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3 **Title:** Long-term nitrogen addition modifies microbial composition and functions for slow
4 carbon cycling and increased sequestration in tropical forest soil

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6 **Running Title:** Effect of nitrogen on soil microbial functions

7

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42 **Abstract**

43 Nitrogen (N) deposition is a component of global change that has considerable impact on
44 belowground carbon (C) dynamics. Plant growth stimulation and alterations of fungal
45 community composition and functions are the main mechanisms driving soil C gains following
46 N deposition in N-limited temperate forests. In N-rich tropical forests, however, N deposition
47 generally has minor effects on plant growth; consequently, C storage in soil may strongly
48 depend on the microbial processes that drive litter and soil organic matter decomposition. Here,
49 we investigated how microbial functions in old-growth tropical forest soil responded to 13
50 years of N addition at four rates: 0 (Control), 50 (Low-N), 100 (Medium-N), and 150 (High-
51 N) kg N ha⁻¹ yr⁻¹. Soil organic carbon (SOC) content increased under High-N, corresponding
52 to a 33% decrease in CO₂ efflux, and reductions in relative abundances of bacteria as well as
53 genes responsible for cellulose and chitin degradation. A 113% increase in N₂O emission was
54 positively correlated with soil acidification and an increase in the relative abundances of
55 denitrification genes (*narG* and *norB*). Soil acidification induced by N addition decreased
56 available P concentrations, and was associated with reductions in the relative abundance of
57 phytase. The decreased relative abundance of bacteria and key functional gene groups for C
58 degradation were related to slower SOC decomposition, indicating the key mechanisms
59 driving SOC accumulation in the tropical forest soil subjected to High-N addition. However,
60 changes in microbial functional groups associated with N and P cycling led to coincidentally
61 large increases in N₂O emissions, and exacerbated soil P deficiency. These two factors partially
62 offset the perceived beneficial effects of N addition on SOC storage in tropical forest soils.
63 These findings suggest a potential to incorporate microbial community and functions into Earth
64 system models considering their effects on greenhouse gas emission, biogeochemical processes
65 and biodiversity of tropical ecosystems.

66 **Keywords:** N deposition; microbial functional community; tropical forest; biogeochemical

67 cycling; global climate change; C and N turnover

68

69 **Introduction**

70 Atmospheric deposition of reactive nitrogen (N) has increased significantly across every
71 continent in the last few decades, with the most rapid increase observed in the northern tropical
72 zone (Galloway et al., 2004; Tian et al., 2015). Investigations of the responses of temperate and
73 boreal forest ecosystems to N deposition are relatively extensive compared to forests in the
74 tropics, where most of them are generally of much shorter duration (Pajares & Bohannan, 2016;
75 Cusack et al., 2016). N fertilization can increase carbon (C) sequestration in temperate and
76 boreal forests by increasing net primary productivity (NPP) and slowing soil organic carbon
77 (SOC) decomposition rates, but the net effect of N deposition on soil C storage in tropical
78 forests remains unclear; this sink is extremely vulnerable to human perturbation, including the
79 indirect effects of land use and climate change (Cusack et al., 2016; Baccini et al., 2017).
80 Tropical forest soils are generally rich in N compared to the natural N-limitation in boreal and
81 temperate ecosystems, and are the largest natural sources of N₂O emissions that are produced
82 primarily by soil microbial nitrification/denitrification processes (Li, Niu, & Yu, 2016; Vitousek,
83 Porder, Houlton, & Chadwick, 2010; Lu & Tian, 2013). In contrast, biologically available P is
84 generally the limiting nutrient in old, highly weathered tropical forest soils (Camenzind,
85 Hattenschwiler, Treseder, Lehman, & Rillig, 2018), and the effects of N addition on P cycling
86 in these fragile environments are poorly understood. Consequently, the N deposition in tropical
87 forest ecosystems may cause changes in soil microbial community composition and metabolic
88 functions, reducing the potential for tropical forests to deliver globally important ecosystem
89 services that regulate global climate and weather patterns.

90 Terrestrial ecosystems are predicted to receive unprecedented quantities of reactive N by
91 the end of this century (Zak et al., 2017). Recent studies on temperate and boreal forests have

92 reported that elevated chronic N deposition significantly affects soil microbial abundance,
93 community structure, and functional gene activity, and that the responses of soil
94 microorganisms are strongly correlated with changes in ecosystem functions including SOC
95 cycling, both directly and indirectly, through multiple plant-soil-microbe interactions (Treseder,
96 2008; Eisenlord et al., 2013; Garcia-Palacios et al., 2015; Boot, Hall, Deneff, & Baron, 2016;
97 Zhang, Chen, & Ruan, 2018; Carrara et al., 2018). Net Primary Productivity is stimulated by
98 N deposition until N saturation is reached; increasing above- and below-ground litter inputs
99 (de Vries, Du, & Butterbach-Bahl, 2014). Nitrogen fertilization may reduce C allocation
100 belowground by modifying root physiology and exudation, and retarding rhizosphere priming
101 effects (Kuzyakov, 2002; Dungait et al., 2012; Janssens et al., 2010; Zhu et al., 2014). Shifts in
102 fungal community composition and functions were reported as a dominant mechanism driving
103 soil C gains following N addition to temperate forest soils (Hassett, Zak, Blackwood, & Pregitzer,
104 2009; Kellner, Luis, Schlitt, & Buscot, 2009; Hesse et al., 2015). Decomposition rates are
105 reduced by the effect of N fertilization on fungal activity as the genes encoding lignocellulolytic
106 enzymes are downregulated (Hesse et al., 2015; Chen et al., 2018), or by inhibiting fungal
107 growth (Waldrop, Zak, Blackwood, Curtis, & Tilman, 2006; Treseder, 2008). However, how
108 anthropogenic N deposition affects soil microbial community structure and metabolic potential
109 in tropical forests, and the subsequent impact on SOC accumulation, remains an open question
110 (Pajares & Bohannan, 2016; Cusack et al., 2016; Janssens et al., 2010). Recent studies reported
111 that N addition increased bacterial biomass, but decreased fungal/bacterial ratios in
112 (sub)tropical forest soils, accompanied by detectable increases in complex organic SOC
113 compounds (Cusack, Silver, Torn, Burton, & Firestone, 2011; Zhou, Wang, Zheng, Jiang, &
114 Luo, 2017), although the opposite effect has also been observed (Li et al., 2015; Wang, Liu, &
115 Bai, 2018).

116 The relatively N-rich status of tropical forests, compared to temperate/boreal forests, may

117 moderate response intensity to N augmentation through atmospheric deposition (Hedin,
118 Brookshire, Menge, & Barron, 2009; Cleveland et al., 2011). Nitrogen dissimilative process
119 rates are usually pronounced in N-rich ecosystems (Levy-Booth, Prescott, & Grayston, 2014),
120 causing higher N₂O losses by denitrification and NO₃⁻ leaching from tropical soils compared
121 to temperate forest soils (Vitousek & Matson, 1988; Zhang, Yu, Zhu, & Cai, 2014). Increasing
122 N availability in tropical forests inhibits biological N fixation (Cusack, Silver, & McDowell,
123 2009), alters nitrification (Han, Shen, Zhang, & Müller, 2018), and increases N₂O emissions
124 (Liu & Greaver, 2009), which increase greenhouse gas warming potential. Furthermore,
125 chronic N deposition causes soil acidification with consequences for the availability and
126 leaching of P, base cations (Mg²⁺, K⁺, and Ca²⁺), and micronutrients (Mo and Zn) that are
127 essential for plant and microorganisms (Lu et al., 2015; Cusack et al., 2016; Zamanian,
128 Zarebanadkouki, & Kuzyakov, 2018). Soil acidification can cause direct effects on plant and
129 microbial community composition, and indirectly through the release of Al³⁺ which has a
130 broad-spectrum toxicity for plants (Kaspari et al., 2017). Wang et al. (2009) reported
131 significantly increased Al³⁺ concentrations under high N addition soon after experimental
132 treatment were started in a tropical forest. Soil acidification may reduce P transport across cell
133 membranes, exacerbating the existing P limitation in weathered tropical forest soils (Kaspari
134 et al., 2017; Li, Niu, & Yu, 2016).

135 Atmospheric N deposited on 'pristine' ecosystems, including old growth tropical forests,
136 may impact ecological functioning in remote locations, where monitoring is sparse and the
137 consequences on biogeochemical cycling are difficult to determine (Holtgrieve et al., 2011).
138 China has become the largest N creator and emitter globally (Liu et al., 2013). Modeled
139 predictions of N deposition are for 105 Tg N y⁻¹ across Asia by 2030 (Zheng et al., 2002; Mo
140 et al., 2008), and deposition rates of up to 73 kg N ha⁻¹ y⁻¹ have been already recorded in tropical
141 old-growth forests in southern China (Liu et al., 2011). Whilst experimental data on the

142 responses of temperate and boreal forests to elevated N deposition is relatively extensive,
143 similar investigations in Chinese tropical forest ecosystems are sparse, and of a much shorter
144 timescale, with the most well-established starting in the 2000s (Liu et al., 2011). However,
145 tropical forest responses to long-term (decadal) atmospheric N fertilization, such as that
146 currently experienced by natural ecosystems in China, and the effects on the functional ecology
147 of such 'pristine' tropical forests is likely to be profound. A recent meta-analysis by de Vries et
148 al. (2014) on ecosystem N retention and C:N responses in forest ecosystem components
149 (canopy, shoots and roots, and soil) suggests that the biological responses to increasing N
150 deposition in tropical forests are different to those of boreal and temperate forests. However,
151 we know very little about the functional ecology of the soil microorganisms that ultimately
152 control the capacity of tropical forests to moderate ecosystem function in response to N
153 deposition, and thereby act as a net SOC sink or nutrient cycling.

154 We exploited an existing 13-year N deposition field experiment in a monsoonal evergreen
155 broadleaf old-growth forest in southern China. Previous research in the region has provided
156 evidence that old-growth forests accumulated SOC over a 24-year period from 1979-2003
157 (Zhou et al., 2006). Previously, we reported that N addition increased rates of plant transpiration
158 (Lu et al., 2018) and N₂O emissions (Zhang et al., 2008), but reduced litter decomposition
159 (Fang, Mo, Peng, Li, & Wang, 2007) and soil respiration (Mo et al., 2008). We also found that
160 N addition aggravated soil acidification and P supply, but increased Al mobility (Lu et al.,
161 2015). However, the extent to which shifts in the microbial functional community underpin
162 these changes was not investigated. Here, we hypothesized that (1) long-term N additions alter
163 microbial community composition by increasing soil acidification; and (2) the effects of N
164 addition on the microbial community composition would translate into changes in microbial
165 function that help explain the observed effects on soil C, N, and P cycling. To test these
166 hypotheses, we used a microarray-based tool (GeoChip 5.0) to profile microbial functional

167 potentials, specifically targeting a wide range of functional genes associated with C, N, and P
168 cycling in soils. This method can be used as a specific and sensitive tool to study microbial
169 functional potentials using correlations between microbial communities and ecosystem
170 processes (He et al., 2010; Zhou et al., 2012; Yang et al., 2013).

171

172 **Materials and Methods**

173 **Field experiment and sampling**

174 An N addition experiment was established in 2003 in a tropical old-growth monsoon evergreen
175 broadleaf forest at the Dinghushan Biosphere Reserve (DHSBR), an UNESCO/MAB site
176 located in the middle of Guangdong Province in southern China (23°10'N and 112°10'E; 250-
177 300 m a.s.l.). The region has a monsoonal humid tropical climate with a mean annual
178 temperature of 21 °C (range 13 °C in January to 28 °C in July), and 1930 mm of precipitation
179 falling in a distinct seasonal pattern; 75% of the precipitation falls from March to August, and
180 6% from December to February. The soil is lateritic red earth formed from sandstone (Oxisol).

181 The experimental design is described in Mo et al. (2008). In brief, twelve plots of four
182 treatments with three replicates were laid out in a completely randomized block design. Each
183 plot was 20 m × 10 m, and was bounded by a 10 m wide buffer strip. N was added as ammonium
184 nitrate (NH₄NO₃) solution at four N levels: Control (CK; zero N), Low-N (LN; 50 kg N ha⁻¹ y⁻¹)
185 ¹), Medium-N (MN; 100 kg N ha⁻¹ y⁻¹), and High-N (HN; 150 kg N ha⁻¹ y⁻¹). Nitrogen was
186 applied below the canopy on 12 occasions at monthly intervals each year at equal rates using a
187 backpack sprayer. . The control soil was treated with an equal volume (20 L) of deionized water.

188 The experimental plots were sampled in October 2015. Prior to soil sampling, CO₂, N₂O,
189 and CH₄ fluxes were collected from each plot from two static chambers that were inserted 5
190 cm into the soil (Zhang et al., 2008). Gas samples were taken using 100 ml plastic syringes at
191 0, 10, 20, and 30 min after chamber closure, and were analyzed in the laboratory within 24 h.

192 Soil samples were taken using a soil corer (0-10 cm deep, 2 cm inner diameter) from 10
193 random points across each plot, and mixed to yield one composite sample per plot. The litter
194 layer was carefully removed before sampling. The soil samples were stored in airtight
195 polypropylene bags and placed in a cool box at 4 °C during transportation to the laboratory.
196 Litter, roots, and stones were carefully removed by hand, and the soil was divided into several
197 subsamples. Subsamples for dissolved organic carbon (DOC), ammonium N ($\text{NH}_4^+\text{-N}$), nitrate
198 N ($\text{NO}_3^-\text{-N}$) and available phosphorus (AP) concentration analyses were stored at 4 °C for no
199 longer than one week. Subsamples for pH, soil organic carbon (SOC), total nitrogen (TN), total
200 phosphorus (TP), and pH analyses were air dried. Subsamples for microbial community
201 composition and functional gene (GeoChip) analysis were stored at -80 °C.

202

203 **Greenhouse gas and soil analyses**

204 Greenhouse gas (CO_2 , N_2O , and CH_4) concentrations of the air sampled in each treatment were
205 measured by gas chromatography (Agilent 4890D, Agilent Co., Santa Clara, CA, USA). Soil
206 pH was measured with a pH meter after shaking the soil in deionized water (1:2.5 w/v)
207 suspensions for 30 min. The SOC and TN content were determined by combustion using a
208 Vario EL III Elemental Analyzer (Elementar, Germany). Ammonium N ($\text{NH}_4^+\text{-N}$) and $\text{NO}_3^-\text{-N}$
209 N concentrations were determined using an autoanalyzer (TRAACS-2000, BRAN+LUEBBE,
210 Germany) following 0.01 M KCL (1:10 w/v) extraction for 30 min. Total P and available P
211 concentrations were measured using the ammonium molybdate method after $\text{H}_2\text{SO}_4\text{-H}_2\text{O}_2\text{-HF}$
212 digestion. The DOC concentration was measured following the method used by Jones and
213 Willett (Jones & Willett, 2006). The field-moist soil samples (equivalent to 15 g oven-dried
214 soil) were extracted with 60 ml of 0.05 mol L^{-1} K_2SO_4 (soil to solution ratio 1:4) for 1 h. The
215 extract was then passed through a 0.45-mm membrane filter and analyzed for DOC using a
216 Multi 3100 N/C TOC analyzer (Analytik Jena, Germany). Microbial community composition

217 was analyzed by phospholipid fatty acid (PLFA) composition according to Frostegård et al.
218 (1991). Changes in microbial community composition was presented as molar percentages
219 (mol %) of the PLFA biomarkers for bacteria or fungi extracted from the soil samples, and the
220 ratio of biomarkers for fungi and bacteria (fungi:bacteria), as previously described by Bossio,
221 Scow, Gunapala, & Graham (1998) and Högberg et al., (2007).

222

223 **Analyses of microbial functional communities**

224 Total DNA was extracted from 0.5 g of well-mixed soil using the PowerSoil kit (MoBio
225 Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. DNA quality
226 and quantity were measured using a NanoDrop ND-1000 Spectrophotometer (NanoDrop
227 Technologies Inc., Wilmington, DE, USA), and with PicoGreen® using a FLUOstar® Optima
228 microplate reader (BMG Labtech, Jena, Germany), respectively. DNA hybridization was
229 performed using GeoChip 5.0 according to Yang et al. (2013). Briefly, DNA samples were
230 labeled with Cy-5 fluorescent dye using a random priming method, and purified with the QIA
231 quick purification kit (Qiagen, Valencia, CA, USA). The DNA was dried in a SpeedVac
232 (ThermoSavant, Milford, MA, USA) at 45 °C for 45 min. GeoChip hybridization was carried
233 out at 42 °C for 16 h on a MAUI® hybridization station (BioMicro, Salt Lake City, UT, USA).
234 After purification, GeoChips were scanned by a NimbleGen MS200 scanner (Roche, Madison,
235 WI, USA) at 633 nm, using a laser power and photomultiplier tube gain of 100% and 75%,
236 respectively.

237

238 **Statistical analyses**

239 Raw GeoChip data were analyzed using a data analysis pipeline as described previously (Yang
240 et al., 2013; He et al., 2010). The data were logarithmically transformed, and then divided by
241 the mean value of each slide. A minimum of two valid values of three biological replicates

242 (samples from the same treatment), were required for each gene. Spots that were flagged, or
243 with a signal to noise ratio less than 2.0 were considered poor in quality and removed from
244 statistical analysis.

245 Detrended correspondence analysis (DCA) was used to assess changes in the overall
246 microbial functional community structure (based on GeoChip data). Adonis was further
247 performed to confirm significant changes in microbial functional community structures in any
248 pair of samples. To determine the relative importance of soil and plant factors in shaping
249 microbial functional community structure, a canonical correspondence analysis (CCA)-based
250 variation partitioning analysis (VPA) was implemented (Ramette & Tiedje, 2007). DCA and
251 VPA statistical analyses were performed in R v.3.2.1 with the vegan package. Matrices of the
252 pairwise distance between functional microbial community structure (Bray-Curtis) and
253 Euclidean distance of environmental variables were also constructed in R using the vegan
254 package.

255 To further link microbial functional genes with environmental variables and
256 biogeochemical processes, partial least squares path modeling (PLS-PM) and Pearson
257 correlations were performed. PLS-PM is a data analysis method for variables that can be
258 summarized by use of latent variables, and the fact that linear relationships exist between latent
259 variables (Sanchez, 2013). Each latent variable included one or more manifest variables, for
260 example, inorganic N including NH_4^+ -N and NO_3^- -N. Each manifest variable has the degree of
261 relative contribution degree shown as an arrow between manifest variable and latent variable
262 in the analysis of path diagram. Models with different structures are evaluated using the
263 Goodness of Fit (GOF) statistics, a measure of their overall predictive power with $\text{GOF} > 0.7$
264 considered acceptable values. The models were constructed using the 'inner plot' function of
265 the R package. PLS-PM was performed based on the reduction of the full models (Kou et al.,
266 2017). The environmental drivers selected in the model were the main predictors according to

267 their contribution for variation based on Random Forest analysis (% of increase of MSE).
268 Random Forest analysis was conducted in R v.3.2.1 using the random Forest package
269 (Delgado-Baquerizo et al., 2016).

270 The significant differences of environmental variables and signal intensities for selected
271 C, N, and P cycling functional genes in soil sampled from four N addition levels were
272 determined by One-Way ANOVA, followed by the Least Significant Difference test using SAS
273 V8 (SAS Inc. 1996). Statistical significance was determined at $p < 0.05$ for all analyses.

274

275 **Results**

276 **Long-term effects of N addition on greenhouse gas emissions and soil properties**

277 Compared with the control treatment (CK), the effects of N addition on GHG (CO₂, N₂O, and
278 CH₄) fluxes were significant under High-N and Medium-N treatments, but not for Low-N
279 (Table 1). CO₂ emission was 33% less under High-N compared with control. As compared with
280 control, CH₄ uptake decreased by 64% and 63% in Medium-N and High-N treatments, but N₂O
281 emission increased by 113% under High-N. All measured soil parameters in the High-N-treated
282 soils apart from DOC and TP were significantly different from CK. Soil organic carbon (SOC)
283 and TN contents and NH₄⁺ concentrations were higher under the High-N treatment. Nitrate
284 (NO₃⁻) concentrations increased under Medium-N and High-N compared to Low-N and CK.
285 Available P and pH were lower under Medium-N and High-N treatments than under Low-N.
286 Bacterial relative abundance based on PLFA biomarker contents was lower under High-N
287 treatment than under Low-N, but there was no significant difference in fungal PLFA biomarker
288 concentrations or fungal: bacterial (F:B) ratios between treatments.

289

290 **Long-term effects of N addition on microbial functional community structure**

291 The α diversity of the microbial functional community decreased with N application rate and

292 was lowest under the High-N treatment (Fig. S1; $p < 0.05$). Nitrogen addition markedly changed
293 soil microbial functional community structure, as indicated by DCA (Fig.1), which was
294 confirmed by non-parametric multivariate statistical tests (Fig.1; $p < 0.001$). Further pairwise
295 comparison revealed that microbial functional community structures under Medium-N and
296 High-N treatments differed from CK (Table S1).

297 A partial CCA-based VPA evaluated the relative contributions of environmental factors to
298 the microbial functional community structure (Fig. S2). A total of 89.9% of the community
299 variations could be explained by all measured variables. Soil and plant variables explained 72.6%
300 and 11.8% of variations, respectively, while their interactions explained 5.50% (Fig. S2).
301 Among soil variables, NO_3^- -N and available P were the most important factors, contributing
302 14.7% and 16.2%, respectively, to variation in microbial functional community structure (Fig.
303 S2).

304

305 **Linking microbial functional genes to soil CO₂ emission**

306 Key genes associated with C degradation were analyzed and related to soil CO₂ emissions.
307 High N decreased the abundances of genes responsible for labile and recalcitrant C (starch,
308 cellulose, and chitin) degradation ($p < 0.05$; Fig. 2). For example, the relative abundances of
309 *apu*, *npiT*, *amyX*, *cellobiase*, *acetylglucosaminidase* and *chitinase* were the lowest under High-
310 N ($p < 0.05$; Fig. S3). PLS path modeling explained 79% of the CO₂ emission variance, and
311 provided the best fit to our data (GOF of 0.77) (Fig. 3a). Bacterial relative abundance showed
312 the largest effect on CO₂ emission via direct (path coefficient = 0.34) and indirect effects (path
313 coefficient = 0.62) on microbial functional C degradation genes (Fig. 3a). There were
314 corresponding strong, positive correlations between bacterial relative abundance and the
315 abundances of genes involved in starch, hemicelluloses, cellulose and chitin degradation, but
316 no similar relationship was observed with fungal relative abundance (Fig. 4). The positive

317 direct effect of C degradation genes (path coefficient = 0.57) on CO₂ emission was the greatest
318 (Fig.3a). Among C degradation genes, strong positive correlations between cellulose and chitin
319 degradation gene abundances and CO₂ production were observed (Fig. S4; $p < 0.05$). This
320 finding was consistent with PLS path modeling that showed large loading factors (Fig. 3a).

321

322 **Linking microbial functional genes to soil N₂O emission**

323 With increasing N levels, the abundance of *gdh* gene (glutamate dehydrogenase) decreased,
324 while that of *ureC* (urease) increased (Fig. 5; $p < 0.05$), suggesting a shift in microbial functional
325 potential toward N mineralization. Both genes associated with nitrification (*amoA* and *hao*)
326 decreased under Medium-N and High-N treatments. High-N increased denitrification genes
327 including *narG* (membrane-bound nitrate reductase), *norB* (nitric oxide reductase), and *nirK*
328 (copper-containing nitrite reductase). Correspondingly, NO₃-N concentrations were related to
329 the increased abundance of genes encoding *NiR* and *nirA* (assimilatory nitrate reduction). The
330 abundance of the N fixation gene (*nifH*) was the lowest under High-N treatment.

331 PLS path modeling explained 89% of the N₂O emission variance (Fig. 3b). There was a
332 direct positive effect of denitrification genes on N₂O emission in the PLS path model that was
333 supported by the Pearson correlations (Fig.S5). This suggested that increased denitrification
334 was the driving force for N₂O production after N amendment. Among soil variables, the
335 positive direct effect of inorganic N concentrations on N₂O emission was the greatest, while
336 pH was related to the largest negative indirect effect (Fig. 3b).

337

338 **Linking microbial functional genes to soil CH₄ uptake**

339 High-N increased the abundance of the gene encoding *mcrA* (methyl coenzyme M reductase),
340 a key enzyme in methanogenesis (Fig. 6a, $p < 0.05$). In contrast, there was a higher abundance
341 of genes encoding *mmoX* (involved in methane oxidation) under the Low-N treatment. PLS

342 path modeling accounted for 81% of the variation in CH₄ uptake (Fig.3c). There were strong
343 positive direct effects of mcrA (methanogenesis) on CH₄ uptake in PLS path modeling (Fig.
344 3c). In general, the direct effect of DOC (path coefficient=0.48) and pH (path coefficient=-0.50)
345 on CH₄ flux was greater than those of SOC (path coefficient=-0.28) and inorganic N
346 concentrations (path coefficient=-0.13) (Fig. 3c).

347

348 **Changes in key genes related to P cycling**

349 The relative abundance of the phytic acid hydrolysis gene (encoding phytase) decreased under
350 Medium-N and High-N additions (Fig. 6b; $p<0.05$). Most genes encoding phytase were derived
351 from Proteobacteria. The decrease of microbial P utilization genes after N amendment
352 suggested that N amendments exacerbate soil P deficiency. This was supported by positive
353 relationships between phytase abundance, and available and total P contents ($p<0.05$; Table
354 S2). We found positive correlations between phytase abundance and soil pH, but negative
355 correlations with inorganic N concentrations ($p<0.05$; Table S2).

356

357 **Discussion**

358 Long-term (13-year) NH_4NO_3 addition in a tropical old-growth forest altered microbial
359 composition, predominantly by decreasing the relative abundance of bacteria (Table 1).
360 Furthermore, microbial functional mechanisms encoded by C, N, and P cycling genes were
361 altered by N addition at high rate, and explained the observed effects on soil C, N, and P cycling
362 at the experimental site (summarized in Fig.7). Therefore, our hypotheses regarding the
363 significant changes in microbial composition and functions after N deposition at rates higher
364 than $100 \text{ kg N ha}^{-1} \text{ yr}^{-1}$ were confirmed. The potential impacts of these findings are discussed
365 in the context of reported phenomena pertinent to the climate change mitigation potential of
366 tropical old growth forests under chronic N addition, i.e. SOC accumulation and increased N
367 loss via denitrification.

368

369 **SOC accumulation in old growth tropical forests under N addition**

370 The positive priming of SOM and increased soil respiration rates caused by increases in NPP
371 and organic matter input after atmospheric CO_2 enrichment of tropical forests reported by Sayer
372 et al. (2011) is apparently contradicted by the observed increase in SOC accumulation after N
373 addition in old growth tropical forest under High-N in this study. No changes in plant growth
374 and litter input were observed at the DHSBR experimental site (Mo et al., 2008). Furthermore,
375 Mo et al. (2008) showed that High-N addition inhibited fine root growth at the same
376 experimental site. Declining root biomass is a key response to N saturation, and has also been
377 reported by Magill et al. (2004) in the chronic N amendment in the Harvard Forest, USA, where
378 similar rates ($50 - 150 \text{ kg N ha}^{-1} \text{ yr}^{-1}$) were applied to hardwood and pine forest soils. Therefore,
379 changes in aboveground NPP or belowground NPP are unlikely to provide a route for increased
380 SOC in the DHSBR tropical forest under increased N.

381 Undisturbed forest soils have large F:B ratios (e.g. 0.3 – 0.5; Frostegård & Bååth, 1996),

382 and large chronic inorganic N input reduces fungal biomass and inhibits lignases (Hassett, Zak,
383 Blackwoo, & Pregitzer, 2009; Frey, Knorr, Parrent, & Simpson, 2004). Zak et al. (2017)
384 recently proposed that reduced decomposition in temperate forests is caused by a shift from
385 basidiomycete fungal activity to bacteria and ascomycete fungi that only can partially oxidize
386 polyphenolic structures in soil. In our study, however, there were no differences between the
387 fungal relative abundance, the F:B ratio (approximately 0.2; Table 1), or the gene abundances
388 of ligninolytic enzymes between N addition levels (Fig. 2). However, the non-experimental N
389 addition (i.e. annual atmospheric addition of 40-50 kg N ha⁻¹ yr⁻¹ recorded at the site) had
390 already reduced the fungal population in the control plots preceding experimental N addition.
391 Indeed, a moderate increase of N addition (15 kg N ha⁻¹ yr⁻¹) for 5 years from 2007-2011 in
392 DHSBR soils increased fungal biomass and F:B ratios, which were also measured using PLFA
393 analysis (Liu et al., 2013). This may suggest that the larger N addition in our study exceeded a
394 biological threshold controlling the activity of soil fungi. Similarly, Frey et al. (2004) reported
395 that active fungal biomass and mycorrhizal activity was 27–69% reduced in the Harvard Forest
396 experiment where background atmospheric N input was relatively low (8 kg N ha⁻¹ yr⁻¹) but
397 bacterial biomass was unchanged, resulting in lower F:B ratios. Högberg et al. (2007) also
398 determined significant decreases in the mol% of fungal biomass and F:B ratio (similarly based
399 on PLFA analysis of 18:2ω6) in a northern European boreal forest after long term (20 + years)
400 N addition (34 – 108 kg N ha⁻¹ yr⁻¹), where the background atmospheric N deposition was
401 minimal (3 kg N ha⁻¹ yr⁻¹). This suggests that chronic N addition has a negative effect on fungal
402 survival in forest soil. The effect of a reduction in rhizosphere exudation as a key mechanism
403 for the substantial (45%) decrease in ectomycorrhizal fungi biomass was suggested by
404 comparison with experimental tree girdling, providing an explanation for the decrease in
405 microbial heterotrophic respiration previously observed in a Chinese pine forest (Fan et al.,
406 2014; Wang et al., 2016). Therefore, increased root and active fungal biomass, as observed by

407 Clemmensun et al. (2013) for N-limited boreal forest ecosystems, are unlikely to be the primary
408 drivers of SOC accumulation in DHSBR tropical forest soils under chronic N addition.

409 High-N addition decreased CO₂ emission by 33% in this study (Table 1), concurring with
410 previous studies that reported that N additions decreased soil respiration in recent studies of
411 tropical forest soils (Fan et al., 2014; Wang et al., 2016), although by comparison, Feng et al.
412 (2017) reported no change. We observed that bacterial relative abundance was decreased under
413 High-N addition (Table 1). Wang et al. (2009) explored the initial effects of N addition in the
414 early stages of the experiment and found that soil microbial biomass C (extracted by
415 chloroform-fumigation) was generally decreased in N addition plots, but significantly
416 decreased in the High-N plots after 2 and 4 years. These observations are contrary to the
417 observations of similar experiments in temperate and boreal forests in general. Furthermore,
418 bacterial relative abundance had the largest total effect of on total soil respiration (CO₂ emission)
419 (Fig.3a; $p < 0.05$). A decrease in microbial abundance provides a straightforward explanation
420 for the measured decrease in CO₂ emissions from tropical forests under High-N addition, as
421 previously proposed by Wei et al. (2008). Direct and indirect effects of each latent variable on
422 target variables were identified using PLSPM analysis. The processes underlying the measured
423 CO₂ emission in the field include soil organic matter, roots and microorganisms derived
424 (Kuayzkov, 2006). Thus, the indirect effect of bacteria on CO₂ may be through changes in the
425 production of C degradation enzymes, and the direct effect of bacteria on CO₂ may be derived
426 from changes in microbial respiration, in response to N addition. Significant relationships
427 between CO₂ emission and the relative abundance of cellulose and chitin degradation genes
428 were observed, respectively (Fig. S4; $p < 0.05$). This indicates that N addition inhibited the
429 production of C degradation enzymes by the soil bacterial biomass, which may drive the
430 accumulation of intact organic compounds in the soil, thereby increasing SOC under High-N
431 conditions. This is further evidence supporting the hypothesis that SOM turnover is controlled

432 by the access of microorganisms and their enzymes, regardless of assumed chemical
433 recalcitrance (Dungait et al., 2012).

434 Tropical forest soils often have a limited capacity to buffer acidification, so N addition
435 can lead to rapid soil acidification (Matson, McDowell, Townsend, & Vitousek, 1999; Lu et al.,
436 2015; Zamanian, Zarebanadkouki, & Kuzyakov, 2018), negatively affecting bacterial growth
437 by an increase in toxic free Al^{3+} (Rousk, Brookes, & Baath, 2009), but also the loss of other
438 nutrients including P and cations (Mg^{2+} , Ca^{2+}) from the soil by leaching. The available P
439 concentrations in High-N treatment reveal a decrease as compared to CK (Table 1). Magill et
440 al. (2004) observed Ca^{2+} , Mg^{2+} and K^+ leaching from the O horizon under N addition, and
441 subsequent declines in foliar Mg:N and Ca:Al in the Harvard Forest, suggesting a positive
442 feedback to decrease biological availability with time. Our results concur with previous studies
443 suggesting bacteria are less tolerant of acidic environments than fungi (Li et al., 2015; Wang,
444 Liu, & Bai, 2018). Overall, our study demonstrates that long-term High-N addition in a tropical
445 forest promotes net C gain by altering microbial community composition and inhibiting organic
446 C degradation functional potential, leading to an accumulation of undegraded organic
447 compounds. However, this C sequestration potential needs to be considered against the changes
448 observed for other aspects of soil biogeochemistry, e.g. macro- and micronutrient cycling, and
449 the wider impacts on ecosystem functions including biodiversity.

450 According to the *in situ* measurements of CH_4 fluxes, the soils under all treatments acted
451 as net sinks of CH_4 , but this was significantly less in the plots treated with high or medium
452 rates of N fertilizer application ($-18.4 \mu g C m^{-2} h^{-1}$ and $-19.1 \mu g C m^{-2} h^{-1}$, respectively)
453 compared to low N addition or the control ($-40.0 \mu g C m^{-2} h^{-1}$, and $-51.2 \mu g C m^{-2} h^{-1}$,
454 respectively) (Table 1). This indicates that the balance between methane methanotrophy and
455 methanogenesis in the tropical forest soils in this study had been affected by N addition. The
456 global meta-analysis performed by Liu & Greaver (2009) showed that N addition increased

457 CH₄ emission by 97% and reduced CH₄ uptake by 38%. Schimel (2000) had previously
458 described that high NH₄⁺ concentrations could inhibit CH₄ oxidation because of competition
459 for methane monooxygenase resulting in increased CH₄ emissions. Methanotrophy indicated by
460 *pmoA* and *mcox* expression was not significantly different from the control apart from *mcox*
461 under LN, but *mcrA* expression (methanogenesis) was increased under HN (Fig.6).
462 Additionally, in this study, the strong positive direct effect of *mcrA* on CH₄ uptake (Fig. 3c)
463 suggests that the decrease of CH₄ uptake may largely be due to increased methanogenesis. The
464 abundance of the *mcrA* gene positively correlates with CH₄ production potential (Ma, Conrad,
465 & Lu, 2012). Accelerated methanogenesis could be partially attributed to increased C
466 availability; we found that labile C (DOC) had a strong direct effect on the *mcrA* gene and CH₄
467 flux (Fig. 3C). This agrees with previous reports that labile C pool fuels the activity of
468 methanogenic populations, resulting in increased CH₄ emissions (Lu et al., 2005; Dorodnikov,
469 knorr, Kuzyakov, & Wilmking, 2011; Zheng et al., 2017).

470

471 **Increased potential N loss in old growth tropical forests under chronic N addition**

472 Soil C and N cycles are closely linked, and often isometric in their stoichiometric expression
473 (Beniston et al., 2015). Therefore, the observation that SOC is accumulating under N addition
474 in tropical forest soils infers that organic N will contribute to increased N mineralization, as
475 attested by the increased relative abundances of *ureC* genes that control ammonification under
476 High-N conditions (Fig. 5), and the positive correlations between ammonification gene
477 abundance and DOC ($r=0.55$, $p<0.05$). However, *gdh* expression decreased under High-N
478 conditions, suggesting the two genes encoding autotrophic ammonia-oxidizing metabolic traits
479 may be expressed in organisms that respond differentially to N addition. Ammonia oxidizing
480 archaea dominate nitrification (Prosser & Nicol, 2012) and *ureC* expression in acid soils (Lu
481 & Jia, 2013), suggesting a switch from bacterial to increasing archaeal functional potential as

482 bacterial abundance decreased under High-N conditions.

483 The N₂O flux from soil mainly originates from nitrification or denitrification (Levy-
484 Boothet al., 2014). The large increase in N₂O emissions (113%; Table 1) under High-N
485 conditions coincided with increased soil acidity, the metabolic potential for NO₃-N reduction
486 in this study (Fig. 3B), and an increased relative abundance of genes involved in denitrification
487 (*narG* and *norB*) and assimilatory N reduction (*NiR* and *nirA*) (Figs. 3b, S5, and S7). The strong
488 direct positive effect of denitrification genes, but not nitrification genes, on N₂O emission (path
489 coefficient =0.67; Fig.3b), suggests that denitrification was a driving force of N₂O emission in
490 this acidic tropical forest soil. Our result agreed with previous studies that N addition can
491 increase N₂O emissions and is correlated with genes involved in denitrification pathway (Corre,
492 Veldkamp, Arnold, & Wright, 2010; Han, Shen, Zhang, & Mülle, 2018). Among denitrification
493 genes, we determined significant contributions from *narG* and *norB* genes to N₂O emissions
494 (Figs. 3b and S5). Previous studies found that *nosZ* gene was positively correlated with soil
495 NO and N₂O production in acidic forests (Yu et al., 2014; Lammel, Feigl, Cerri, & Nusslein,
496 2015), but the abundance of *nosZ* was not significantly different from the control in our study.
497 Relative abundances of *nirK* were reported to increase under enhanced NO₃⁻-N concentrations
498 (Morales, Cosart, & Holben, 2010), and low pH (Rütting, Huygens, Boeckx, Staelens, &
499 Klemedtsson, 2013). Nitrogenous gas emissions (N₂O/N₂ emission ratios) can be influenced
500 by measurable soil properties in tropical soils including parent materials, pH, soil moisture,
501 and redox potential (Rütting, Huygens, Boeckx, Staelens, & Klemedtsson, 2013; Zhang, Cai,
502 Cheng, & Zhu, 2009; Liu, Morkved, Frostegård, & Bakken, 2010; Stone, Kan, & Plante, 2015;
503 Kang, Mulder, Duan, & Dorsch, 2017). Therefore, the variations between soil N processes and
504 gene expression response to N additions are complex and barely predictable (Levy-Boothet al.,
505 2014; Chen et al., 2019). Indeed, in our experiment, *nirS* decreased, again suggesting that
506 ubiquitous soil processes including denitrification are performed by a range of microorganisms

507 that respond differently to stresses including pH change. The decreased abundance of
508 nitrification genes that coincided with the increased abundances of denitrification genes and
509 N₂O emissions indicate a potentially high N loss, likely counteracting net N accumulation in
510 this study. Most significantly, considering the high warming potential of N₂O, the stimulated
511 N₂O emissions potentially offset the perceived beneficial effect of N addition on soil C
512 accumulation in tropical old growth forest soils, and suggest that the effects of chronic N
513 deposition on remote ecosystems must be considered in models predicting feedbacks to climate
514 change.

515

516 In conclusion, this study revealed profound interrelationships between the response of soil
517 microbial functional potentials and soil C, N, and P cycling to chronic N addition in ‘pristine’
518 tropical forests. Changes in bacterial relative abundance significantly affected CO₂ emissions,
519 mainly via indirect effects on microbial functional C degradation genes leading to SOC
520 accumulation. We revealed the metabolic potential for the increased expression of microbial
521 nitrogen functional genes driving increased N₂O fluxes from these ecosystems. Limitations on
522 P availability caused by soil acidification through reduced *phytase* expression were also
523 identified. This new understanding of the effect of human-induced atmospheric N deposition
524 must be factored into Earth system models considering the GHG sink capacity of tropical
525 forests, and the effect of air pollution from agriculture and industry on the biogeochemical
526 processes and biodiversity of tropical ecosystems. Further studies are necessary to investigate
527 the effect of climatic variation at a range of timescales (seasonal and decadal) to investigate the
528 impact of N addition on the dynamics of microbial communities and functions, and their roles
529 in mediating soil biogeochemistry, under a more extensive range of environmental conditions
530 experienced by the soil microbial community.

531

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537

538 **Conflict of interest**

539 The authors declare that they have no conflict of interest.

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805 **Table 1** Effect of 13 years N fertilizer addition to soil in a tropical forest at different rates on carbon, nitrogen and phosphorus pools and soil
 806 microbial composition and greenhouse gas emission.

	Treatment (kg N ha⁻¹ yr⁻¹)			
	CK 0 (control)	Low-N 50	Medium-N 100	High-N 150
Greenhouse gas emissions				
CO ₂ (mg CO ₂ m ⁻² h ⁻¹)	91.8 ± 4.05 a	88.2 ± 2.27 ab	85.4 ± 1.40 ab	61.2 ± 0.86 b
CH ₄ (µg C m ⁻² h ⁻¹)	-51.2 ± 7.66 b	-40.0 ± 3.93 b	-18.4 ± 3.88 a	-19.1 ± 4.06 a
N ₂ O (µg N m ⁻² h ⁻¹)	29.2 ± 2.14 b	41.7 ± 5.16 ab	39.6 ± 9.28 ab	62.2 ± 9.48 a
Soil chemistry				
pH (H ₂ O)	3.9 ± 0.03 a	3.8 ± 0.05 ab	3.8 ± 0.02 b	3.7 ± 0.01 b
Soil organic carbon (SOC, g C ⁻¹ kg ⁻¹)	25.4 ± 3.44 b	29.6 ± 2.54 ab	30.2 ± 0.46 ab	31.9 ± 0.77 a
Dissolved organic carbon (DOC, mg C ⁻¹ g ⁻¹)	0.5 ± 0.04 a	0.6 ± 0.04 a	0.6 ± 0.04 a	0.6 ± 0.01 a
Total nitrogen (TN, g N ⁻¹ kg ⁻¹)	1.8 ± 0.14 b	2.3 ± 0.28 ab	2.2 ± 0.07 ab	2.5 ± 0.08 a
Nitrate N (NO ₃ ⁻ -N, mg N ⁻¹ kg ⁻¹)	2.4 ± 0.27 c	9.6 ± 1.49 b	16.1 ± 2.83 a	17.7 ± 1.21 a
Ammonium N (NH ₄ ⁺ -N, mg N ⁻¹ kg ⁻¹)	4.0 ± 0.18 b	5.0 ± 0.53ab	5.1 ± 0.72 ab	7.4 ± 1.66 a
Available phosphorus (AP, mg P kg ⁻¹)	0.5 ± 0.05 a	0.4 ± 0.08 ab	0.4 ± 0.02 b	0.4 ± 0.01 b
Total phosphorus (TP, mg P g ⁻¹)	0.2 ± 0.03 a	0.2 ± 0.01a	0.2 ± 0.01a	0.2 ± 0.01a
Soil microbial composition (phospholipid fatty acids)				
Bacterial abundance (mol %)	52.4 ± 0.24 a	52.0 ± 0.31 a	51.4 ± 0.24 ab	50.0 ± 0.24 b
Fungal abundance (mol %)	10.0 ± 0.51 a	9.3 ± 0.16 a	9.9 ± 0.14 a	9.7 ± 0.39 a
Fungal: Bacterial ratio (F:B)	0.19± 0.01 a	0.18± 0.00 a	0.19± 0.00 a	0.19± 0.01 a

807 Mean values are presented (n=3) ± 1 standard error of the mean are followed by lower-case letters that indicate significant difference among
 808 treatments (*p*<0.05).

809 **Figure captions**

810

811 **Figure 1.** Detrended correspondence analysis of soil microbial functional community based on
812 GeoChip data after analysis of tropical forest soils treated with different rates of N fertilizer for
813 13 years. Treatment definitions: CK (zero N addition; control); LN (Low-N, 50 kg N ha⁻¹ yr⁻¹);
814 MN (Medium-N, 100 kg N ha⁻¹ yr⁻¹); HN (High-N, 150 kg N ha⁻¹ yr⁻¹).

815

816 **Figure 2.** Normalized signal intensity of detected genes indicating the degradation of different
817 organic compounds in tropical soils treated with different rates of N fertilizer addition (CK,
818 LN, MN and HN; see Figure 1 for treatment definitions) for 13 years. Signal intensities were
819 summed and normalized by the probe number for each substance. Different lower-case letters
820 indicate significant differences among treatments; error bars indicate standard error of the mean
821 (n=3).

822

823 **Figure 3.** Partial least squares path analysis for greenhouse gas (GHG) fluxes (a) CO₂, (b) N₂O
824 and (c) CH₄ from a tropical forest soil, showing the relationships between selected
825 biogeochemical processes, microbial community composition and functional gene abundances.
826 GOF = goodness of fit. GHG pools are shown in blue, microbial functional genes in black and
827 environmental variables in yellow green. The blue arrows are direct effect of environmental
828 and microbial variables on GHG fluxes, and the black arrows indicate the indirect path. The
829 numbers listed within arrows are standardized path coefficient.

830

831 **Figure 4.** Pearson correlations between abundances of C degradation genes indicating potential
832 decomposition of organic C compounds in a tropical forest soil and relative abundances of
833 bacteria and fungi.

834

835 **Figure 5.** The normalized average signal intensity of detected functional genes for N cycling
836 after N fertilizer addition for 13 years at different rates (CK, LN, MN, HN; see Figure 1 for
837 treatment definitions). Signal intensities were averaged and normalized by the probe number
838 for each gene. Different lowercase letters indicate significant differences among treatments;
839 error bars indicate standard error of the mean (n=3).

840

841 **Figure 6.** The normalized average signal intensity of detected genes indicating CH₄ (a) and P
842 (b) cycling genes in tropical forest soils treated with different rates of N fertilizer for 13 years
843 (CK, LN, MN, HN; see Figure 1 for treatment definitions). Signal intensities were averaged
844 and normalized by the probe number for each gene. Different lower-case letters indicate
845 significant differences among treatments; error bars indicate standard error of the mean (n=3).

846

847 **Figure 7.** A conceptual diagram illustrating the positive (+) or negative (-) impact of high N
848 addition on the potential activity of microbial functional genes that control C and N cycling in
849 tropical forest soils after N addition for 13 years. Greenhouse gas pools are shown in green,
850 substrate pools in yellow, microbial functional genes in orange and biological processes in blue.

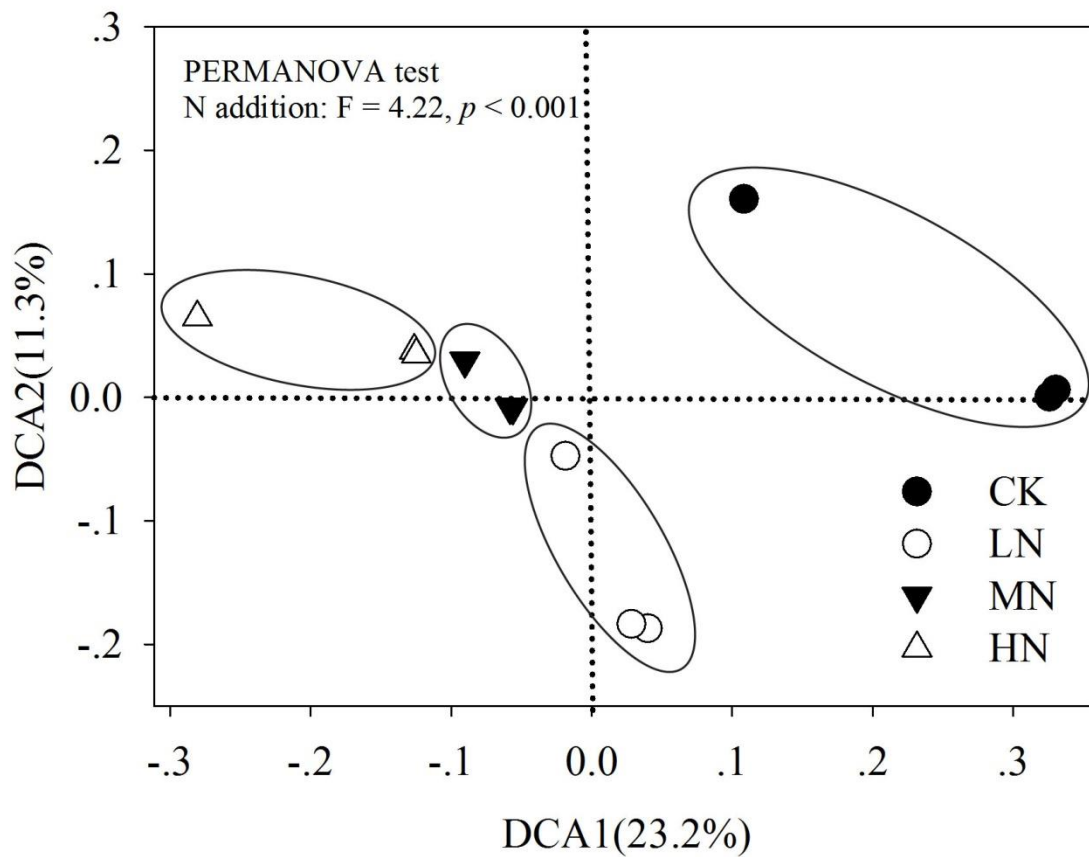
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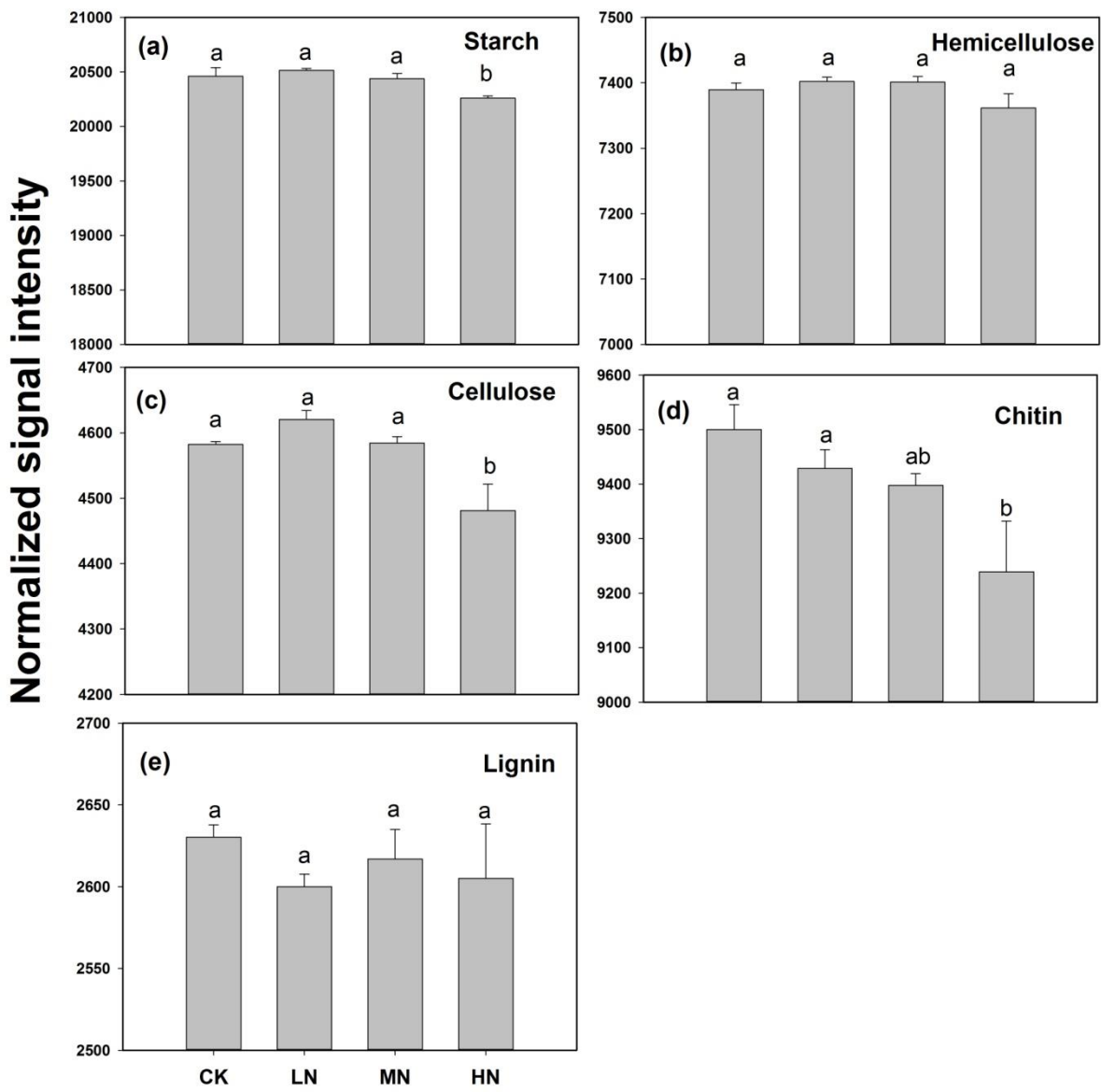
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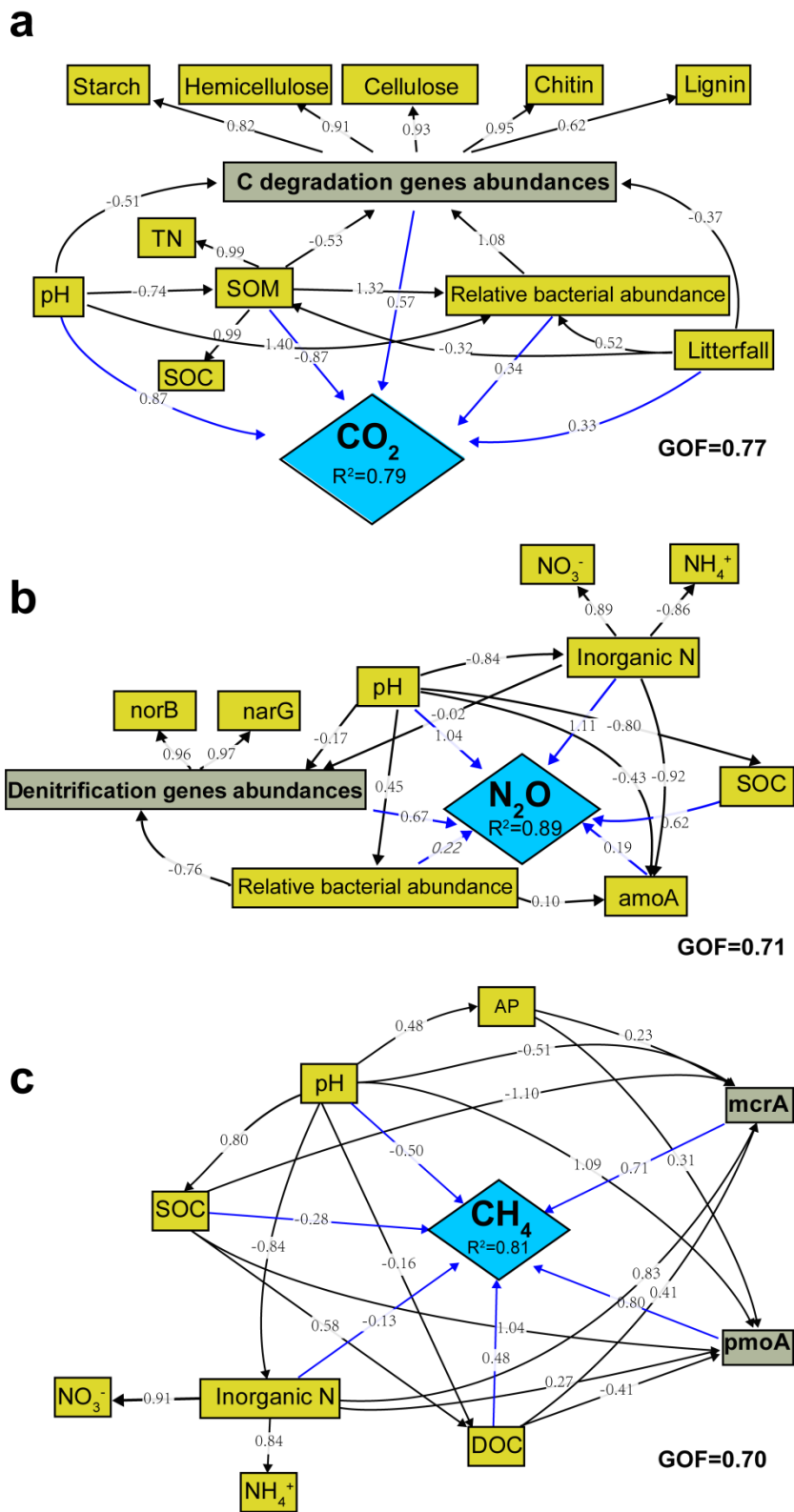
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Figure 1



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Figure 2



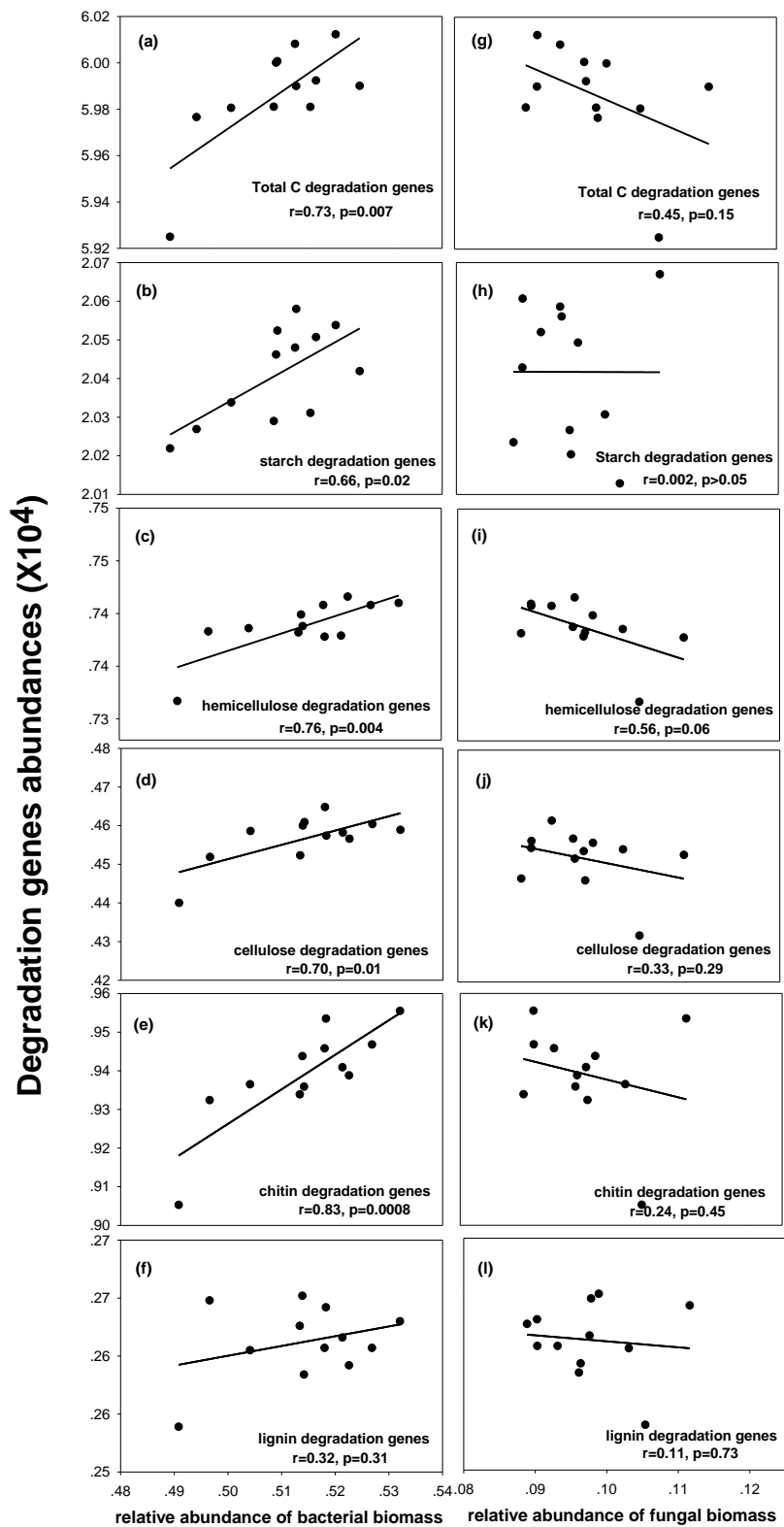
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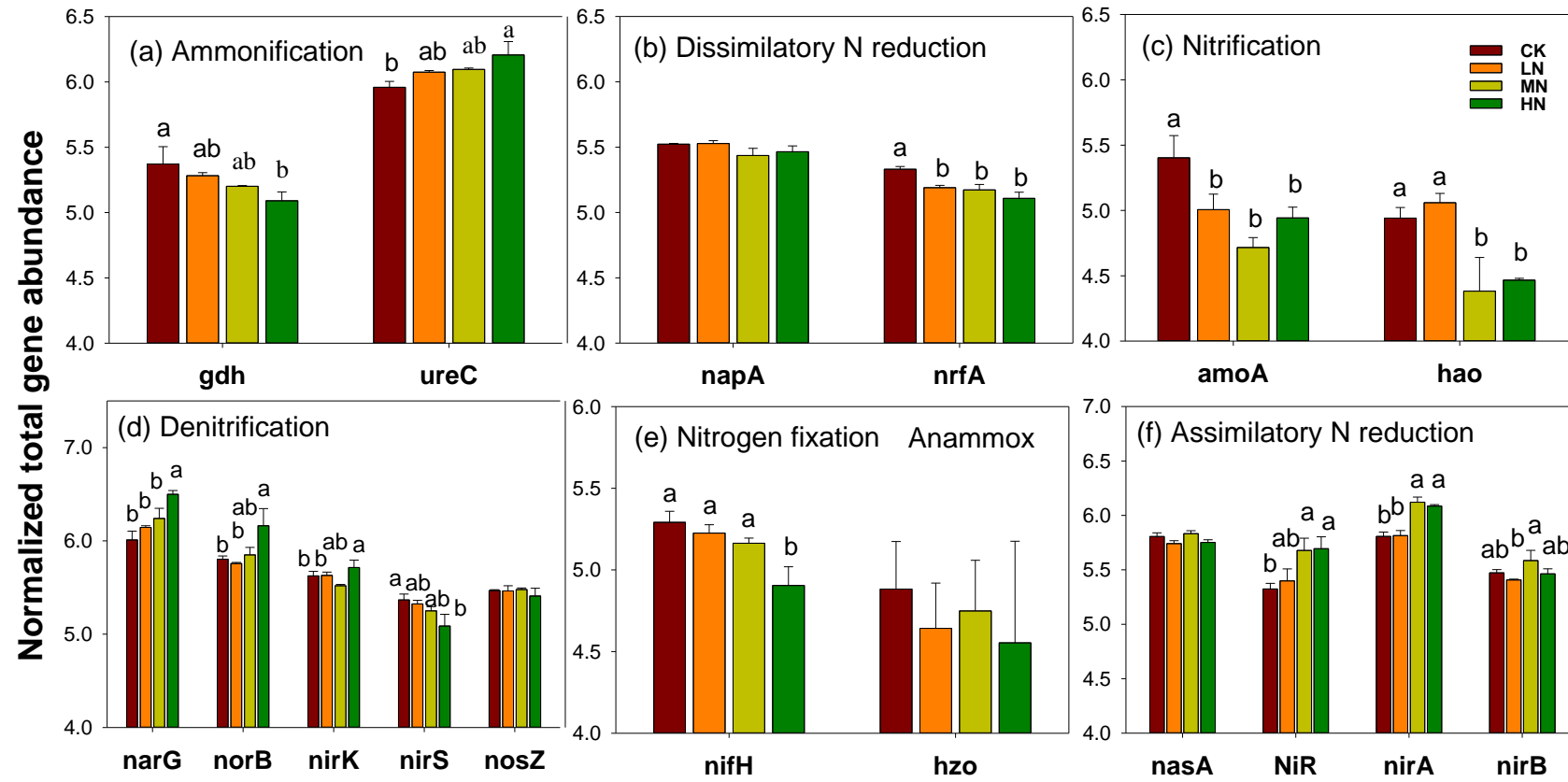
894 **Figure 3**

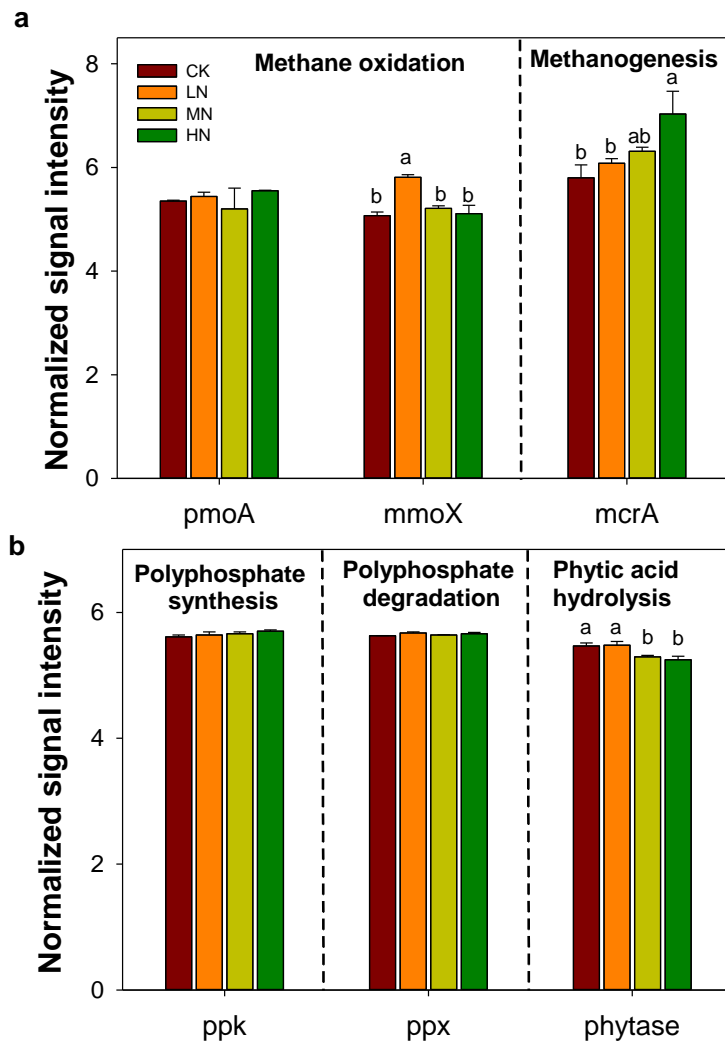
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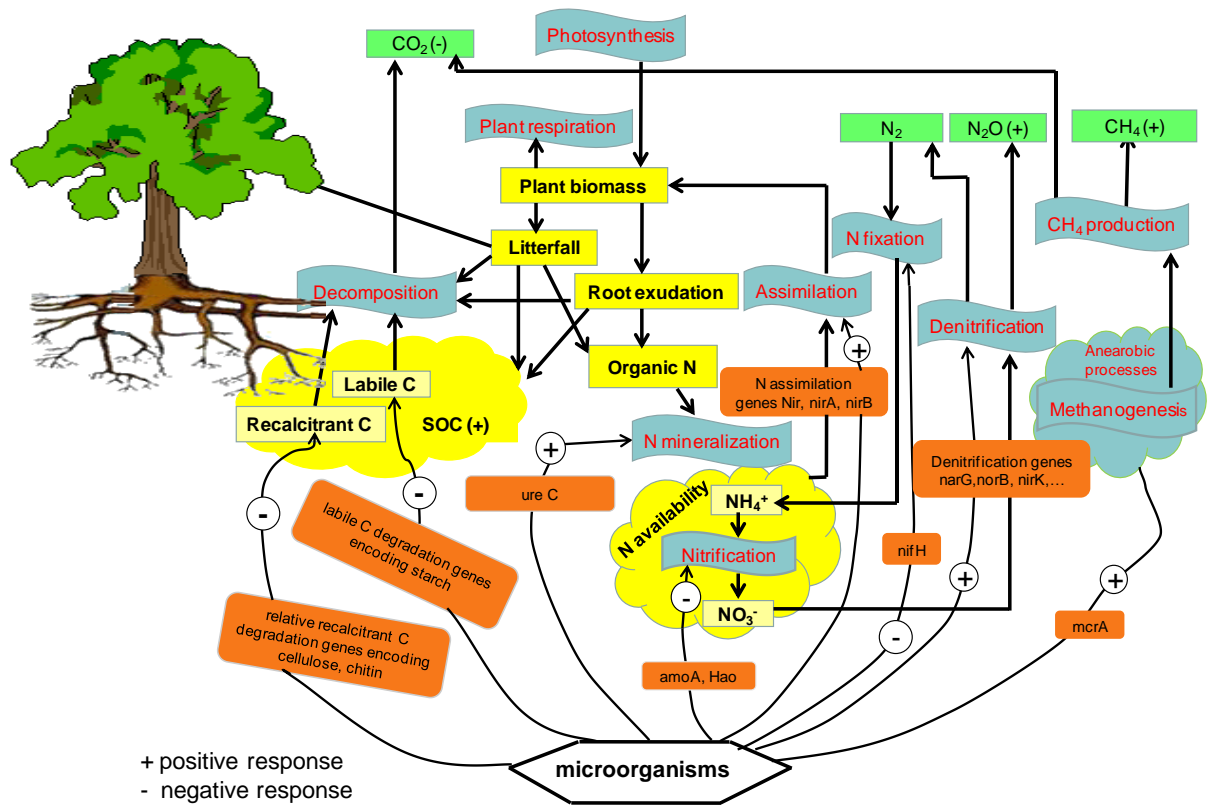






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Figure 6



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Figure 7