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Peer Review File

Strengthened resource limitation driven via accelerated microbial growth dampens response to elevated CO2 in a mature forest

Corresponding Author: Dr Mingyue Yuan

This file contains all editorial decision letters in order by version, followed by all author rebuttals in order by version.

Version 0:

Decision Letter:

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Dear Dr Yuan,

Your manuscript titled "Strengthened resource limitation driven via accelerated microbial growth dampens response to elevated CO2 in a mature forest" has now been seen by 2 reviewers, whose comments are appended below. You will see that they find your work of some potential interest. However, they have raised substantial concerns that must be addressed. In light of these comments, extensive revisions will be required before we can further consider the manuscript for publication. We would, however, be interested in considering a revised version that fully addresses these serious concerns and the editorial thresholds as follows:

* Provide a compelling case that enhanced resource limitation which is driven by microbial growth reduces ecosystem responses to elevated CO2 in a mature forest, including full consideration of variables affecting the results of the microcosm experiment, for example, both litter quality and quantity under elevated CO2.
* Clearly justify the experiment design, as raised by both reviewers.
* Clearly explain the methods, particularly justifying the statistical metrics used in the study.

We hope you will find the reviewers' comments useful as you decide how to proceed. If additional work allows you to either incorporate or refute these criticisms, we will be happy to look at a substantially revised manuscript. If you choose to take up this option, please either highlight all changes in the manuscript text file, or provide a list of the changes to the manuscript with your responses to the reviewers.

When resubmitting, please provide a point-by-point response to the reviewers' comments. Please submit your responses as a separate file, distinct from your cover letter where you can add responses to the Editors' comments that you do not want to be made available to the reviewers. Word files are preferred. We recommend that any figures, tables or graphs that are included in the response to reviewers are also included in the main article or Supplementary Information.

Please bear in mind that we will be reluctant to approach the reviewers again in the absence of substantial revisions.

If the revision process takes significantly longer than three months, we will be happy to reconsider your paper at a later date, as long as nothing similar has been accepted for publication at Communications Earth & Environment or published elsewhere in the meantime.

We are committed to providing a fair and constructive peer-review process. Please do not hesitate to contact us if you wish to discuss the revision in more detail.

Please use the following link to submit your revised manuscript, point-by-point response to the reviewers' comments with a list of your changes to the manuscript text (which should be in a separate document to any cover letter), a tracked-changes version of the manuscript (as a PDF file) and any completed checklist:

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Please do not hesitate to contact us if you have any questions or would like to discuss the required revisions further. Thank you for the opportunity to review your work.

Best regards,

Mengjie Wang

Associate Editor, Communications Earth & Environment
Consulting Editor, Communications Sustainability
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REVIEWER COMMENTS:

Reviewer #1 (Remarks to the Author):

Yuan et al. present a well-written and stimulating study with novel insights. The experimental combination of a limiting-factor assay and a litter-addition microcosm is an excellent, complementary laboratory approach to test how eCO_2 affects microbial nutrient limitation. The results broadly support the hypothesis that eCO_2 increases productivity and microbial growth, thereby further strengthening resource limitation and dampening carbon sequestration. However, limited replication (n = 3) and some microbial indicators lacking repeated measures reduce statistical power (relatively large SEs) and may weaken the confidence in some specific conclusions. Despite statistical limitations from modest replication, this manuscript is a strong and thoughtful contribution and provides an exemplary laboratory analysis of microbial resource limitation under eCO_2. I offer a few points for authors to consider and address.
Major points
1.Consider litter quantity versus litter quality and realism of the lab simulation. In the field, eCO_2 can change both litter quantity and litter quality according to many previous studies. Please discuss whether and how changes in litter quality under eCO_2 could affect the link between field observations and the microcosm results (Lines 287–289: “to simulate an input of ca.10× the annual litterfall …” and “The C:N of the added Eucalyptus litter was ca. 50 and the C:P ratio was ca. 1700.”). Why was a 10× annual input chosen? The rationale notes a reference to 5 years of eCO_2, while the soils are from year 10—clarify and justify this choice or discuss sensitivity to different magnitudes/qualities of litter input.

2.Temporal resolution of microbial measurements and claims about community composition.
Are the microbial abundance and biomass data in Fig. 3 from a single field sampling? Microbial biomass and activity can vary substantially through time; be cautious when asserting “microbial community structure did not change” if field sampling was single-timepoint. Line 295–296 (“measured over different timepoints during the 6-week incubation”) is ambiguous—please clarify sampling schedule and which datasets are single versus repeated measures. Note that PLFA in Fig. 4 is clearly at day 7 and day 42; enzyme assays also have timepoints—this temporal structure should be clearly summarised, and the significant Lab × Day interactions discussed.

3.Use of PLFA physiological/stress indicators.
Classic PLFA metrics (e.g., GP:GN, Cy:Pre, Sat:Mono ratios) have been used as stress/physiological indicators. Consider whether these or other PLFA-derived ratios could help interpret physiological or nutritional states in your soils and whether they are applicable here.

4.Ecoenzymatic stoichiometry and testing microbial C vs P limitation.
Do the authors align with the ecoenzymatic stoichiometry framework (Sinsabaugh 2012)? Would enzyme-activity stoichiometric ratios help to strengthen evidence that microbes under eCO_2 shift from C- to P-limited states (or vice versa)? Consider presenting or discussing enzyme stoichiometry to corroborate the interpretation.

5.Statistical choices, one-tailed tests and use of 85% CI.
The manuscript uses one-tailed tests and sometimes reports 85% confidence intervals. Is the choice of lower confidence thresholds driven by limited statistical power, or by inability to compute effect sizes? The authors should explicitly justify these choices and (where feasible) emphasise the most robust, high-confidence results while down-weighting conclusions that rest on weaker evidence.
Minor / editorial comments
Line 166: check figure citation — is 'Fig. 3c' correct, or should this cite an Extended Figure (e.g., Extended Fig. 2)? Search for similar mis-citations.

Line 276: avoid beginning a sentence with an acronym/abbreviation.

Typos/spacing: Line 341 add a space between "800" and "µl"; Line 351 add a space before "SOM"; please scan the manuscript for similar formatting errors.

Line 342: the text reads "200ul fluorometric" — the unit/number here seems inconsistent. Please check whether this should read "200 µL of fluorometric substrate" or another correct volume (your methods should be unambiguous).

Line 393: consider changing "CO2 effects" to "The eCO_2 effects" (or otherwise clarify grammar).

The abbreviation "Lab" used in figures is not defined in the text (I infer "laboratory addition"). Please explain all abbreviations when first used.

Reviewer #2 (Remarks to the Author):

This manuscript presents a timely and well-executed investigation into the soil microbial mechanisms governing ecosystem responses to elevated CO2 in a phosphorus-limited mature forest. The study leverages the unique, long-term EucFACE facility to address a critical question in global change ecology: why does increased C availability under rising CO2 often fail to result in greater ecosystem C sequestration? The authors provide compelling evidence that a shift in microbial physiology towards a more copiotrophic life history strategy exacerbates microbial co-limitation by C and P, intensifying plant-microbe competition and short-circuiting the C cycle. The experimental design is robust, employing complementary approaches (limiting factor assay and microcosm experiment) to test their hypotheses. The findings are novel and have significant implications for predicting terrestrial C feedbacks. However, the manuscript requires revision to clarify certain methodological details and to better integrate the findings with the proposed mechanistic framework.

1. The pilot experiment for the LFA is crucial but briefly described. Please provide more detail in the main text.
2. For the microcosm experiment, the litter addition rate is stated as "23 mg ground litter per gram fresh soil" to simulate "10x the annual litterfall." Please justify this seemingly very high rate.
3. The use of one-tailed t-tests and 85% confidence intervals to declare significance is unconventional. While the rationale (limited replication, environmental variability) is understood, it requires stronger justification.

Specific comments:
Line 74: The statement "these lines of evidence imply that soil microorganisms in the ecosystem were P-limited" seems too strong given that the supporting evidence is indirect. The cited studies indicate P sequestration in the organic pool and limited plant response, but do not provide direct evidence of microbial P limitation.

line 125-127: "Together, these results suggest that the additional plant-derived C under eCO2 assimilated by microorganisms was primarily used to support fast growing copiotrophs (Box 1),with limited impact on decomposer functioning and biomass accumulation.", The authors suggest that the additional plant-derived C under eCO2 was primarily used to support fast-growing copiotrophs, with limited impact on decomposer functioning and biomass accumulation. While this interpretation is consistent with the reported results, it is largely inferential. The authors should clarify that this is a hypothesis or provide supporting evidence from microbial community composition or functional data to strengthen the claim.
Line 136 : "However, in our study, eCO2 did not significantly affect microbial community structure (Fig. 3)." However, in Box 1, the authors emphasize differences in microbial life-history strategies. I suggest clarifying how these life-history differences relate to both community structure and microbial growth under eCO2. Moreover, the authors rely on PLFA analysis to characterize soil microbial taxa, which has limited taxonomic resolution. Therefore, it is not appropriate to conclude that overall community structure did not change; PLFA may only reflect changes at the level of broad microbial groups rather than finer-scale community composition.
Line 252: "The limiting resource for microbial growth was determined using a limiting factor assay (LFA)", The author used the LFA to identify the limiting factor for soil microbes; however, this result may primarily reflect the short-term, rapid response of microbes to environmental changes, rather than long-term resource limitation in the ecosystem. I suggest the authors clarify this limitation and discuss how the LFA results relate to microbial responses to nutrient additions over

different incubation periods.

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Version 1:

Decision Letter:

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Dear Dr Yuan,

Your manuscript titled "Strengthened resource limitation driven via accelerated microbial growth dampens response to elevated CO2 in a mature forest" has now been seen by our reviewers, whose comments appear below. In light of their advice we are delighted to say that we are happy, in principle, to publish a suitably revised version in Communications Earth & Environment.

We therefore invite you to revise your paper one last time to address the remaining concerns of our reviewers. At the same time we ask that you edit your manuscript to comply with our format requirements and to maximise the accessibility and therefore the impact of your work.

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We hope to hear from you within two weeks; please let us know if you need more time.

Best regards,

Mengjie Wang

Associate Editor, Communications Earth & Environment
Consulting Editor, Communications Sustainability
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REVIEWERS' COMMENTS:

Reviewer #1 (Remarks to the Author):

I appreciate the authors' careful consideration of my feedback and have well integrated the comments. Their point-by-point responses and the implemented revisions have effectively resolved my concerns. I am satisfied with the current version of the manuscript and recommend it for acceptance.

Reviewer #2 (Remarks to the Author):

I thank the authors for their detailed and thoughtful responses to my comments. All my concerns have been adequately addressed.The revisions and clarifications provided have substantially strengthened the manuscript. I congratulate them for their achievements.

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Version 2:

Decision Letter:

Dear Dr Yuan,

We are delighted to accept your manuscript titled "Strengthened resource limitation driven via accelerated microbial growth dampens response to elevated CO2 in a mature forest" for publication in Communications Earth & Environment. Thank you for choosing to publish your interesting work with us.

We would like to encourage you to write a "Behind the paper" blog post for our Earth & Environment Community website. You will receive an invitation letter.

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Providing great service is very important to us. We would greatly appreciate any comments you have about your experience at Communications Earth & Environment. We look forward to publishing your paper, and we hope to work with you again in the future.

Best regards,

Mengjie Wang

Associate Editor, Communications Earth & Environment
Consulting Editor, Communications Sustainability
Bluesky: @commsearth.nature.com; @commssustain.nature.com

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Reviewer #1 (Remarks to the Author):

Yuan et al. present a well-written and stimulating study with novel insights. The experimental combination of a limiting-factor assay and a litter-addition microcosm is an excellent, complementary laboratory approach to test how eCO_2 affects microbial nutrient limitation. The results broadly support the hypothesis that eCO_2 increases productivity and microbial growth, thereby further strengthening resource limitation and dampening carbon sequestration. However, limited replication (n = 3) and some microbial indicators lacking repeated measures reduce statistical power (relatively large SEs) and may weaken the confidence in some specific conclusions. Despite statistical limitations from modest replication, this manuscript is a strong and thoughtful contribution and provides an exemplary laboratory analysis of microbial resource limitation under eCO_2. I offer a few points for authors to consider and address.

We appreciate the reviewer's recognition of our work, and the constructive suggestions for how it could be further improved. The ambitious infrastructure used to create eCO_2 in plots of a size relevant for a forest ecosystem is prohibitively expensive, allowing only for a limited design of the field experiment from which the soils were sampled. This is typical for the few existing CO_2 enrichment ecosystem scale experiments, which all require substantial infrastructure and running expenses. To enhance the power of our inferences, we therefore employed a two-tiered approach to infer the limiting resources for microbial decomposers, combining both a short-term assay (termed "LFA"), with the microbial responses in a relatively longer-term microcosm experiment using leaf litter from the study site. We believe this dual approach enhances the robustness and credibility of our findings, as we are able to combine these two lines of evidence to make inferences about responses of microbial resource limitations under eCO_2. As also raised by the reviewer, due to logistical complexities, a common limitation of large-scale FACE experiments is the inherent constraints of statistical power (King *et al.*, 2004). To maximize the robustness of our inferences, we followed a sampling regime which took three soil cores from four replicate subplots within each treatment ring (i.e. 12 cores per treatment ring) to capture inherent variation associated with the field experiment. The 12 cores from within each treatment ring were subsequently pooled prior to laboratory incubations. This information has been added in l.259-262 in the manuscript.

In each plot, soil was sampled from 4 subplots (i.e., twelve 5 cm-diameter cores per treatment ring) from 0-10 cm depth and then homogenised, maintaining the original plot replication (2 treatments x 3 replicates). The 12 cores from within each treatment ring were subsequently pooled and sieved to < 2mm, transported and stored at 4 °C in the dark until further analysis.

We have addressed all other specific comments point by point, below:

Major points

1.Consider litter quantity versus litter quality and realism of the lab simulation. In the field, eCO_2 can change both litter quantity and litter quality according to many previous studies. Please

discuss whether and how changes in litter quality under eCO_2 could affect the link between field observations and the microcosm results (Lines 287–289: "to simulate an input of ca.10× the annual litterfall …" and "The C:N of the added Eucalyptus litter was ca. 50 and the C:P ratio was ca. 1700."). Why was a 10× annual input chosen? The rationale notes a reference to 5 years of eCO_2, while the soils are from year 10—clarify and justify this choice or discuss sensitivity to different magnitudes/qualities of litter input.

The reviewer raises valid points, and we agree that it is reasonable to expect that both litter quantity and quality could change under eCO_2. For example, a meta-analysis of different studies showed that eCO_2 generally reduced litter N content (Norby *et al.*, 2001). However, this has not happened at out study site, where neither the quantity of litter (i.e., litterfall) nor litter quality (nutrient composition) changed. In fact, to date, the litterfall (Jiang *et al.*, 2020), litter N and P content (Nielsen *et al.*, 2024), and litter C/N and C/P ratios have all been unaffected by eCO_2 in our study system. Consistent with this, the resorption and re-translocation of N and P has not changed (Crous *et al.*, 2019). In a recent assessment from 2022, soil P availability was also not found to change in response to eCO_2 (nor did it change in previous years) (Pihlblad *et al.*, 2023), further indirectly indicating that there was no change in the quality of litter input. An overview of specific results are presented in the following table:

Table. Results for variables related to litter quantity and quality in EucFACE, expressed as mean (se, n=3).

	Ambient CO_2	Elevated CO_2	Reference
Annual total litterfall (g C m^{-2} yr^{-1}) (2012-2015)	277 (23)	303 (27)	(Jiang *et al.*, 2020)
Annual total litterfall (g C m^{-2} yr^{-1}) (2013-2022)	264 (20)	279 (25)	Unpublished data
N concentration of litter (%)	1.1 (0.06)	1.3 (0.23)	(Nielsen *et al.*, 2024)
P concentration of litter (%)	0.01 (0.01)	0.03 (0.01)	(Nielsen *et al.*, 2024)
Litter C/N ratio	49.6 (2.6)	49.0 (2.8)	(Crous *et al.*, 2019)
Litter C/P ratio	1740 (107)	1692 (107)	(Crous *et al.*, 2019)
N retranslocation (dimensionless)	0.29 (0.04)	0.28 (0.05)	(Crous *et al.*, 2019)
P retranslocation (dimensionless)	0.52 (0.03)	0.49 (0.04)	(Crous *et al.*, 2019)
Soil extractable P (PO_4^{3-}, mg/kg) (Collected in 2022)	0.96 (0.18)	0.92 (0.21)	unpublished data

Although there is no evidence that changes in litter quantity or quality have occurred in response to eCO_2 at our study site, we agree that this is relevant information to provide, as it also justifies the litter additions used in the microcosm experiment. This text was added in l. 322-324.

The same litter was used in all microcosms, as previous studies at the same site did not find changes in either the quantity of litterfall or its elemental composition (e.g., litter C/N ratio, litter C/P ratio) under eCO_2[11,70,71].

We also thank both reviewers for raising this point regarding the amount of litter added in our microcosm experiment. In fact, 320g C m^{-2} yr^{-1} corresponds to approximately 5× that of the mean annual total litterfall (sum of e.g., leaf, twig, bark) . For this addition, we considered a scenario reflecting an extreme annual total litterfall magnitude of approximately 1500 g C m^{-2} yr^{-1} within a subtropical Eucalyptus forest. Observational data from the study sites shows very high interannual variability in both leaf and total litterfall. For instance, the total litterfall varied from 214 g C m^{-2} yr^{-1} (in 2017) to 340 g C m^{-2} yr^{-1} (in 2022). The observed annual maxima was approximately 190-210 g C m^{-2} yr^{-1} for leaf litter, and 325-340 g C m^{-2} yr^{-1} for total litterfall. Eucalyptus forests in Australia are structurally and physiologically adapted to episodic disturbance, including drought, extreme wind events, and fire. The systems exhibit nonlinear canopy responses, such that rare compound disturbances can produce litterfall magnitudes far exceeding those observed during typical interannual variability. We now have added this information, and our motivation of for the litter addition used, in the MS (l.311-321).

For the laboratory litter addition, 23 mg ground litter was added per gram fresh soil, corresponding to an input of ca. 5x the annual total litterfall (ca. 320 g C m^{-2} yr^{-1}, measured 2013-2021; unpublished data, Catriona Macdonald). This large litter addition was used to simulate conditions under an extreme litterfall scenario (see supplementary methods for details). Eucalyptus forests in Australia are structurally and physiologically adapted to episodic disturbance, including drought, extreme wind events, and fire. These systems exhibit nonlinear canopy responses, such that rare compound disturbances can produce litterfall magnitudes far exceeding those observed during typical interannual variability. A total litterfall of 1500 g C m^{-2} yr^{-1} therefore represents a low-frequency, high-impact disturbance pulse consistent with the evolutionary disturbance regime of the ecosystem.

2.Temporal resolution of microbial measurements and claims about community composition.

Are the microbial abundance and biomass data in Fig. 3 from a single field sampling? Microbial biomass and activity can vary substantially through time; be cautious when asserting “microbial community structure did not change” if field sampling was single-timepoint. Line 295–296 (“measured over different timepoints during the 6-week incubation”) is ambiguous—please clarify sampling schedule and which datasets are single versus repeated measures. Note that PLFA in Fig. 4 is clearly at day 7 and day 42; enzyme assays also have timepoints—this temporal structure should be clearly summarised, and the significant Lab × Day interactions discussed.

Thanks for these suggestions. We have added “from field sampling” in the legend of figure 3, in order to clarify this.

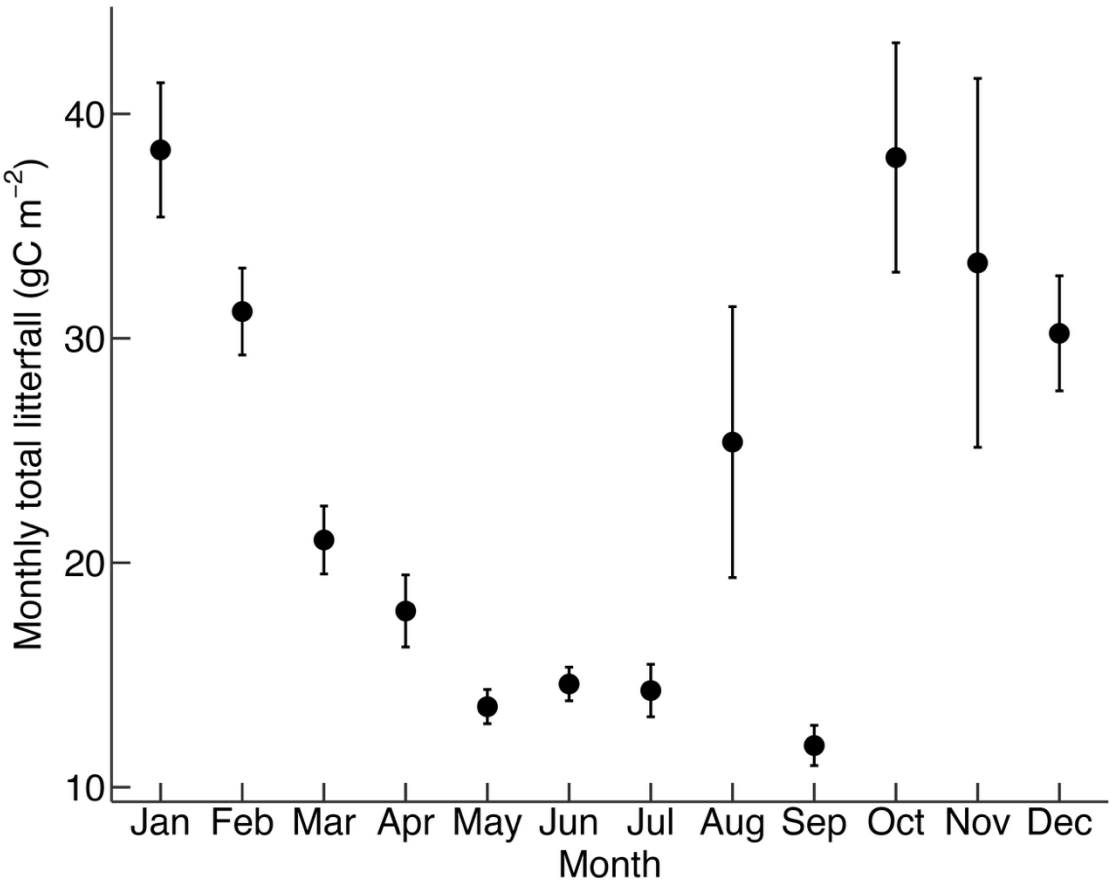

Figure. Average monthly total litterfall at EucFACE facility, collected in litter baskets in the field between Oct 2012 to December 2015. Values shown as mean ± se (n=6). Data source: Jiang, M., Medlyn, B.E., Drake, J.E. et al. The fate of carbon in a mature forest under carbon dioxide enrichment. Nature 580, 227–231 (2020). https://doi.org/10.1038/s41586-020-2128-9.

We agree with the reviewer's point that microbial biomass and community composition may vary through time/season. However, previous studies have found that changes are more dependent on substrate availability/nutrient conditions rather than environmental variability (Leff *et al.*, 2015; Castañeda-Gómez *et al.*, 2020). Our sampling took place in November, just after a large litterfall event in the field (refer to the figure above), therefore it was a time which had a high potential to capture differences in microbial biomass and/or community structure, had they occurred. However, we did not find any difference. Our finding also was consistent with earlier findings from the same site, where the composition of the microbial community did not change in response to eCO_2, or across different seasons (Castañeda-Gómez *et al.*, 2020). We have added this information in the MS (l.141-147).

This suggested that in the studied mature forest, microbial communities became more active without undergoing compositional changes. This finding was consistent with earlier findings from the same site after two years of eCO_2, which showed the microbial community composition

(also assessed by both PLFA and DNA) was not affected by eCO_2 or seaonality[42]. These results were also consistent with the general observations from other FACE experiments, where greater plant-derived inputs to soil under eCO_2 enhance microbial activities, but did not substantially alter the composition of the soil decomposer community[43].

We also thank the reviewer for their suggestion regarding the timepoints and the Lab × Day interaction. The specific timepoints sampled for the microcosm experiment have now been added in l.331-336.

After the additions, the microcosms were incubated at 18 ± 1 °C for six weeks, corresponding to the average annual soil temperature in the field65. Soil microbial growth, respiration and extracellular enzyme activities were measured at several timepoints during the 6-week incubation (see below). Microbial growth and respiration were measured on Days 1, 2, 4, 7, 14, 21 and 42, while enzyme activities were measured on Days 1,4, 7, 14 and 42.

Regarding the Lab × Day interaction, our statistical analysis showed that the effect of time depended on the litter and nutrient additions. Specifically, the response of microbial biomass to litter alone declined toward the end of the incubation (42 days after addition), whereas the responses to litter with N and litter with N and P additions continued to increase. The microbial biomass response to litter and P addition remained unchanged over time. This pattern was consistent with microbial growth dynamics: growth in response to litter alone declined over time, while growth in response to litter with N and litter with P additions remained relatively constant, allowing microbial biomass in these treatments to accumulate. Furthermore, although the growth response to litter with P addition also declined over time, microbial biomass did not change, suggesting that when C and P limitations were alleviated, biomass remained relatively stable. This is also consistent with the findings on microbial carbon use efficiency (CUE): although microbial CUE declined in response to resource additions containing litter, the decline in response to litter with P addition was weaker compared to other treatments, as well as under elevated CO_2. We now have incorporated this in l.177-187 of the MS:

Microbial carbon use efficiency decreased in response to resource additions, in both in the limiting factor assay and litter addition experiment (Extended data Fig.2j-l), indicating a reduced potential for soil C sequestration via soil microorganisms when new C-rich resources are supplied[52]. This could be because labile C stimulates microbial respiration, while growth remains limited by other nutrients[19,53]. Notably, the reduction of microbial CUE responses to litter and litter with P additions in soils under eCO_2 were weaker than in soils under ambient CO_2 (Extended data Fig.2j-l). Microbial biomass also increased in response to litter and litter with P additions. The increase in response to litter declined over time, while the increase in response to litter and P remained unchanged (Fig. 4). These results imply that soil microorganisms under eCO_2 have a higher capacity to use and assimilate C when the limiting resources, i.e., C and/or P are available, matching our inferences of exacerbated C and P limitation under eCO_2 from the LFA.

3.Use of PLFA physiological/stress indicators.

Classic PLFA metrics (e.g., GP:GN, Cy:Pre, Sat:Mono ratios) have been used as stress/physiological indicators. Consider whether these or other PLFA-derived ratios could help interpret physiological or nutritional states in your soils and whether they are applicable here.

This is a good suggestion. We had tested all the metrics suggested (e.g., GP:GN, Cy:Pre, Cis/Trans, Sat:Mono ratios) and found that the GP:GN ratio decreased under eCO_2, suggesting a lower stress status for the bacterial community. We also found that microbial C limitation was exacerbated under eCO_2, which was attributed to a shift in the microbial community toward one dominated by a more copiotrophic life-history strategy, as evidenced by a faster microbial growth rate. Furthermore, the increased microbial growth rate under eCO_2 was driven mainly by bacterial growth. All these lines of evidence suggested that the shift of the microbial community toward a more copiotrophic life-history strategy was predominately responsible for the exacerbated microbial limitation. We have updated figure 2 to show this, and added a discussion of these results in l.158-166 as below:

PLFA metrics such as a higher ratio of gram-positive to gram-negative bacteria (GP/GN) have been used as indicators of physiological stress or starvation for the soil microbial community[46]. Gram-positive bacteria are typically more abundant when the C source consists of more recalcitrant compounds, whereas GN bacteria dominate when more labile C is available[38,47]. In our study, we found that GP/GN tended to decrease under eCO_2 (Figure 2), suggesting a lower stress status for the bacterial community. In general, a low stress status is positively correlated to copiotrophs[38,48]. Therefore, the decrease in GP/GN could be additional evidence that the exacerbated C limitation under eCO_2 was driven by a shift of the microbial physiology towards one dominated by a more copiotrophic life history strategy.

4.Ecoenzymatic stoichiometry and testing microbial C vs P limitation.

Do the authors align with the ecoenzymatic stoichiometry framework (Sinsabaugh 2012)? Would enzyme-activity stoichiometric ratios help to strengthen evidence that microbes under eCO_2 shift from C- to P-limited states (or vice versa)? Consider presenting or discussing enzyme stoichiometry to corroborate the interpretation.

Thanks for the suggestion. Yes, indeed, ecoenzymatic stoichiometry has been a popular method to infer microbial resource limitation, offering a high throughput method. However, the use of enzyme ratios (or vector analyses) to infer growth limiting resources for resident microbes has recently been challenged. For instance, BG/(NAG+LAP) is often used to infer relative C or N limitation. However, it has been found that when chitin, peptidoglycan, or protein are more abundant compared to cellulose in the environment, microbes may also use NAG and LAP to gain C (Mori, 2020). In addition, while the production of AP is induced by shortage of phosphate, the production of BG, NAG and LAP are all induced by substrate supply (i.e., production increases when substrate load increases). The ability for which the potential activity

of NAG, LAP, BG and AP can provide a representative picture of the extreme diversity of enzymes microbes use to harvest resources also remains unknown, and, as such, is unlikely that the molar ratios of these selected enzymes can provide unambiguous insights about the general production of C-, N-, or P-acquisitioning enzymes, unless this can be demonstrated by experimental validation (Mori, 2025). The few published attempts to test the ability of enzyme ratios (or vector analysis) to identify the limiting factor for microbial growth have found that they clearly cannot (Rosinger *et al.*, 2019; Zheng *et al.*, 2022). Nevertheless, the use of enzymes to infer resource limitation is widespread, motivating the incorporation of these results also within the context of our study. We have therefore estimated ecoenzymatic stoichiometry as well as vector analysis. Matching recent experimental demonstrations that enzymes cannot be used to infer growth limiting resources for soil microbes, the enzyme ratio based assessment provide a misleading representation of microbial resource limitations also here. The vector analyses suggests a limitation by N, yet when we added N (in the microcosm experiment), the results show that N limitation was enhanced. Also, it seemed like the litter additions (with or without N and P) tended to shift microbial nutrient limitation towards relatively stronger microbial C limitation. These results were paradoxical in terms of the enzyme stoichiometry theory, but match well with recent research. As such, we agree with the reviewer that the disconnect with the enzyme-based assessment to alternative means to test growth limiting nutrients is well worth reporting, and this has been added to the MS(l.399-411):

Ecoenzymatic ratios have often been used to infer microbial resource limitation[40]. As such, ratios were calculated as BG/(NAG + LAP), BG/AP, and (NAG+LAP)/AP, respectively. Vector analysis was conducted by calculating BG/(NAG + LAP), against BG/AP as the distance and angle from the origin[84], where a vector > 45° indicated a relatively stronger P limitation, whereas a vector < 45° indicated relatively stronger N limitation. These results indicated that soil microorganisms under both ambient CO_2 and eCO_2 were limited more by N, and the microbial N limitation tended to be enhanced by the addition of litter combined with N and P (Extended data Fig. 6). The litter additions (with or without N and P) also tended to shift microbial nutrient limitation towards stronger microbial C limitation. These results were paradoxical, since N addition would be expected to alleviate N limitation and litter addition would be expected to alleviate C limitation. However, aligning with our results, some previous studies have demonstrated that enzymes cannot be used to infer growth limiting resources for soil microbes[85-88]. Therefore, ecoenzymatic stoichiometry was not used to identify microbial resource limitation in this study.

5.Statistical choices, one-tailed tests and use of 85% CI.

The manuscript uses one-tailed tests and sometimes reports 85% confidence intervals. Is the choice of lower confidence thresholds driven by limited statistical power, or by inability to compute effect sizes? The authors should explicitly justify these choices and (where feasible) emphasise the most robust, high-confidence results while down-weighting conclusions that rest on weaker evidence.

The reviewer raises an excellent point. We understand that 85% CI does not appear to match standard statistical tradition. In fact, this is a slight misunderstanding. Typical pair-wise comparisons are conducted at a set type-I error level (alpha level), that often is selected to be 5%. Albeit not intuitively obvious, such an alpha=0.05 level is equivalent to comparing two 85% CIs (Payton *et al.*, 2000), which was clarified in our method in l.455-460.

It has been suggested that reporting means alongside confidence intervals provides a more informative assessment of uncertainty and effect size than relying solely on p-values[11,17,90]. Typical pair-wise comparisons are conducted at a set type-I error level (alpha level), that often is selected to be 5%. Albeit not intuitively obvious, such an alpha=0.05 level is equivalent to comparing two 85% CIs.

Minor / editorial comments

Line 166: check figure citation — is 'Fig. 3c' correct, or should this cite an Extended Figure (e.g., Extended Fig. 2)? Search for similar mis-citations.

Thanks for drawing our attention to this, it has now been corrected in l.174.

Line 276: avoid beginning a sentence with an acronym/abbreviation.

Thanks for the suggestions, we have checked through the manuscript and made the correction in l.31, l.133 and l.177.

Typos/spacing: Line 341 add a space between "800" and "µl"; Line 351 add a space before "SOM"; please scan the manuscript for similar formatting errors.

We have checked through the manuscript and made the correction in l.388 and l.398.

Line 342: the text reads "200ul fluorometric" — the unit/number here seems inconsistent. Please check whether this should read "200 µL of fluorometric substrate" or another correct volume (your methods should be unambiguous).

We have corrected this in l. 389.

Line 393: consider changing "CO_2 effects" to "The eCO_2 effects" (or otherwise clarify grammar).

Implemented in l.453.

The abbreviation "Lab" used in figures is not defined in the text (I infer "laboratory addition"). Please explain all abbreviations when first used.

We have addressed this in l. 306.

Reviewer #2 (Remarks to the Author):

This manuscript presents a timely and well-executed investigation into the soil microbial mechanisms governing ecosystem responses to elevated CO_2 in a phosphorus-limited mature forest. The study leverages the unique, long-term EucFACE facility to address a critical question in global change ecology: why does increased C availability under rising CO_2 often fail to result in greater ecosystem C sequestration? The authors provide compelling evidence that a shift in microbial physiology towards a more copiotrophic life history strategy exacerbates microbial co-limitation by C and P, intensifying plant-microbe competition and short-circuiting the C cycle. The experimental design is robust, employing complementary approaches (limiting factor assay and microcosm experiment) to test their hypotheses. The findings are novel and have significant implications for predicting terrestrial C feedbacks. However, the manuscript requires revision to clarify certain methodological details and to better integrate the findings with the proposed mechanistic framework.

We thank the reviewer for their positive evaluation of our work and for the helpful suggestions to further improve it. We have addressed the reviewer's comments point-by-point, below:

1. The pilot experiment for the LFA is crucial but briefly described. Please provide more detail in the main text.

We thank the reviewer for the suggestion. Considering the limited space in the main text, we have added a note in the Introduction (l.81-83) referring readers to the Methods section for more detailed information about the pilot experiment:

Prior to this, a pilot experiment was conducted to determine the optimal concentration of resources addition and incubation time for the LFA (see Methods for details).

2. For the microcosm experiment, the litter addition rate is stated as "23 mg ground litter per gram fresh soil" to simulate "10x the annual litterfall." Please justify this seemingly very high rate.

The reviewer raises a good point, which we also addressed in response to Reviewer #1. To reiterate, we chose the amount of litter added based on an estimation of extreme litterfall events (please see full response, above). This is now explained in l.311-321 of the MS.

3. The use of one-tailed t-tests and 85% confidence intervals to declare significance is unconventional. While the rationale (limited replication, environmental variability) is understood, it requires stronger justification.

We agree with the reviewer that this required further explanation (please also refer to response to Reviewer #1 above). To reiterate, typical pair-wise comparisons are conducted at a set type-I error level (alpha level), that often is selected to be 5%. Albeit not intuitively obvious, such an alpha=0.05 level is equivalent to comparing two 85% CIs. This has been incorporated into the manuscript in l.455-460.

Specific comments:

Line 74: The statement “these lines of evidence imply that soil microorganisms in the ecosystem were P-limited” seems too strong given that the supporting evidence is indirect. The cited studies indicate P sequestration in the organic pool and limited plant response, but do not provide direct evidence of microbial P limitation.

The reviewer makes a good point. This was rephrased as “These lines of evidence suggested that soil microorganisms in the ecosystem may be P-limited. This might contribute to competition for P between plants and soil microorganisms, which in turn might substantially affect the microbial mediation of the ecosystem responses to eCO_2” in l.74-76.

line 125-127: “Together, these results suggest that the additional plant-derived C under eCO2 assimilated by microorganisms was primarily used to support fast growing copiotrophs (Box 1),with limited impact on decomposer functioning and biomass accumulation.”, The authors suggest that the additional plant-derived C under eCO_2 was primarily used to support fast-growing copiotrophs, with limited impact on decomposer functioning and biomass accumulation. While this interpretation is consistent with the reported results, it is largely inferential. The authors should clarify that this is a hypothesis or provide supporting evidence from microbial community composition or functional data to strengthen the claim.

We appreciate the reviewer’s perspective and agree that the claim needed to be strengthened. However, there is no clear definition of copiotrophs in studies using environmental samples so far, as growth at the level of individual species or taxa was not assessed in similar studies. Moreover, we did not observe changes in microbial PLFA composition under eCO_2. Therefore, we concluded that the shift toward a more copiotrophic state was more like a physiological change rather than a compositional change, which led to increased C limitation under eCO_2.

We re-examined the PLFA data (following Reviewer #1’s suggestion regarding classic PLFA metrics) and found that the ratio of gram-positive to gram-negative bacteria decreased under eCO_2. Given that gram-negative bacteria are generally considered more copiotrophic than gram-positive bacteria (due to their differences in stress tolerance and C quality preferences), we believe this provides complementary evidence for the community becoming more copiotrophic, even though overall microbial community composition did not change. We have incorporated this new finding into the manuscript in l.158-166.

Line 136: “However, in our study, eCO_2 did not significantly affect microbial community structure (Fig. 3).” However, in Box 1, the authors emphasize differences in microbial life-history strategies. I suggest clarifying how these life-history differences relate to both community structure and microbial growth under eCO_2. Moreover, the authors rely on PLFA analysis to characterize soil microbial taxa, which has limited taxonomic resolution. Therefore, it is not appropriate to conclude that overall community structure did not change; PLFA may only reflect changes at the level of broad microbial groups rather than finer-scale community composition.

We understand the reviewers' point regarding the resolution of microbial PLFA. A previous study from the EucFACE site used a hybrid approach, combining PLFA and DNA analyses to assess the soil fungal community (Castañeda-Gómez *et al.*, 2020). Both assessments found no effect of eCO2 on fungal community composition, suggesting that there was also no clear change in microbial composition at a finer taxonomic resolution. We have adjusted the text in l.142-145:

This finding was consistent with the findings from a previous study conducted at the same site, which showed the microbial community composition (assessed by both PLFA and DNA) was not affected by eCO_2 or seaonality[42].

Line 252: "The limiting resource for microbial growth was determined using a limiting factor assay (LFA)", The author used the LFA to identify the limiting factor for soil microbes; however, this result may primarily reflect the short-term, rapid response of microbes to environmental changes, rather than long-term resource limitation in the ecosystem. I suggest the authors clarify this limitation and discuss how the LFA results relate to microbial responses to nutrient additions over different incubation periods.

We thank the reviewer for this insightful comment and agree that clarifying the interpretation of LFA results is important. In fact, we intentionally use the instantaneous growth response in LFA, because it can capture the limiting factor for microbial growth in the sampled soils, thus reflecting the *in situ* status. We have added this in l.276-279:

The limiting resource for microbial growth was determined using a limiting factor assay (LFA)[30,68], where a rapid increase in microbial growth is expected following the addition of the growth limiting resource. The short incubation time was used to capture the limiting factor for microbial growth in soils resembling the *in situ* resource availability.

Secondly, to minimize potential bias related to incubation time in resource addition experiments, we used a two-tiered experimental design, combining results from the short-term LFA with a longer-term microcosm experiment (using standard litter from the field as the C source). The results showed that both approaches yielded consistent results. We have added this in l.306-308:

To complement the LFA, the litter addition microcosm experiment ("Lab") was performed to test microbial resource limitation using a different type of C source with a relatively longer incubation.

Our main focus was to compare microbial resource limitation in soils under ambient CO_2 and eCO_2, and to test how this could affect plant–microbe interactions and, consequently, ecosystem productivity under eCO_2. Therefore, we sampled soils early in the growing season (Duursma et al., 2016), when plant productivity was rapidly increasing and litterfall input was high (also refer to the figure in the response to Reviewer 1). During this period, interactions involving resource exchange and competition between plants and microbes were likely most pronounced. We have now added this in l.255-258:

November is the early growing season at the study site, when plant productivity is rapidly increasing and the litterfall peaks (Supplementary figure)[11,67]. As such, resource exchange/competition between plants and microorganisms was likely most pronounced during this period, giving the highest potential to detect the changes in microbial resource limitation under eCO_2.

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