

Letter of Responses

Dear Dr. Rammig,

Thanks for handling our manuscript “bg-2015-319”. All reviewers’ comments are constructive and useful for improving our manuscript. We appreciate the opportunity to re-submit the manuscript. It has been revised thoroughly based on the reviewers’ comments.

The original reviewers’ comments are in italic and colored blue, and our responses follow. All line numbers indicated in the responses are those in the marked-up revision.

Sincerely,
Junyi Liang

First of all, because both reviewers and one of the public comments concerned about data independency and/or organization, we re-organized our data and re-conducted the analysis. After the reorganization, conclusions have not changed. We present the re-organized data and results in the revised version. Here are details on the data organization and the comparison of results between the two versions.

➤ **Data organization**

In the revised version, we provide all the organized data and individual effect sizes and variances in an xlsx file (i.e., Database S1). In addition, we have revised the Methods section (lines 106 – 113). To keep data independency and including as much information in individual studies as possible, if multiple measurements were conducted across years/dates, they were averaged by

$$M = \sum_{i=1}^j \frac{M_i}{j}$$

with standard deviation

$$SD = \sqrt{\frac{\sum_{i=1}^j SD_i^2 (n_i - 1) n_i}{(\sum_{i=1}^j n_i - 1) \sum_{i=1}^j n_i}}$$

where j is the number of results, M_i , SD_i and n_i are the mean, SD and sample size of the i th sampling data, respectively. By following previous meta-analyses in ecology (e.g., de Graaff et al. 2006; Luo et al. 2006, van Groenigen et al. 2006, Hungate et al. 2009), if additional treatments applied (e.g., nitrogen addition), they were treated as independent studies because the additional treatments could change the system (see the followed example #3).

Here are three representative examples how data were organized.

1. Hungate et al. (2004): we derived BNF data from individual years via personal communication. Means and variances in both control and CO₂ enriched treatment were calculated by averaging across the six years using the above functions (“Average” in the table). Similarly, short-term (years 2 and 3) and long-term (years 4 – 7) means and variances were calculated, respectively.

Treatment Peroid (years)	Mean _C	Mean _E	SD _C	SD _E	N _C	N _E	Where Used (Figure)
2	0.2904	0.3537	0.0767	0.1120	8	8	
3	0.9119	0.9225	0.3676	0.9723	8	8	
4	0.4285	0.2971	0.4541	0.2944	8	8	
5	0.1862	0.0998	0.1327	0.1040	8	8	
6	0.3813	0.2110	0.2617	0.1707	8	8	
7	0.4229	0.2477	0.1939	0.2046	8	8	
Average	0.4369	0.3553	0.1081	0.1672	8	8	1, 2(DEF), 3
Short-term	0.6012	0.6381	0.1814	0.4728	8	8	2(ABC)
Long-term	0.3547	0.2139	0.1365	0.0975	8	8	2(ABC)

2. Zak et al. (2007, 2011): In Zak et al. (2007), litter N content (LNP) in 2004 were reported (forest floor in their Table 2). In Zak et al. (2011), LNP in 2006, 2007, 2008 were resported (in their Fig. 2b). Because all the treatment periods were longer than 3 years, no short-term means and variances were calculated. Similarly, in studies where all measurements were taken during ≤ 3 -year period, no long-term means and variances were calculated.

Treatment Peroid (years)	Mean _C	Mean _E	SD _C	SD _E	N _C	N _E	Where Used (Figure)
7	8.4100	9.0500	3.1004	3.9318	3	3	
10	1.7945	2.4847	0.2869	0.3028	3	3	
11	1.8313	2.3374	0.3028	0.3028	3	3	
12	2.0521	2.4479	0.3347	0.3188	3	3	
Average	3.5220	4.0800	0.6708	0.8459	3	3	1, 2(DEF), 3
Short-term	*	*	*	*	*	*	*
Long-term	3.5220	4.0800	0.6708	0.8459	3	3	2(ABC)

3. Arnone & Gordon (1999): In addition to CO₂ treatment, they had nodulation and nitrogen addition. In their Table 4, they provided nitrogen pools in different tissues. The following table shows the results of total plant nitrogen pool (TPNP). Because of the additional treatments, we treated them as independent studies.

Treatment Peroid (years)	Other treatments	Mean _C	Mean _E	SD _C	SD _E	N _C	N _E	Where Used (Figure)
0.13	No	18.5000	27.6000	12.0748	10.2191	5	3	1, 2, 3
0.13	Nodulation	50.9000	55.6000	4.0249	14.5344	5	5	1, 2, 3
0.13	Nodulation+ Nitrogen	75.9000	119.3000	17.8885	35.8535	5	3	1, 2, 3

➤ **Results comparison before and after data re-organization**

Data re-organization does not change our conclusions. All response directions have not been changed. The changes in statistical significance ($P < 0.05$) are shown in the following table (no change: NC; from significant to insignificant: S2I; from insignificant to significant: I2S).

	Fig. 1	Fig. 2				Fig. 3		
		Short-term	Long-term	Without N	With N	Forest	Grassland	Cropland
APNP	NC	NC	NC	NC	NC	NC	NC	NC
BPNP	NC	NC	NC	NC	NC	NC	NC	NC
TPNP	NC	NC	NC	NC	NC	NC	NC	NC
LNP	NC	NC	NC	NC	NC	NC		NC
SNP	NC	NC	NC	NC	NC	NC	NC	NC
Fixation	NC	NC	NC	NC	NC	NC	NC	NC
Mineralization	NC	NC	NC	NC	NC	NC	NC	NC
Nitrification	NC	NC	NC	NC	NC	NC	NC	NC
Denitrification	NC	NC	NC	NC	NC		NC	NC
N ₂ O emission	I2S	NC	NC	NC	NC	NC	NC	NC
Leaching	NC	NC	NC	NC	NC	NC	NC	
TIN	NC	NC	NC	NC	NC	S2I	NC	NC
NH ₄ ⁺	S2I	NC	S2I	NC	S2I	NC	NC	NC
NO ₃ ⁻	S2I	NC	NC	NC	I2S	NC	NC	NC
NH ₄ ⁺ /NO ₃ ⁻ (*)	NC	NC	NC	NC	NC	NC	NC	NC

* NH₄⁺/NO₃⁻ ratio was not calculated in the previous version. The calculation in the revised version shows that CO₂ enrichment significantly increases soil NH₄⁺/NO₃⁻ ratio, which is consistent with, and actually improve, our discussion in the previous version.

➤ **Other changes**

We have done additional analyses based on reviewers' comments. We have added a new figure (i.e., Fig. 2) in the revised version. Inspired by reviewer 3, Fig. 2A has been added to show the response of BNF measured by different methods to CO₂ enrichment. Fig. 2B shows the responses of nodule mass and number in legume species to CO₂ enrichment (Fig. S3 in the previous version). Figs. 2 and 3 in the previous version have been combined and shown as Fig. 3 in the revised version. Values in other figures have been changed according to the new analyses. In addition, we have deleted Table S1. Instead, Database S1, which includes all organized data and individual effect sizes and variances, is provided.

Responses to Reviewer #1

In this study Liang et al. reviewed the to date available data on CO₂-effects on ecosystem N cycling, and in particular the proposed Progressive Nitrogen Limitation (PNL). This study is a valuable contribution to the field and can possibly inform experimentalists where there are needs for further measurements and modellers on the directions of processes in response to elevated [CO₂]. I recommend some minor (or major, depending on general comment #2) changes to the manuscript.

Response: We appreciate the constructive and valuable comments from the reviewer 1. Please see detailed responses and revisions below that have greatly improved our manuscript.

General comments

1) One thing that strikes me, is that one of the key findings, the effect that elevated [CO₂] has on gaseous N emissions, is missing in both the abstract and the conclusions. Also the lack of comparison with for instance Zaehle et al. 2011, where the opposite effect was seen. Although not a significant response for the experiments without N fertilisation, there is still a trend towards higher emissions in the data. This I find as an important missing part of the manuscript that is not really covered/discussed, and also the implications for the ecosystems where N addition is most common, croplands. As elevated [CO₂] have been suggested to be positive for crop productivity, the implications from realising the potential benefits from the CO₂-fertilisation could be that we are contributing to climate change even more.

Response: We agree with the reviewer that the response of N₂O emission is an important aspect in N cycle itself and in regulating climate change as a greenhouse gas. We have now added gaseous N emission in abstract (i.e., lines 21 – 23: “*However, CO₂ enrichment significantly increased the N influx via biological N fixation and the loss via N₂O emission, but decreased the N efflux via leaching*”; lines 25 – 27: “*Overall, our analyses suggest that the extra N supply by the increased biological N fixation and decreased leaching may potentially alleviate PNL under elevated CO₂ conditions despite of the increases in plant N sequestration and N₂O emission*”) and conclusions (i.e., lines 371 – 373: “*In addition, CO₂ enrichment increased N₂O emission, especially with extra N addition. The increased N₂O emission can partially offset the mitigation of climate change by stimulated plant CO₂ assimilation*”).

Zaehle et al. (2011), via modeling, found that elevated CO₂ reduced radiative forcing of N₂O. However, our data-based synthesis and another meta-analysis (van Groenigen et al. 2011) show that elevated CO₂ enhances N₂O emission. In their model, less availability of N substrates for nitrification and denitrification due to enhanced plant N sequestration attributed to the reduced N₂O emission. Our synthesis shows that inorganic N does not decrease. Especially with additional N application, enhanced denitrification by CO₂ enrichment results in greater N₂O emission. We have discussed in the revised version (lines 328 – 335): “*Our results are consistent with a previous synthesis (van Groenigen et al. 2011). The increased N₂O emission can partially offset the mitigation of climate change by stimulated plant CO₂ assimilation as the warming potential by N₂O is as 296 time as that by CO₂. However, a recent modeling study by Zaehle et al. (2011) has generated an opposite result that CO₂ enrichment reduced radiative forcing of N₂O. In their model, less availability of N substrates for nitrification and denitrification due to enhanced plant N sequestration attributed to the reduced N₂O emission. Our synthesis shows that inorganic N does not decrease. Especially with additional N application, enhanced denitrification by CO₂ enrichment results in greater N₂O emission.*”

2) As mentioned in the open discussion, the fact that several of the studies are from the same group may compromise the results. If the data sets are not independent, they should be treated differently. Have you performed such a check? If not, then this would be required and possibly also redo the analysis where data that comes from the same experiment are treated as one. Either way, this has to be mentioned and possibly be discussed in the manuscript.

Response: As described at the beginning, we have re-organized our data and re-conducted the analysis. After the reorganization, conclusions have not changed. We present the re-organized data and results in the revised version. Please see details provided at the beginning.

3) In Table S1 it would have been useful to also include the number of observations for each of the processes or pools, preferably in the head. Also, adding the ecosystem type that the study represent. It would also been useful to include the numbers as percent change instead of just a binary x. The latter just a recommendation to make the study more attractive.

Response: We have incorporated the suggestions made by the reviewer. We now provide all the organized data and individual effect sizes, variances and the number of observations in Database S1.

Specific comments

The precision in your resulting percentage changes do not reflect the uncertainty in the data. I think the precision is too high.

Response: We appreciate that the reviewer looked into details on our manuscript. We followed standard protocol in doing meta-analysis using the software Metawin 2.1 (Rosenberg et al., 2000). We used mean and 95% bootstrapping confidence interval in Figs. 1 – 3. In the revised version, we have provided all the organized data and individual effect sizes and variances.

The sentence on line 17, page 16962 seems to be unfinished.

Response: We changed the sentence “*The N efflux via leaching reduced under elevated CO₂ condition*” to “*Results showed that the N efflux via leaching reduced under elevated CO₂ condition*” (lines 224 – 225).

In the sentence starting on line 21, page 16962, it seems to me that you are making a general statement, but discussing an observed influx and reduction in leaching. See point 4 below.

Response: We changed the sentence to “*The net effect of the responses of N processes to CO₂ enrichment resulted in more N retention in ecosystems, especially in plant tissues and litter*” (lines 231 – 232).

On line 11 on page 16963, it says Birth, I think you mean Birch.

Response: Corrected (line 268).

On page 1694, the sentence that starts on line 11, improve is a value laden word. As it is, the word improve is not connected to the increased turnover as I suspect you are referring to. Also in the same sentence, are you talking about the microbial flora or the micro-fauna? And in the following sentence you are making a very general statement that management practices on croplands may increase the N mineralisation. Where in your findings is the data to support that? The statement is true, but it is not related to elevated [CO₂]. Either remove the sentence or relate to the study.

Response: We have revised the sentences to “*Second, tillage can alter the soil conditions (e.g., increasing O₂ content), which can potentially favor N mineralization under enriched CO₂ (Wienhold and Halvorson, 1999; Bardgett and Wardle, 2010). These findings suggest that CO₂*

enrichment can stimulate the N transfer from organic to inorganic forms in managed croplands.”
(lines 304 – 308)

The paragraph on page 16965 starting on line 15, there a few questions regarding that paragraph.

1) would not any change in pH result in a shift in microbial community function?

Response: we have discussed the possible changes in microbial communities and their associated ecosystem functions (lines 349 – 355): *“The lowered pH could have a significant effect on soil microbial communities and their associated ecosystem functions. For example, the fungal/bacterial ratio increases with the decrease in pH (de Vries et al., 2006; Rousk et al., 2009). The increased fungal/bacterial ratio may result in lower N mineralization because of the higher C/N ratio of fungi and lower turnover rates of fungal-feeding fauna (de Vries et al., 2006; Rousk and Bååth, 2007). In other words, the increased fungal/bacterial ratio may slow down the N turnover from organic to inorganic forms.”*

2) I find the mix between can and could a bit confusing.

Response: We changed “can” to “could” in line 348.

3) And there is probably a missing “a” in the sentence on line 21.

Response: Corrected (line 349).

4) On line 22, I would recommend changing “the” to “a”, because this is a general statement and not linked to one observation. The same reasoning applies on line 23.

Response: The word “the” has been deleted. The sentence in the revised version is *“For example, fungal/bacterial ratio increases with the decrease in pH”* (lines 351 – 352).

On line 6 page 16966, I would change sequestration to something more general like cycle or balance, as these results do not point in a specific direction.

Response: We changed “sequestration” to “cycle” (line 362).

References Zaehle, S., Ciais, P., Friend, A. D., and Prieur, V.: Carbon benefits of anthropogenic reactive nitrogen offset by nitrous oxide emissions, Nat. Geosci., 4, 601–605, doi:10.1038/ngeo1207, 2011.

Response: Thanks again for the constructive comments and useful information.

Responses to Reviewer #3

The authors present an extensive meta-analysis on the effects of elevated atmospheric CO₂ levels on terrestrial N cycle processes. The aim is to investigate how the responses in various N cycle processes control the occurrence of progressive N limitation (PNL) under CO₂ perturbation. The major deductions from the analysis are that through increased biological N fixation (BNF)

and decreased ecosystem N losses PNL may be alleviated, and that the soil NH₄⁺ to NO₃⁻ balance may shift.

I believe that a general assessment of terrestrial N process dynamics, in equilibrium and under perturbation, has the potential to be of tremendous value to biogeochemists, in particular to global modellers who perennially have to deal with lack of experimental evidence for their process hypotheses on large spatio-temporal scales. The effect of nutrient limitation on carbon sequestration responses to global change is (and has been) a very relevant issue in today's research, and I think the manuscript comes at good time, and to a fitting journal. The suggested shift in soil NH₄/NO₃ seems like a novel hypothesis that could have some interesting follow-ups.

Unfortunately, I have several concerns with the presented manuscript detailed below. Based on these, I cannot recommend publication unless some of the fundamental issues are addressed.

Response: We appreciate the constructive, valuable, and detailed comments from the reviewer. Please see detailed responses and revisions below that have greatly improved our manuscript.

Major concern:

The assertion that BNF significantly increased under elevated CO₂ is a very strong statement, and it has been my understanding thus far that this is not clear at all. I am not convinced that this hypothesis is supported by the presented meta-analysis for two reasons: (1) 15 of 29 studies included in the analysis of BNF responses have experiment durations of under 1 year. Or, by the authors' definition of ≤ 3 years, all but 6 to 7 studies are short-term studies. Yet, the conclusions drawn from the analysis are used to speculate about the long-term (decade-scale!) controls on PNL. The Serraj & Sinclair (2003) experiment only lasted for a few days. The Hungate et al. (2004) paper shows why this is a problem, as their CO₂ effect on BNF diminished over the years. The effect of phenology and multi-year forest succession in natural ecosystems has long been part of the theory of controls on BNF (Vitousek & Howarth, 1991). I am assuming that Figure 2 is somewhat meant to address this issue, but I do not find it very helpful.

Response: The reviewer has raised a very important point regarding the investigation of biological nitrogen fixation (BNF). We have carefully considered the valuable suggestions proposed by the reviewer and studied those relevant papers again. Below we describe our understanding.

We agree with the reviewer that long-term BNF response is more important in controlling progressive nitrogen limitation (PNL). However, the diminished BNF over time as shown in Hungate et al. (2004), was site-specific and due to molybdenum (Mo) deficiency in N-fixing plants in sandy acidic soils as discussed in their paper. In other studies, however, the response of BNF to CO₂ enrichments varies. Therefore, meta-analysis was necessary to derive more general response of BNF to CO₂ enrichment from those diverse studies.

We have divided our database into short-term (≤ 3 years) and long-term (> 3 years) categories. BNF studies in the long-term category lasted from 4 to 7 years. We agree with the reviewer that the relatively small number of long-term BNF studies (12 data lines from 8 studies) may have some influences on the results. We would argue, however, that the influences should only limit to the response magnitude. The response direction (i.e., increased BNF), derived from random model of meta-analysis (mean \pm 95% confidence interval), is supported by evidence at gene level from long-term experiments. Tu et al. (2015) found the abundance of *nifH* gene amplicons, which is a widely used marker for analyzing biological nitrogen fixation, was significantly

enhanced by 12-year CO₂ enrichment in a grassland (BioCON). In addition, an unpublished work by He and coauthors (including myself), using GeoChip 3.0 technique, documents that the abundance of *nifH* were significantly enhanced by CO₂ enrichment in six long-term free air CO₂ enrichment (FACE) experiments across US (i.e., BioCON, Duke, ORNL, MaizeFACE, SoyFACE, PHACE). These results provide supportive evidence for our conclusion.

Because of limited qualified experiments, ecologists have tried to derive more general patterns through synthesizing the limited studies. These meta-analyses have been very useful to both experimental and modeling communities. The values of these meta-analyses at least include (1) improving scientific understanding, and (2) highlighting scientific gaps that should be addressed in future studies. For example, Rustad et al. (2001) synthesized effects of warming on soil respiration, net nitrogen mineralization and aboveground plant growth using 32 studies. They divided their database into categories based on experiment duration, biome, and vegetation type. The study numbers of these categories were relatively small. For example, the study numbers from tundra, low tundra, forest and grassland were 3, 14, 9 and 6, respectively. This study has been very valuable to the community (e.g., citation number from google scholar is 1313). One more recent example is that a paper published in *Ecology Letters* by McCary et al. (2016) used 5 to 15 studies in categories for their meta-analysis.

We have added a paragraph in the revised version to discuss the influences of relatively small number of studies to highlight potential limitations but also encourage long-term studies in the investigation of BNF (lines 243 – 258): “*Since biological N fixation provides at least 30% of nitrogen requirement across natural biomes (Asner et al., 2001; Galloway et al., 2004), our results suggest that the positive response of biological N fixation to CO₂ enrichment plays an important role in alleviating PNL. PNL was proposed to characterize long-term dynamics of carbon-nitrogen coupling in response to rising atmospheric CO₂ concentration. Thus, it is critical to understand long-term response of biological N fixation to elevated CO₂. In this paper, we synthesize 12 studies that lasted 4 – 7 years and binned them in a long-term category (> 3 years). On average of those long-term studies, CO₂ enrichment increased biological N fixation by 26.2%. The increased biological N fixation is supported by evidence at gene level from long-term experiments. For example, Tu et al. (2015) found the abundance of *nifH* gene amplicons, which is a widely used marker for analyzing biological N fixation, was significantly enhanced by 12-year CO₂ enrichment in a grassland (BioCON). However, our synthesis showed a relatively wide 95% confidence interval from 2.54% to 59.8%. The wide range can be partially attributed to the relatively small study numbers. In addition, most studies incorporated in the current synthesis were conducted in temperate regions. Thus, longer-term studies, as well as studies in other regions (e.g., boreal and tropical) are critically needed to reveal more general patterns in the future.*”

(2) The mixing of agricultural experiments and experiments carried out in natural environments is problematic because of the very different nutrient regimes in these systems, which makes general conclusions about N cycle processes suspect. This is well illustrated by the Lam et al. 2012 BFS reference, where elevated CO₂ is reported to have increased BNF by 109 kg N ha⁻¹ over a span of 4 months. Such BNF rates are at least one order of magnitude above those in natural ecosystems. Even if only the response ratio was assimilated into the analysis, this does not make for a sound assessment of the BNF response in the terrestrial biosphere as a whole, which the manuscript is ultimately looking to provide.

Response: We appreciate the insightful comments, which brings an interesting concept to scale up how much BNF CO₂ enrichment can stimulate globally. Although the suggestion by the reviewer is excellent, the meta-analysis technique used in the current study (and other similar syntheses), is not capable of quantifying the BNF response in the terrestrial biosphere as a whole. In order to do such, many weighting factors, such as biome area and BNF in each biome (as suggested by the reviewer), and more data points across the globe are needed. What meta-analysis is able to do is that synthesizing available data to derive more general conclusions in comparison with individual studies. Log-transformed response ratio has been mathematically proven an efficient way to synthesize data from different studies. It has been widely applied in ecological studies, in which a variety of variables have been synthesized. For example, carbon pool size can differ by several orders of magnitude across ecosystems. Luo et al. (2006) showed that the log-transformed response ratio of carbon pool size to CO₂ enrichment followed a normal distribution, allowing a general statistical assessment of the response of carbon pool sizes to CO₂ enrichment.

Even though the difference in magnitude is not a problem in the meta-analysis, different types of ecosystems may respond to CO₂ enrichment in different ways. Therefore, we have divided our database into three categories, forest, grassland, and cropland (Fig. 3G – I and related text).

Since the BNF responses are a major part of the authors' arguments, I see this part as a strong weakness of the manuscript. I think that, if we want to do our understanding of BNF justice, an analysis should only include long-term experiments. It may well be that as of right now, we do not have enough experiments on BNF responses to eCO₂ to perform a meta-analysis.

Response: We do agree with the reviewer that in order to best address BNF under elevated CO₂, long-term experiments are ideal. The issue is, as pointed out by the reviewer (and by us in the responses above), the limited number of qualified experiments. As we responded above, we have discussed the contributions and potential limitations, highlighting that long-term studies in different regions are critically needed. Please see detailed responses to the two concerns above.

I would also like to encourage the authors to consider that vast parts of the terrestrial biosphere are not covered by N fixing vegetation, while obviously such species were always the subjects of the meta-analysed experiments.

Response: We agree that the distribution of N fixing plants varies across ecosystems. However, previous studies have shown that BNF (including **both symbolic and non-symbolic**) is important in most, if not all, ecosystems. Cleveland et al. (1999) showed that BNF exists in all 23 vegetation types, from polar desert/alpine tundra to tropical deciduous forest. In addition, at least 30% of nitrogen requirement across natural biomes is met via BNF (Asner et al. 2001; Galloway et al. 2004). Even taking fertilization in agricultural systems into consideration, the number is still about 15% (Cleveland et al. 1999; Galloway et al. 2004). For the example mentioned above, *nifH* genes have been detected in all grassland (i.e., BioCON, PHASE), forest (i.e., Duke, ORNL,) and cropland (MaizeFACE, SoyFACE) systems.

We agree that the distribution of BNF across ecosystems is asymmetric. In the revised version, we have discussed that “*although a general trend of PNL alleviation has been found in this study, the alleviation potential may vary across different ecosystems due to asymmetric distribution of biological N fixation*” (lines 259 – 261).

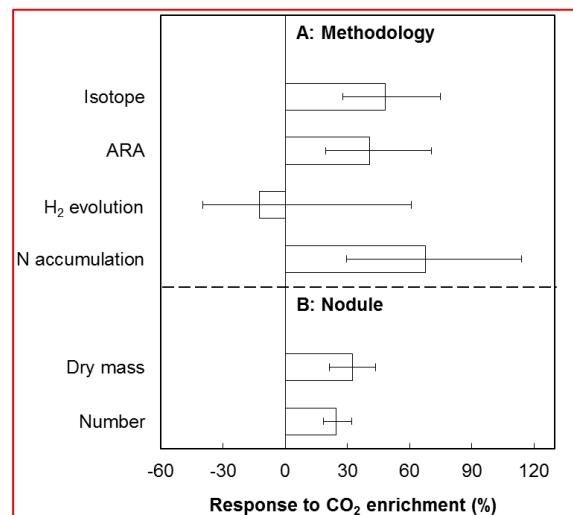
Further concerns:

- Although the methodology is described in good detail in the MS, it is not clear how the individual studies contributed to the overall results. To this end, I agree with previous comments that Table S1 should show the individual effect sizes, not just markers. For example, I am unsure how the Hungate et al. (2004) response was treated. A mean+variation would certainly not be appropriate in this case.

Response: As described at the beginning, in the revised version, we provide all the organized data and individual effect sizes and variances in Database S1. Please see detailed examples provided at the beginning.

- Many of the BNF experiments used the acetylene reduction method. This is very common to estimate BNF, however, as most studies point out, this method only determines Nitrogenase activity, which is not the same as BNF (Cleveland et al., 1999). This is a potential issue: Cabrerizo et al. (2001) report enhanced Nitrogenase activity, but no effect on BNF!

Response: We appreciate the constructive comments. We have done an additional analysis (Fig. 2A in the revised version) as suggested by the reviewer. In our database, four methods were used to estimate BNF, including isotope, acetylene reduction assay (ARA), H₂ evolution and N accumulation. The numbers for the four methods were 36, 39, 3 and 9, respectively. In the four methods, both ARA and H₂ evolution (the two methods were compared in Cabrerizo et al., 2001) measure nitrogenase activity (Hunt and Layzell, 1993), whereas isotope and N accumulation methods directly measure BNF. We found that all but H₂ evolution method showed significantly positive response to CO₂ enrichment. The insignificant response by H₂ evolution method was likely because of the small study numbers (i.e., 3). Specifically, both direct methods (i.e., isotope and N accumulation) showed positive effect of CO₂ enrichment. Therefore, methodology seems not an issue in the current synthesis. In the revised version, we have added a paragraph to discuss the methodology (lines 280 – 290): “Methodology may potentially influence the results. Cabrerizo et al. (2001) found that CO₂ enrichment increased nitrogenase activity measured by acetylene reduction assay (ARA), but not specific N fixation measured by H₂ evolution method. In studies synthesized here, four methods were used to estimate biological N fixation, including isotope, ARA, H₂ evolution and N accumulation. Among them, ARA and H₂ evolution measure nitrogenase activity (Hunt and Layzell, 1993) whereas isotope and N accumulation methods directly measure biological N fixation. All but H₂ evolution method showed significantly positive response to CO₂ enrichment (Fig. 2A). The insignificant response by H₂ evolution method was likely because of the small study numbers (i.e., 3). In addition,



biological N fixation by ARA, isotope and N accumulation showed similar response magnitude (Fig. 2A), suggesting consistency among the three methods. However, further assessment on H₂ evolution method is needed.”

- I appreciate the differentiation between forests, grasslands, and croplands. However, since we are talking about the N cycle and nutrient limitation, there is strong reason to consider a zonal separation as well (boreal, temperate, tropical), see Vitousek & Howarth (1991). This may be offset by the fact that most experiments were carried out under controlled conditions (Greenhouses, growth chambers,...), but then again, how much can we expect to learn from these experiments about the overall terrestrial biosphere?

Response: We agree that zonal separation is an important aspect to explore, especially for modelers. Unfortunately, however, almost all manipulative experiments, especially CO₂ experiments, during the past decades were conducted in temperate regions (most in North America and Western Europe). This limitation has been shown in other meta-analytical studies (e.g., Lu et al. 2013). In the revised version, we have discussed the limitation (lines 254 – 258): *“However, our synthesis showed a relatively wide 95% confidence interval from 2.54% to 59.8%. The wide range can be partially attributed to the relatively small study numbers. In addition, most studies incorporated in the current synthesis were conducted in temperate regions. Thus, longer-term studies, as well as studies in other regions (e.g., boreal and tropical) are critically needed to reveal more general patterns in the future.”*

Minor concerns:

- There are some inaccuracies in Table S1: I believe the Billings et al. (2003) experiment ran for 1 and 2 years, not 4.

Response: We appreciate that the reviewer looked into details on our manuscript. Billings et al. (2003) sampled soil in October 1999 and February 2001 from Nevada Desert FACE experiment, which started in April 1997. Thus, the soil samples had experienced field CO₂ enrichment for 2.5 and 3.8 years, respectively. In their Table 1, they presented the average data across the two samples. We treated the CO₂ treatment period as 3.8 years, which was consistent with our overall criteria (i.e., using the longest treatment time when averaging multiple data points).

The Hofmockel & Schlesinger entry should read "2007", not "2002". The "Tobia" entry should read "Tobita".

Response: Corrected in Database S1.

- I strongly recommend an English language check for the entire MS for readability.

Response: We have checked the language carefully in the revised version.

References not from the MS or supplement:

Vitousek, P. M., and R. W. Howarth (1991), Nitrogen limitation on land and in the sea: How can it occur?, Biogeochemistry, 13(2), 87-115.

Cleveland, C. C., et al. (1999), Global patterns of terrestrial biological nitrogen (N₂) fixation in natural ecosystems, Global Biogeochemical Cycles, 13(2), 623-645.

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14 **Abstract:** Nitrogen (N) cycle has the potential to regulate climate change through its influence
15 on carbon (C) sequestration. Although extensive researches have been done to explore whether
16 or not progressive N limitation (PNL) occurs under CO₂ enrichment, a comprehensive
17 assessment of the processes that regulate PNL is still lacking. Here, we quantitatively
18 synthesized the responses of all major processes and pools in terrestrial N cycle with meta-
19 analysis of CO₂ experimental data available in the literature. The results showed that CO₂
20 enrichment significantly increased N sequestration in plant and litter pools but not in soil pool.
21 Thus, the mechanisms that drive ~~basis of~~ PNL occurrence partially exists. However, CO₂
22 enrichment ~~also~~ significantly increased the N influx via biological N fixation and the loss via
23 N₂O emission, but decreased the N efflux via leaching. In addition, no general diminished CO₂
24 fertilization effect on plant growth ~~over time~~ was observed over time up to the longest
25 experiment of 13 years. Overall, our analyses suggest that the extra N supply by the increased
26 biological N fixation and decreased leaching may potentially alleviate PNL under elevated CO₂
27 conditions despite of the increases in plant N sequestration and N₂O emission. Moreover, our
28 synthesis showed that CO₂ enrichment increased soil ammonium (NH₄⁺) to nitrate (NO₃⁻) ratio.
29 The changed NH₄⁺/NO₃⁻ ratio and ~~the consequent~~ biological processes, may result in changes
30 in soil microenvironment, community structures and above-belowground interactions, which
31 could potentially affect the terrestrial biogeochemical cycles and the feedback to climate change.
32

33 **1 Introduction**

34 Fossil-fuel burning and deforestation have led to substantial increase in atmospheric carbon
35 dioxide (CO₂) concentrations, which could stimulate plant growth (IPCC, 2013). The stimulated
36 plant growth by CO₂ fertilization and the resulting terrestrial carbon (C) storage could partially
37 mitigate the further increase in CO₂ concentrations and associated climate warming (IPCC,
38 2013). However, the stimulated plant growth by CO₂ enrichment may be constrained by nitrogen
39 (N), an essential element for molecular compounds of amino acids, proteins, ribonucleic acids
40 (RNAs) and deoxyribonucleic acids (DNAs) in organisms (Rastetter et al., 1997; Oren et al.,
41 2001; Luo et al., 2004; Reich et al., 2006; Norby et al., 2010; Reich and Hobbie, 2013). A
42 popular hypothesis of the N constraint to the CO₂ fertilization effect is progressive N limitation
43 (PNL) (Luo et al., 2004).

44 Progressive N limitation postulates that the stimulation of plant growth by CO₂ enrichment
45 results in more N sequestered in plant, litter and soil organic matter (SOM) so that, the N
46 availability for plant growth progressively declines in soils over time (Luo et al., 2004). The
47 reduced N availability then in turn constrains the further CO₂ fertilization effect on plant growth
48 on long-term scales. However, whether and to what extent PNL occurs are dependent on the
49 balance of N demand and supply (Luo et al., 2004; Finzi et al., 2006; Walker et al., 2015). If the
50 N supply meets the N demand, PNL may not occur. Otherwise, the CO₂ fertilization effect on
51 plant growth may diminish over time. The PNL hypothesis has been tested in individual
52 ecosystems during the past decade (e.g., Finzi et al., 2006; Moore et al., 2006; Reich et al., 2006;
53 Norby et al., 2010). Some of the site-level studies support (Reich et al., 2006; Norby et al., 2010),
54 while the others refute PNL (Finzi et al., 2006; Moore et al., 2006). To date, no general pattern of
55 PNL across ecosystems has yet been revealed.

56 Since the key determining PNL occurrence is that whether N supply meets N demand (Luo et
57 al., 2004), it is important to understand how N supply changes under elevated CO₂. The change
58 in N supply for plant growth under elevated CO₂ is determined by the responses of multiple N
59 cycle processes, including biological N fixation, mineralization, nitrification, denitrification, and
60 leaching (Chapin III et al., 2011). In addition, the responses of these processes to CO₂
61 enrichment may be influenced by external N addition, such as N deposition and fertilization
62 (Reay et al., 2008). Thus, synthesizing the responses of processes that regulate PNL to CO₂
63 enrichment may help reveal the general pattern of PNL in terrestrial ecosystems.

64 In the current study, the main objective was to explore the general pattern of the N limitation
65 to plant growth under enriched CO₂ conditions. To do so, two questions were asked: (i) How do
66 the major processes in terrestrial N cycle respond to CO₂ enrichment? (ii) Does the CO₂
67 fertilization effect on plant growth diminish over time? To answer these questions, two sets of
68 data from literature were collected (Table S1, Table 1). With the first dataset, we quantitatively
69 synthesized the effects of CO₂ enrichment on all the major processes and pools in N cycle using
70 meta-analysis. These variables included N sequestered in organic components (i.e., plant tissues,
71 litter and soil organic matter (SOM)), biological N fixation, net mineralization, nitrification,
72 denitrification, leaching, and total inorganic N (TIN), ammonium (NH₄⁺) and nitrate (NO₃⁻)
73 contents in soils. The responses of the N processes to short- vs. long-term CO₂ treatment were
74 also explored. In addition, the responses of the N processes to CO₂ enrichment under without vs.
75 with N addition conditions were compared. With the second dataset in which the decadal plant
76 growth in free air CO₂ enrichment (FACE) experiments were collected, we explored whether
77 CO₂ fertilization effect on plant growth diminishes over time.

78

79

80 2 Materials and Methods

81 2.1 Data collection

82 For dataset one, a comprehensive literature search with the terms of “CO₂ enrichment (or CO₂
83 increase)”, “nitrogen” and “terrestrial” was conducted using the online search connection *Web of*
84 *Science* in Endnote. Then papers meeting the following two criteria were selected to do the
85 further analyses: (i) including both control and CO₂ enrichment treatments, and the ambient and
86 elevated CO₂ concentrations were around the current and predicted atmospheric CO₂
87 concentrations by Intergovernmental Panel on Climate Change (IPCC, 2013), respectively (Fig.
88 S1); (ii) including or from which we could calculate at least one of the major nitrogen (N) pools
89 or processes: soil TIN content, soil NH₄⁺ content, soil NO₃⁻ content, aboveground plant N pool
90 (APNP), belowground plant N pool (BPNP), total plant N pool (TPNP), litter N pool (LNP), soil
91 N pool (SNP), N fixation, nodule mass and/or number, net mineralization, nitrification,
92 denitrification, and inorganic N leaching. Overall, there were 175 papers included in the first
93 dataset (Table S1, References S1). For each paper, means, variations (standard deviation (*SD*),
94 standard error (*SE*) or confidence interval (*CI*)) and sample sizes of the variables in both control
95 and CO₂ enrichment treatments were collected.

96 For those studies that provided *SE* or *CI*, *SD* was computed by

$$97 \quad SD = SE\sqrt{n} \quad \text{Eq. (1)}$$

$$98 \quad \text{or } SD = (CI_u - CI_l)\sqrt{n}/2u_p \quad \text{Eq. (2)}$$

99 where *n* is the sample size, *CI_u* and *CI_l* are the upper and lower limits of *CI*, and *u_p* is the
100 significant level and equal to 1.96 and 1.645 when $\alpha = 0.05$ and 0.10, respectively. In some
101 studies, tissue N concentration and biomass were reported, we multiplied the two parts as N
102 pools. When both APNP and BPNP were provided (or calculated), the two were added together

103 to represent the TPNP. When data from multiple soil layers were provided, they were summed if
 104 they were area-based (i.e., m² land), or averaged if they were weight-based (i.e., g⁻¹ soil). In
 105 studies where the respective contents of NH₄⁺ and NO₃⁻ were reported, the TIN was calculated
 106 by adding the two together. For all the variables, if more than one result were reported ~~in the~~
 107 same year during the experiment period, they were averaged by

$$M = \sum_{i=1}^j \frac{M_i}{j} \quad \text{Eq. (3)}$$

109 with standard deviation

$$SD = \sqrt{\frac{\sum_{i=1}^j SD_i^2 (n_i - 1) n_i}{(\sum_{i=1}^j n_i - 1) \sum_{i=1}^j n_i}} \quad \text{Eq. (4)}$$

111 where j is the number of results, M_i , SD_i and n_i are the mean, SD and sample size of the i th
 112 sampling data, respectively (Liang et al., 2013). If additional treatments applied (e.g., nitrogen
 113 addition), they were treated as independent studies.

114 Because treatment time and N addition may affect the responses of the N processes to CO₂
 115 enrichment, the dataset was divided into different categories: (i) short-term (≤ 3 years) vs. long-
 116 term (> 3 years), and (ii) without N addition vs. with N addition. Moreover, the dataset was also
 117 divided into forest, grassland, and cropland to explore possible differences among ecosystems.

118 For the dataset two, 15 available time courses of plant growth were collected from 7 decadal-
 119 long FACE experiments (Table 1). The ecosystems included 9 forests, 5 grasslands and 1 desert.
 120 Because of the limited data, we included variables that can represent plant growth on a way or
 121 another, for example, net primary production (NPP), biomass, and leaf production. These data
 122 were collected to reveal whether the effect of CO₂ enrichment on plant growth diminishes over
 123 treatment time as proposed by PNL (Luo et al., 2004). In the 7 studies, the treatment lasted from
 124 7 to 13 years, and at least 6 years' production measurements were reported. For each data, the

125 percentage change in NPP (or biomass or leaf production) by CO₂ enrichment was calculated.
 126 Then a linear regression between the percentage change and the treatment year was conducted. A
 127 significantly negative slope indicates that the effect of CO₂ enrichment on plant production
 128 diminishes over time. A non-significant slope was treated as 0. After deriving all the slopes, the
 129 frequency distribution of the slopes were fitted by a Gaussian function:

$$130 \quad y = y_0 + ae^{-\frac{(x-\mu)^2}{2\sigma^2}} \quad \text{Eq. (5)}$$

131 where x is the mean value of each individual interval, and y is the frequency of each interval. y_0
 132 is the base frequency. μ and σ are the mean and *SD* of the distribution.

133

134 **2.2 Meta-analysis**

135 With the first dataset, the effect of CO₂ enrichment for each line of data of the N variables was
 136 estimated using the natural logarithm transformed response ratio (*RR*) (Hedges et al., 1999;
 137 Liang et al., 2013):

$$138 \quad \log_e RR = \log_e (X_E / X_C) \quad \text{Eq. (6)}$$

139 where X_E and X_C are the variable values under enriched CO₂ and control conditions, respectively.
 140 The variation of the logged *RR* was

$$141 \quad V = \left(\frac{SD_C^2}{n_C X_C^2} + \frac{SD_E^2}{n_E X_E^2} \right) \quad \text{Eq. (7)}$$

142 where SD_C and SD_E are the standard deviation of X_C and X_E , and n_C and n_E are the sample sizes
 143 of X_C and X_E .

144 Then random-effects model was used to calculate the weighted mean. In the random-effects
 145 model, the weighted mean was calculated as

$$146 \quad M_{weighted} = \frac{\sum_{j=1}^k W_j^* M_j}{\sum_{j=1}^k W_j^*} \quad \text{Eq. (8)}$$

147 with the variance as

$$148 \quad V_{weighted} = \frac{1}{\sum_{j=1}^k W_j^*} \quad \text{Eq. (9)}$$

149 where k is the number of studies, M_j is the $Ln(RR)$ in study j , and W_j^* is the weighting factor
150 which consists of between- and within-study variances (Rosenberg et al., 2000; Liang et al.,
151 2013). The 95% lower and upper limits ($LL_{weighted}$ and $UL_{weighted}$) for the weighted mean were
152 computed as

$$153 \quad LL_{weighted} = M_{weighted} - 1.96 \times \sqrt{V_{weighted}} \quad \text{Eq. (10)}$$

154 and

$$155 \quad UL_{weighted} = M_{weighted} + 1.96 \times \sqrt{V_{weighted}} \quad \text{Eq. (11)}$$

156 The weighted mean and corresponding 95% bootstrapping CI (999 iterations) for each
157 variable and category were calculated in MetaWin 2.1 (details are described in the software
158 handbook by Rosenberg et al., 2000). The results were back-transformed and represented as
159 percentage change by $(RR - 1) \times 100\%$. The response was considered significant if the 95% CI
160 did not overlap with zero.

161

162 3 Results

163 The meta-analysis from the first dataset showed that CO₂ enrichment significantly increased N
164 sequestered in plants and litter but not in SOM (Figs. 1A, S2). Whereas CO₂ enrichment had
165 little overall effects on N mineralization, nitrification and denitrification, it significantly
166 increased biological N fixation by 41.0244.3% (with 95% CI from 28.6829.5% to 55.5061.8%).
167 The increased biological N fixation was consistent when using various methods except H₂
168 evolution (Fig. 2A). In legume species, CO₂ enrichment significantly increased nodule mass and
169 number (Fig. 2B). In addition, CO₂ enrichment increased N₂O emission by 10.7% (with 95% CI
170 from 2.0% to 22.3%), but reduced leaching (i.e., -25.5441.8% with 95% CI from -35.4658.9% to
171 -13.2924.3%) (Fig. 1B). ~~In addition~~ Although, CO₂ enrichment did not change inorganic N in
172 soils, it increased soil NH₄⁺/NO₃⁻ ratio by 16.9% (with 95% CI from 5.4% to 30.2%) ~~the content~~
173 ~~of NH₄⁺ by 6.46% (with 95% CI from 0.55% to 13.17%), but decreased that of NO₃⁻ by 11.09%~~
174 ~~(with 95% CI from -17.65% to -4.99%), leading to a neutral change in TIN in soils~~ (Fig. 1C).

175 Treatment time had no effect on most of the variables (overlapped 95% CIs for short- and
176 long-term treatments) except nitrification, which was not changed by short-term treatment, but
177 was significantly reduced (-21.7723.4% with 95% CI from -29.5830.4% to -9.9312.1%) by long-
178 term CO₂ enrichment (Fig. 23B). In addition, it seemed that the responses of NH₄⁺ ~~and~~ /NO₃⁻
179 ratio was ~~were~~ strengthened over time, representing neutral response to short-term CO₂
180 enrichment, but significantly positive and negative responses to long-term CO₂ enrichment,
181 respectively (Fig. 2C3C). The effects of CO₂ enrichment ~~could be~~ were influenced by N addition
182 (Fig. 23D – F). For example, nitrification was significantly reduced by CO₂ enrichment without
183 N addition by 19.253% (with 95% CI from -39.7640.5% to -1.430.65%), but was not changed
184 with N addition. Denitrification and N₂O emission responded to CO₂ enrichment neutrally

185 without N addition, but significantly positively with N addition (~~30.54% with 95% CI from 0.70%~~
186 ~~to 113.22% for denitrification, and 9.33% with 95% CI from 0.23% to 20.49%~~; Fig. 2E3E).

187 Additionally, the responses of some variables to CO₂ enrichment were dependent on ecosystem
188 type (Fig. 33G-I). APNP responded to CO₂ enrichment positively in forests and croplands, but
189 neutrally in grasslands (Fig. 3A3G). ~~The n~~Net mineralization had no response to CO₂ enrichment
190 in forests or grasslands, while it was significantly increased in croplands (Fig. 3H3B). Moreover,
191 the change in the TIN was ~~negative, neutral~~ in forests, grassland, and but positive ~~in forests,~~
192 ~~grassland, and in~~ croplands, respectively (Fig. 3I3C). In addition, positive response of
193 NH₄⁺/NO₃⁻ was only observed in grasslands (Fig. 3I).

194 The results from the second dataset showed that CO₂ enrichment significantly increased plant
195 growth in most of the decadal FACE experiments (Fig. 4). In addition, the CO₂ fertilization
196 effect over treatment time on plant growth did not change in 11 experiments ($P > 0.05$),
197 decreased in 2 experiments (slope < 0 , $P < 0.05$), and increased in 2 experiments (slope > 0 , $P <$
198 0.05), respectively (Table 1, Fig. 4). Overall, the slope of the response of plant growth vs.
199 treatment time was not significantly different from 0 (i.e., $-0.37\% \text{ year}^{-1}$ with 95% CI from -1.84%
200 year^{-1} to $1.09\% \text{ year}^{-1}$; Fig. 4).

201

202 **4 Discussion**

203 The current study carried out two syntheses on the responses of terrestrial N cycle and plant
204 growth to CO₂ enrichment to reveal the general pattern of PNL and the underlying processes that
205 regulate PNL.

206

207 **4.1 PNL alleviation**

208 In PNL hypothesis, a prerequisite for PNL occurrence is that more N is sequestered in plant,
209 litter and SOM (Luo et al., 2004). Our results showed that elevated CO₂ significantly increased N
210 retentions in plant tissues and litter, which is consistent with previous meta-analyses (de Graaff
211 et al., 2006; Luo et al., 2006). Thus, the basis of PNL occurrence partially exists. However, the
212 results from the second dataset showed no general diminished CO₂ fertilization effect on plant
213 growth on the decadal scale, which disagrees with the expectation of PNL hypothesis, suggesting
214 that N supply under elevated CO₂ may meet the N demand. In this study, we have identified two
215 processes that increase N supply under elevated CO₂, biological N fixation and leaching.

216 CO₂ enrichment significantly enhanced the N influx to terrestrial ecosystems through
217 biological N fixation, which reduces dinitrogen (N₂) to NH₄⁺ (Fig. 1B). The enhanced biological
218 N fixation could result from the stimulated activities of the symbiotic (Fig. [S32B](#)) and free-lived
219 heterotrophic N-fixing bacteria (Hoque et al., 2001). In addition, the competition between N₂-
220 fixing and non-N₂-fixing species could also contribute to enhance the biological N fixation on
221 the ecosystem level (Poorter and Navas, 2003; Batterman et al., 2013). A review by Poorter and
222 Navas (2003) suggests that elevated CO₂ could strengthen the competition of N₂-fixing dicots
223 when nutrient level is low.

224 ~~The Results showed that the~~ N efflux via leaching reduced under elevated CO₂ condition (Fig.
225 1B). This could be attributed to the decrease in the primary N form in leaching, NO₃⁻ (Chapin III
226 et al., 2011), and the increased root growth which may immobilize more free N in soils (Luo et
227 al., 2006; Iversen, 2010). In contrast, gaseous N loss through N₂O emission increased under
228 elevated CO₂ in comparison with that under ambient CO₂. But the increase was only observed
229 when additional N was applied.

230 ~~The increased N influx via biological N fixation and the reduced N loss through leaching~~
231 ~~result in~~ The net effect of the responses of N processes to CO₂ enrichment resulted in more N
232 retention in ~~the biosphere ecosystems, especially in plant tissues and litter~~ (Fig. S2). Because the
233 product of biological N fixation (i.e., NH₄⁺) and the primary form for N leaching loss (i.e., NO₃⁻)
234 can be directly used by plants, the effects of CO₂ enrichment on the two processes directly
235 increase the N availability for plant growth, potentially alleviating PNL (Fig. 5). ~~In addition, the~~
236 ~~increased N retention by CO₂ enrichment is primarily in plant tissues and litter but not in SOM~~
237 ~~(Figs. 1, S2).~~ The increased N in plant tissues can be re-used by plant for multiple times via
238 resorption (Norby et al., 2000; Norby et al., 2001), and consequently reduce the N demand from
239 soils. This may be another mechanism that alleviates PNL (Walker et al., 2015). Therefore, the
240 increased N availability by the increased N fixation and reduced N leaching could potentially
241 support net accumulation of organic matter in terrestrial ecosystems (Rastetter et al., 1997; Luo
242 and Reynolds, 1999).

243 Since biological N fixation provides at least 30% of nitrogen requirement across natural
244 biomes (Asner et al., 2001; Galloway et al., 2004), our results suggest that the positive response
245 of biological N fixation to CO₂ enrichment plays an important role in alleviating PNL. PNL was
246 proposed to characterize long-term dynamics of carbon-nitrogen coupling in response to rising

247 atmospheric CO₂ concentration. Thus, it is critical to understand long-term response of biological
248 N fixation to elevated CO₂. In this paper, we synthesize 12 studies that lasted 4 – 7 years and
249 binned them in a long-term category (> 3 years). On average of those long-term studies, CO₂
250 enrichment increased biological N fixation by 26.2%. The increased biological N fixation is
251 supported by evidence at gene level from long-term experiments. For example, Tu et al. (2015)
252 found the abundance of *nifH* gene amplicons, which is a widely used marker for analyzing
253 biological N fixation, was significantly enhanced by 12-year CO₂ enrichment in a grassland
254 (BioCON). However, our synthesis showed a relatively wide 95% confidence interval from 2.54%
255 to 59.8%. The wide range can be partially attributed to the relatively small study numbers. In
256 addition, most studies incorporated in the current synthesis were conducted in temperate regions.
257 Thus, longer-term studies, as well as studies in other regions (e.g., boreal and tropical) are
258 critically needed to reveal more general patterns in the future.

259 Although a general trend of PNL alleviation has been found in this study, the alleviation
260 potential may vary across different ecosystems~~varies among sites~~due to asymmetric distribution
261 of biological N fixation (Cleveland et al., 1999). In addition, the PNL alleviation~~and~~ may also be
262 influenced by other factors. While most of the long-term experiments did not show diminished
263 CO₂ fertilization effect, the CO₂ fertilization effect on plant production decreased in two sites
264 (i.e., ORNL and Aspen-Birch) (Fig. 4). Plant growth is usually influenced by multiple
265 environmental factors (e.g., nutrients, water, light, ozone, etc.). The undiminished CO₂
266 fertilization effect in most studies indicates that resources (including N) limitations are not
267 aggravated, suggesting that no PNL occurs in these sites. However, in ORNL and Aspen-~~Birth~~
268 Birch (without O₃ treatment), the diminished CO₂ fertilization effect could be attributed to
269 limitation of N, or other resources, or their combined. For example, reduced N availability has

270 been identified as one of the primary factors that lead to the diminished CO₂ fertilization effect
271 on NPP in ORNL FACE experiment (Norby et al., 2010). In Aspen-Birch community, however,
272 deceleration of leaf area increase due to canopy closure is responsible for the diminished CO₂
273 fertilization effect without O₃ addition (Talhelm et al., 2012). With O₃ addition, O₃ significantly
274 reduces the canopy development, resulting in relatively open canopy during the experiment
275 period. In addition, the negative effect of O₃ addition increases over time, leading to the apparent
276 increase in the CO₂ fertilization effect (Fig. 4) (Talhelm et al., 2012).

277

278 **4.2 Dependence of the responses of N cycle processes upon methodology, treatment** 279 **duration, N addition and ecosystem types**

280 Methodology may potentially influence the results. Cabrerizo et al. (2001) found that CO₂
281 enrichment increased nitrogenase activity measured by acetylene reduction assay (ARA), but not
282 specific N fixation measured by H₂ evolution method. In studies synthesized here, four methods
283 were used to estimate biological N fixation, including isotope, ARA, H₂ evolution and N
284 accumulation. Among them, ARA and H₂ evolution measure nitrogenase activity (Hunt and
285 Layzell, 1993) whereas isotope and N accumulation methods directly measure biological N
286 fixation. All but H₂ evolution method showed significantly positive response to CO₂ enrichment
287 (Fig. 2A). The insignificant response by H₂ evolution method was likely because of the small
288 study numbers (i.e., 3). In addition, biological N fixation by ARA, isotope and N accumulation
289 showed similar response magnitude (Fig. 2A), suggesting consistency among the three methods.
290 However, further assessment on H₂ evolution method is needed.

291 The responses of biological N fixation and leaching to CO₂ enrichment are barely influenced
292 by treatment duration, N addition, or ecosystem types (Figs. 2,3), suggesting that the alleviation

293 of PNL by the increased biological N fixation and decreased leaching generally occurs in
294 terrestrial ecosystems. However, the responses of other N cycle processes that affect N
295 availability are dependent on treatment duration, N addition, and/or ecosystem types (Figs. 2,3).

296 N mineralization, in addition to biological N fixation, is a major source of available N in soils.
297 The meta-analysis showed no change in the net N mineralization in response to CO₂ enrichment,
298 which is consistent with the results by de Graaff et al. (2006). However, the response of net
299 mineralization was dependent upon ecosystem types, showing no change in forests and
300 grasslands, but significantly increase in croplands (Fig. 3B3H). There may be two reasons for the
301 stimulated net mineralization in croplands. First, N fertilization, which is commonly practiced in
302 croplands, can increase the substrate quantity and quality for the mineralization (Barrios et al.,
303 1996; Chapin III et al., 2011; Booth et al., 2005; Lu et al., 2011; Reich and Hobbie, 2013).

304 Second, tillage ~~could~~ can alter/improve the soil conditions (e.g., increasing O₂ content), which
305 can potentially favor N mineralization under enriched CO₂ and stimulate the turnover rate of soil
306 fauna, increasing N mineralization (Wienhold and Halvorson, 1999; Bardgett and Wardle, 2010).
307 These findings suggest that CO₂ enrichment can managements (in croplands) may stimulate the
308 N transfer from organic to inorganic forms in managed croplands.

309 Unlike leaching, the response of nitrification is dependent upon treatment duration (Fig. 23).
310 Nitrification was not changed by short-term treatment, but was significantly reduced by long-
311 term CO₂ enrichment (Fig. 23). One possible reason for the reduced nitrification by the long-
312 term CO₂ enrichment is cumulative effect of hydrological change. CO₂ enrichment generally
313 reduces the stomatal conductance and the consequent water loss via plant transpiration, leading
314 to an increase in soil water content (Niklaus et al., 1998; Tricker et al., 2009; van Groenigen et
315 al., 2011; Keenan et al., 2013). A synthesis by van Groenigen et al. (2011) shows that CO₂

316 enrichment increases soil water content by 2.6% –10.6%. The increased soil water content may
317 result in less oxygen (O₂) content in soils, which could potentially constrain nitrification.

318 In addition, the response of gaseous N loss depends on N addition (Fig. 23). Reduced
319 nitrification was only observed under without N addition (Fig. 2E3E). With N addition, no
320 response of nitrification to CO₂ enrichment was observed (Fig. 2E3E). Additionally, the response
321 of denitrification to CO₂ enrichment shifted from neutral without N addition to significantly
322 positive with N addition (Fig. 2E3E). One possible reason is that N addition provides more N
323 substrate to nitrifying and denitrifying bacteria (Keller et al., 1988; Stehfest and Bouwman, 2006;
324 Russow et al., 2008). The strengthening trends of both nitrification and denitrification lead to a
325 shift of the response of N₂O emission to CO₂ enrichment from neutral without N addition to
326 significantly positive with N addition (Fig. 2E3E). Our results indicate that CO₂ enrichment
327 significantly increases gaseous N loss when additional N is applied.

328 Our results are consistent with a previous synthesis (van Groenigen et al. 2011). The increased
329 N₂O emission can partially offset the mitigation of climate change by stimulated plant CO₂
330 assimilation as the warming potential by N₂O is as 296 time as that by CO₂. However, a recent
331 modeling study by Zaehle et al. (2011) has generated an opposite result that CO₂ enrichment
332 reduced radiative forcing of N₂O. In their model, less availability of N substrates for nitrification
333 and denitrification due to enhanced plant N sequestration attributed to the reduced N₂O emission.
334 Our synthesis shows that inorganic N does not decrease. Especially with additional N application,
335 enhanced denitrification by CO₂ enrichment results in greater N₂O emission.

336

337 **4.3 Changes in soil microenvironment, community structures and above-belowground** 338 **interactions**

339 The meta-analysis showed that the two major forms of soil available N, NH_4^+ and NO_3^- , respond
340 to long-term CO_2 enrichment in opposing manners (Fig. ~~2C3C~~). ~~One the one hand,~~ While the
341 enhanced biological N fixation by CO_2 enrichment ~~significantly increased~~ tended to increase
342 NH_4^+ content in soil. ~~On the other hand,~~ the reduced nitrification decreased NO_3^- content in soils,
343 leading to significant increase in $\text{NH}_4^+/\text{NO}_3^-$ ratio (Fig. ~~2B,3~~-C).

344 Although the total available N does not change under elevated CO_2 , the altered proportion of
345 NH_4^+ over NO_3^- in soils may have long-term effects on soil microenvironment and associated
346 aboveground-belowground linkages that control C cycle (Bardgett and Wardle, 2010). On the
347 one hand, plants would release more hydrogen ion (H^+) to regulate the charge balance when
348 taking up more NH_4^+ . As a result, the increased NH_4^+ absorption ~~can~~ could acidify the
349 rhizosphere soil (Thomson et al., 1993; Monsant et al., 2008). The lowered pH could have a
350 significant effect on soil microbial ~~community~~ communities and their associated ecosystem
351 functionseomposition. For example, ~~the~~ fungal/bacterial ratio increases with the decrease in pH
352 (de Vries et al., 2006; Rousk et al., 2009). The increased fungal/bacterial ratio may result in
353 lower N mineralization because of the higher C/N ratio of fungi and lower turnover rates of
354 fungal-feeding fauna (de Vries et al., 2006; Rousk and Bååth, 2007). In other words, the
355 increased fungal/bacterial ratio may slow down the N turnover from organic to inorganic forms.
356 On the other hand, the increased $\text{NH}_4^+/\text{NO}_3^-$ ratio may increase the N use efficiency because it is
357 more energetically expensive for plants to utilize NO_3^- than NH_4^+ (Chapin III et al., 2011; Odum
358 and Barrett, 2005; Lambers et al., 2008). In addition, since the preferences for plant absorption of
359 different forms of N are different (Chapin III et al., 2011; Odum and Barrett, 2005), the increased
360 $\text{NH}_4^+/\text{NO}_3^-$ ratio may benefit some plant species while depress others, and consequently alter the
361 community structures over time. These diverse changes in soil microenvironment and microbial

362 and plant community compositions could further affect the terrestrial C sequestration cycle on
363 long temporal scales, on which more studies are needed.

364

365 **5 Summary**

366 This synthesis provides a comprehensive assessment of the effects of CO₂ enrichment on
367 terrestrial N cycle, which helps improve the understanding of the N limitation to plant growth
368 under elevated CO₂. Our results indicate that elevated CO₂ stimulates N influx via biological N
369 fixation but reduces N loss via leaching, increasing N availability for plant growth. The extra N
370 supply by the enhanced biological N fixation and reduced leaching may meet the increased N
371 demand under elevated CO₂, potentially alleviating PNL. In addition, CO₂ enrichment increased
372 N₂O emission, especially with extra N addition. The increased N₂O emission can partially offset
373 the mitigation of climate change by stimulated plant CO₂ assimilation. Moreover, the changes in
374 the soil microenvironment, ecosystem communities and above-belowground interactions induced
375 by the different responses of NH₄⁺ and NO₃⁻ to CO₂ enrichment may have long-term effects on
376 terrestrial biogeochemical cycles and climate change, on which further studies are needed.

377

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383

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544

545 **Supporting Information captions**

546 **Figure S1** Distributions of the experimental duration (**A**) and the CO₂ concentrations under
547 ambient (**B**) and elevated (**C**) treatments and their difference (**D**) for the 175 collected studies.
548 Red dashed lines represent the mean values.

549
550 **Figure S2** Summary of the effect of CO₂ enrichment on ecosystem level N budget. Square boxes
551 are nitrogen pools, ovals are nitrogen processes. Red dashed boxes mean the sum of the pools in
552 the boxes. “+”, “-”, and “ns” mean the response to CO₂ enrichment are positive, negative, and
553 not significant, respectively. Please see **Figure 1** for abbreviations.

554
555 ~~**Figure S3** Responses of the nodule biomass and number in legume plants to CO₂ enrichment~~
556 ~~(Mean ± 95% confidence interval).~~

557
558 ~~**Table-Database S1** Variables-Database extracted from ~~each~~ papers listed in References S1.~~

559
560 **References S1** Papers from which the first dataset was extracted.

561

562 **Table 1.** Results on the effect of CO₂ enrichment on ecosystem NPP (or biomass or leaf production) in decadal-long free air CO₂
563 enrichment (FACE) experiments over treatment time. The values of the slope, R^2 and P in the linear regression in **Fig. 4** are shown.
564 The lower and upper n (i.e., n and N) in Refs. Schneider et al., 2004; McCarthy et al., 2010; Reich and Hobbie, 2013 mean without
565 and with N addition, respectively. The lower and upper o (i.e., o and O) in Ref. Talhelm et al., 2012 mean without and with O₃
566 treatment, respectively.

Experiment	Ecosystem type	Treatment years	Variable	Slope	R^2	P	Reference
Duke_n	Forest	8	NPP	0.50	0.25	0.21	McCarthy et al., 2010
Duke_N	Forest	8	NPP	-1.39	0.27	0.29	McCarthy et al., 2010
ORNL	Forest	11	NPP	-1.42	0.38	0.04	Norby et al., 2010
BioCON_n	Grassland	13	Biomass	0.42	0.05	0.48	Reich and Hobbie, 2013
BioCON_N	Grassland	13	Biomass	0.23	0.01	0.76	Reich and Hobbie, 2013
NZ	Grassland	10	Biomass	0.95	0.05	0.53	Ross et al., 2013
Swiss_n	Grassland	10	Harvestable biomass	0.30	0.01	0.75	Schneider et al., 2004
Swiss_N	Grassland	10	Harvestable biomass	1.66	0.47	0.03	Schneider et al., 2004
NDFD	Desert	9	Standing biomass	-9.54	0.15	0.40	Smith et al., 2014
Aspen_o	Forest	7	Leaf production	-0.07	0.00	0.97	Talhelm et al., 2012
Aspen_O	Forest	7	Leaf production	0.09	0.00	0.93	Talhelm et al., 2012
AspenBirch_o	Forest	7	Leaf production	-5.27	0.77	0.01	Talhelm et al., 2012
AspenBirch_O	Forest	7	Leaf production	6.48	0.82	0.00	Talhelm et al., 2012
AspenMaple_o	Forest	7	Leaf production	-9.16	0.40	0.13	Talhelm et al., 2012
AspenMaple_O	Forest	7	Leaf production	1.11	0.11	0.46	Talhelm et al., 2012

567

568 **Figure captions**

569 **Figure 1.** Results of a meta-analysis on the responses of nitrogen pools and processes to
570 CO₂ enrichment. In (A), APNP, BPNP, TPNP, LNP, and SNP are the abbreviations for
571 aboveground plant nitrogen pool, belowground plant nitrogen pool, total plant nitrogen
572 pool, litter nitrogen pool, and soil nitrogen pool, respectively. In (C), TIN, NH₄⁺ and
573 NO₃⁻ are total inorganic nitrogen, ammonium, and nitrate in soils, respectively. The error
574 bars represent 95% confidence intervals.

575

576 **Figure 2.** Responses of biological N fixation measured by different methods (A) and
577 nodule dry mass and number in legume species (B). ARA: acetylene reduction assay.
578 Mean ± 95% confidence interval.

579

580 **Figure 3.** Responses of terrestrial nitrogen pools and processes to CO₂ enrichment (Mean
581 ± 95% confidence interval) as regulated by experimental durations (A – C; short-term: ≤
582 3 years vs. long-term: > 3 years), nitrogen addition (D – F), and ecosystem types (G – I).
583 Please see Figure 1 for abbreviations.

584

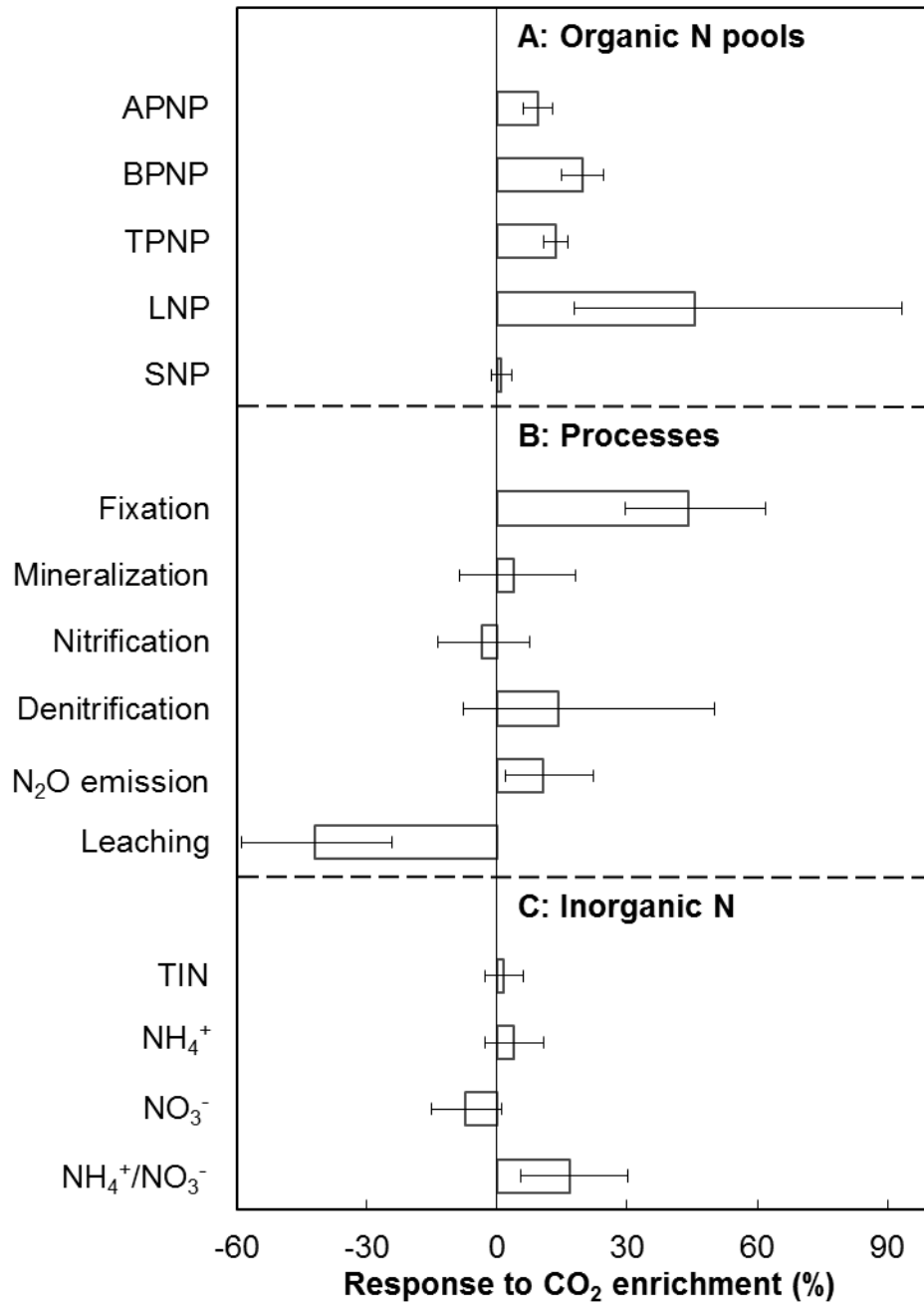
585 **Figure 4.** Time courses of CO₂ effects on ecosystem NPP (or biomass or leaf production)
586 in decadal-long FACE experiments. Please see **Table 1** for details of experiments,
587 references and statistical results. Only statistically significant ($P < 0.05$) regression lines
588 are shown. The panel at the right-low corner shows the distribution of the slopes (-0.37%
589 year⁻¹ with 95% CI from -1.84% year⁻¹ to 1.09% year⁻¹).

590

591 **Figure 5.** Mechanisms that alleviate PNL. PNL hypothesis posits that the stimulated
592 plant growth by CO₂ enrichment leads to more N sequestered in long-lived plant tissues,
593 litter and soil organic matter (SOM) so that, the N availability for plant growth
594 progressively declines over time, and plant growth is downregulated (grey symbols). The
595 current synthesis indicates that the basis of PNL occurrence partially exists (i.e., more N
596 sequestered in plant tissues and litter; black symbols). ~~However,~~Despite of the increases
597 in plant N sequestration and N₂O emission, stimulated biological N fixation and reduced
598 N leaching can replenish the N availability, potentially alleviating PNL (blue boxes and
599 arrows). Upward, downward, and horizontal arrows mean increase, decrease, and no
600 change, respectively.

601

602 **Figure 1.**

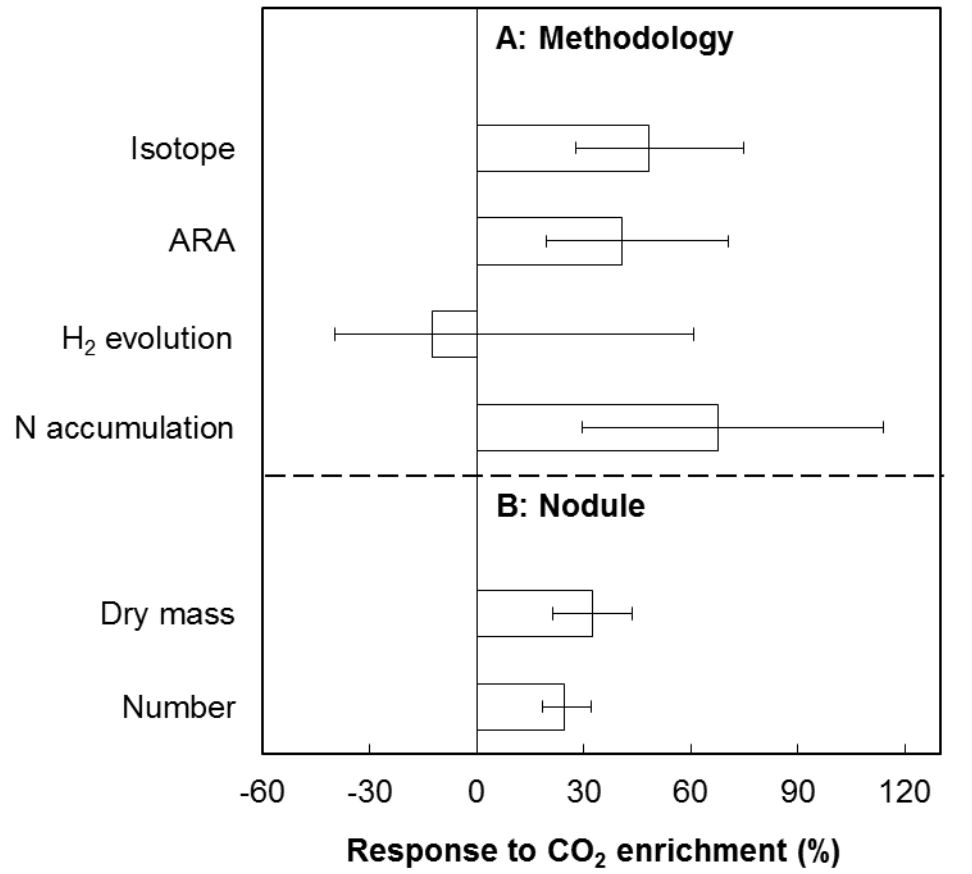


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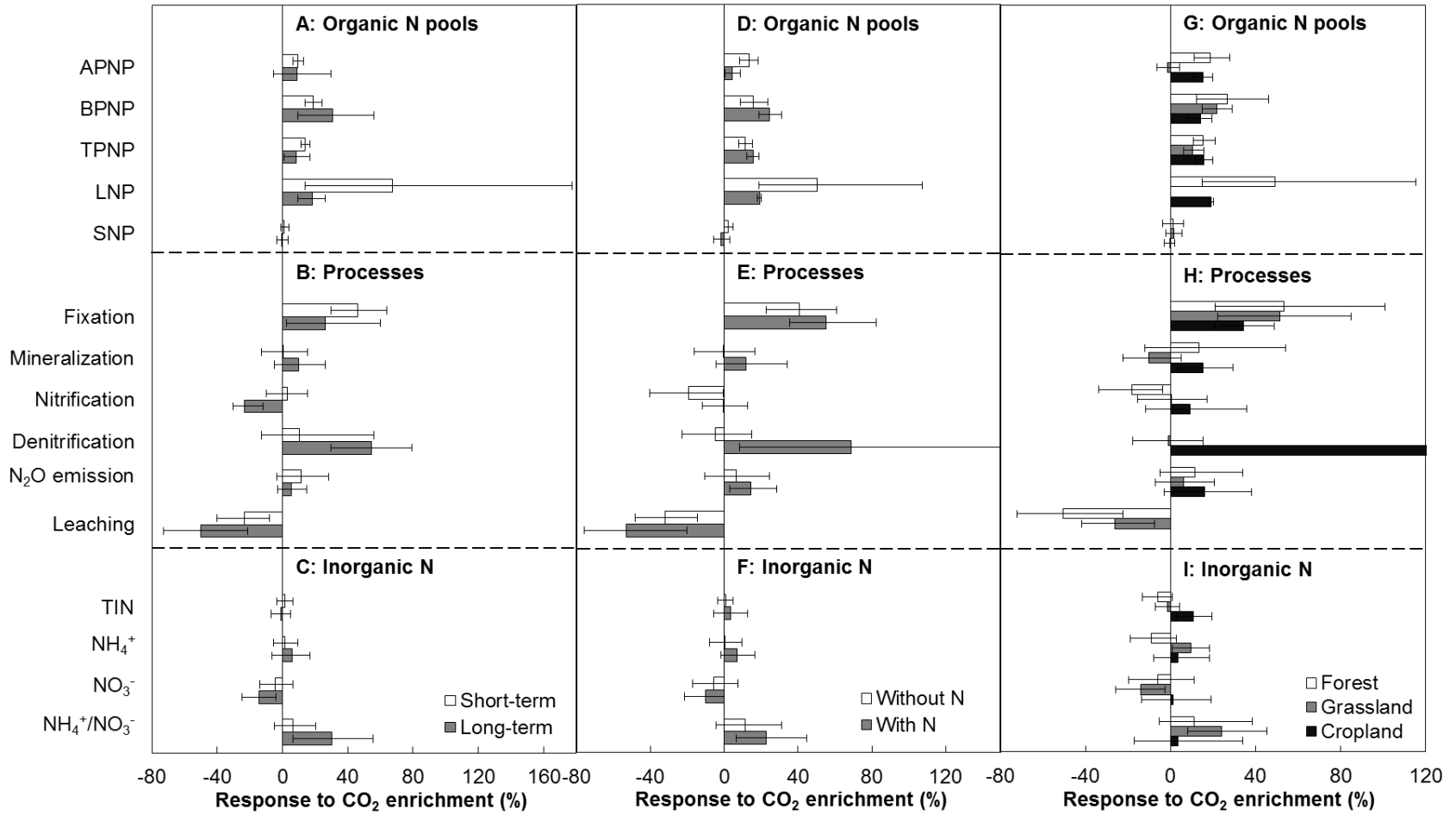
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Figure 2.

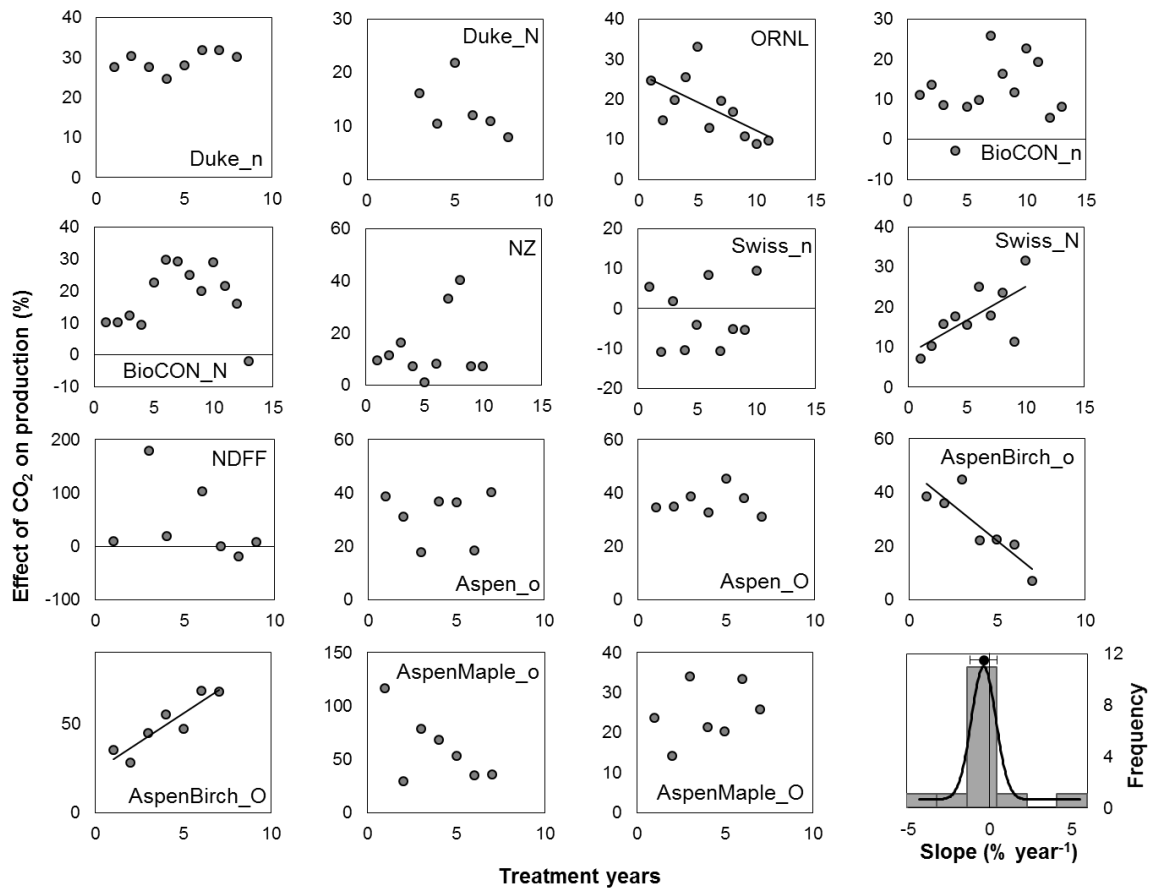


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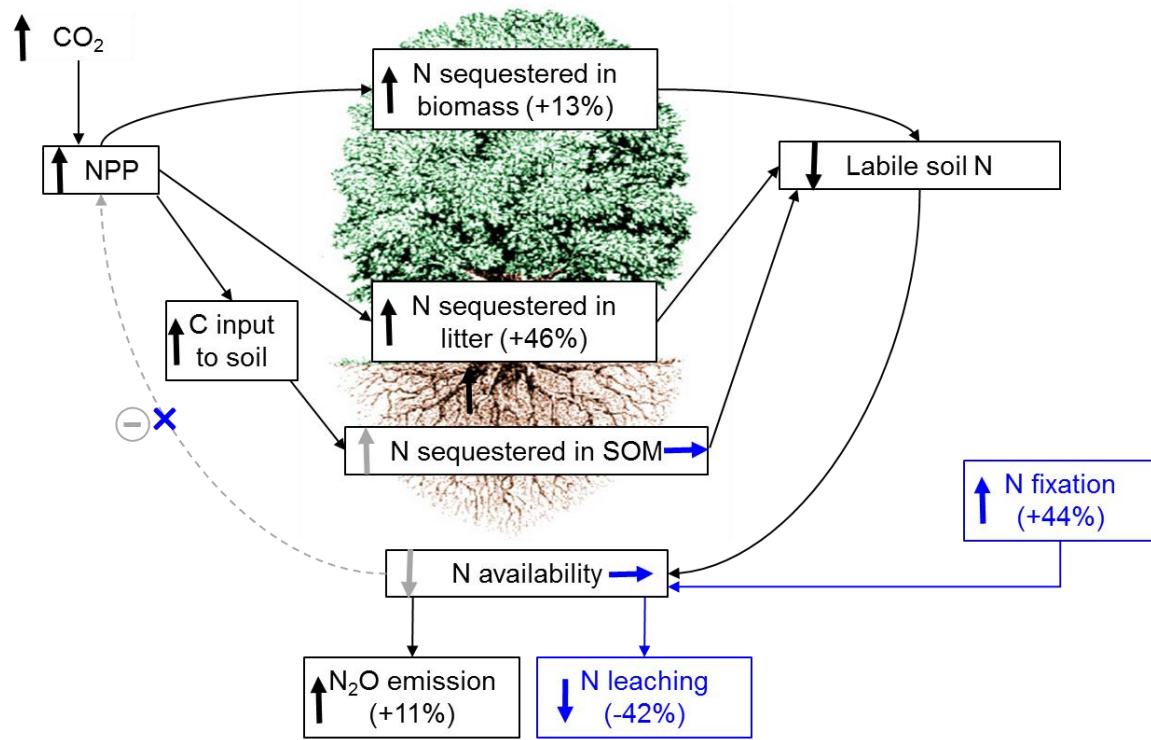
610 **Figure 4.**



611

612

613 **Figure 5.**



614

615