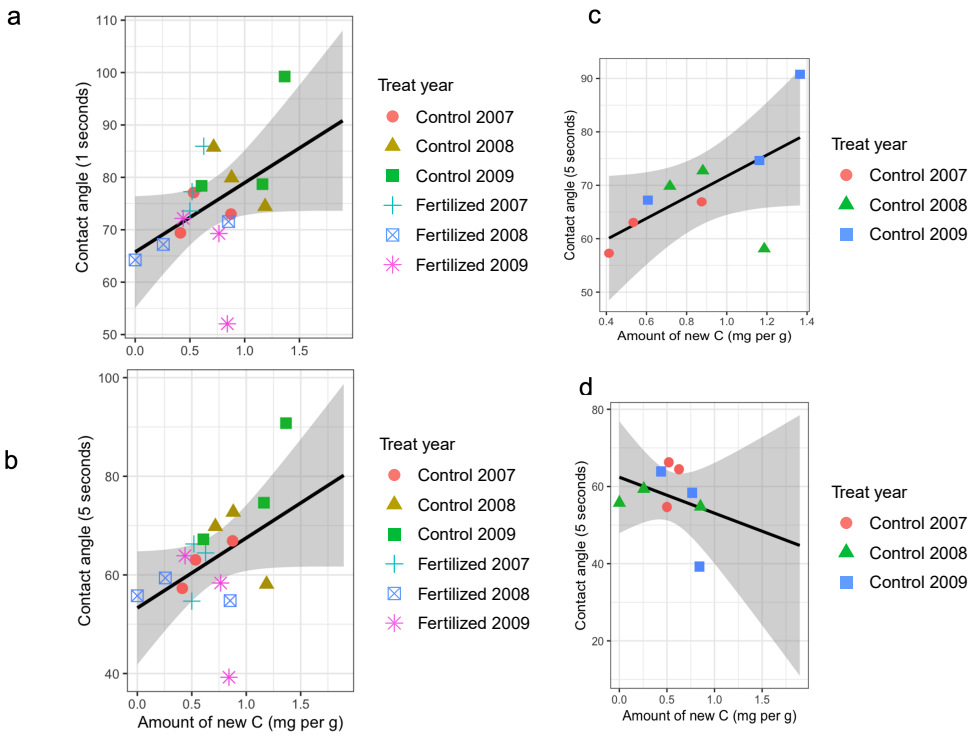
Done.

Comment:

Line 271: I think Figure 2 could be cleaned up a bit so the legend and axis labels look nicer and do not contain underscores.

Answer:

Fair enough! The figure has improved: 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:

Line 285: Why is it total fungal EMF and saprotrophic fungal communities? Is this because all other trophic groups and non-assigned to trophic group fungi were removed? Did you also look at EMF alone and saprotrophs alone?

Answer:

The way it is written is indeed confusing. We did not exclude any data in this analysis. We used all the fungal guilds present in the communities. I change that part to:

The total fungal communities were significantly influenced…

Comment:

Line 301-304: These details seem like they should be the first part of section 3.4, where the molecular results are first introduced.
Line 305-310: Already stated verbatim in section 3.3?

Answer:

Changed! I remove one of the repeated sections. Thank you for noticing this repetition of the results. It´s peculiar… neither of the co-authors nor the other reviewers noticed it!!

Comment:

Line 311-316: Already stated at lines 290-294.

Answer:

Changed! Thank you for noticing this repetition of the results

Comment:

Line 321. Missing period after '(Fig 3b)'

Answer:

Fixed.

Comment:

Line 331-333: What linear model was used? Please add these details into the methods section.

Answer:

The method ***lm: Fitting Linear Models*** in the package stats in R uses the QR decomposition:

Sharma, A., Paliwal, K. K., Imoto, S., & Miyano, S. (2013). Principal component analysis using QR decomposition. International Journal of Machine Learning and Cybernetics, 4(6), 679-683.

Comment:

Line 333-334: I do not believe you mean to say the 'proportion of EMF to total Basidiomycota', as it sounds like you calculated a ratio of the two, but rather, I think you mean to to say, 'the proportion of EMF and the proportion of EMF increased over-time…'. I would also write Basidiomycota and Ascomycota versus 'mycetes'

Answer:

Fixed.

Comment:

Section 3.5: You only need to site Fig. 4 one time.

Answer:

Fixed.

Comment:

Line 358: The idea of fungal succession is important, but it need not be independent from changes in environmental conditions across time. I personally do not feel this qualification is necessary here. It derails the momentum of your story so early on! If you feel it is essential to already provide a caveat in this first paragraph of the discussion, I encourage you make one that does not derail the traction of the main conclusion of this work. You could say something more powerful like: 'Whether shifts in EMF were due to selection of later succession fungal taxa as the forest aged versus variation in climatic conditions remains unclear, but is ultimately not particularly important in terms of understanding how shifts in EMF relate to soil organic matter cycling'.

Answer:

I added the sentence suggested.

Comment:

Line 394: I believe this result is robust around T. asterphora responding positively to N additions, but I am willing to hedge it is geographically unique. Van der Linde et al. (2018. Nature) suggest an N depo optimum around 9 kg N ha yr-1 for this taxon, which is quite low for many parts of Central Europe where the work was conducted. Since your work was more northern and in a boreal forest, I bet the results are quite different from more southern, temperate forests. This is just a comment for consideration. I do not think there is need to comment on this in the text.

Answer:

Interesting observation. I agree that it might reflect a geographical pattern. This study was done in Sweden where N deposition levels are not as high as central and southern Europe. In a forest more south than the present study, Almeida et al. (2019) found that *T. asterophora* tended to respond to N fertilization but it was less marked than in the present study. Probably, this species reaches an optimum but it can only tolerate so much N so it decreases as N deposition keeps increasing further south.

Comment:

Line 401: Could you explain why the N tolerance component of this sentence helps to explain this result in little more detail?

Answer:

It doesn't help to explain.

Previously we presented some references saying that Amphinena is N tolerant so in this particular sentence where we compared T. fibrillosa with Amphinena probably we wanted to remark it that the later is N tolerant. I have remove that word.

Comment:

Line 404: You found the hydrophobicity did not change until the third year in the control mesh bags, which is also when proportions of EMF dramatically increased to make this group dominant. This supports the idea that the increase in hydrophobicity was due to EMF accumulation. It could be worth stating this also in this paragraph.

Answer:

Agreed! We added text:

As expected, hydrophobicity increased over time in respect to the reference material (non-incubated maize-sand mixture), and this increase occurred only in the unfertilized controls ***at the last sampling when the fungal communities in the mesh bags were dominated by EMF.***

Comment:

Line 421-441: Such an interesting paragraph! The explanation around yeasts and their ergosterol content is particularly compelling.

Answer:

Thanks!! It is tricky with this method to measure fungal biomass since it is hard to discriminate between yeast and filamentous fungi. Luckily we had sequencing data to help us discuss that.

Line 442-473: Another very interesting paragraph! Good ideas to explain the role of Piloderma species in soil C cycling.

Answer:

Thanks! Lately I think I have found *Piloderma* when opening some new meshbags (amended with Fe-oxide; they seem to love it for some reason). There were thick yellow rhizomorphs that clumped together and were very hard to break apart and homogenize for the

extraction. In one of the papers where they extract the yellow pigment they actually discuss that it was hard to dissolve even in organic solvents. Indeed, I noticed that those yellow little balls did not dissolve when extracting the ergosterol of this new samples I had. I think that that could be one of the reasons we do not see a change in ergosterol over time in the current paper. Probably *Piloderma* is hard to break apart to have a successful extraction of its ergosterol. Unfortunately, this in anecdotical evidence and I cannot discuss it in the manuscript ☹

Comment:

Figure 1. Is this the average contact angle at 1 and 5 s combined?

Answer:

No it is not, what it is meant by average is the number of samples used to measure the contact angle (n=3). The legend is very misleading I reckon now. I have changed it:

Contact angle (CA) comparisons between a) control treatment and reference material b) fertilization treatment and reference material and c) control and fertilized treatments. Bars represent standard deviation (n=3).

Comment:

Figure 2. It could help to provide a sentence describing what CA at 1 s and 5 s means; otherwise, the figure is a little tricky to interpret as a stand-alone component.

Answer:

Some clarification to figure legend was added:

Figure 2: Correlation between the amount of new C and the hydrophobicity of the meshbag contents measured as the contact angle (CA). The C.A was determined directly after placement of the water drop at 1 second (1s) a) and 5 seconds (5s) b, c, d).

Some clarification was also added in the figure legend 1:

Figure 1: Contact angle (CA) comparisons between a) control treatment and reference material b) fertilization treatment and reference material and c) control and fertilized treatments. Shown is the intial CA (ini), determined directly after placement of the water drop and CA determined 1 second (1s) and 5 seconds (5s) after placement of the water drop. Bars represent standard deviation (n=3).

Comment:

Figure 3b: It is hard to differentiate between Tomentella stuposa and Tylospora asterophora and then between Piloderma olivaceum and Amphinema byssoides and then between Suillus lutes and Amphinema sp 5. Could you select colors with more contrast?

Answer:

Done. The graph has changed. Now it has different color codes and also information about the exploration type and hydrophobicity as suggested by another reviewer.

Comment:

Figure 4: Are the vectors the coefficients of the linear combos of the initial variables used to make the PCA? I would also write OTU instead of species.

Answer:

They are the initial variables.