

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

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14

15 **Abstract:** The nitrogen (N) cycle has the potential to regulate climate change through its
16 influence on carbon (C) sequestration. Although extensive research has explored whether or not
17 progressive N limitation (PNL) occurs under CO₂ enrichment, a comprehensive assessment of
18 the processes that regulate PNL is still lacking. Here, we quantitatively synthesized the responses
19 of all major processes and pools in the terrestrial N cycle with meta-analysis of CO₂
20 experimental data available in the literature. The results showed that CO₂ enrichment
21 significantly increased N sequestration in the plant and litter pools but not in the soil pool,
22 partially supporting one of the basic assumptions in the PNL hypothesis that elevated CO₂ results
23 in more N sequestered in organic pools. However, CO₂ enrichment significantly increased the N
24 influx via biological N fixation and the loss via N₂O emission, but decreased the N efflux via
25 leaching. In addition, no general diminished CO₂ fertilization effect on plant growth was
26 observed over time up to the longest experiment of 13 years. Overall, our analyses suggest that
27 the extra N supply by the increased biological N fixation and decreased leaching may potentially
28 alleviate PNL under elevated CO₂ conditions in spite of the increases in plant N sequestration
29 and N₂O emission. Moreover, our syntheses indicate that CO₂ enrichment increases soil
30 ammonium (NH₄⁺) to nitrate (NO₃⁻) ratio. The changed NH₄⁺/NO₃⁻ ratio and subsequent
31 biological processes may result in changes in soil microenvironments, above-belowground
32 community structures and associated interactions, which could potentially affect the terrestrial
33 biogeochemical cycles. In addition, our data synthesis suggests that more long-term studies,
34 especially in regions other than temperate ones, are needed for comprehensive assessments of the
35 PNL hypothesis.

36

37 **1 Introduction**

38 Fossil-fuel burning and deforestation have led to substantial increase in atmospheric carbon
39 dioxide (CO₂) concentrations, which could stimulate plant growth (IPCC, 2013). The plant
40 growth stimulated by CO₂ fertilization and the resulting terrestrial carbon (C) storage could
41 partially mitigate the further increase in CO₂ concentrations and associated climate warming
42 (IPCC, 2013). However, this effect may be constrained by the availability of nitrogen (N), an
43 essential element for molecular compounds of amino acids, proteins, ribonucleic acids (RNAs)
44 and deoxyribonucleic acids (DNAs) in organisms (Rastetter et al., 1997; Oren et al., 2001; Luo et
45 al., 2004; Reich et al., 2006; Norby et al., 2010; Reich and Hobbie, 2013). A popular hypothesis
46 of the N constraint to the CO₂ fertilization effect is progressive N limitation (PNL) (Luo et al.,
47 2004).

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

58 Since the key determining PNL occurrence is that whether N supply meets N demand (Luo et
59 al., 2004), it is important to understand how N supply changes under elevated CO₂. The change

60 in the N supply for plant growth under elevated CO₂ is determined by the responses of multiple
61 N cycling processes, including biological N fixation, mineralization, nitrification, denitrification,
62 and leaching (Chapin III et al., 2011). In addition, the responses of these processes to CO₂
63 enrichment may be influenced by external N addition, such as N deposition and fertilization
64 (Reay et al., 2008). Thus, synthesizing the responses of processes that regulate PNL to CO₂
65 enrichment may help reveal the general pattern of PNL in terrestrial ecosystems.

66 In the current study, the main objective was to synthesize data published in the literature on the
67 N limitation to plant growth under enriched CO₂ conditions. Our data synthesis was designed to
68 answer two questions: (i) How do the major processes in the terrestrial N cycle respond to CO₂
69 enrichment? (ii) Does the CO₂ fertilization effect on plant growth diminish over time? To answer
70 these questions, two sets of data from the literature were collected (Table S1, Table 1). With the
71 first dataset, we quantitatively examined the effects of CO₂ enrichment on all the major
72 processes and pools in the N cycle using meta-analysis. These processes and pools included N
73 sequestered in organic components (i.e., plant tissues, litter and soil organic matter (SOM)),
74 biological N fixation, net mineralization, nitrification, denitrification, leaching, and total
75 inorganic N (TIN), ammonium (NH₄⁺) and nitrate (NO₃⁻) contents in soils. We separated the first
76 dataset according to the experimental durations to explore the responses of the N processes to
77 short- vs. long-term CO₂ treatments. In addition, the responses of the N processes to CO₂
78 enrichment were compared between without and with N addition conditions. The second dataset
79 was compiled for the plant growth in decadal free air CO₂ enrichment (FACE) experiments.
80 With the dataset, we explored whether the CO₂ fertilization effect on plant growth diminishes or
81 not over time.

82 2 Materials and Methods

83 2.1 Data collection

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

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

$$99 \quad SD = SE\sqrt{n} \quad \text{Eq. (1)}$$

$$100 \quad \text{or } SD = (CI_u - CI_l)\sqrt{n}/2u_p \quad \text{Eq. (2)}$$

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

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

$$110 \quad M = \sum_{i=1}^j \frac{M_i}{j} \quad \text{Eq. (3)}$$

111 with standard deviation

$$112 \quad SD = \sqrt{\frac{\sum_{i=1}^j SD_i^2 (n_i - 1) n_i}{(\sum_{i=1}^j n_i - 1) \sum_{i=1}^j n_i}} \quad \text{Eq. (4)}$$

113 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
 114 sampling data, respectively (Liang et al., 2013). If additional treatments applied (e.g., N addition),
 115 they were treated as independent studies.

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

121 For the second dataset, 15 available time series of plant growth were collected from 7 decadal
 122 FACE experiments (Table 1). The ecosystems included 9 forests, 5 grasslands and 1 desert.
 123 Because of the limited data, we included variables that can represent plant growth in one way or
 124 another, for example, net primary production (NPP), biomass, and leaf production. These data
 125 were collected to reveal whether the effect of CO₂ enrichment on plant growth diminishes over
 126 treatment time as proposed by the PNL hypothesis (Luo et al., 2004). In the 7 studies, the

127 treatment lasted from 7 to 13 years, and at least 6 years' production measurements were reported.
 128 For each data, the percentage change in NPP (or biomass or leaf production) by CO₂ enrichment
 129 was calculated. Then, a linear regression between the percentage change and the treatment year
 130 was conducted. A significantly negative slope indicates that the effect of CO₂ enrichment on the
 131 plant production diminishes over time. A non-significant slope was treated as 0. After deriving
 132 all the slopes, the frequency distribution of the slopes were fitted by a Gaussian function:

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

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

136

137 **2.2 Meta-analysis**

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

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

142 where X_E and X_C are the variable values under enriched CO₂ and control conditions, respectively.

143 The variation of the $\log RR$ was

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

145 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
 146 of X_C and X_E .

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

149
$$M_{weighted} = \frac{\sum_{j=1}^k W_j^* M_j}{\sum_{j=1}^k W_j^*}$$
 Eq. (8)

150 with the variance as

151
$$V_{weighted} = \frac{1}{\sum_{j=1}^k W_j^*}$$
 Eq. (9)

152 where k is the number of studies, M_j is the $Ln(RR)$ in study j , and W_j^* is the weighting factor
153 which consists of between- and within-study variances (Rosenberg et al., 2000; Liang et al.,
154 2013). The 95% lower and upper limits ($LL_{weighted}$ and $UL_{weighted}$) for the weighted mean were
155 computed as

156
$$LL_{weighted} = M_{weighted} - 1.96 \times \sqrt{V_{weighted}}$$
 Eq. (10)

157 and

158
$$UL_{weighted} = M_{weighted} + 1.96 \times \sqrt{V_{weighted}}$$
 Eq. (11)

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

164

165 **3 Results**

166 The meta-analysis of the first dataset showed that CO₂ enrichment significantly increased N
167 sequestered in plants and litter but not in SOM (Figs. 1A, S2). Whereas CO₂ enrichment had
168 little overall effects on N mineralization, nitrification and denitrification, it significantly
169 increased biological N fixation by 44.3% (with 95% CI from 29.5% to 61.8%). The increased
170 biological N fixation was consistent when using various methods except H₂ evolution (Fig. 2A).
171 In legume species, CO₂ enrichment significantly increased nodule mass and number (Fig. 2B). In
172 addition, CO₂ enrichment increased N₂O emission by 10.7% (with 95% CI from 2.0% to 22.3%),
173 but reduced leaching (i.e., -41.8% with 95% CI from -58.9% to -24.3%) (Fig. 1B). Although CO₂
174 enrichment did not change the total inorganic N availability in soils, it increased the soil
175 NH₄⁺/NO₃⁻ ratio by 16.9% (with 95% CI from 5.4% to 30.2%) (Fig. 1C).

176 Treatment time had no effect on most of the variables (overlapped 95% CIs for short- and
177 long-term treatments) except nitrification, which was not changed by short-term treatment, but
178 was significantly reduced (-23.4% with 95% CI from -30.4% to -12.1%) by long-term CO₂
179 enrichment (Fig. 3B). In addition, it seemed that the responses of the NH₄⁺/NO₃⁻ ratio was
180 strengthened over time, representing a neutral response to short-term CO₂ enrichment, but
181 significantly positive and negative responses to long-term CO₂ enrichment (Fig. 3C). The effects
182 of CO₂ enrichment were influenced by N addition (Fig. 3D – F). For example, nitrification was
183 significantly reduced by CO₂ enrichment without N addition by 19.3% (with 95% CI from -40.5%
184 to -0.65%), but was not changed with N addition. Denitrification and N₂O emission responded to
185 CO₂ enrichment neutrally without N addition, but significantly positively with N addition (Fig.
186 3E). Additionally, the responses of some variables to CO₂ enrichment were dependent on
187 ecosystem type (Fig. 3G – I). APNP responded to CO₂ enrichment positively in forests and

188 croplands, but neutrally in grasslands (Fig. 3G). Net mineralization had no response to CO₂
189 enrichment in forests or grasslands, while it was significantly increased in croplands (Fig. 3H).
190 Moreover, the change in the TIN was neutral in forests, grassland, but positive, in croplands,
191 respectively (Fig. 3I). In addition, a positive response of the NH₄⁺/NO₃⁻ ratio was only observed
192 in grasslands (Fig. 3I).

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

200

201 **4 Discussion**

202 In this study, we carried out two syntheses on the responses of the terrestrial N cycle and plant
203 growth to CO₂ enrichment to test whether PNL generally occurs across ecosystems.

204

205 **4.1 PNL alleviation**

206 According to the PNL hypothesis, a prerequisite for PNL occurrence is that more N is
207 sequestered in plant, litter and SOM (Luo et al., 2004). Our results showed that elevated CO₂
208 significantly increased N retention in plant tissues and litter, which is consistent with previous
209 meta-analyses (de Graaff et al., 2006; Luo et al., 2006). Thus, there seems to be evidence for
210 some basic assumptions of the PNL hypothesis. However, the results from the second dataset did

211 not show a general diminished CO₂ fertilization effect on plant growth on the decadal scale,
212 which disagrees with the expectation of the PNL hypothesis, suggesting that N supply under
213 elevated CO₂ may meet the N demand. In this study, we have identified two processes that
214 increase N supply under elevated CO₂, i.e., biological N fixation and leaching.

215 CO₂ enrichment significantly enhanced the N influx to terrestrial ecosystems through
216 biological N fixation, which reduces dinitrogen (N₂) to NH₄⁺ (Fig. 1B). The enhanced biological
217 N fixation may have resulted from the stimulated activities of symbiotic (Fig. 2B) and free-living
218 heterotrophic N-fixing bacteria (Hoque et al., 2001). In addition, the competition between N₂-
219 fixing and non-N₂-fixing species may have contributed to enhance the biological N fixation at
220 the ecosystem level (Poorter and Navas, 2003; Batterman et al., 2013).

221 In addition, the N efflux via leaching was reduced under elevated CO₂ conditions (Fig. 1B).
222 This could be attributed to the decrease in NO₃⁻, which is the primary N form in leaching,
223 (Chapin III et al., 2011), and the increased root growth which may immobilize more inorganic N
224 in soils (Luo et al., 2006; Iversen, 2010). In contrast, gaseous N loss through N₂O emission
225 increased under elevated CO₂, although this increase was only observed when additional N was
226 applied.

227 The net effect of the responses of N processes to CO₂ enrichment resulted in higher N
228 retention in ecosystems, especially within plant tissues and litter (Fig. S2). Because the product
229 of biological N fixation (i.e., NH₄⁺) and the primary form for N leaching loss (i.e., NO₃⁻) can be
230 directly used by plants, the effects of CO₂ enrichment on the two processes directly increase the
231 N availability for plant growth, potentially alleviating PNL (Fig. 5). The increased N in plant
232 tissues can be re-used by plants via resorption (Norby et al., 2000; Norby et al., 2001), and
233 consequently reduce the N demand from soils. This may be another mechanism that alleviates

234 PNL (Walker et al., 2015). Therefore, the increased N availability from increased N fixation and
235 reduced N leaching could potentially support net accumulation of organic matter in terrestrial
236 ecosystems (Rastetter et al., 1997; Luo and Reynolds, 1999).

237 Since biological N fixation provides at least 30% of the N requirement across natural biomes
238 (Asner et al., 2001; Galloway et al., 2004), our results suggest that the positive response of
239 biological N fixation to CO₂ enrichment plays an important role in alleviating PNL. The PNL
240 hypothesis was proposed to characterize long-term dynamics of C-N coupling in response to
241 rising atmospheric CO₂ concentration. Thus, it is critical to understand the long-term response of
242 biological N fixation to elevated CO₂. In this paper, we have synthesized 12 studies that lasted 4
243 – 7 years and binned them in a long-term category (> 3 years). On average, in those long-term
244 studies, CO₂ enrichment increased biological N fixation by 26.2%. The increased biological N
245 fixation is supported by evidence at gene level from long-term experiments. For example, Tu et
246 al. (2015) found that the abundance of *nifH* gene amplicons, which is a widely used marker for
247 analyzing biological N fixation, was significantly enhanced by 12 years of CO₂ enrichment in a
248 grassland (BioCON). However, our synthesis showed a relatively wide 95% confidence interval
249 from 2.54% to 59.8%. The wide range can be partially attributed to the relatively small number
250 of studies. In addition, most studies incorporated in the current synthesis were conducted in
251 temperate regions. Thus, longer-term studies, as well as studies in other regions (e.g., boreal and
252 tropical) are critically needed to reveal more general patterns in the future.

253 In this study, it is suggested that the general trend of the N cycle changes under elevated CO₂
254 converges towards increased soil N supply for plant growth, which in theory could alleviate PNL.
255 However, the PNL alleviation potential may vary across different ecosystems due to asymmetric
256 distributions of biological N fixation (Cleveland et al., 1999). In addition, PNL alleviation may

257 also be influenced by other factors. While a diminished CO₂ fertilization effect on plant growth
258 was not observed in most of the long-term experiments, it occurred in two sites (i.e., ORNL and
259 Aspen-Birch) (Fig. 4). Plant growth is usually influenced by multiple environmental factors (e.g.,
260 nutrients, water, light, ozone). The undiminished CO₂ fertilization effect in most studies indicates
261 that resource limitation (including N) was not aggravated, suggesting that no PNL occurred in
262 these sites. However, in the ORNL and Aspen-Birch (without O₃ treatment) experiments, the
263 diminished CO₂ fertilization effect on plant growth was potentially driven by limitation of N, or
264 other resources, or their combined effect. For example, reduced N availability has been identified
265 as one of the primary factors that lead to the diminished CO₂ fertilization effect on NPP in the
266 ORNL FACE experiment (Norby et al., 2010). In the Aspen-Birch community, however, the
267 deceleration of leaf area increases due to canopy closure was responsible for the diminished CO₂
268 fertilization effect on plant growth without O₃ addition (Talhelm et al., 2012). With O₃ addition,
269 O₃ significantly reduced the canopy development, resulting in a relatively open canopy during
270 the experiment period. In addition, the negative effect of O₃ addition increased over time, leading
271 to the apparent increase in the CO₂ fertilization effect (Fig. 4) (Talhelm et al., 2012).

272

273 **4.2 Dependence of the responses of N cycling processes upon methodology, treatment**

274 **duration, N addition and ecosystem types**

275 Experimental methodology may potentially influence findings. Cabrerizo et al. (2001) found that
276 CO₂ enrichment increased the nitrogenase activity measured by acetylene reduction assay (ARA),
277 but not the specific N fixation measured by the H₂ evolution method. In the studies synthesized
278 here, four methods were used to estimate biological N fixation, including isotope, ARA, H₂
279 evolution and N accumulation. Among them, ARA and H₂ evolution measure nitrogenase

280 activity (Hunt and Layzell, 1993) whereas isotope and N accumulation methods directly measure
281 biological N fixation. All but the H₂ evolution method showed a significantly positive response
282 to CO₂ enrichment (Fig. 2A). The insignificant response shown by the H₂ evolution method was
283 likely because of the small study numbers (i.e., 3). In addition, the biological N fixation
284 measured by ARA, isotope and N accumulation showed similar response magnitudes (Fig. 2A),
285 suggesting consistency among the three methods. However, further assessment on the H₂
286 evolution method is needed.

287 The responses of some N cycling processes that affect N availability are dependent on
288 treatment duration, N addition, and/or ecosystem types (Fig. 3).

289 N mineralization, in addition to biological N fixation, is a major source of available N in soils.
290 Our meta-analysis showed no change in the net N mineralization in response to CO₂ enrichment,
291 which is consistent with the results by de Graaff et al. (2006). However, the response of net
292 mineralization was dependent upon ecosystem types, showing no change in forests and
293 grasslands, but significant increases in croplands (Fig. 3H). There may be two reasons for the
294 stimulated net mineralization in croplands. First, N fertilization, which is commonly practiced in
295 croplands, can increase the substrate quantity and quality for mineralization (Barrios et al., 1996;
296 Chapin III et al., 2011; Booth et al., 2005; Lu et al., 2011; Reich and Hobbie, 2013). Second,
297 tillage can alter soil conditions (e.g., increasing O₂ content), which can potentially favor the N
298 mineralization under enriched CO₂ (Wienhold and Halvorson, 1999; Bardgett and Wardle, 2010).
299 These findings suggest that CO₂ enrichment can stimulate the N transfer from organic to
300 inorganic forms in managed croplands.

301 Unlike leaching, the response of nitrification was dependent upon treatment duration (Fig. 3).
302 Nitrification was not changed by short-term treatment, but was significantly reduced by long-

303 term CO₂ enrichment (Fig. 3). One possible reason for the reduced nitrification with long-term
304 CO₂ enrichment is the cumulative effect of hydrological changes. CO₂ enrichment is assumed to
305 reduce stomatal conductance and, consequently, water loss via plant transpiration, leading to an
306 increase in soil water content (Niklaus et al., 1998; Tricker et al., 2009; van Groenigen et al.,
307 2011; Keenan et al., 2013). A synthesis by van Groenigen et al. (2011) shows that CO₂
308 enrichment increases soil water content by 2.6%–10.6%. Increased soil water content may result
309 in less oxygen (O₂) concentration in soils, which could potentially constrain nitrification.

310 In addition, the response of gaseous N loss was dependent on N addition (Fig. 3). The reduced
311 nitrification was only observed under conditions without N addition (Fig. 3E). With N addition,
312 no response of nitrification to CO₂ enrichment was observed (Fig. 3E). Additionally, the
313 response of denitrification to CO₂ enrichment shifted from neutral, without N addition, to
314 significantly positive with N addition (Fig. 3E). One possible reason is that N addition provides
315 more N substrate for nitrifying and denitrifying bacteria (Keller et al., 1988; Stehfest and
316 Bouwman, 2006; Russow et al., 2008). The strengthening trends of both nitrification and
317 denitrification led to a shift of the response of N₂O emission to CO₂ enrichment from neutral
318 without N addition to significantly positive with N addition (Fig. 3E). Our results indicate that
319 CO₂ enrichment significantly increases gaseous N loss when additional N is applied. This is
320 consistent with a previous synthesis (van Groenigen et al. 2011). Increased N₂O emissions can
321 partially offset the mitigation of climate change by the stimulated plant CO₂ assimilation as the
322 warming potential of N₂O is 296 times that of CO₂. However, a recent modeling study by Zaehle
323 et al. (2011) found an opposite result showing that CO₂ enrichment reduced emissions of N₂O. In
324 their model, elevated CO₂ enhanced plant N sequestration and consequently, decreased the N
325 availability for nitrification and denitrification in soils, which led to the reduced N₂O emissions.

326 However, our synthesis shows that inorganic N does not decrease. Especially with additional N
327 application, enhanced denitrification by CO₂ enrichment results in a greater N₂O emission.

328

329 **4.3 Changes in soil microenvironment, community structures and above-belowground** 330 **interactions**

331 The meta-analysis showed that the two major forms of soil available N, NH₄⁺ and NO₃⁻,
332 responded to long-term CO₂ enrichment in opposing manners (Fig. 3C). While the enhanced
333 biological N fixation by CO₂ enrichment tended to increase the NH₄⁺ content in soils, the
334 reduced nitrification decreased the NO₃⁻ content in soils, leading to a significant increase in the
335 NH₄⁺/NO₃⁻ ratio (Fig. 3C).

336 Although the total available N did not change under elevated CO₂, the altered proportion of
337 NH₄⁺ over NO₃⁻ in soils may have long-term effects on soil microenvironment and associated
338 aboveground-belowground linkages that control the C cycle (Bardgett and Wardle, 2010). On the
339 one hand, plants would release more hydrogen ion (H⁺) to regulate the charge balance when
340 taking up more NH₄⁺. As a result, the increased NH₄⁺ absorption could acidify the rhizosphere
341 soil (Thomson et al., 1993; Monsanto et al., 2008). The lowered pH could have significant effects
342 on soil microbial communities and their associated ecosystem functions. For example,
343 fungal/bacterial ratio increases with the decrease in pH (de Vries et al., 2006; Rousk et al., 2009).
344 The increased fungal/bacterial ratio may result in lower N mineralization because of the higher
345 C/N ratio of fungi and the lower turnover rates of fungal-feeding fauna (de Vries et al., 2006;
346 Rousk and Bååth, 2007). In other words, the increased fungal/bacterial ratio may slow down the
347 N turnover from organic to inorganic forms. On the other hand, the increased NH₄⁺/NO₃⁻ ratio
348 may increase the N use efficiency because it is more energetically expensive for plants to utilize

349 NO_3^- than NH_4^+ (Chapin III et al., 2011; Odum and Barrett, 2005; Lambers et al., 2008). In
350 addition, since the preferences for plant absorption of different forms of N are different (Chapin
351 III et al., 2011; Odum and Barrett, 2005), the increased $\text{NH}_4^+/\text{NO}_3^-$ ratio may benefit some plant
352 species while depress others, and consequently alter the community structures over time. These
353 diverse changes in soil microenvironment and microbial and plant community compositions
354 could further affect the terrestrial C cycle on long temporal scales, on which more studies are
355 needed.

356

357 **5 Summary**

358 This study synthesizes data in the literature on the effects of CO_2 enrichment on the terrestrial N
359 cycle to improve our understanding of the N limitation to plant growth under elevated CO_2 . Our
360 results indicate that elevated CO_2 stimulates N influx via biological N fixation but reduces N loss
361 via leaching, leading to increased N supply for plant growth. The additional N supply via the
362 enhanced biological N fixation and the reduced leaching may partially meet the increased N
363 demand under elevated CO_2 , potentially alleviating PNL. In addition, our analysis indicates that
364 increased N_2O emissions may partially offset the mitigation of climate change by stimulated
365 plant CO_2 assimilation. Moreover, changes in soil microenvironments, ecosystem communities
366 and above-belowground interactions induced by the different responses of NH_4^+ and NO_3^- to
367 CO_2 enrichment may have long-term effects on the terrestrial biogeochemical cycles and climate
368 change.

369

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376

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537

538 **Supporting Information captions**

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

542

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

547

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

549

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

551

552 **Table 1.** Results on the effect of CO₂ enrichment on ecosystem NPP (or biomass or leaf production) in decadal-long free air CO₂
553 enrichment (FACE) experiments over treatment time. The values of the slope, R^2 and P in the linear regression in **Fig. 4** are shown.
554 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
555 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
556 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
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	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

557

558 **Figure captions**

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

565

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

569

570 **Figure 3.** Responses of terrestrial nitrogen pools and processes to CO₂ enrichment (Mean
571 ± 95% confidence interval) as regulated by experimental durations (A – C; short-term: ≤
572 3 years vs. long-term: > 3 years), nitrogen addition (D – F), and ecosystem types (G – I).
573 Please see Figure 1 for abbreviations.

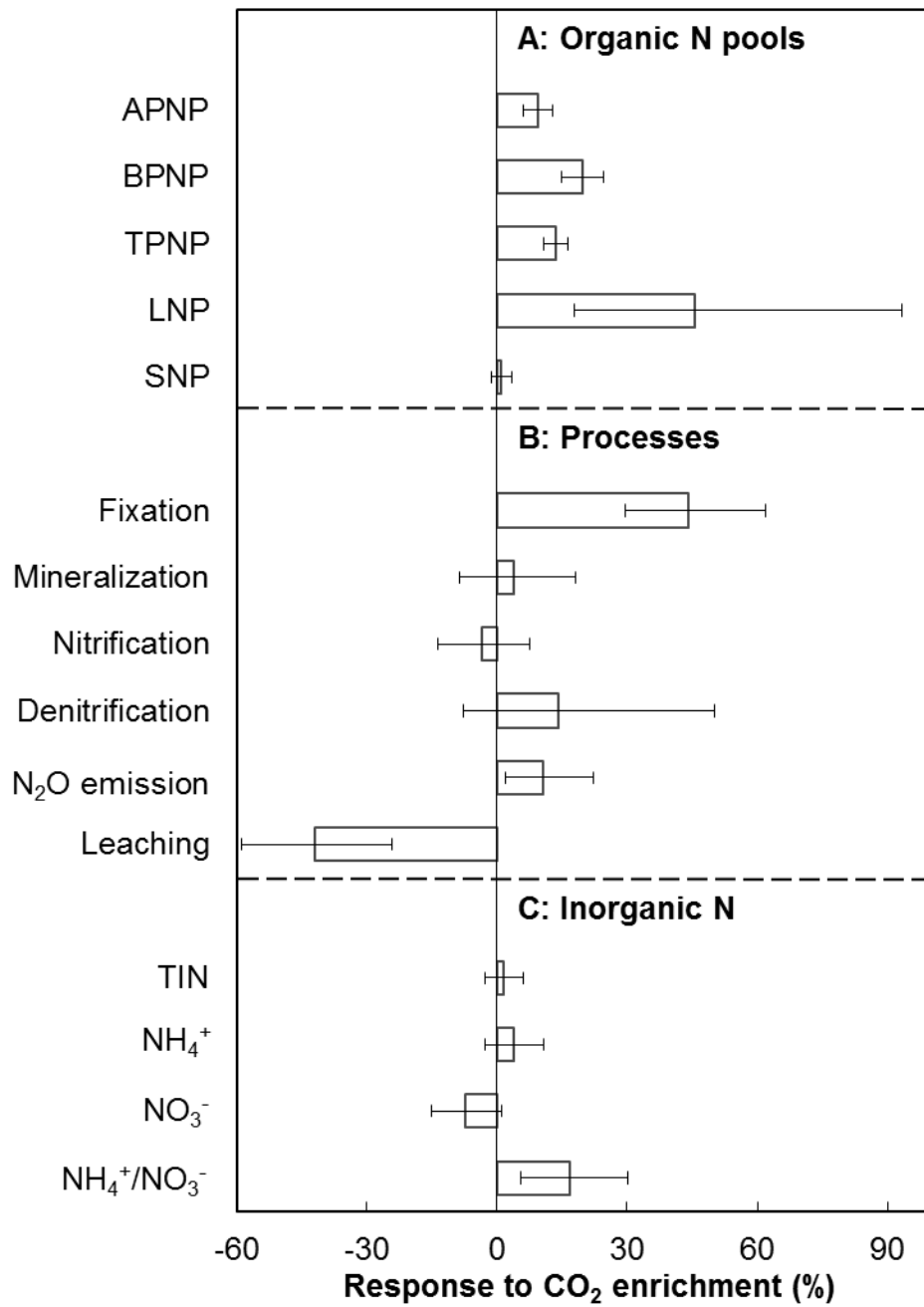
574

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

580

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

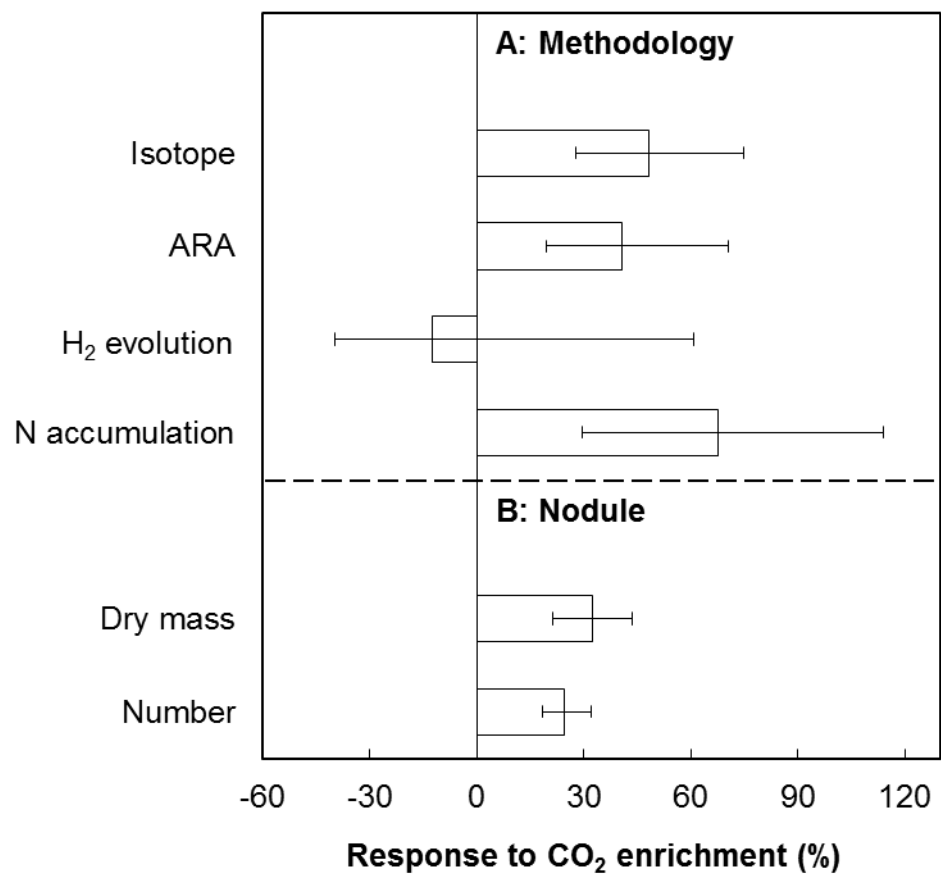
592 **Figure 1.**



593

594

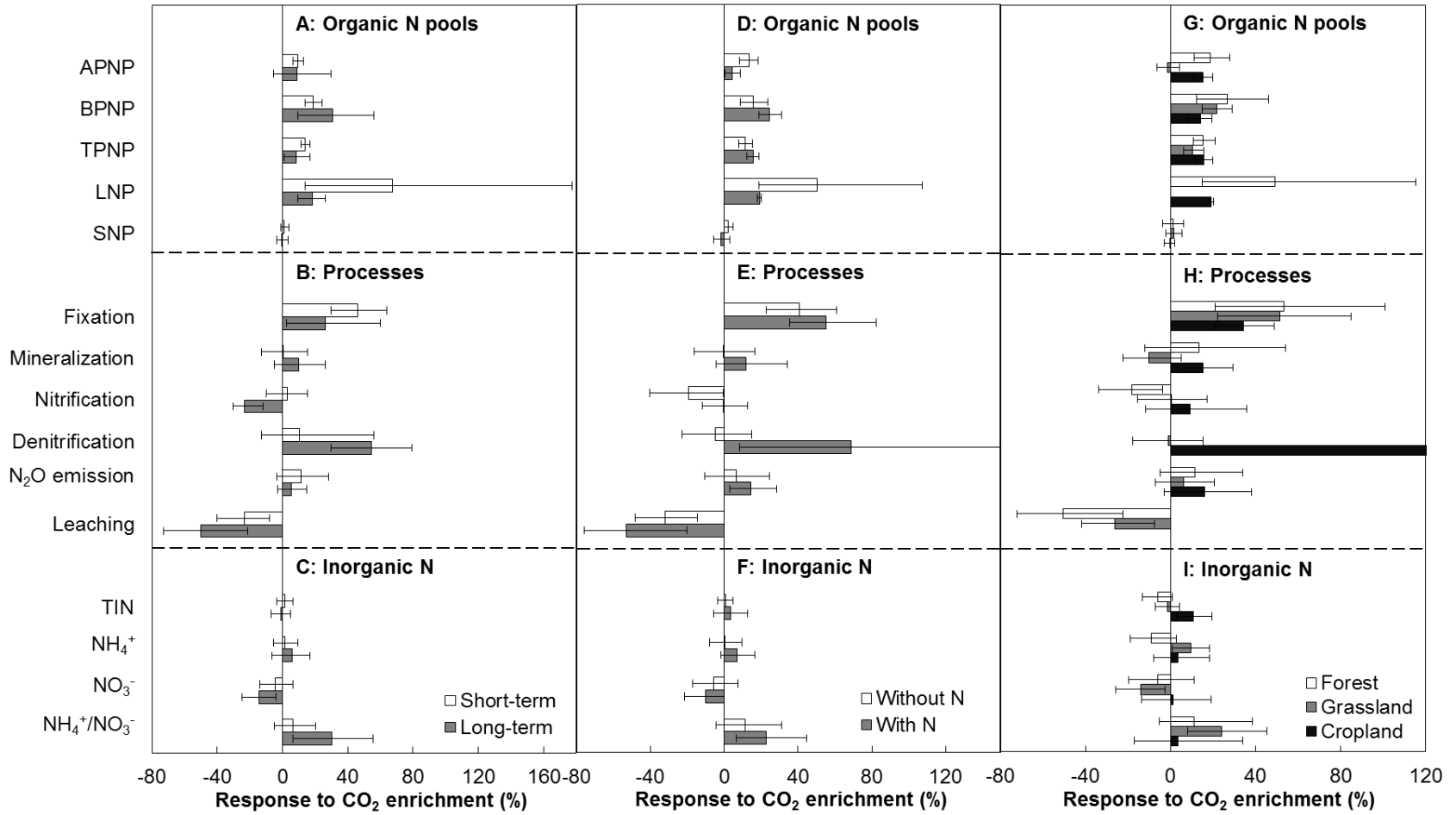
595 **Figure 2.**



596

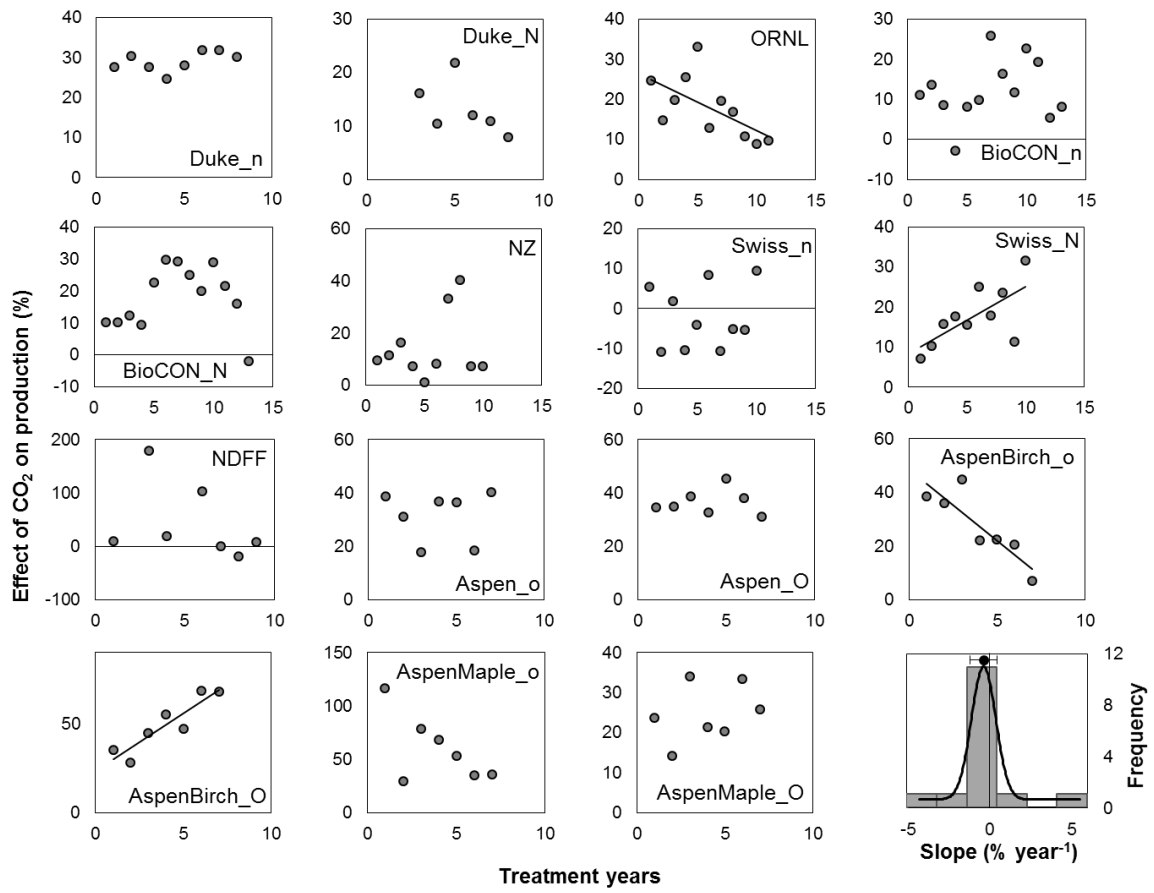
597

598 **Figure 3.**



599

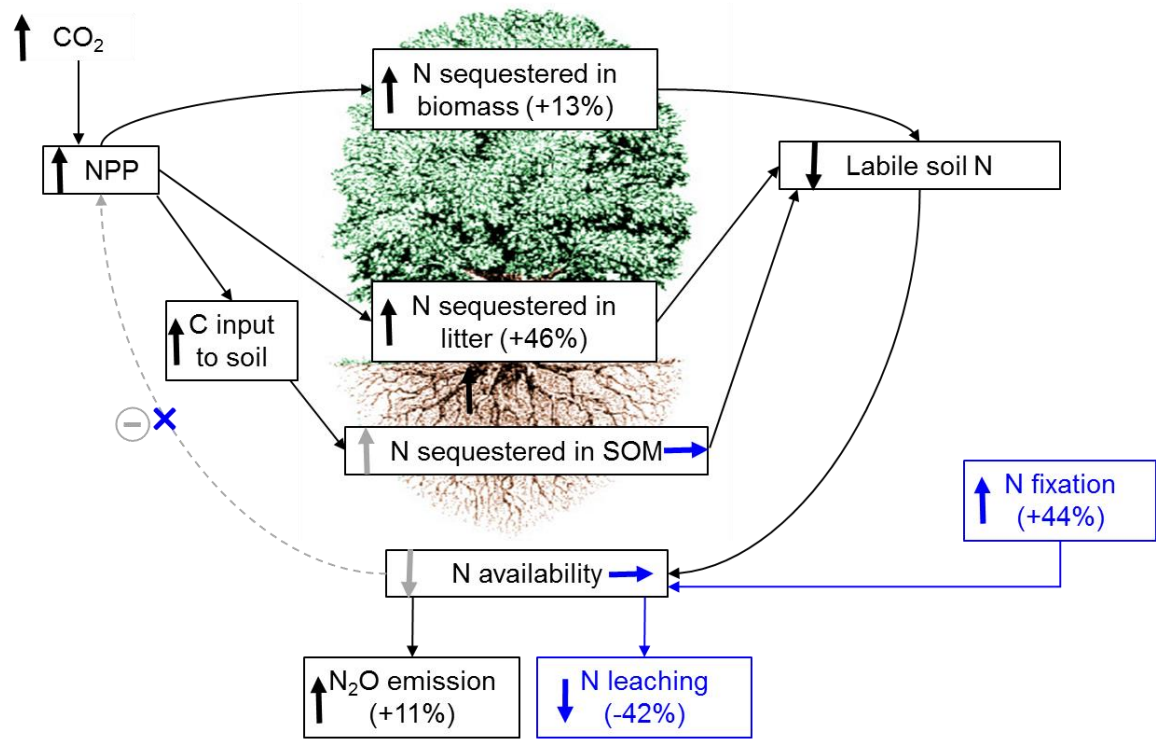
600 **Figure 4.**



601

602

603 **Figure 5.**



604

605