

1 **Processes regulating progressive nitrogen limitation under elevated carbon dioxide: A**  
2 **meta-analysis**

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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 CO<sub>2</sub> 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 CO<sub>2</sub> experimental data available in the literature. The results showed that CO<sub>2</sub>  
20 enrichment significantly increased N sequestration in plant and litter pools but not in soil pool.  
21 Thus, the mechanisms that drive PNL occurrence partially exist. However, CO<sub>2</sub> enrichment  
22 significantly increased the N influx via biological N fixation and the loss via N<sub>2</sub>O emission, but  
23 decreased the N efflux via leaching. In addition, no general diminished CO<sub>2</sub> fertilization effect  
24 on plant growth was observed over time up to the longest experiment of 13 years. Overall, our  
25 analyses suggest that the extra N supply by the increased biological N fixation and decreased  
26 leaching may potentially alleviate PNL under elevated CO<sub>2</sub> conditions despite of the increases in  
27 plant N sequestration and N<sub>2</sub>O emission. Moreover, our synthesis showed that CO<sub>2</sub> enrichment  
28 increased soil ammonium (NH<sub>4</sub><sup>+</sup>) to nitrate (NO<sub>3</sub><sup>-</sup>) ratio. The changed NH<sub>4</sub><sup>+</sup>/NO<sub>3</sub><sup>-</sup> ratio and  
29 subsequent biological processes may result in changes in soil microenvironment, community  
30 structures and above-belowground interactions, which could potentially affect the terrestrial  
31 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 (CO<sub>2</sub>) concentrations, which could stimulate plant growth (IPCC, 2013). The stimulated  
36 plant growth by CO<sub>2</sub> fertilization and the resulting terrestrial carbon (C) storage could partially  
37 mitigate the further increase in CO<sub>2</sub> concentrations and associated climate warming (IPCC,  
38 2013). However, the stimulated plant growth by CO<sub>2</sub> 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 CO<sub>2</sub> fertilization effect is progressive N limitation  
43 (PNL) (Luo et al., 2004).

44 Progressive N limitation postulates that the stimulation of plant growth by CO<sub>2</sub> 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 CO<sub>2</sub> 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 CO<sub>2</sub> 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 CO<sub>2</sub>. The change  
58 in N supply for plant growth under elevated CO<sub>2</sub> 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 CO<sub>2</sub>  
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 CO<sub>2</sub>  
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 CO<sub>2</sub> conditions. To do so, two questions were asked: (i) How do  
66 the major processes in terrestrial N cycle respond to CO<sub>2</sub> enrichment? (ii) Does the CO<sub>2</sub>  
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 CO<sub>2</sub> 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 (NH<sub>4</sub><sup>+</sup>) and nitrate (NO<sub>3</sub><sup>-</sup>)  
73 contents in soils. The responses of the N processes to short- vs. long-term CO<sub>2</sub> treatment were  
74 also explored. In addition, the responses of the N processes to CO<sub>2</sub> 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 CO<sub>2</sub> enrichment (FACE) experiments were collected, we explored whether  
77 CO<sub>2</sub> fertilization effect on plant growth diminishes over time.

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## 80 2 Materials and Methods

### 81 2.1 Data collection

82 For dataset one, a comprehensive literature search with the terms of “CO<sub>2</sub> enrichment (or CO<sub>2</sub>  
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 CO<sub>2</sub> enrichment treatments, and the ambient and  
86 elevated CO<sub>2</sub> concentrations were around the current and predicted atmospheric CO<sub>2</sub>  
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 NH<sub>4</sub><sup>+</sup> content, soil NO<sub>3</sub><sup>-</sup> 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 CO<sub>2</sub> enrichment treatments were collected.

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

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

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

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

102 to represent the TPNP. When data from multiple soil layers were provided, they were summed if  
103 they were area-based (i.e., m<sup>-2</sup> land), or averaged if they were weight-based (i.e., g<sup>-1</sup> soil). In  
104 studies where the respective contents of NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> were reported, the TIN was calculated  
105 by adding the two together. For all the variables, if more than one result were reported during the  
106 experiment period, they were averaged by

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

107 with standard deviation

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

108 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  
109 sampling data, respectively (Liang et al., 2013). If additional treatments applied (e.g., nitrogen  
110 addition), they were treated as independent studies.

111 Because treatment time and N addition may affect the responses of the N processes to CO<sub>2</sub>  
112 enrichment, the dataset was divided into different categories: (i) short-term ( $\leq 3$  years) vs. long-  
113 term ( $> 3$  years), and (ii) without N addition vs. with N addition. Moreover, the dataset was also  
114 divided into forest, grassland, and cropland to explore possible differences among ecosystems.

115 For the dataset two, 15 available time courses of plant growth were collected from 7 decadal-  
116 long FACE experiments (Table 1). The ecosystems included 9 forests, 5 grasslands and 1 desert.  
117 Because of the limited data, we included variables that can represent plant growth on a way or  
118 another, for example, net primary production (NPP), biomass, and leaf production. These data  
119 were collected to reveal whether the effect of CO<sub>2</sub> enrichment on plant growth diminishes over  
120 treatment time as proposed by PNL (Luo et al., 2004). In the 7 studies, the treatment lasted from  
121 7 to 13 years, and at least 6 years' production measurements were reported. For each data, the

122 percentage change in NPP (or biomass or leaf production) by CO<sub>2</sub> enrichment was calculated.  
 123 Then a linear regression between the percentage change and the treatment year was conducted. A  
 124 significantly negative slope indicates that the effect of CO<sub>2</sub> enrichment on plant production  
 125 diminishes over time. A non-significant slope was treated as 0. After deriving all the slopes, the  
 126 frequency distribution of the slopes were fitted by a Gaussian function:

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

127 where  $x$  is the mean value of each individual interval, and  $y$  is the frequency of each interval.  $y_0$   
 128 is the base frequency.  $\mu$  and  $\sigma$  are the mean and *SD* of the distribution.

129

## 130 **2.2 Meta-analysis**

131 With the first dataset, the effect of CO<sub>2</sub> enrichment for each line of data of the N variables was  
 132 estimated using the natural logarithm transformed response ratio (*RR*) (Hedges et al., 1999;  
 133 Liang et al., 2013):

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

134 where  $X_E$  and  $X_C$  are the variable values under enriched CO<sub>2</sub> and control conditions, respectively.  
 135 The variation of the logged *RR* was

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

136 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  
 137 of  $X_C$  and  $X_E$ .

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

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

140 with the variance as

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

141 where  $k$  is the number of studies,  $M_j$  is the  $Ln(RR)$  in study  $j$ , and  $W_j^*$  is the weighting factor  
142 which consists of between- and within-study variances (Rosenberg et al., 2000; Liang et al.,  
143 2013). The 95% lower and upper limits ( $LL_{weighted}$  and  $UL_{weighted}$ ) for the weighted mean were  
144 computed as

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

145 and

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

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

151



### 152 **3 Results**

153 The meta-analysis from the first dataset showed that CO<sub>2</sub> enrichment significantly increased N  
154 sequestered in plants and litter but not in SOM (Figs. 1A, S2). Whereas CO<sub>2</sub> enrichment had  
155 little overall effects on N mineralization, nitrification and denitrification, it significantly  
156 increased biological N fixation by 44.3% (with 95% CI from 29.5% to 61.8%). The increased  
157 biological N fixation was consistent when using various methods except H<sub>2</sub> evolution (Fig. 2A).  
158 In legume species, CO<sub>2</sub> enrichment significantly increased nodule mass and number (Fig. 2B). In  
159 addition, CO<sub>2</sub> enrichment increased N<sub>2</sub>O emission by 10.7% (with 95% CI from 2.0% to 22.3%),  
160 but reduced leaching (i.e., -41.8% with 95% CI from -58.9% to -24.3%) (Fig. 1B). Although CO<sub>2</sub>  
161 enrichment did not change inorganic N in soils, it increased soil NH<sub>4</sub><sup>+</sup>/NO<sub>3</sub><sup>-</sup> ratio by 16.9% (with  
162 95% CI from 5.4% to 30.2%) (Fig. 1C).

163 Treatment time had no effect on most of the variables (overlapped 95% CIs for short- and  
164 long-term treatments) except nitrification, which was not changed by short-term treatment, but  
165 was significantly reduced (-23.4% with 95% CI from -30.4% to -12.1%) by long-term CO<sub>2</sub>  
166 enrichment (Fig. 3B). In addition, it seemed that the responses of NH<sub>4</sub><sup>+</sup>/NO<sub>3</sub><sup>-</sup> ratio was  
167 strengthened over time, representing neutral response to short-term CO<sub>2</sub> enrichment, but  
168 significantly positive and negative responses to long-term CO<sub>2</sub> enrichment (Fig. 3C). The effects  
169 of CO<sub>2</sub> enrichment were influenced by N addition (Fig. 3D – F). For example, nitrification was  
170 significantly reduced by CO<sub>2</sub> enrichment without N addition by 19.3% (with 95% CI from -40.5%  
171 to -0.65%), but was not changed with N addition. Denitrification and N<sub>2</sub>O emission responded to  
172 CO<sub>2</sub> enrichment neutrally without N addition, but significantly positively with N addition (Fig.  
173 3E). Additionally, the responses of some variables to CO<sub>2</sub> enrichment were dependent on  
174 ecosystem type (Fig. 3G – I). APNP responded to CO<sub>2</sub> enrichment positively in forests and

175 croplands, but neutrally in grasslands (Fig. 3G). Net mineralization had no response to CO<sub>2</sub>  
176 enrichment in forests or grasslands, while it was significantly increased in croplands (Fig. 3H).  
177 Moreover, the change in the TIN was neutral in forests, grassland, but positive, in croplands,  
178 respectively (Fig. 3I). In addition, positive response of NH<sub>4</sub><sup>+</sup>/NO<sub>3</sub><sup>-</sup> was only observed in  
179 grasslands (Fig. 3I).

180 The results from the second dataset showed that CO<sub>2</sub> enrichment significantly increased plant  
181 growth in most of the decadal FACE experiments (Fig. 4). In addition, the CO<sub>2</sub> fertilization  
182 effect over treatment time on plant growth did not change in 11 experiments ( $P > 0.05$ ),  
183 decreased in 2 experiments (slope  $< 0$ ,  $P < 0.05$ ), and increased in 2 experiments (slope  $> 0$ ,  $P <$   
184  $0.05$ ), respectively (Table 1, Fig. 4). Overall, the slope of the response of plant growth vs.  
185 treatment time was not significantly different from 0 (i.e.,  $-0.37\% \text{ year}^{-1}$  with 95% CI from  $-1.84\%$   
186  $\text{year}^{-1}$  to  $1.09\% \text{ year}^{-1}$ ; Fig. 4).

187

## 188 **4 Discussion**

189 The current study carried out two syntheses on the responses of terrestrial N cycle and plant  
190 growth to CO<sub>2</sub> enrichment to reveal the general pattern of PNL and the underlying processes that  
191 regulate PNL.

192

### 193 **4.1 PNL alleviation**

194 In PNL hypothesis, a prerequisite for PNL occurrence is that more N is sequestered in plant,  
195 litter and SOM (Luo et al., 2004). Our results showed that elevated CO<sub>2</sub> significantly increased N  
196 retentions in plant tissues and litter, which is consistent with previous meta-analyses (de Graaff  
197 et al., 2006; Luo et al., 2006). Thus, the basis of PNL occurrence partially exists. However, the  
198 results from the second dataset showed no general diminished CO<sub>2</sub> fertilization effect on plant  
199 growth on the decadal scale, which disagrees with the expectation of PNL hypothesis, suggesting  
200 that N supply under elevated CO<sub>2</sub> may meet the N demand. In this study, we have identified two  
201 processes that increase N supply under elevated CO<sub>2</sub>, biological N fixation and leaching.

202 CO<sub>2</sub> enrichment significantly enhanced the N influx to terrestrial ecosystems through  
203 biological N fixation, which reduces dinitrogen (N<sub>2</sub>) to NH<sub>4</sub><sup>+</sup> (Fig. 1B). The enhanced biological  
204 N fixation could result from the stimulated activities of the symbiotic (Fig. 2B) and free-lived  
205 heterotrophic N-fixing bacteria (Hoque et al., 2001). In addition, the competition between N<sub>2</sub>-  
206 fixing and non-N<sub>2</sub>-fixing species could also contribute to enhance the biological N fixation on  
207 the ecosystem level (Poorter and Navas, 2003; Batterman et al., 2013). A review by Poorter and  
208 Navas (2003) suggests that elevated CO<sub>2</sub> could strengthen the competition of N<sub>2</sub>-fixing dicots  
209 when nutrient level is low.

210 Results showed that the N efflux via leaching reduced under elevated CO<sub>2</sub> condition (Fig. 1B).  
211 This could be attributed to the decrease in the primary N form in leaching, NO<sub>3</sub><sup>-</sup> (Chapin III et al.,  
212 2011), and the increased root growth which may immobilize more free N in soils (Luo et al.,  
213 2006; Iversen, 2010). In contrast, gaseous N loss through N<sub>2</sub>O emission increased under elevated  
214 CO<sub>2</sub> in comparison with that under ambient CO<sub>2</sub>. But the increase was only observed when  
215 additional N was applied.

216 The net effect of the responses of N processes to CO<sub>2</sub> enrichment resulted in more N retention  
217 in ecosystems, especially in plant tissues and litter (Fig. S2). Because the product of biological N  
218 fixation (i.e., NH<sub>4</sub><sup>+</sup>) and the primary form for N leaching loss (i.e., NO<sub>3</sub><sup>-</sup>) can be directly used by  
219 plants, the effects of CO<sub>2</sub> enrichment on the two processes directly increase the N availability for  
220 plant growth, potentially alleviating PNL (Fig. 5). The increased N in plant tissues can be re-used  
221 by plant for multiple times via resorption (Norby et al., 2000; Norby et al., 2001), and  
222 consequently reduce the N demand from soils. This may be another mechanism that alleviates  
223 PNL (Walker et al., 2015). Therefore, the increased N availability by the increased N fixation  
224 and reduced N leaching could potentially support net accumulation of organic matter in  
225 terrestrial ecosystems (Rastetter et al., 1997; Luo and Reynolds, 1999).

226 Since biological N fixation provides at least 30% of nitrogen requirement across natural  
227 biomes (Asner et al., 2001; Galloway et al., 2004), our results suggest that the positive response  
228 of biological N fixation to CO<sub>2</sub> enrichment plays an important role in alleviating PNL. PNL was  
229 proposed to characterize long-term dynamics of carbon-nitrogen coupling in response to rising  
230 atmospheric CO<sub>2</sub> concentration. Thus, it is critical to understand long-term response of biological  
231 N fixation to elevated CO<sub>2</sub>. In this paper, we synthesize 12 studies that lasted 4 – 7 years and  
232 binned them in a long-term category (> 3 years). On average of those long-term studies, CO<sub>2</sub>

233 enrichment increased biological N fixation by 26.2%. The increased biological N fixation is  
234 supported by evidence at gene level from long-term experiments. For example, Tu et al. (2015)  
235 found the abundance of *nifH* gene amplicons, which is a widely used marker for analyzing  
236 biological N fixation, was significantly enhanced by 12-year CO<sub>2</sub> enrichment in a grassland  
237 (BioCON). However, our synthesis showed a relatively wide 95% confidence interval from 2.54%  
238 to 59.8%. The wide range can be partially attributed to the relatively small study numbers. In  
239 addition, most studies incorporated in the current synthesis were conducted in temperate regions.  
240 Thus, longer-term studies, as well as studies in other regions (e.g., boreal and tropical) are  
241 critically needed to reveal more general patterns in the future.

242       Although a general trend of PNL alleviation has been found in this study, the alleviation  
243 potential may vary across different ecosystems due to asymmetric distribution of biological N  
244 fixation (Cleveland et al., 1999). In addition, the PNL alleviation may also be influenced by  
245 other factors. While most of the long-term experiments did not show diminished CO<sub>2</sub>  
246 fertilization effect, the CO<sub>2</sub> fertilization effect on plant production decreased in two sites (i.e.,  
247 ORNL and Aspen-Birch) (Fig. 4). Plant growth is usually influenced by multiple environmental  
248 factors (e.g., nutrients, water, light, ozone, etc.). The undiminished CO<sub>2</sub> fertilization effect in  
249 most studies indicates that resources (including N) limitations are not aggravated, suggesting that  
250 no PNL occurs in these sites. However, in ORNL and Aspen-Birch (without O<sub>3</sub> treatment), the  
251 diminished CO<sub>2</sub> fertilization effect could be attributed to limitation of N, or other resources, or  
252 their combined. For example, reduced N availability has been identified as one of the primary  
253 factors that lead to the diminished CO<sub>2</sub> fertilization effect on NPP in ORNL FACE experiment  
254 (Norby et al., 2010). In Aspen-Birch community, however, deceleration of leaf area increase due  
255 to canopy closure is responsible for the diminished CO<sub>2</sub> fertilization effect without O<sub>3</sub> addition

256 (Talhelm et al., 2012). With O<sub>3</sub> addition, O<sub>3</sub> significantly reduces the canopy development,  
257 resulting in relatively open canopy during the experiment period. In addition, the negative effect  
258 of O<sub>3</sub> addition increases over time, leading to the apparent increase in the CO<sub>2</sub> fertilization effect  
259 (Fig. 4) (Talhelm et al., 2012).

260

#### 261 **4.2 Dependence of the responses of N cycle processes upon methodology, treatment** 262 **duration, N addition and ecosystem types**

263 Methodology may potentially influence the results. Cabrerizo et al. (2001) found that CO<sub>2</sub>  
264 enrichment increased nitrogenase activity measured by acetylene reduction assay (ARA), but not  
265 specific N fixation measured by H<sub>2</sub> evolution method. In studies synthesized here, four methods  
266 were used to estimate biological N fixation, including isotope, ARA, H<sub>2</sub> evolution and N  
267 accumulation. Among them, ARA and H<sub>2</sub> evolution measure nitrogenase activity (Hunt and  
268 Layzell, 1993) whereas isotope and N accumulation methods directly measure biological N  
269 fixation. All but H<sub>2</sub> evolution method showed significantly positive response to CO<sub>2</sub> enrichment  
270 (Fig. 2A). The insignificant response by H<sub>2</sub> evolution method was likely because of the small  
271 study numbers (i.e., 3). In addition, biological N fixation by ARA, isotope and N accumulation  
272 showed similar response magnitude (Fig. 2A), suggesting consistency among the three methods.  
273 However, further assessment on H<sub>2</sub> evolution method is needed.

274 The responses of biological N fixation and leaching to CO<sub>2</sub> enrichment are barely influenced  
275 by treatment duration, N addition, or ecosystem types (Fig. 3), suggesting that the alleviation of  
276 PNL by the increased biological N fixation and decreased leaching generally occurs in terrestrial  
277 ecosystems. However, the responses of other N cycle processes that affect N availability are  
278 dependent on treatment duration, N addition, and/or ecosystem types (Fig. 3).

279 N mineralization, in addition to biological N fixation, is a major source of available N in soils.  
280 The meta-analysis showed no change in the net N mineralization in response to CO<sub>2</sub> enrichment,  
281 which is consistent with the results by de Graaff et al. (2006). However, the response of net  
282 mineralization was dependent upon ecosystem types, showing no change in forests and  
283 grasslands, but significantly increase in croplands (Fig. 3H). There may be two reasons for the  
284 stimulated net mineralization in croplands. First, N fertilization, which is commonly practiced in  
285 croplands, can increase the substrate quantity and quality for the mineralization (Barrios et al.,  
286 1996; Chapin III et al., 2011; Booth et al., 2005; Lu et al., 2011; Reich and Hobbie, 2013).  
287 Second, tillage can alter the soil conditions (e.g., increasing O<sub>2</sub> content), which can potentially  
288 favor N mineralization under enriched CO<sub>2</sub> (Wienhold and Halvorson, 1999; Bardgett and  
289 Wardle, 2010). These findings suggest that CO<sub>2</sub> enrichment can stimulate the N transfer from  
290 organic to inorganic forms in managed croplands.

291 Unlike leaching, the response of nitrification is dependent upon treatment duration (Fig. 3).  
292 Nitrification was not changed by short-term treatment, but was significantly reduced by long-  
293 term CO<sub>2</sub> enrichment (Fig. 3). One possible reason for the reduced nitrification by the long-term  
294 CO<sub>2</sub> enrichment is cumulative effect of hydrological change. CO<sub>2</sub> enrichment generally reduces  
295 the stomatal conductance and the consequent water loss via plant transpiration, leading to an  
296 increase in soil water content (Niklaus et al., 1998; Tricker et al., 2009; van Groenigen et al.,  
297 2011; Keenan et al., 2013). A synthesis by van Groenigen et al. (2011) shows that CO<sub>2</sub>  
298 enrichment increases soil water content by 2.6% –10.6%. The increased soil water content may  
299 result in less oxygen (O<sub>2</sub>) content in soils, which could potentially constrain nitrification.

300 In addition, the response of gaseous N loss depends on N addition (Fig. 3). Reduced  
301 nitrification was only observed under without N addition (Fig. 3E). With N addition, no response

302 of nitrification to CO<sub>2</sub> enrichment was observed (Fig. 3E). Additionally, the response of  
303 denitrification to CO<sub>2</sub> enrichment shifted from neutral without N addition to significantly  
304 positive with N addition (Fig. 3E). One possible reason is that N addition provides more N  
305 substrate to nitrifying and denitrifying bacteria (Keller et al., 1988; Stehfest and Bouwman, 2006;  
306 Russow et al., 2008). The strengthening trends of both nitrification and denitrification lead to a  
307 shift of the response of N<sub>2</sub>O emission to CO<sub>2</sub> enrichment from neutral without N addition to  
308 significantly positive with N addition (Fig. 3E). Our results indicate that CO<sub>2</sub> enrichment  
309 significantly increases gaseous N loss when additional N is applied.

310 Our results are consistent with a previous synthesis (van Groenigen et al. 2011). The increased  
311 N<sub>2</sub>O emission can partially offset the mitigation of climate change by stimulated plant CO<sub>2</sub>  
312 assimilation as the warming potential by N<sub>2</sub>O is as 296 time as that by CO<sub>2</sub>. However, a recent  
313 modeling study by Zaehle et al. (2011) has generated an opposite result that CO<sub>2</sub> enrichment  
314 reduced radiative forcing of N<sub>2</sub>O. In their model, less availability of N substrates for nitrification  
315 and denitrification due to enhanced plant N sequestration attributed to the reduced N<sub>2</sub>O emission.  
316 Our synthesis shows that inorganic N does not decrease. Especially with additional N application,  
317 enhanced denitrification by CO<sub>2</sub> enrichment results in greater N<sub>2</sub>O emission.

318

### 319 **4.3 Changes in soil microenvironment, community structures and above-belowground** 320 **interactions**

321 The meta-analysis showed that the two major forms of soil available N, NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup>, respond  
322 to long-term CO<sub>2</sub> enrichment in opposing manners (Fig. 3C). While the enhanced biological N  
323 fixation by CO<sub>2</sub> enrichment tended to increase NH<sub>4</sub><sup>+</sup> content in soil, the reduced nitrification  
324 decreased NO<sub>3</sub><sup>-</sup> content in soils, leading to significant increase in NH<sub>4</sub><sup>+</sup>/NO<sub>3</sub><sup>-</sup> ratio (Fig. 3C).



325 Although the total available N does not change under elevated CO<sub>2</sub>, the altered proportion of  
326 NH<sub>4</sub><sup>+</sup> over NO<sub>3</sub><sup>-</sup> in soils may have long-term effects on soil microenvironment and associated  
327 aboveground-belowground linkages that control C cycle (Bardgett and Wardle, 2010). On the  
328 one hand, plants would release more hydrogen ion (H<sup>+</sup>) to regulate the charge balance when  
329 taking up more NH<sub>4</sub><sup>+</sup>. As a result, the increased NH<sub>4</sub><sup>+</sup> absorption could acidify the rhizosphere  
330 soil (Thomson et al., 1993; Monsant et al., 2008). The lowered pH could have a significant effect  
331 on soil microbial communities and their associated ecosystem functions. For example,  
332 fungal/bacterial ratio increases with the decrease in pH (de Vries et al., 2006; Rousk et al., 2009).  
333 The increased fungal/bacterial ratio may result in lower N mineralization because of the higher  
334 C/N ratio of fungi and lower turnover rates of fungal-feeding fauna (de Vries et al., 2006; Rousk  
335 and Bååth, 2007). In other words, the increased fungal/bacterial ratio may slow down the N  
336 turnover from organic to inorganic forms. On the other hand, the increased NH<sub>4</sub><sup>+</sup>/NO<sub>3</sub><sup>-</sup> ratio may  
337 increase the N use efficiency because it is more energetically expensive for plants to utilize NO<sub>3</sub><sup>-</sup>  
338 than NH<sub>4</sub><sup>+</sup> (Chapin III et al., 2011; Odum and Barrett, 2005; Lambers et al., 2008). In addition,  
339 since the preferences for plant absorption of different forms of N are different (Chapin III et al.,  
340 2011; Odum and Barrett, 2005), the increased NH<sub>4</sub><sup>+</sup>/NO<sub>3</sub><sup>-</sup> ratio may benefit some plant species  
341 while depress others, and consequently alter the community structures over time. These diverse  
342 changes in soil microenvironment and microbial and plant community compositions could  
343 further affect the terrestrial C cycle on long temporal scales, on which more studies are needed.

344

## 345 **5 Summary**

346 This synthesis provides a comprehensive assessment of the effects of CO<sub>2</sub> enrichment on  
347 terrestrial N cycle, which helps improve the understanding of the N limitation to plant growth

348 under elevated CO<sub>2</sub>. Our results indicate that elevated CO<sub>2</sub> stimulates N influx via biological N  
349 fixation but reduces N loss via leaching, increasing N availability for plant growth. The extra N  
350 supply by the enhanced biological N fixation and reduced leaching may meet the increased N  
351 demand under elevated CO<sub>2</sub>, potentially alleviating PNL. In addition, CO<sub>2</sub> enrichment increased  
352 N<sub>2</sub>O emission, especially with extra N addition. The increased N<sub>2</sub>O emission can partially offset  
353 the mitigation of climate change by stimulated plant CO<sub>2</sub> assimilation. Moreover, the changes in  
354 the soil microenvironment, ecosystem communities and above-belowground interactions induced  
355 by the different responses of NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> to CO<sub>2</sub> enrichment may have long-term effects on  
356 terrestrial biogeochemical cycles and climate change, on which further studies are needed.

357

358 *Acknowledgements.* We thank two anonymous reviewers for their valuable comments and  
359 suggestions. This study was financially supported by the US Department of Energy, Terrestrial  
360 Ecosystem Sciences grant DE SC0008270 and Biological Systems Research on the Role of  
361 Microbial Communities in Carbon Cycling Program grants DE-SC0004601 and DE-SC0010715.  
362 Authors declare no conflict of interest.

363

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524

525 **Supporting Information captions**

526 **Figure S1** Distributions of the experimental duration (**A**) and the CO<sub>2</sub> concentrations under  
527 ambient (**B**) and elevated (**C**) treatments and their difference (**D**) for the 175 collected studies.  
528 Red dashed lines represent the mean values.

529

530 **Figure S2** Summary of the effect of CO<sub>2</sub> enrichment on ecosystem level N budget. Square boxes  
531 are nitrogen pools, ovals are nitrogen processes. Red dashed boxes mean the sum of the pools in  
532 the boxes. “+”, “-”, and “ns” mean the response to CO<sub>2</sub> enrichment are positive, negative, and  
533 not significant, respectively. Please see **Figure 1** for abbreviations.

534

535 **Database S1** Database extracted from papers listed in References S1.

536

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

538

539 **Table 1.** Results on the effect of CO<sub>2</sub> enrichment on ecosystem NPP (or biomass or leaf production) in decadal-long free air CO<sub>2</sub>  
540 enrichment (FACE) experiments over treatment time. The values of the slope,  $R^2$  and  $P$  in the linear regression in **Fig. 4** are shown.  
541 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  
542 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 O<sub>3</sub>  
543 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	<b>0.04</b>	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	<b>0.03</b>	Schneider et al., 2004
NDFE	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	<b>0.01</b>	Talhelm et al., 2012
AspenBirch_O	Forest	7	Leaf production	6.48	0.82	<b>0.00</b>	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

544

545 **Figure captions**

546 **Figure 1.** Results of a meta-analysis on the responses of nitrogen pools and processes to  
547 CO<sub>2</sub> enrichment. In (A), APNP, BPNP, TPNP, LNP, and SNP are the abbreviations for  
548 aboveground plant nitrogen pool, belowground plant nitrogen pool, total plant nitrogen  
549 pool, litter nitrogen pool, and soil nitrogen pool, respectively. In (C), TIN, NH<sub>4</sub><sup>+</sup> and  
550 NO<sub>3</sub><sup>-</sup> are total inorganic nitrogen, ammonium, and nitrate in soils, respectively. The error  
551 bars represent 95% confidence intervals.

552

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

556

557 **Figure 3.** Responses of terrestrial nitrogen pools and processes to CO<sub>2</sub> enrichment (Mean  
558 ± 95% confidence interval) as regulated by experimental durations (A – C; short-term: ≤  
559 3 years vs. long-term: > 3 years), nitrogen addition (D – F), and ecosystem types (G – I).  
560 Please see Figure 1 for abbreviations.

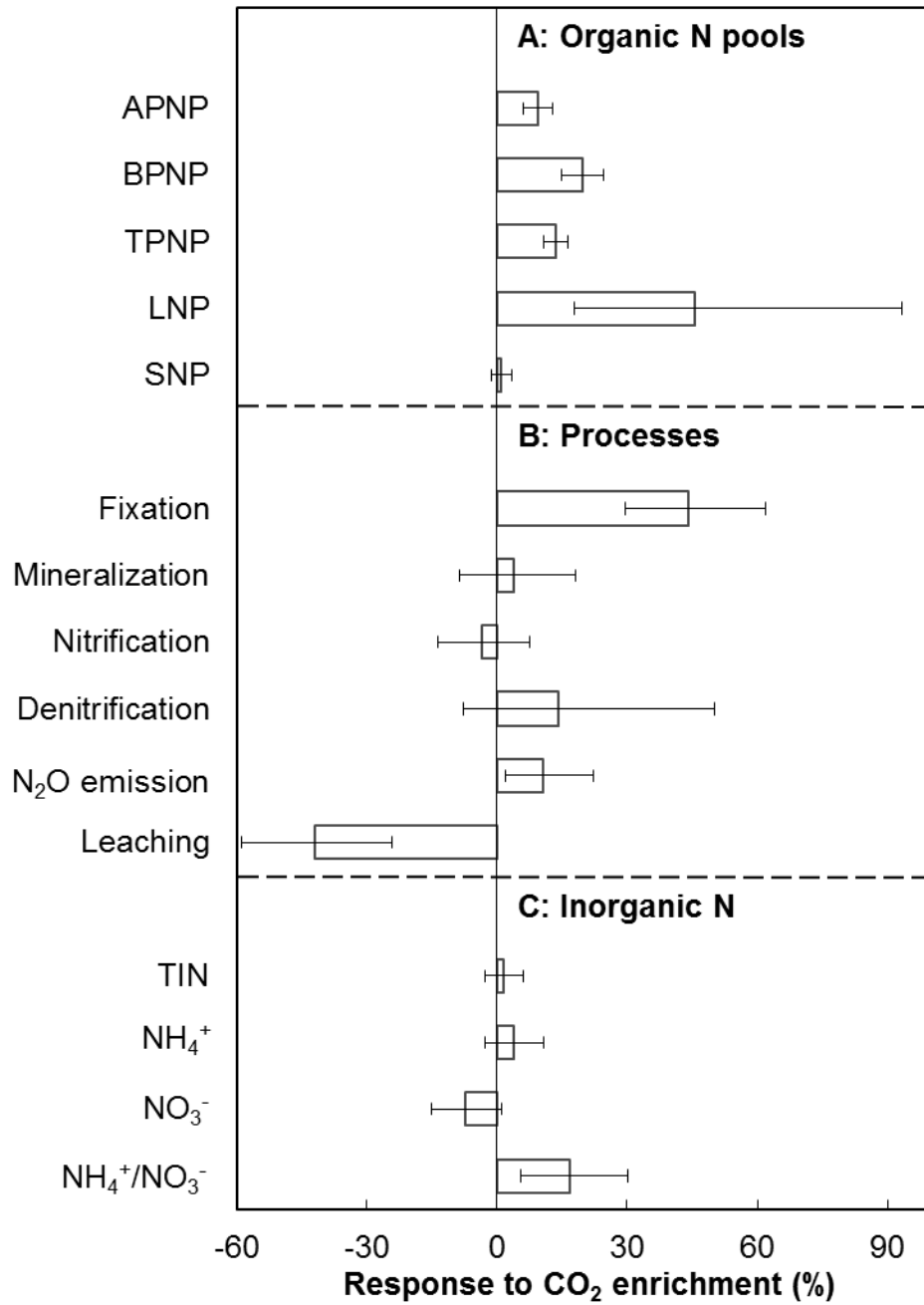
561

562 **Figure 4.** Time courses of CO<sub>2</sub> effects on ecosystem NPP (or biomass or leaf production)  
563 in decadal-long FACE experiments. Please see **Table 1** for details of experiments,  
564 references and statistical results. Only statistically significant ( $P < 0.05$ ) regression lines  
565 are shown. The panel at the right-low corner shows the distribution of the slopes (-0.37%  
566 year<sup>-1</sup> with 95% CI from -1.84% year<sup>-1</sup> to 1.09% year<sup>-1</sup>).

567

568 **Figure 5.** Mechanisms that alleviate PNL. PNL hypothesis posits that the stimulated  
569 plant growth by CO<sub>2</sub> enrichment leads to more N sequestered in long-lived plant tissues,  
570 litter and soil organic matter (SOM) so that, the N availability for plant growth  
571 progressively declines over time, and plant growth is downregulated (grey symbols). The  
572 current synthesis indicates that the basis of PNL occurrence partially exists (i.e., more N  
573 sequestered in plant tissues and litter; black symbols). Despite of the increases in plant N  
574 sequestration and N<sub>2</sub>O emission, stimulated biological N fixation and reduced N leaching  
575 can replenish the N availability, potentially alleviating PNL (blue boxes and arrows).  
576 Upward, downward, and horizontal arrows mean increase, decrease, and no change,  
577 respectively.  
578

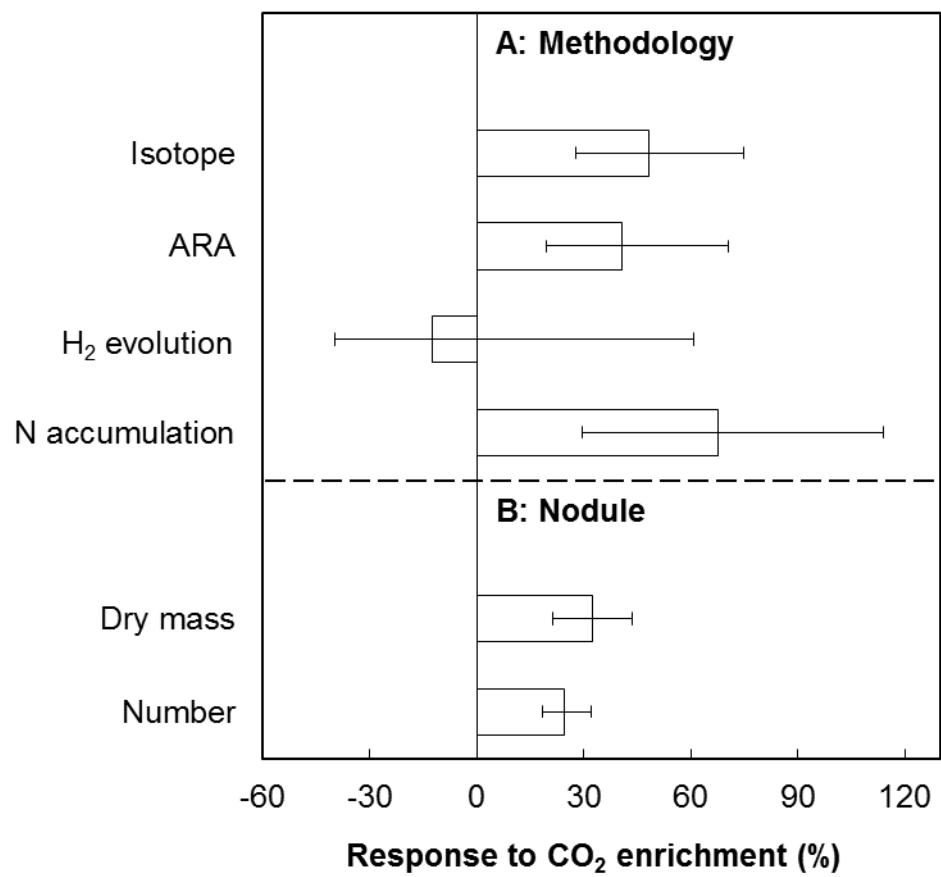
579 **Figure 1.**



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581

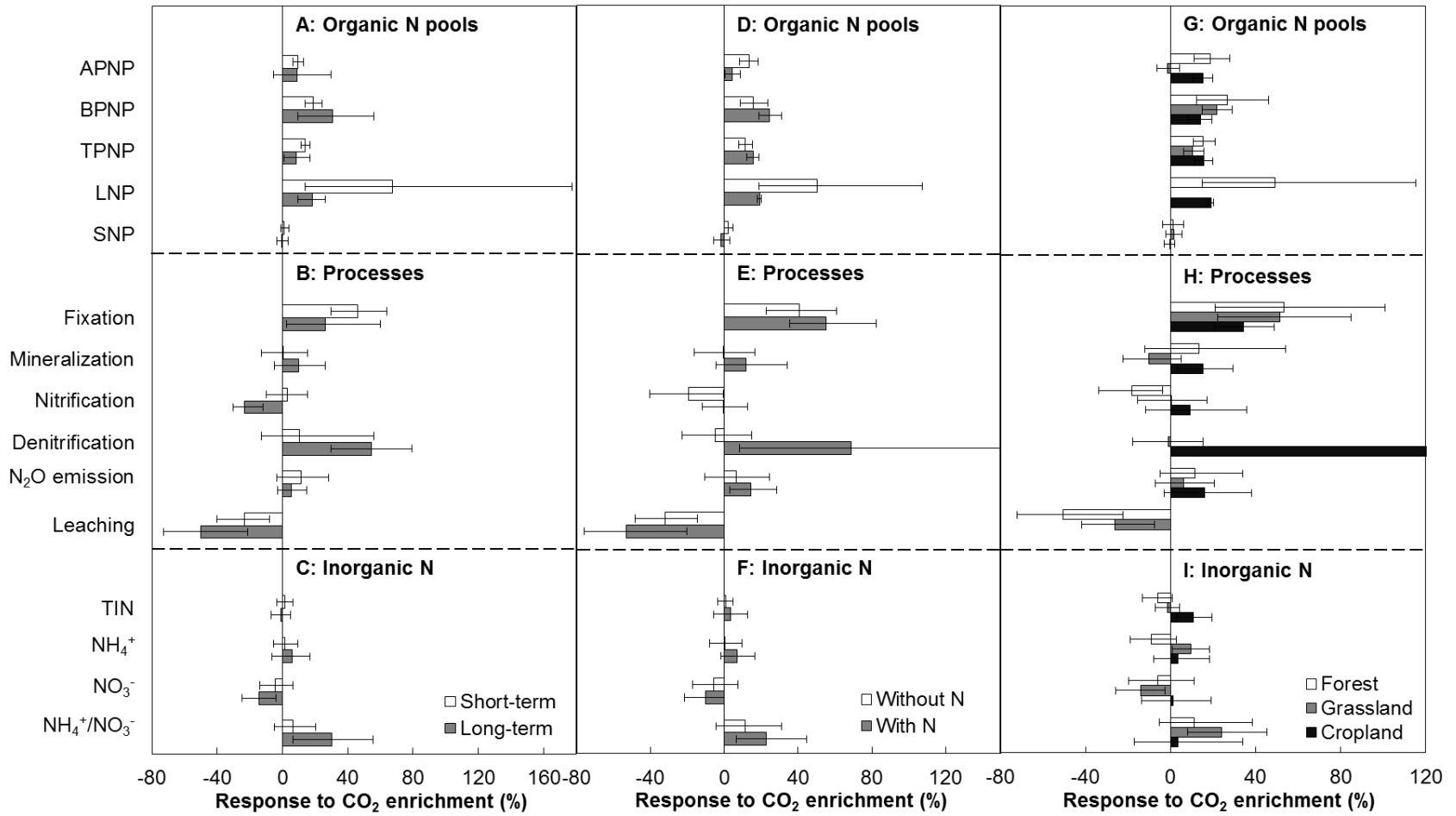
582 **Figure 2.**



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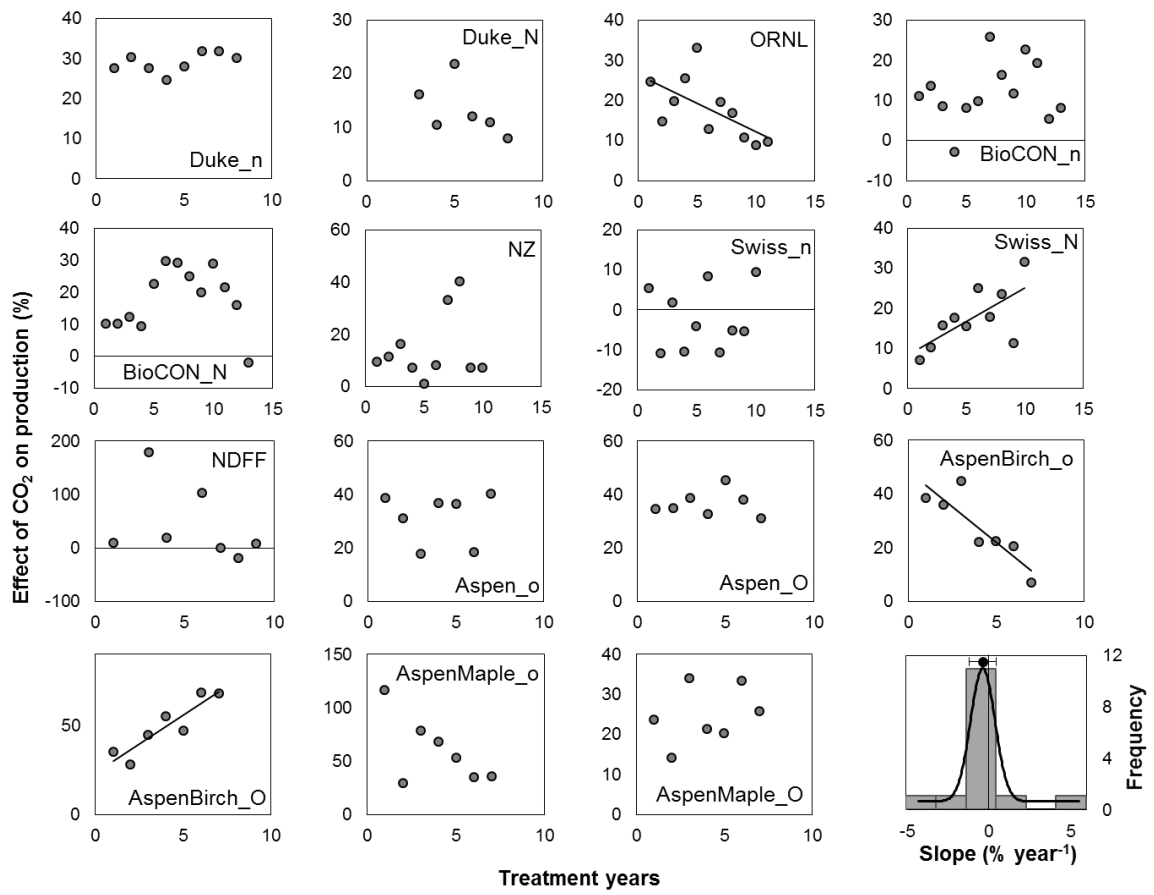
585 **Figure 3.**



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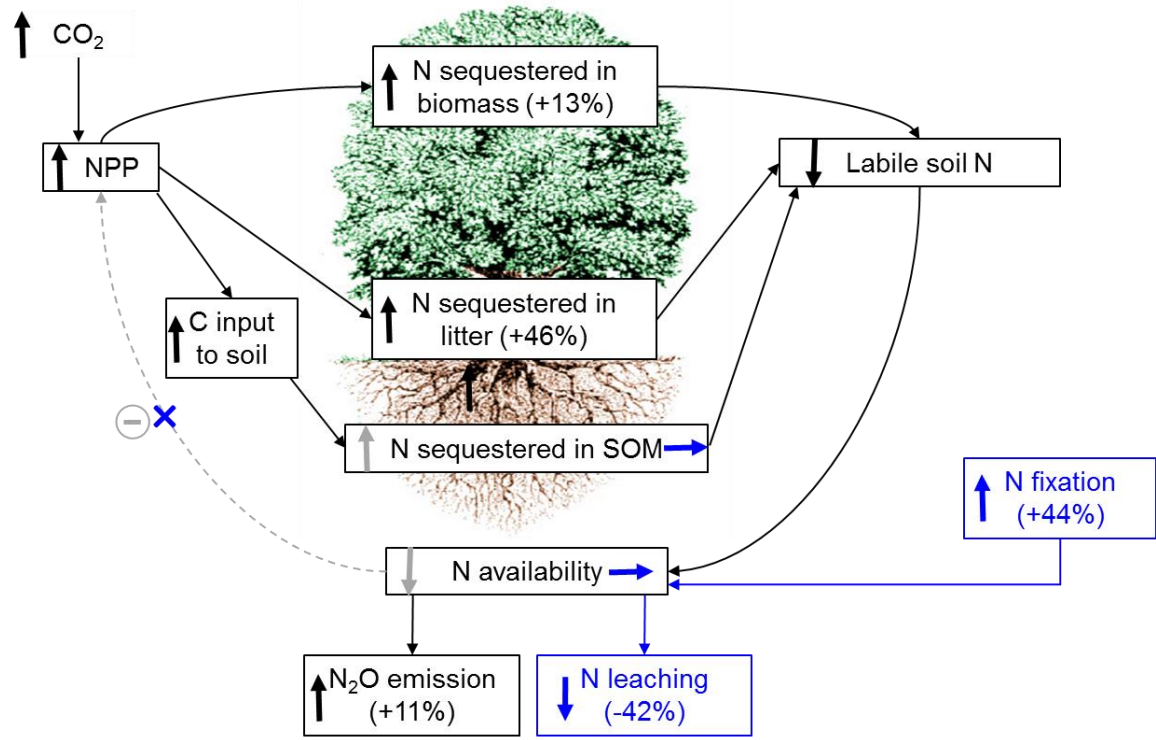
587 **Figure 4.**



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590 **Figure 5.**



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