Article type: Primary Research Article

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

Running Title: Effect of nitrogen on soil microbial functions

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Abstract

Nitrogen (N) deposition is a component of global change that has considerable impact on belowground carbon (C) dynamics. Plant growth stimulation and alterations of fungal community composition and functions are the main mechanisms driving soil C gains following N deposition in N-limited temperate forests. In N-rich tropical forests, however, N deposition generally has minor effects on plant growth; consequently, C storage in soil may strongly depend on the microbial processes that drive litter and soil organic matter decomposition. Here, we investigated how microbial functions in old-growth tropical forest soil responded to 13 years of N addition at four rates: 0 (Control), 50 (Low-N), 100 (Medium-N), and 150 (High-N) kg N ha^{-1} yr^{-1}. Soil organic carbon (SOC) content increased under High-N, corresponding to a 33% decrease in CO_2 efflux, and reductions in relative abundances of bacteria as well as genes responsible for cellulose and chitin degradation. A 113% increase in N_2O emission was positively correlated with soil acidification and an increase in the relative abundances of denitrification genes (*narG* and *norB*). Soil acidification induced by N addition decreased available P concentrations, and was associated with reductions in the relative abundance of phytase. The decreased relative abundance of bacteria and key functional gene groups for C degradation were related to slower SOC decomposition, indicating the key mechanisms driving SOC accumulation in the tropical forest soil subjected to High-N addition. However, changes in microbial functional groups associated with N and P cycling led to coincidentally large increases in N_2O emissions, and exacerbated soil P deficiency. These two factors partially offset the perceived beneficial effects of N addition on SOC storage in tropical forest soils. These findings suggest a potential to incorporate microbial community and functions into Earth system models considering their effects on greenhouse gas emission, biogeochemical processes and biodiversity of tropical ecosystems.

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

cycling; global climate change; C and N turnover

Introduction

Atmospheric deposition of reactive nitrogen (N) has increased significantly across every continent in the last few decades, with the most rapid increase observed in the northern tropical zone (Galloway et al., 2004; Tian et al., 2015). Investigations of the responses of temperate and boreal forest ecosystems to N deposition are relatively extensive compared to forests in the tropics, where most of them are generally of much shorter duration (Pajares & Bohannan, 2016; Cusack et al., 2016). N fertilization can increase carbon (C) sequestration in temperate and boreal forests by increasing net primary productivity (NPP) and slowing soil organic carbon (SOC) decomposition rates, but the net effect of N deposition on soil C storage in tropical forests remains unclear; this sink is extremely vulnerable to human perturbation, including the indirect effects of land use and climate change (Cusack et al., 2016; Baccini et al., 2017). Tropical forest soils are generally rich in N compared to the natural N-limitation in boreal and temperate ecosystems, and are the largest natural sources of N_2O emissions that are produced primarily by soil microbial nitrification/denitrification processes (Li, Niu, & Yu, 2016; Vitousek, Porder, Houlton, & Chadwick, 2010; Lu & Tian, 2013). In contrast, biologically available P is generally the limiting nutrient in old, highly weathered tropical forest soils (Camenzind, Hattenschwiler, Treseder, Lehman, & Rillig, 2018), and the effects of N addition on P cycling in these fragile environments are poorly understood. Consequently, the N deposition in tropical forest ecosystems may cause changes in soil microbial community composition and metabolic functions, reducing the potential for tropical forests to deliver globally important ecosystem services that regulate global climate and weather patterns.

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

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

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

moderate response intensity to N augmentation through atmospheric deposition (Hedin, Brookshire, Menge, & Barron, 2009; Cleveland et al., 2011). Nitrogen dissimilative process rates are usually pronounced in N-rich ecosystems (Levy-Booth, Prescott, & Grayston, 2014), causing higher N_2O losses by denitrification and NO_3^- leaching from tropical soils compared to temperate forest soils (Vitousek & Matson, 1988; Zhang, Yu, Zhu, & Cai, 2014). Increasing N availability in tropical forests inhibits biological N fixation (Cusack, Silver, & McDowell, 2009), alters nitrification (Han, Shen, Zhang, & Müller, 2018), and increases N_2O emissions (Liu & Greaver, 2009), which increase greenhouse gas warming potential. Furthermore, chronic N deposition causes soil acidification with consequences for the availability and leaching of P, base cations (Mg^{2+}, K^+, and Ca^{2+}), and micronutrients (Mo and Zn) that are essential for plant and microorganisms (Lu et al., 2015; Cusack et al., 2016; Zamanian, Zarebanadkouki, & Kuzyakov, 2018). Soil acidification can cause direct effects on plant and microbial community composition, and indirectly through the release of Al^{3+} which has a broad-spectrum toxicity for plants (Kaspari et al., 2017). Wang et al. (2009) reported significantly increased Al^{3+} concentrations under high N addition soon after experimental treatment were started in a tropical forest. Soil acidification may reduce P transport across cell membranes, exacerbating the existing P limitation in weathered tropical forest soils (Kaspari et al., 2017; Li, Niu, & Yu, 2016).

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

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

We exploited an existing 13-year N deposition field experiment in a monsoonal evergreen broadleaf old-growth forest in southern China. Previous research in the region has provided evidence that old-growth forests accumulated SOC over a 24-year period from 1979-2003 (Zhou et al., 2006). Previously, we reported that N addition increased rates of plant transpiration (Lu et al., 2018) and N_2O emissions (Zhang et al., 2008), but reduced litter decomposition (Fang, Mo, Peng, Li, & Wang, 2007) and soil respiration (Mo et al., 2008). We also found that N addition aggravated soil acidification and P supply, but increased Al mobility (Lu et al., 2015). However, the extent to which shifts in the microbial functional community underpin these changes was not investigated. Here, we hypothesized that (1) long-term N additions alter microbial community composition by increasing soil acidification; and (2) the effects of N addition on the microbial community composition would translate into changes in microbial function that help explain the observed effects on soil C, N, and P cycling. To test these hypotheses, we used a microarray-based tool (GeoChip 5.0) to profile microbial functional

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

Materials and Methods

Field experiment and sampling

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

The experimental design is described in Mo et al. (2008). In brief, twelve plots of four treatments with three replicates were laid out in a completely randomized block design. Each plot was 20 m × 10 m, and was bounded by a 10 m wide buffer strip. N was added as ammonium nitrate (NH_4NO_3) solution at four N levels: Control (CK; zero N), Low-N (LN; 50 kg N ha^{-1} y^{-1}), Medium-N (MN; 100 kg N ha^{-1} y^{-1}), and High-N (HN; 150 kg N ha^{-1} y^{-1}). Nitrogen was applied below the canopy on 12 occasions at monthly intervals each year at equal rates using a backpack sprayer. . The control soil was treated with an equal volume (20 L) of deionized water.

The experimental plots were sampled in October 2015. Prior to soil sampling, CO_2, N_2O, and CH_4 fluxes were collected from each plot from two static chambers that were inserted 5 cm into the soil (Zhang et al., 2008). Gas samples were taken using 100 ml plastic syringes at 0, 10, 20, and 30 min after chamber closure, and were analyzed in the laboratory within 24 h.

Soil samples were taken using a soil corer (0-10 cm deep, 2 cm inner diameter) from 10 random points across each plot, and mixed to yield one composite sample per plot. The litter layer was carefully removed before sampling. The soil samples were stored in airtight polypropylene bags and placed in a cool box at 4 °C during transportation to the laboratory. Litter, roots, and stones were carefully removed by hand, and the soil was divided into several subsamples. Subsamples for dissolved organic carbon (DOC), ammonium N (NH_4^+-N), nitrate N (NO_3^--N) and available phosphorus (AP) concentration analyses were stored at 4 °C for no longer than one week. Subsamples for pH, soil organic carbon (SOC), total nitrogen (TN), total phosphorus (TP), and pH analyses were air dried. Subsamples for microbial community composition and functional gene (GeoChip) analysis were stored at -80 °C.

Greenhouse gas and soil analyses

Greenhouse gas (CO_2, N_2O, and CH_4) concentrations of the air sampled in each treatment were measured by gas chromatography (Agilent 4890D, Agilent Co., Santa Clara, CA, USA). Soil pH was measured with a pH meter after shaking the soil in deionized water (1:2.5 w/v) suspensions for 30 min. The SOC and TN content were determined by combustion using a Vario EL III Elemental Analyzer (Elementar, Germany). Ammonium N (NH_4^+-N) and NO_3^--N concentrations were determined using an autoanalyzer (TRAACS-2000, BRAN+LUEBBE, Germany) following 0.01 M KCL (1:10 w/v) extraction for 30 min. Total P and available P concentrations were measured using the ammonium molybdate method after H_2SO_4-H_2O_2-HF digestion. The DOC concentration was measured following the method used by Jones and Willett (Jones & Willett, 2006). The field-moist soil samples (equivalent to 15 g oven-dried 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 extract was then passed through a 0.45-mm membrane filter and analyzed for DOC using a Multi 3100 N/C TOC analyzer (Analytik Jena, Germany). Microbial community composition

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

Analyses of microbial functional communities

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

Statistical analyses

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

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

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

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

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

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

Results

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

Compared with the control treatment (CK), the effects of N addition on GHG (CO_2, N_2O, and CH_4) fluxes were significant under High-N and Medium-N treatments, but not for Low-N (Table 1). CO_2 emission was 33% less under High-N compared with control. As compared with control, CH_4 uptake decreased by 64% and 63% in Medium-N and High-N treatments, but N_2O emission increased by 113% under High-N. All measured soil parameters in the High-N-treated soils apart from DOC and TP were significantly different from CK. Soil organic carbon (SOC) and TN contents and NH_4^+ concentrations were higher under the High-N treatment. Nitrate (NO_3^-) concentrations increased under Medium-N and High-N compared to Low-N and CK. Available P and pH were lower under Medium-N and High-N treatments than under Low-N. Bacterial relative abundance based on PLFA biomarker contents was lower under High-N treatment than under Low-N, but there was no significant difference in fungal PLFA biomarker concentrations or fungal: bacterial (F:B) ratios between treatments.

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

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

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

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

Linking microbial functional genes to soil CO_2 emission

Key genes associated with C degradation were analyzed and related to soil CO_2 emissions. High N decreased the abundances of genes responsible for labile and recalcitrant C (starch, cellulose, and chitin) degradation ($p< 0.05$; Fig. 2). For example, the relative abundances of *apu*, *npiT*, *amyX*, *cellobiase*, *acetylglucosaminidase* and *chitinase* were the lowest under High-N ($p<0.05$; Fig. S3). PLS path modeling explained 79% of the CO_2 emission variance, and provided the best fit to our data (GOF of 0.77) (Fig. 3a). Bacterial relative abundance showed the largest effect on CO_2 emission via direct (path coefficient = 0.34) and indirect effects (path coefficient = 0.62) on microbial functional C degradation genes (Fig. 3a). There were corresponding strong, positive correlations between bacterial relative abundance and the abundances of genes involved in starch, hemicelluloses, cellulose and chitin degradation, but no similar relationship was observed with fungal relative abundance (Fig. 4). The positive

direct effect of C degradation genes (path coefficient = 0.57) on CO_2 emission was the greatest (Fig.3a). Among C degradation genes, strong positive correlations between cellulose and chitin degradation gene abundances and CO_2 production were observed (Fig. S4; $p<0.05$). This finding was consistent with PLS path modeling that showed large loading factors (Fig. 3a).

Linking microbial functional genes to soil N_2O emission

With increasing N levels, the abundance of *gdh* gene (glutamate dehydrogenase) decreased, while that of *ureC* (urease) increased (Fig. 5; $p<0.05$), suggesting a shift in microbial functional potential toward N mineralization. Both genes associated with nitrification (*amoA* and *hao*) decreased under Medium-N and High-N treatments. High-N increased denitrification genes including *narG* (membrane-bound nitrate reductase), *norB* (nitric oxide reductase), and *nirK* (copper-containing nitrite reductase). Correspondingly, NO_3-N concentrations were related to the increased abundance of genes encoding *NiR* and *nirA* (assimilatory nitrate reduction). The abundance of the N fixation gene (*nifH*) was the lowest under High-N treatment.

PLS path modeling explained 89% of the N_2O emission variance (Fig. 3b). There was a direct positive effect of denitrification genes on N_2O emission in the PLS path model that was supported by the Pearson correlations (Fig.S5). This suggested that increased denitrification was the driving force for N_2O production after N amendment. Among soil variables, the positive direct effect of inorganic N concentrations on N_2O emission was the greatest, while pH was related to the largest negative indirect effect (Fig. 3b).

Linking microbial functional genes to soil CH_4 uptake

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

342 path modeling accounted for 81% of the variation in CH_4 uptake (Fig.3c). There were strong
343 positive direct effects of mcrA (methanogenesis) on CH_4 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 CH_4 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

Discussion

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

SOC accumulation in old growth tropical forests under N addition

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

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

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

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

High-N addition decreased CO_2 emission by 33% in this study (Table 1), concurring with previous studies that reported that N additions decreased soil respiration in recent studies of tropical forest soils (Fan et al., 2014; Wang et al., 2016), although by comparison, Feng et al. (2017) reported no change. We observed that bacterial relative abundance was decreased under High-N addition (Table 1). Wang et al. (2009) explored the initial effects of N addition in the early stages of the experiment and found that soil microbial biomass C (extracted by chloroform-fumigation) was generally decreased in N addition plots, but significantly decreased in the High-N plots after 2 and 4 years. These observations are contrary to the observations of similar experiments in temperate and boreal forests in general. Furthermore, bacterial relative abundance had the largest total effect of on total soil respiration (CO_2 emission) (Fig.3a; $p<0.05$). A decrease in microbial abundance provides a straightforward explanation for the measured decrease in CO_2 emissions from tropical forests under High-N addition, as previously proposed by Wei et al. (2008). Direct and indirect effects of each latent variable on target variables were identified using PLSPM analysis. The processes underlying the measured CO_2 emission in the field include soil organic matter, roots and microorganisms derived (Kuayzkov, 2006). Thus, the indirect effect of bacteria on CO_2 may be through changes in the production of C degradation enzymes, and the direct effect of bacteria on CO_2 may be derived from changes in microbial respiration, in response to N addition. Significant relationships between CO_2 emission and the relative abundance of cellulose and chitin degradation genes were observed, respectively (Fig. S4; $p<0.05$). This indicates that N addition inhibited the production of C degradation enzymes by the soil bacterial biomass, which may drive the accumulation of intact organic compounds in the soil, thereby increasing SOC under High-N conditions. This is further evidence supporting the hypothesis that SOM turnover is controlled

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

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

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

457 CH_4 emission by 97% and reduced CH_4 uptake by 38%. Schimel (2000) had previously
458 described that high NH_4^+ concentrations could inhibit CH_4 oxidation because of competition
459 for methane monoxygenase resulting in increased CH_4 emissions. Methanotrophy indicated by
460 *pmoA* and *mmox* expression was not significantly different from the control apart from *mmox*
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 CH_4 uptake (Fig. 3c)
463 suggests that the decrease of CH_4 uptake may largely be due to increased methanogenesis. The
464 abundance of the *mcrA* gene positively correlates with CH_4 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 CH_4
467 flux (Fig. 3C). This agrees with previous reports that labile C pool fuels the activity of
468 methanogenic populations, resulting in increased CH_4 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

bacterial abundance decreased under High-N conditions.

The N_2O flux from soil mainly originates from nitrification or denitrification (Levy-Boothet al., 2014). The large increase in N_2O emissions (113%; Table 1) under High-N conditions coincided with increased soil acidity, the metabolic potential for NO_3-N reduction in this study (Fig. 3B), and an increased relative abundance of genes involved in denitrification (*narG* and *norB*) and assimilatory N reduction (*NiR* and *nirA*) (Figs. 3b, S5, and S7). The strong direct positive effect of denitrification genes, but not nitrification genes, on N_2O emission (path coefficient =0.67; Fig.3b), suggests that denitrification was a driving force of N_2O emission in this acidic tropical forest soil. Our result agreed with previous studies that N addition can increase N_2O emissions and is correlated with genes involved in denitrification pathway (Corre, Veldkamp, Arnold, & Wright, 2010; Han, Shen, Zhang, & Mülle, 2018). Among denitrification genes, we determined significant contributions from *narG* and *norB* genes to N_2O emissions (Figs. 3b and S5). Previous studies found that *nosZ* gene was positively correlated with soil NO and N_2O production in acidic forests (Yu et al., 2014; Lammel, Feigl, Cerri, & Nusslein, 2015), but the abundance of *nosZ* was not significantly different from the control in our study. Relative abundances of *nirK* were reported to increase under enhanced NO_3^--N concentrations (Morales, Cosart, & Holben, 2010), and low pH (Rütting, Huygens, Boeckx, Staelens, & Klemedtsson, 2013). Nitrogenous gas emissions (N_2O/N_2 emission ratios) can be influenced by measurable soil properties in tropical soils including parent materials, pH, soil moisture, and redox potential (Rütting, Huygens, Boeckx, Staelens, & Klemedtsson, 2013; Zhang, Cai, Cheng, & Zhu, 2009; Liu, Morkved, Frostegård, & Bakken, 2010; Stone, Kan, & Plante, 2015; Kang, Mulder, Duan, & Dorsch, 2017). Therefore, the variations between soil N processes and gene expression response to N additions are complex and barely predictable (Levy-Boothet al., 2014; Chen et al., 2019). Indeed, in our experiment, *nirS* decreased, again suggesting that ubiquitous soil processes including denitrification are performed by a range of microorganisms

that respond differently to stresses including pH change. The decreased abundance of nitrification genes that coincided with the increased abundances of denitrification genes and N_2O emissions indicate a potentially high N loss, likely counteracting net N accumulation in this study. Most significantly, considering the high warming potential of N_2O, the stimulated N_2O emissions potentially offset the perceived beneficial effect of N addition on soil C accumulation in tropical old growth forest soils, and suggest that the effects of chronic N deposition on remote ecosystems must be considered in models predicting feedbacks to climate change.

In conclusion, this study revealed profound interrelationships between the response of soil microbial functional potentials and soil C, N, and P cycling to chronic N addition in 'pristine' tropical forests. Changes in bacterial relative abundance significantly affected CO_2 emissions, mainly via indirect effects on microbial functional C degradation genes leading to SOC accumulation. We revealed the metabolic potential for the increased expression of microbial nitrogen functional genes driving increased N_2O fluxes from these ecosystems. Limitations on P availability caused by soil acidification through reduced *phytase* expression were also identified. This new understanding of the effect of human-induced atmospheric N deposition must be factored into Earth system models considering the GHG sink capacity of tropical forests, and the effect of air pollution from agriculture and industry on the biogeochemical processes and biodiversity of tropical ecosystems. Further studies are necessary to investigate the effect of climatic variation at a range of timescales (seasonal and decadal) to investigate the impact of N addition on the dynamics of microbial communities and functions, and their roles in mediating soil biogeochemistry, under a more extensive range of environmental conditions experienced by the soil microbial community.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (grant nos. 31770560, 41571130041 and 41731176), the National Key R&D Program of China (grant no. 2017YFA0604803), and the Youth Innovation Research Team Project (grant no.LENOM2016Q0004). Yakov Kuzyakov is very thankful for the Changjiang Scholar Award.

Conflict of interest

The authors declare that they have no conflict of interest.

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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 microbial composition and greenhouse gas emission.

	Treatment (kg N ha^{-1} yr^{-1})			
	CK	Low-N	Medium-N	High-N
	0 (control)	50	100	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_2O (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_2O)	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

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

Figure captions

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

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

Figure 3. Partial least squares path analysis for greenhouse gas (GHG) fluxes (a) CO_2, (b) N_2O and (c) CH_4 from a tropical forest soil, showing the relationships between selected biogeochemical processes, microbial community composition and functional gene abundances. GOF = goodness of fit. GHG pools are shown in blue, microbial functioal genes in black and environmental variables in yellow green. The blue arrows are direct effect of environmental and microbial variables on GHG fluxes, and the black arrows indicate the indirect path. The numbers listed within arrows are standardized path coefficient.

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

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

Figure 6. The normalized average signal intensity of detected genes indicating CH_4 (a) and P (b) cycling genes in tropical forest soils treated with different rates of N fertilizer for 13 years (CK, LN, MN, HN; see Figure 1 for treatment definitions). Signal intensities were averaged and normalized by the probe number for each gene. Different lower-case letters indicate significant differences among treatments; error bars indicate standard error of the mean (n=3).

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

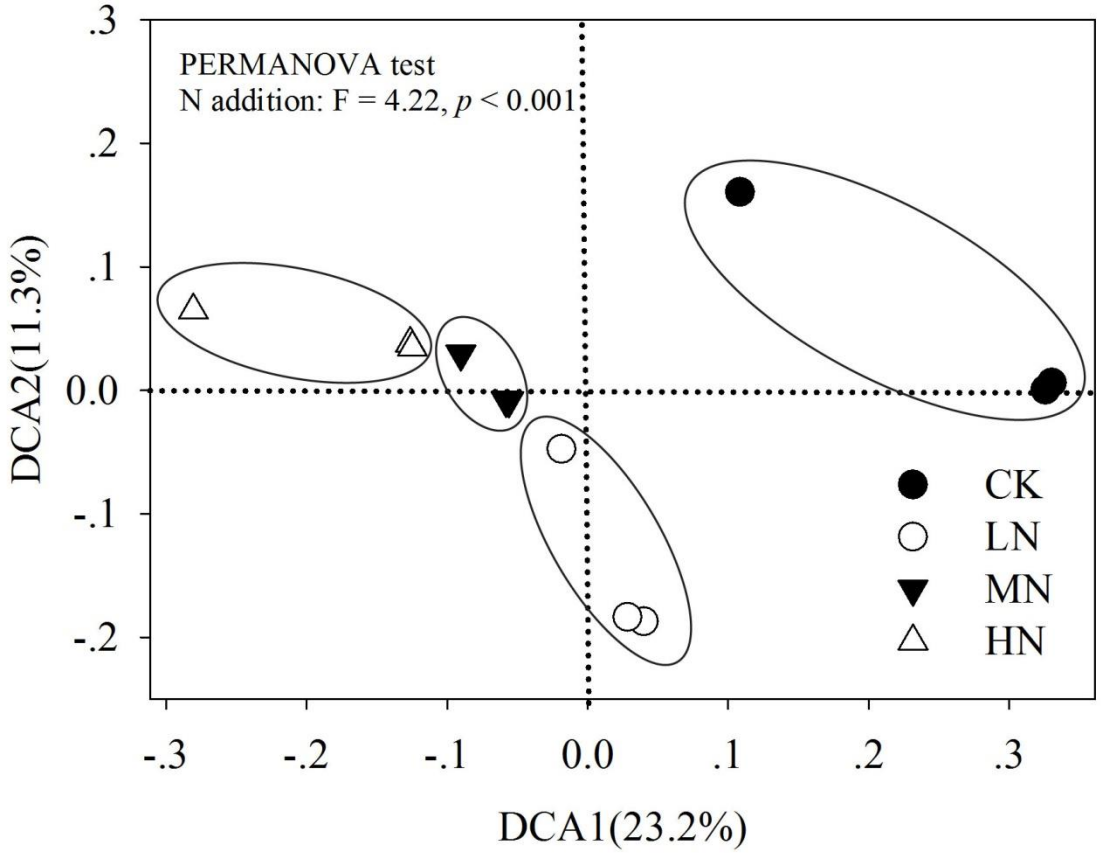
PERMANOVA test
N addition: F = 4.22, p < 0.001
CK
LN
MN
HN
DCA2(11.3%)
DCA1(23.2%)
.3
.2
.1
0.0
-.1
-.2
-.3
-.2
-.1
0.0
.1
.2
.3

Figure 1

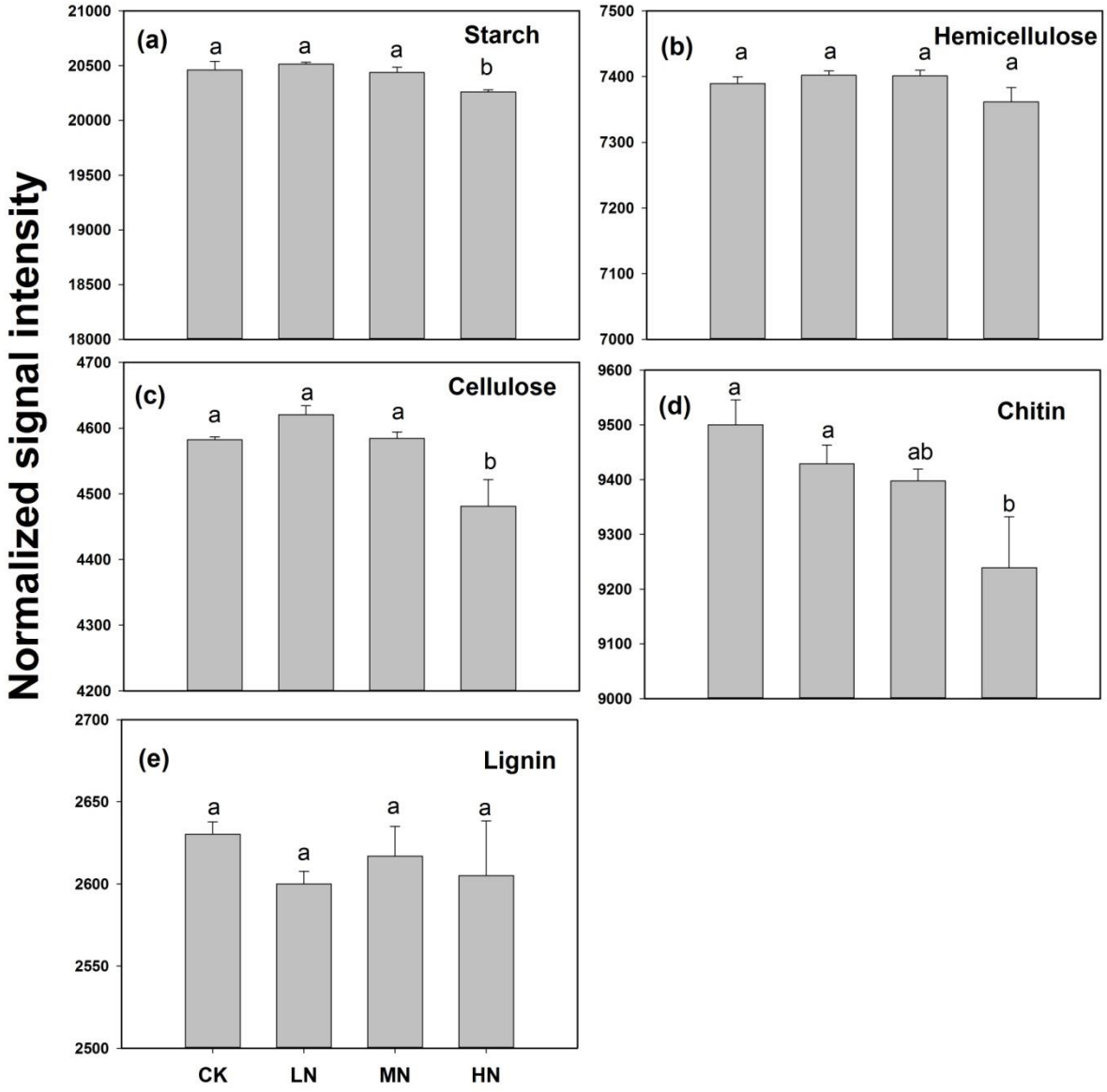
Normalized signal intensity
(a)
Starch
a
a
a
b
21000
20500
20000
19500
19000
18500
18000
(b)
Hemicellulose
a
a
a
a
7500
7400
7300
7200
7100
7000
(c)
Cellulose
a
a
a
b
4700
4600
4500
4400
4300
4200
(d)
Chitin
a
a
ab
b
9600
9500
9400
9300
9200
9100
9000
(e)
Lignin
a
a
a
a
2700
2650
2600
2550
2500
CK
LN
MN
HN

Figure 2

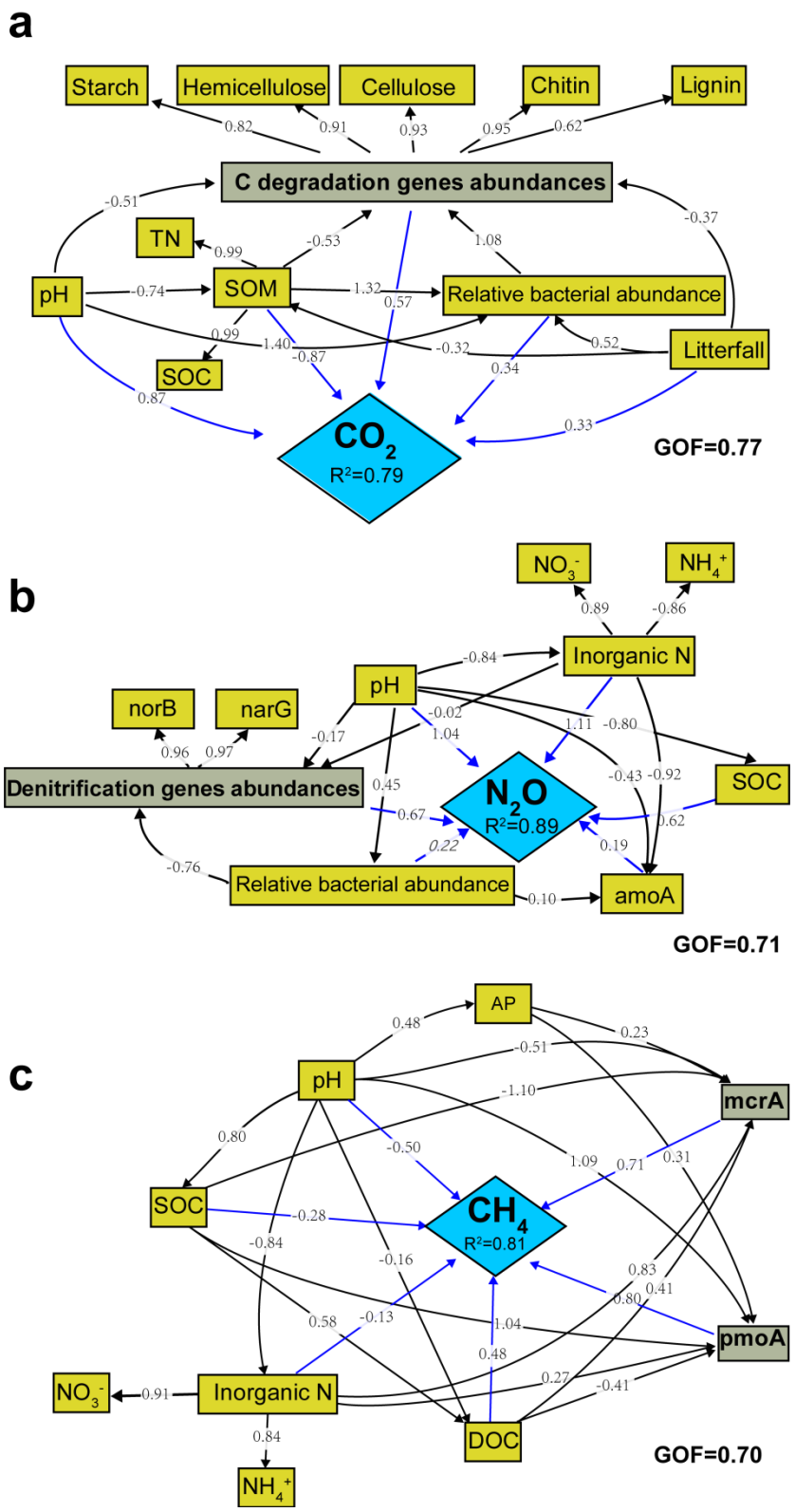
a
Starch
Hemicellulose
Cellulose
Chitin
Lignin
C degradation genes abundances
TN
pH
SOM
Relative bacterial abundance
Litterfall
SOC
CO2
R²=0.79
GOF=0.77
b
NO3-
NH4+
Inorganic N
pH
norB
narG
Denitrification genes abundances
N2O
R²=0.89
SOC
Relative bacterial abundance
amoA
GOF=0.71
c
AP
pH
mcrA
SOC
CH4
R²=0.81
pmoA
NO3-
Inorganic N
DOC
NH4+
GOF=0.70

Figure 3

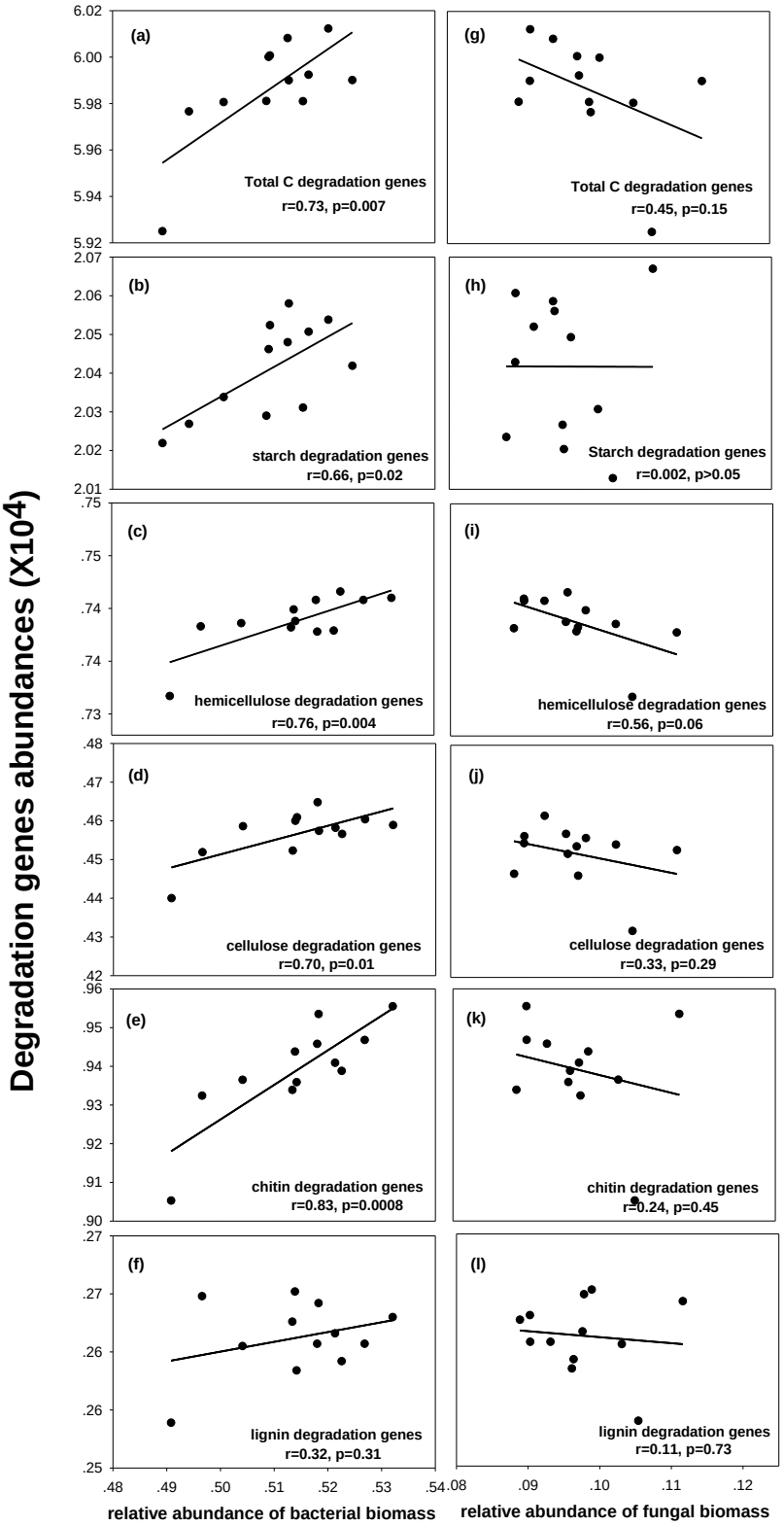
Degradation genes abundances (X10^4)
(a)
Total C degradation genes
r=0.73, p=0.007
(g)
Total C degradation genes
r=0.45, p=0.15
(b)
starch degradation genes
r=0.66, p=0.02
(h)
Starch degradation genes
r=0.002, p>0.05
(c)
hemicellulose degradation genes
r=0.76, p=0.004
(i)
hemicellulose degradation genes
r=0.56, p=0.06
(d)
cellulose degradation genes
r=0.70, p=0.01
(j)
cellulose degradation genes
r=0.33, p=0.29
(e)
chitin degradation genes
r=0.83, p=0.0008
(k)
chitin degradation genes
r=0.24, p=0.45
(f)
lignin degradation genes
r=0.32, p=0.31
(l)
lignin degradation genes
r=0.11, p=0.73
relative abundance of bacterial biomass
relative abundance of fungal biomass

Figure 4

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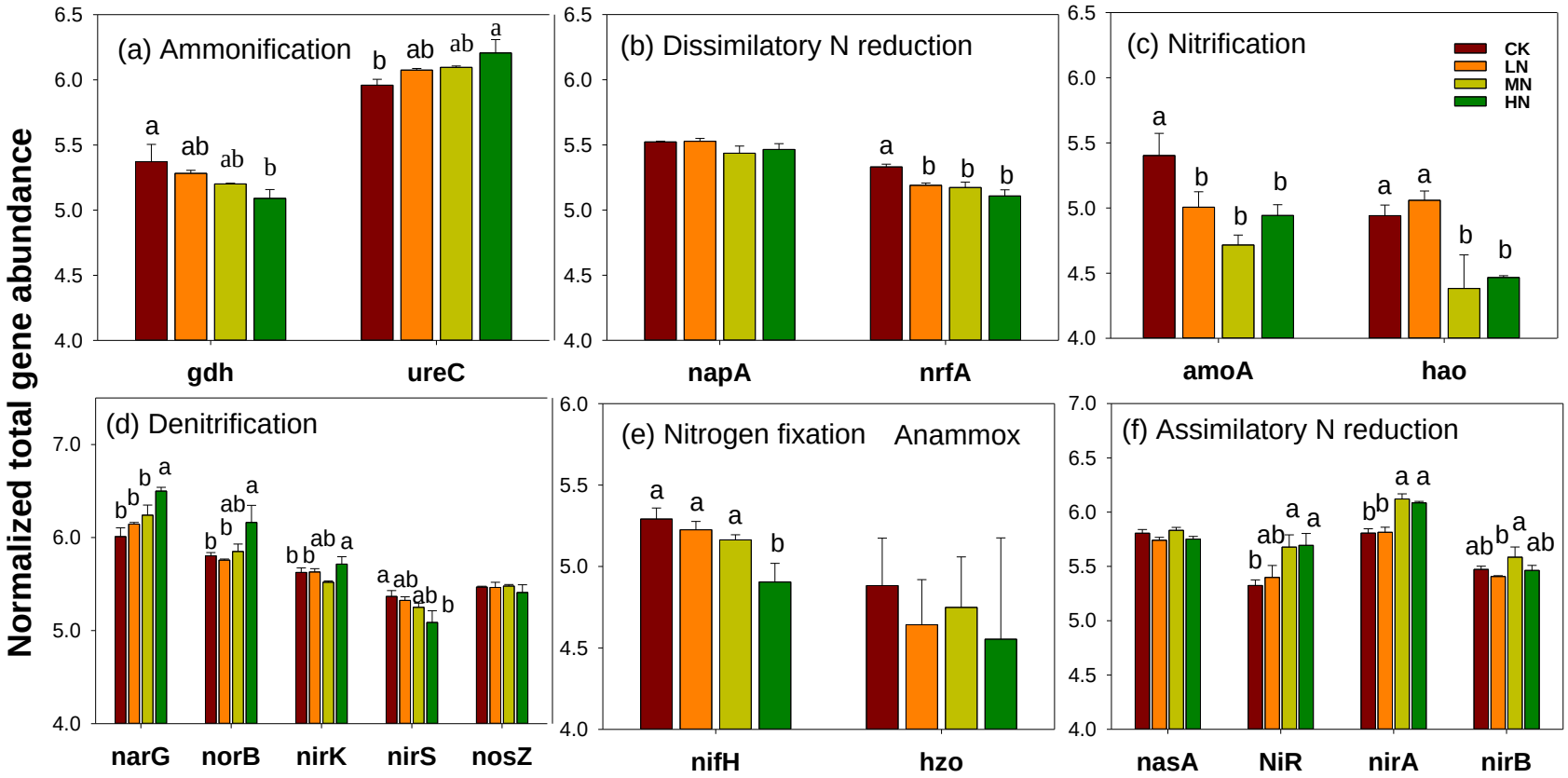

904

905 **Figure 5**

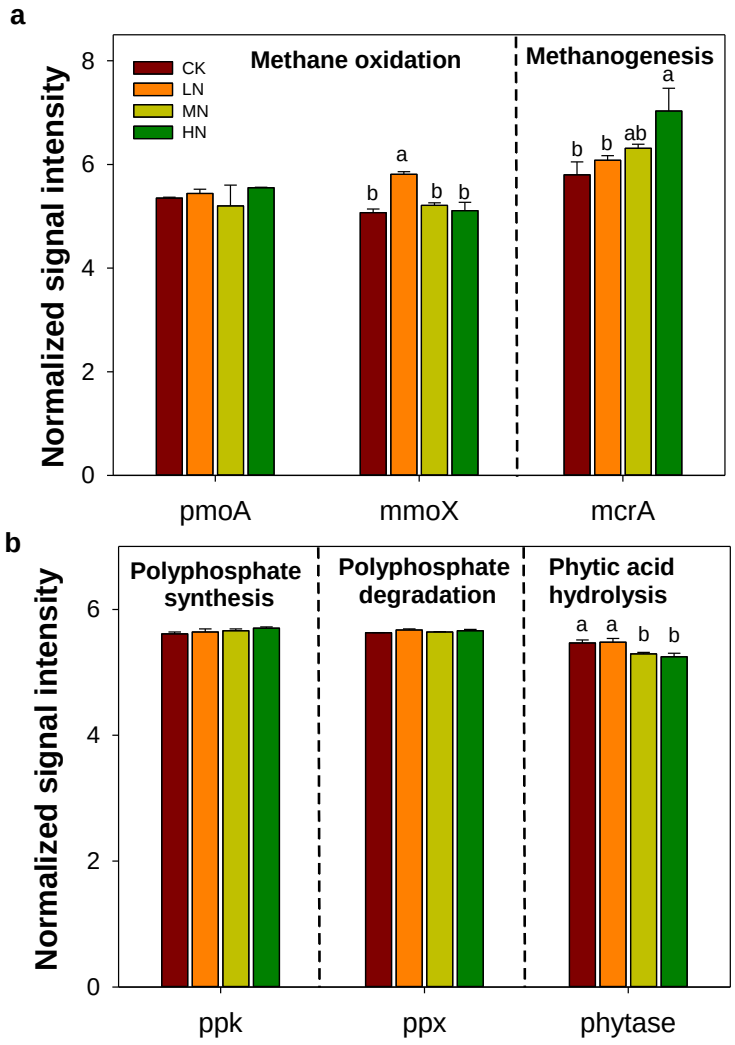
a
Normalized signal intensity
Methane oxidation
Methanogenesis
CK
LN
MN
HN
pmoA
mmoX
mcrA
b
Normalized signal intensity
Polyphosphate synthesis
Polyphosphate degradation
Phytic acid hydrolysis
ppk
ppx
phytase

Figure 6

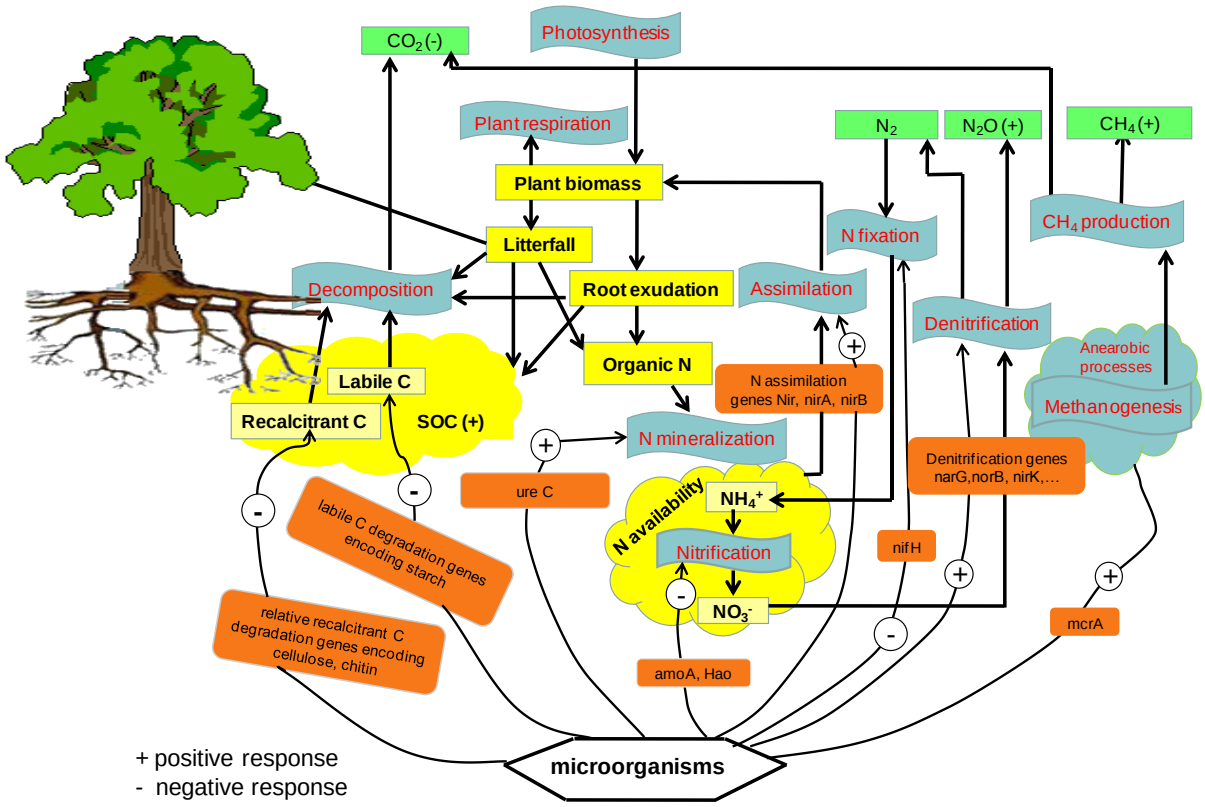
CO_2 (-)
Photosynthesis
Plant respiration
N_2
N_2O (+)
CH_4 (+)
Plant biomass
Litterfall
N fixation
CH_4 production
Decomposition
Root exudation
Assimilation
Denitrification
Anearobic processes
Methanogenesis
Organic N
N assimilation genes Nir, nirA, nirB
Labile C
Recalcitrant C
SOC (+)
N mineralization
ure C
Denitrification genes narG,norB, nirK,...
N availability
NH_4^+
Nitrification
nifH
labile C degradation genes encoding starch
relative recalcitrant C degradation genes encoding cellulose, chitin
NO_3^-
mcrA
amoA, Hao
microorganisms
+ positive response
- negative response

Figure 7