

Peer Review File

Manuscript Title: High CO₂ dampens then amplifies N-induced diversity loss over 24 years

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referees' comments:

Referee #2 (Remarks to the Author):

This ms reports the results of a long-running experiment that combines elevated CO₂, N and a gradient of plant diversity. It finds that elevated CO₂ initially decreases losses of diversity due to increased N, but then accelerates it.

The work is important in various ways:

It reports long term results from one of (the) longest running FACE+ experiments

It reports an important new result where elevated CO₂ exacerbates loss of diversity from N pollution

I see no technical issues with the MS but have some suggestions that I think would improve it

The proposed causality is that enrichment increases aboveground biomass production that increases competition for light. Initially eCO₂ reduces effect of N, then increases it. However, I think there is only one supplementary graph on biomass. The story would be much clearer with some additional graphs and analyses that address each step in the causal chain, including biomass.

It took me a while to work out the flow from left to right for the 3 columns in your key graphs – a supplementary simplified version that explains how one panel relates to the next could help? Changes in differences are quite hard to get your head around!

Minor points

- I have used abbreviations in this review to save time as I am writing it during travel to a conference but it is not nice to read. All writing guides find that abbreviations reduce readability and should be used minimally.

- Focus more on effect sizes and confidence intervals than P values

- Your design and analysis was clear to me (but I am familiar with the design from earlier papers) but a 'reproducible research document' walking the reader through the basic analyses and key results is increasingly encouraged (I am literally about to give a workshop encouraging authors to submit them using Quarto for my journal!). They are really very easy to produce now using various languages with Quarto (or the older, similar R markdown).

Referee #3 (Remarks to the Author):

Reich and colleagues present an analysis of a 24 years long experiment in which nitrogen and CO₂ were manipulated in a grassland ecosystem in Minnesota, focusing on trends in community metrics such as diversity and evenness. This is in some ways a follow up on the 10-year reporting on the experiment, which was published in places such as Science at the time. Perhaps the most interesting part of the dataset (aside from its very existence, given the challenges in maintaining any experiment for over two decades!) is the ways in which “initial” responses from the 10-year window now appear to be transient responses that have been reversed in some cases, something that only a multi-decadal study could have recovered. The conceit of the design here, which I agree with, is that nitrogen is often manipulated in plant ecology but CO₂ rarely so, and so the opportunity to observe the ways in which N and CO₂ enrichment interact in driving diversity trends over time is especially critical in this era of global change. At the 24- year window reported on here, it now appears that CO₂ enrichment amplifies the loss of diversity that N additions trigger, and given global change predictions this is an important message for anyone concerned with the future of the world’s grasslands.

As a reader, I think there are three main contributions this manuscript makes. First, this contribution is relatively unique in reporting on a FACE experiment in a grassland system over such an extended period of time. The issue of the interactive effects of CO₂ and N is a central issue in plant and ecosystem ecology in an era of global change, and so just the basic trends in community diversity and composition over time are likely to be of widespread interest, along with the conservation implications of the findings. Second, if the data are made available for readers to analyze in new ways in the future, this updated dataset has the potential to be an important piece of ecological infrastructure for exploring global change topics, especially given the lack of datasets that do what has been done here. Based on the links in the ms (which I was able to follow), the raw data are accessible, which bodes well in this respect. Third, the discussion of mechanisms underlying the diversity loss are interesting and plausible, but are perhaps not the strongest part of the manuscript relative to the first two points. I think they are appropriate given everything else the ms has to address, but readers interested in the mechanics of competition and coexistence in plant communities will likely be unsatisfied with this as a definitive statement as to WHY N and CO₂ enrichment lead to diversity loss, though the inferences around light competition certainly are plausible to me as an outsider to this system.

One area that could be improved in the mechanisms section is to deepen the level of discussion to the actual biology/ physiology of the species. Much of the discussion of mechanism focuses essentially on the terms in the phenomenological statistical models. For example, in lines 145-146 the emphasis is on “shifting interactive effects of CO₂ x N on biomass and thus on light” or lines 139-141 “eCO₂ regulation on the +N effects on light explained half the interannual variation in the eCO₂ regulation of the +N effects on both richness and evenness”. These kinds of lines center the discussion on model terms rather than the biology that the models are representing, and put the meat of the discussion on terms in a coarse correlational model rather than on the actual mechanisms that presumably underlie such complex and interactive effects. If it is possible to unpack these complex interactions more I think this part of the paper will be substantially more

interesting and accessible to a general audience- the conceptual framework (or predictions even) for this section, especially around the interactions between the main effects, are unlikely to be that easy for readers to bring to the table without more help from the authors, and the same is true for the more nuanced results here.

One final small commentary- while I applaud the acknowledgement of the undergraduate workers who played an important role in this long-term project (lines 556-7), the fact that they were unnamed felt somewhat callous and perhaps even a little intentionally exploitive in the era we are in today. Perhaps a supporting supplement could be put together that lists them all, as best you are able to reconstruct? I'm sure many would appreciate it, and I think it sends a better message to current undergraduates about the value of the work that they might be doing in ecology labs around the world. I also expect your funders would be happy to see such a long list of early career researchers the work has impacted over the decades.

Referee #4 (Remarks to the Author):

The manuscript submitted to Nature by Reich et al. 'N-induced diversity loss dampened then amplified by high CO₂ over 24 years', is a remarkable contribution to our emerging knowledge on long term effects on grassland ecosystems from anthropogenic factors.

A. Summary of key results. The experiment motivates that grasslands will lose diversity in the near future due to the two harmful factors: Nitrogen deposition and elevated atmospheric CO₂. Both factors have a fertilising effect for the vegetation and therefore reduce biodiversity of the grassland plants due to the light limitation, which increases as plants grow in response to both factors.

B. Originality and significance. The presented study is original and significant in its' long duration through 24 years. During the first many years of the experiment, the effects of N and CO₂ were not in the same direction, but in the later years the competition enhanced, giving a larger than just additive loss in the combined N and eCO₂ treatment. The ultimate consequence was that some species were driven to extinction. This emphasizes the very high value of long-term field experiments as the conclusion about the consequences for grasslands is altered as compared to a shorter term.

C. Data and methodology. The presentation of results and methodology is of high quality as well as the performed work. The field experiment had controlled sown diversity and density, and was set up with N fertilisation (4 gN m⁻² yr⁻¹) and elevated atmospheric CO₂ (ambient air + 180 µmol CO₂ mol⁻¹) and with all combinations of treatments. This is a highly valuable set up which has overcome the large technical challenges and costs of elevated CO₂ fumigation treatment.

D. Appropriate use of statistics and treatment of uncertainties is in my judgement overall well performed (using the program R): linear mixed effects model for treatment effects (also with co-variates) and correlations analysis to account for repeated sampling, for soil parameters and rainfall variation among years data was transformed for reaching normal distribution when appropriate.

E. The conclusions are robust

F. Suggested improvements. I have a suggestion to add a diagram showing the experimental set-up, which is explained in the methodology section, but I feel it would help the reader a lot to see this is a diagram, either as a 'geographical' map or photo of the site or as a scheme with fx. colours showing the treatments, their combinations and n.

It might be said in the article that when light is limiting, it is in fact the space which is the constraining factor, therefore a different density of the plants could resolve the light limitation, which in essence is what happens when species or individuals are eradicated. In the methodology section it can also be explained in a few more words that 'ring' for CO₂ is a physical structure that adds the gas to the experiment, it has a ring shaped structure for practical reasons.

In figure 1 caption, I miss a few explaining words to explain the 'dampening' and 'amplifying' as these are mentioned in b, c, e and f – what is having the dampening effect (eCO₂) needs to be said. Also in figure 1 c and f and figure 2 c I think the shadow should be the darker grey, as it is for the elevated CO₂ situation, which is sown with the darker colour in the subfigure beside (sub figure b and e), just to keep this consistent for more easy reading.

G. References are appropriate

H. The text is clearly written with no flaws or mistakes in language.

Signed by Louise C. Andresen alias Louise Rütting

Author Rebuttals to Initial Comments:

Referees' comments in **blue non-italics font**, our replies in *black italics*.

Referees' comments:

Referee #2 (Remarks to the Author):

This ms reports the results of a long-running experiment that combines elevated CO₂, N and a gradient of plant diversity. It finds that elevated CO₂ initially decreases losses of diversity due to increased N, but then accelerates it.

The work is important in various ways:

It reports long term results from one of (the) longest running FACE+ experiments. It reports an important new result where elevated CO₂ exacerbates loss of diversity from N pollution

We appreciate ref#2 seeing the importance of this work.

I see no technical issues with the MS but have some suggestions that I think would improve it

Thank you for these helpful ideas.

The proposed causality is that enrichment increases aboveground biomass production that increases competition for light. Initially eCO₂ reduces effect of N, then increases it. However, I think there is only one supplementary graph on biomass. The story would be much clearer with some additional graphs and analyses that address each step in the causal chain, including biomass.

We now discuss present and discuss biomass more prominently. We have added biomass in revised Figure 2. We now show how biomass responses to CO₂ and N treatments changed over time; and how light is related to biomass. Moreover, the best mixed model (according to BIC) across all years and treatments found that biomass was the most influential factor on light, and that neither CO₂ nor N had a further influence on light once biomass was accounted for. Collectively these results are strong evidence for the importance of biomass, and treatment influences on biomass, regulating light and hence diversity.

It took me a while to work out the flow from left to right for the 3 columns in your key graphs – a supplementary simplified version that explains how one panel relates to the next could help? Changes in differences are quite hard to get your head around!

We have added a new Extended Data Fig 1 explaining more directly to readers how each panel relates to the other.

Minor points

- I have used abbreviations in this review to save time as I am writing it during travel to a conference but it is not nice to read. All writing guides find that abbreviations reduce readability and should be used minimally.

We were uncertain whether the comment about abbreviations was aimed only at the referee's own review or also at our manuscript. Regardless, if the Editor agrees with the referee's sentiments about abbreviations, we will be fine with removing our abbreviations for our treatments; and always use elevated CO₂ instead of eCO₂ and enriched N instead of +N.

- Focus more on effect sizes and confidence intervals than P values

Thank you for this suggestion. We have modified text in the results section to include percent changes in richness, evenness and light due to +N in both early and later years of the experiment. As the main trends in the manuscript are primarily focused on describing 3-way interactions between N, CO₂ and year, we present unstandardized effect sizes, i.e., difference in mean effects of treatments, at different time points. Given the often non-linear temporal trends in response to N and/or CO₂ over time, we feel that reporting effect sizes of just main effects of N and/or CO₂ might miss important trends and similarly a single coefficient for a three way interaction term may not be useful by itself.

While standardized effect sizes such as Cohen's d are very useful, there are concerns about estimating such effect sizes in complex models (e.g., mixed models/ multi-level models, repeated measures data, models with significant higher order interaction terms) as mentioned in Nakagawa and Cuthill 2007 Biological Reviews (<https://doi.org/10.1111/j.1469-185X.2007.00027.x>). Specifically for mixed models, there is considerable debate about estimation of population SD and dfs which will also influence estimation of effect sizes and confidence intervals. It seems more straightforward to us to report the significance from the full mixed model and then simply report the magnitude of effects of different combinations of treatments at different times to illustrate those.

- Your design and analysis was clear to me (but I am familiar with the design from earlier papers) but a 'reproducible research document' walking the reader through the basic analyses and key results is increasingly encouraged (I am literally about to give a workshop encouraging authors to submit them using Quarto for my journal!). They are really very easy to produce now using various languages with Quarto (or the older, similar R markdown).

Thank you for this suggestion. We have prepared a 'reproducible research document' and are going to store it in the same EDI location as the data, where both will be publicly available and accessible.

Referee #3 (Remarks to the Author):

Reich and colleagues present an analysis of a 24 years long experiment in which nitrogen and CO₂ were manipulated in a grassland ecosystem in Minnesota, focusing on trends in community metrics such as diversity and evenness. This is in some ways a follow up on the 10-year reporting on the experiment, which was published in places such as Science at the time. Perhaps the most interesting part of the dataset (aside from its very existence, given the challenges in maintaining any experiment for over two decades!) is the ways in which “initial” responses from the 10-year window now appear to be transient responses that have been reversed in some cases, something that only a multi-decadal study could have recovered. The conceit of the design here, which I agree with, is that nitrogen is often manipulated in plant ecology but CO₂ rarely so, and so the opportunity to observe the ways in which N and CO₂ enrichment interact in driving diversity trends over time is especially critical in this era of global change. At the 24- year window reported on here, it now appears that CO₂ enrichment amplifies the loss of diversity that N additions trigger, and given global change predictions this is an important message for anyone concerned with the future of the world's grasslands.

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We appreciate the detailed outlining of the different contributions of this work. Upon prompting from this comment and those of referee #2 we have added biomass to Figure

2 and presented and explained more about the relationships between light and aboveground biomass, and their cascading impacts on diversity. We also added to our discussion of species abundances and the way that dominant species biomass increased under elevated CO₂ and enriched N, leading to the community biomass increases and associated light competition changes and consequent outcomes for diversity.

One area that could be improved in the mechanisms section is to deepen the level of discussion to the actual biology/ physiology of the species. Much of the discussion of mechanism focuses essentially on the terms in the phenomenological statistical models. For example, in lines 145-146 the emphasis is on “shifting interactive effects of CO₂ x N on biomass and thus on light” or lines 139-141 “eCO₂ regulation on the +N effects on light explained half the interannual variation in the eCO₂ regulation of the +N effects on both richness and evenness”. These kinds of lines center the discussion on model terms rather than the biology that the models are representing, and put the meat of the discussion on terms in a coarse correlational model rather than on the actual mechanisms that presumably underlie such complex and interactive effects. If it is possible to unpack these complex interactions more I think this part of the paper will be substantially more interesting and accessible to a general audience- the conceptual framework (or predictions even) for this section, especially around the interactions between the main effects, are unlikely to be that easy for readers to bring to the table without more help from the authors, and the same is true for the more nuanced results here.

These are ambitious suggestions, which we applaud. In the revised submission we now focus a bit more on how dominant species increased biomass under elevated CO₂ and enriched N treatments, contributing to the increased shade and outcome of light competition. In our original submission we had hoped that Figure 4 and ED Fig. 5 would help in this regard. Fig 4 links species characteristics in monocultures with their relative abundance in mixture and shows the importance of high abundance of species that tend to cast more shade. In the text we now also point out that this is true whether we average over the entire experiment or look by individual years. Figure ED 5 shows which species are most sensitive to competition in mixture (in terms of local extinctions) to treatment combinations over time. Additionally, by now emphasizing more about biomass and its links to light availability, we connect dominant species which produce more biomass and cast more shade with reductions in diversity. We now present data about and discuss the raised issues more; in lines 154-186 and 194-216 but space limitation makes it difficult to go much more deeply into how individual species and species interactions might be contributing to the observed changes over time.

One final small commentary- while I applaud the acknowledgement of the undergraduate workers who played an important role in this long-term project (lines 556-7), the fact that they were unnamed felt somewhat callous and perhaps even a little intentionally exploitive in the era we are in today. Perhaps a supporting supplement could be put together that lists them all, as best you are able to reconstruct? I'm sure

many would appreciate it, and I think it sends a better message to current undergraduates about the value of the work that they might be doing in ecology labs around the world. I also expect your funders would be happy to see such a long list of early career researchers the work has impacted over the decades.

We appreciate this suggestion and now name all of the undergraduates (other than ≈4% due to missing records in the second year of the study – we are still searching for those) in the reproducible research document which will be a permanent document that will be available online along with the data and code.

Referee #4 (Remarks to the Author):

The manuscript submitted to Nature by Reich et al. 'N-induced diversity loss dampened then amplified by high CO₂ over 24 years', is a remarkable contribution to our emerging knowledge on long term effects on grassland ecosystems from anthropogenic factors.

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D. Appropriate use of statistics and treatment of uncertainties is in my judgement overall well performed (using the program R): linear mixed effects model for treatment effects (also with co-variates) and correlations analysis to account for repeated sampling, for soil parameters and rainfall variation among years data was transformed for reaching normal distribution when appropriate.

E. The conclusions are robust

F. Suggested improvements. I have a suggestion to add a diagram showing the experimental set-up, which is explained in the methodology section, but I feel it would help the reader a lot to see this is a diagram, either as a 'geographical' map or photo of the site or as a scheme with fx. colours showing the treatments, their combinations and n.

Thank you for this suggestion. We have put such a diagram in the 'reproducible research document' mentioned above.

It might be said in the article that when light is limiting, it is in fact the space which is the constraining factor, therefore a different density of the plants could resolve the light limitation, which in essence is what happens when species or individuals are eradicated.

This might be a 'chicken or egg' question. If the referee is suggesting that crowding is the reason for shading, we agree, but feel that the resource shortfall is what more directly influences plant performance (especially as we found much less power to explain our results from other resources that are less available due to crowding, such as water and nitrogen).

In the methodology section it can also be explained in a few more words that 'ring' for CO₂ is a physical structure that adds the gas to the experiment, it has a ring shaped structure for practical reasons.

Great idea to explain this. We have added text to the Methods to do so.

In figure 1 caption, I miss a few explaining words to explain the 'dampening' and 'amplifying' as these are mentioned in b, c, e and f – what is having the dampening effect (eCO₂) needs to be said.

Thanks; we now explain this with an added sentence in the caption. We also explain the links among panels in this Figure in the new Extended Data Figure 1.

Also in figure 1 c and f and figure 2 c I think the shadow should be the darker grey, as it is for the elevated CO₂ situation, which is sown with the darker colour in the subfigure beside (sub figure b and e), just to keep this consistent for more easy reading.

Thank you for this suggestion. Done.

G. References are appropriate

H. The text is clearly written with no flaws or mistakes in language.

Signed by Louise C. Andresen alias Louise Rütting

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Reviewer Reports on the First Revision:

Referees' comments:

Referee #2 (Remarks to the Author):

Thank you for the revisions which strengthen the paper. I have no further action points (just a few responses to your review points fyi).

- Thank you for the extra detail on the mechanisms and causality.
- The comment on abbreviations was for the paper – but I suspect if you don't make changes the journal will not – for all their talk about general audiences etc. this basic point of writing is rarely enforced. Strange.
- Estimates and intervals. I take the point on estimating effects and intervals (and that statisticians are not practicing what they preach by still making it usually easier to get P values than estimates and intervals) but that applies at least as much to P values too (lme4 does not even give them by default!) which are fundamentally less desirable as well. Thanks for the citation on the topic.
- Thanks for the reproducible research document – the ideal is to deposit the code file (Qmd, Rmd etc.), data and rendered document. FYI, my journal is about to move to requesting the rendered document as a pdf of submission (for editors and reviewers) and all three documents on publication.

I have one comment on another review point (from another reviewer) – see below –

Re. whether it is light or space...experiments that increase light without reducing biomass (Hautier et al. 2009; Escalinien et al. 2022) would seem to suggest it is light not space?

Referee #3 (Remarks to the Author):

I appreciate the efforts that the authors have made to address my varied suggestions- I think the ms is much improved by the revisions. I don't have any further concerns at this time.

Referee #4 (Remarks to the Author):

The authors have obtained an important and clear paper, I recommend publication