Nitrogen deposition enhances soil organic carbon and microbial residual carbon in a tropical forest

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Abstract

Background: Two carbon (C) sources are of particular interest for soil organic carbon (SOC) storage under nitrogen (N) deposition: 1) glomalin-related soil protein (GRSP) and 2) microbial residual carbon (MRC) derived from microbial metabolites and residues. Both soil C sources are purported to have long residence times, but their contribution to SOC may be modified by changing soil N status.

Methods: We assessed how N deposition influences GRSP and MRC as sources of SOC using soils from a long-term (11 years) N-addition site in a tropical forest in south China. We assessed differences in MRC and GRSP, microbial biomarkers, soil physico-chemical properties, and particle-size fractions between N-fertilized soils and controls.

Results: Total GRSP, MRC, and SOC concentrations were higher under N-addition, but soil microbial biomass and community composition were largely unaffected, indicating that higher GRSP and MRC concentrations resulted from long-term accumulation and reduced microbial degradation. However, the relative contributions of GRSP or MRC to SOC were unchanged or lower than the controls due to a greater contribution of other C sources to SOC which were largely unstabilized by association with soil minerals.

Conclusion: Tropical forests have great potential for SOC sequestration in response to N deposition which may help mitigate climate change. However, most of the additional SOC in N-fertilized soils was not associated with soil minerals, and thus prone to decomposition.

Keywords:

Soil organic carbon, Nitrogen deposition, Glomalin-related soil protein, Microbial residual carbon, Tropical forests

Abbreviations

SOC: soil organic carbon
AMF: arbuscular mycorrhizal fungi
GRSP: glomalin-related soil protein
EE-GRSP: easily-extractable glomalin-related soil protein
T-GRSP: total glomalin-related soil protein
ACE: autoclaved-citrate extractable protein
PLFAs: phospholipid fatty acids
GluN: glucosamine
MurN: muramic acid
GalN: galactosamine
MRC: microbial residual carbon
POC: particulate organic carbon
MAOC: mineral-associated organic carbon

Introduction

Human activities are responsible for a rapid increase in global nitrogen (N) deposition (Dirnboeck et al. 2017), which affects carbon (C) and N cycling in terrestrial ecosystems by altering plant photosynthetic rates and soil C pools (IPCC 2014). The impacts of N deposition on a given ecosystem depend to a large extent on initial N availability and the fate of deposited N (Gurmesa et al. 2016). In N-limited ecosystems, low rates of N deposition may stimulate plant growth and C storage, whereas N deposition in ecosystems with high N availability can cause acidification, cation depletion and plant nutrient imbalance (Lu et al. 2018). In lowland tropical forests, N availability is relatively high compared with that in temperate or boreal forests (Wright et al. 2011), and although there is evidence that atmospheric N deposition in tropical forests can accelerate soil acidification (Lu et al. 2014), enhance leaching losses, and increase plant transpiration (Lu et al. 2018), recent work also suggests that N deposition in N-rich lowland tropical forests can also enhance soil C storage (Lu et al. 2021). Tropical forest soils play a particularly important role in global C cycling, as they account for one third of global soil organic C (SOC) storage (Grace et al. 2001; Guo and Gifford 2010; Phillips et al. 1998) with rapid turnover of organic matter under warm and humid tropical conditions (Ramankutty et al. 2002). Thus, as N deposition in tropical forests is projected to increase dramatically in the coming decades (Lu et al. 2021), understanding the mechanisms by which N deposition promotes additional SOC storage in tropical forests is globally relevant.

Nitrogen deposition can affect SOC storage by altering soil physico-chemical properties such as aggregate stability (Bai et al. 2010) and pH (Mo et al. 2008), which in turn influence microbial communities, microbial activity, and the amount of SOC available for microbial breakdown (Lu et al. 2021). Nitrogen addition can enhance SOC storage by boosting forest productivity, and because greater N-availability can reduce microbial mineralization of soil organic matter (Hui et al. 2020; Mo et al. 2008). Assessing changes in different SOC fractions, such as particulate organic C (POC) and mineral-associated organic carbon (MAOC) can elucidate how N inputs influence the stabilization or mineralization of soil organic matter (Cotrufo et al. 2019; Fang et al. 2014; Lavallee et al. 2019). POC is mainly derived from plants (Golchin et al. 1994) and persists in soils through chemical recalcitrance of soil organic molecules, physical protection in aggregates, and microbial inhibition (Cotrufo et al. 2019). By contrast, MAOC primarily comprises N-rich microbial products and persists in soils because of chemical bonding to minerals and physical protection in small aggregates (Koegel-Knabner et al. 2008). In general, POC is more vulnerable to disturbance and has faster turnover rates than MAOC (Poeplau et al. 2018), and N deposition can alter the relative contributions of these fractions to total SOC. For example, Chen et al. (2020) found that N fertilization enhances the accumulation of MAOC in temperate forest

soils and Fang et al. (2014) observed a lower ratio of POC to MAOC in soils subject to low rates of N addition (20 kg N ha^{-1} y^{-1}). Thus, long-term N addition might increase tropical forest SOC stocks by enhancing inputs and limiting microbial utilization of SOC (Hui et al. 2020; Lu et al. 2021).

Microorganisms play a critical role in SOC storage because they mediate C cycling through decomposition of organic matter. Two microbial sources of C are of particular interest for SOC storage in tropical forest soils: First, turnover of the soil microbial biomass produces microbial residual carbon (MRC), including necromass, microbial metabolites and other residues (Khan et al. 2016; Ma et al. 2017), part of which can be stabilized as SOC (Liang et al. 2017). Second, glomalin-related soil protein (GRSP) is largely composed of proteinaceous compounds of primarily microbial origin and contribute substantially to stable soil organic matter in many soils (Holátko et al. 2021). Although often associated with arbuscular mycorrhizal fungi (AMF; Steinberg & Rillig 2003; Wright and Upadhyaya 1998), recent work suggests that GRSP is not predominantly of fungal origin (Gillespie et al. 2011), but produced by complex interactions among plants, AMF, and other soil microorganisms (Holátko et al. 2021). Here, we use the term GRSP to refer to an operationally defined pool of extractable compounds without any assumptions about their origin (Holátko et al. 2021). Both GRSP and MRC are relatively stable and can make considerable contributions to SOC storage in tropical soils (Dong et al. 2015; Staddon et al. 2003). Indeed, many studies have noted strong relationships between total SOC stocks and GRSP (Fokom et al. 2012; Wang et al. 2018; Wilson et al. 2009; Zhang et al. 2015) or MRC content (Ma et al. 2017; Yuan et al. 2020). Nitrogen deposition could therefore promote SOC storage by stimulating microbial turnover and C inputs such as GRSP and MRC (Ma et al. 2020; Ma et al. 2017; Zhang et al. 2015), or by reducing the microbial turnover of organic C (Carreiro et al. 2000; Chen et al. 2013; Li et al. 2014; Xiao et al. 2018).

Inputs of GRSP from interactions between plants, AMF and other associated organisms might be particularly important for SOC storage in tropical forests, because most trees form mutualistic associations with AMF (Alexander and Lee 2005; Gillespie et al. 2011; McGuire et al. 2008). The fungal partner utilizes an estimated 10-20% of net photosynthate, depositing chitin and GRSP into soil which contributes to the formation of SOM (Treseder and Allen 2002; Wilson et al. 2009). Although GRSP only accounts for 4-5% of total SOC (Lovelock et al. 2004; Rillig et al. 2001), it can be preserved for a long time due to its low water solubility and resistance to degradation (Staddon et al. 2003). In addition, the viscous nature of GRSP can protect labile SOC by facilitating the formation of soil aggregates (Rillig 2004; Rillig et al. 2003; Wu et al. 2014). Generally, total GRSP (T-GRSP) can be separated into two fractions: easily-extractable GRSP (EE-GRSP), which may be newly produced or recently modified and is relatively labile, and difficult-to-extract GRSP (DE-GRSP), which is thought to be more recalcitrant (Koide and Peoples 2013; Rillig 2004; Wright and Upadhyaya 1996;

Wu et al. 2014). Although the quantification of GRSP is subjected to experimental artefacts and dilution dependence (Moragues-Saitua et al. 2019; Redmile-Gordon et al. 2013), comparative differences in EE-GRSP and DE-GRSP inputs may explain altered SOC storage in tropical forest soils under N deposition. Changes in GRSP inputs into soil under N deposition depend on duration and level of N addition (Garcia et al. 2008) and the ecosystem (Treseder and Turner 2007). Elevated N availability enhances GRSP concentration in temperate forests (Garcia et al. 2008; Sun et al. 2018), subtropical forests (Zhang et al. 2015) and farmland (Wang et al. 2017b). However, others found no effect of N addition on GRSP in a temperate boreal forest (Treseder et al. 2007) or tilled silt-loam soil (Wuest et al. 2005). Given that GRSP is often found in higher concentrations in tropical soils than in temperate systems (Holátko et al. 2021) but N additions generally reduce AMF abundance in tropical forests (Sheldrake et al. 2018; Treseder 2004), it is possible that GRSP production will decline with N deposition in tropical forest soils. Alternatively, as GRSP is a potential source of N to microbes, high N availability could also reduce the microbial mineralization of GRSP, resulting in greater standing stocks (Treseder and Turner 2007).

Although GRSP plays an essential role in stabilizing SOC pools, MRC accounts for 25-40% of total SOC (Khan et al. 2016; Ma et al. 2017). MRC consists of numerous distinct compounds including amino sugars, such as glucosamine (GluN), muramic acid (MurN), and galactosamine (GalN), which act as biomarkers of MRC (Amelung 2001). GluN is primarily of fungal origin, whereas MurN indicates bacterial residual C (Amelung 2001; Liang et al. 2011). GalN is probably a major component of extracellular polymeric substances produced by a range of microorganisms (Joergensen 2018). Given the distinct origin of MRC biomarkers, N deposition likely has varied impacts on the contribution of MRC to SOC pools. For example, the total contribution of MRC to SOC increases with N addition in subtropical forests due to reduced microbial mineralization of residual C (Ma et al. 2017) or greater fungal biomass (Fokom et al. 2012). However, the contribution of fungal MRC can also decline under elevated N because of a shift from fungal- to bacterial-dominated microbial communities (Ma et al. 2020; Zhang et al. 2016). Studies in plantations and tropical forests in South China indicate that high rates of N deposition increase soil C storage by enhancing MRC (Ma et al. 2017) or GRSP concentration (Ma et al. 2017; Zhang et al. 2015). However, Yuan et al. (2020) found no change in the contribution of MRC to SOC with N addition in a secondary coastal tropical forest. Such inconsistencies among studies demonstrate that our understanding of the contribution of microbial C sources to SOC, and their response to N deposition, is still deficient.

Here, we took advantage of an 11-year N-addition experiment in a tropical forest to assess how N deposition influences SOC storage and investigate the impacts of N deposition on these understudied

microbial sources of C. We aimed to test the following hypotheses:

(H1) Total SOC, GRSP, and MRC concentration will be greater in soils under N addition than in controls;

(H2) N addition will enhance the contribution of GRSP and MRC to total SOC.

Material and Methods

Study site and soil collection

Soils were collected from a long-term fertilization experiment at the Xiaoliang Research Station of Tropical Coastal Ecosystems, Chinese Academy of Sciences (21°270'N, 110°540'E), Southwestern Guangdong Province, China. The soil at the study site is a ferralsol (according to the FAO soil classification system) with a pH of *c*. 4 and low phosphorus (P) availability but sufficient N availability compared with other tropical sites (Table 1; Lu et al. 2021). Initially, the site was developed as a *Eucalyptus exserta* plantation in 1959, but an additional 312 species were planted between 1964 and 1975 (Mo et al. 2019; Wang et al. 2017a), resulting in a present-day forest community with the diversity and structural complexity typical of secondary forests in the region (Yu and Peng 1996). In August 2009, a factorial N- and P-addition experiment was established using a completely randomized block design with five replicate blocks. The present study focuses on the N-addition treatment (+N) and controls (CK), which were each randomly assigned to one 10 m × 10 m plot per block (Zhao et al. 2014). From September 2009, the equivalent of 100 kg N ha^{-1} $year^{-1}$ was applied to the +N plots by fertilizing every two months. For the fertilizer application, 476.6 g NH_4NO_3 (equal to 166.6 g N) was dissolved in 30 L groundwater and applied as close as possible to the soil surface using a backpack sprayer; 30 L groundwater was applied to each control plot. The amounts of N added corresponded to N-addition experiments in forests nearby (Lu et al. 2010), and detailed information about the site and experimental design are given in Wang et al. (2014).

In December 2019, we took five randomly located soil cores to 0-5 cm and 5-10 cm depth in each plot. The soils were sieved through a 2-mm mesh to remove stones and roots. Each soil sample was separated into two subsamples; one was air-dried for analyses of soil physico-chemical properties, and the other was stored at -20℃ for microbial analyses.

Physico-chemical properties

Soil ammonium-N (NH_4^+-N) and nitrate-N (NO_3^--N) were extracted by 2 M KCl (Page et al. 1982), 'available' soil phosphorus (P_{extr}) was extracted by the Bray 1 method (0.03 M NH_4F and 0.025 M HCl (Bray and Kurtz 1945); and total phosphorus (TP) and total nitrogen (TN) were analyzed using a micro-Kjeldahl digestion; the nutrient concentrations were then analyzed by automated discrete analyzers (BluVison™, SKALAR, Breda, the

Netherlands). Soil pH was determined in a 1:2.5 soil:water slurry using a glass pH electrode (FiveGO™, METTLER TOLEDO, Zurich, Switzerland). SOC concentration was determined by dry combustion (Delta V advantage, Thermo Fisher Scientific, Waltham, MA, USA). The physico-chemical properties of the soils are summarized in Table 1.

Measurement of litter inputs

Naturally-senesced leaves, branches, flowers, and fruits were collected using one litter trap (1 m ×1 m) per control and N-addition plot from January to December in 2019. Litter samples were collected in paper bags, oven-dried at 60℃, and weighed. The litter input per plot was calculated by summing the weight of collected litter during the whole year and is presented as g m^{-2} y^{-1}. The litter inputs in control (801±108 g m^{-2} y^{-1}) and N-addition plots (880±104 g m^{-2} y^{-1}) did not differ significantly ($P > 0.05$).

Particle-size fractionation

Soil particle-size fractions were assessed using a wet-sieving method (Fang et al. 2014), adapted from Cambardella and Elliott (1992). Briefly, aliquots of 10 g air-dried soil samples were placed in flasks with 40 ml of 5 g L^{-1} $(NaPO_3)_6$ solution and shaken for 15 h at a rate of 90 rpm. The samples were then washed through a 53 μm mesh sieve into a receptacle to separate two size fractions of ≥ 53 μm and < 53 μm, which were assumed to be mainly composed of mineral-associated organic matter. All particle-size samples were dried at 60℃ and weighed. SOC concentration in each fraction was determined by dry combustion (Delta V advantage, Thermo Fisher Scientific, Waltham, MA, USA). Organic C contained in the ≥ 53 μm and < 53 μm fractions was considered particulate organic C (POC) and mineral-associated organic C (MAOC), respectively, following Lavallee et al. (2019).

Phospholipid fatty acids (PLFAs)

To determine soil microbial community structure and microbial biomass, we performed phospholipid fatty acid (PLFAs) analyses according to Bossio and Scow (1998). Briefly, 8 g of freeze-dried soil sample was extracted in a chloroform–methanol–phosphate buffer (1:2:0.8 v/v/v), and phospholipids were separated from neutral lipids and glycolipids on a 0.5 g silica gel solid-phase extraction column, successively eluted with chloroform, acetone, and methanol. The methanol fraction (containing phospholipids) was subjected to mild alkaline methanolysis to transform the fatty acids into free methyl esters. Peaks were determined by comparison with a 19:0 internal standard using gas chromatography (GC7890, Agilent, California, USA) and assigned following standard nomenclature (Tunlid et al. 1989). The biomass of single fatty acids was calculated based on the content of the 19:0 internal standard and expressed as nmol g^{-1} soil dry weight. Fungi were represented by the PLFAs 18:2ω6c, 18:3ω3c, and 16:1ω5c (Frostegård and Bååth 1996), whereas AMF

were represented by 16:1ω5c. Bacterial biomass was calculated from the PLFAs i14:0, i15:0, a15:0, i16:0, i17:0 a17:0, 16:1ω7c, 18:1ω9c, 18:1ω7c, 15:0 and 17:0 (Bossio et al. 2006). The total PLFA biomass of the soil microbes was calculated as the sum of fungi, bacteria, and PLFA biomarkers 14:0, 16:0, 16:1ω5c, 17:1ω8c. The relative abundances of bacteria, fungi, and AMF was expressed as the ratio of their biomass to the total PLFAs biomass. Finally, the ratio of fungal to bacterial PLFAs (F/B ratio) was calculated to represent changes in soil microbial community structure (Bardgett et al. 1996; Frostegård and Bååth 1996).

Soil amino sugars

To determine MRC, we first extracted amino sugars based using the method described by Indorf et al. (2011) with minor modifications (Mou et al. 2020). An aliquot of 0.5 g of air-dried soil (< 2 mm) was hydrolyzed for 6 h at 105℃ with 10 ml 6 M HCl. Then the samples were uniformly mixed, cooled to room temperature, and filtered. An aliquot of 0.5 ml supernatant was evaporated to dryness (40-45℃) by nitrogen gas to remove HCl. We then added 0.5 ml ultrapure water to the residues, dried them with nitrogen gas again, and re-dissolved them in 2 ml ultrapure water to be stored at -20℃ until analysis. The concentrations of the amino sugars glucosamine (GluN), galactosamine (GalN) and muramic acid (MurN) were determined using a high-performance liquid chromatograph (Dionex Ultimate 3000, Thermo Fisher Scientific, Waltham, MA, USA) equipped with an octadecylsilylated silica gel column (Acclaim120 C18; 150 mm, 4.6 mm, 3 μm; Thermo Fisher Scientific, Waltham, MA, USA) after pre-column derivatization with ortho-phthaldialdehyde. Individual amino sugars (GluN, MurN, GalN) were identified and quantified according to the chromatogram of standard solutions containing mixed amino sugars. The soil concentrations of individual and total amino sugars were expressed as μg g^{-1} of dry soil.

Since GluN is present in both fungal and bacterial cell walls, fungal GluN (F-GluN) was calculated by subtracting bacterial GluN from total GluN. To determine bacterial GluN, it is assumed that MurN and GluN are present in the bacterial cell wall in a 1:2 molar ratio (Engelking et al., 2008). We, therefore, calculated fungal GluN as:

$$\text{F-GluN } (\mu g\ g^{-1}) = \text{total GluN } (\mu g\ g^{-1}) - 2\times\text{MurN } (\mu g\ g^{-1})\times(179.2/251.2)$$

where F-GluN is the fungal GluN and 179.2 and 251.2 are the molecular weights of GluN and MurN, respectively (Shao et al. 2017). Fungal and bacterial MRC was subsequently calculated as follows:

$$\text{Fungal MRC} = \text{F-GluN}\times 9$$

$$\text{Bacterial MRC} = \text{MurN}\times 45$$

where 9 and 45 are conversion factors (Appuhn and Joergensen 2006).

Determination of GRSP

To quantify GRSP, we used an improved method (Zhang et al. 2014) based on the Bradford protein assay (Wright and Upadhyaya 1996) to determine two GRSP fractions: easily-extractable glomalin (EE-GRSP) and total glomalin (T-GRSP). In brief, EE-GRSP and T-GRSP were extracted from 1.0 g air-dried sieved soil (2-mm mesh) using 8 ml of 20 mM sodium citrate (pH = 7.0) or 50 mM sodium citrate (pH = 8.0), respectively. For EE-GRSP, the soil extracts were autoclaved for 30 min at 121℃ and then centrifuged at 10,000 × g for 10 min. For T-GRSP, the soil extracts were autoclaved for 60 min at 121℃ and centrifuged at 10,000 × g for 10 min. The extraction for T-GRSP was repeated four times until the solution was straw-colored. The supernatants were combined and stored at 4℃ until Bradford analysis (Rillig 2004; Wright and Upadhyaya 1996). The optical density of each GRSP fraction was determined spectrophotometrically (Multiskan^{TM} FC, Thermo Fisher Scientific, Waltham, MA, USA). We determined the GRSP concentrations of soils from optical density using bovine serum albumin as a standard.

Data analyses

To investigate the effects of N addition and soil depth on each soil physico-chemical property, particle-size fractions, microbial community parameters, and MRC or GRSP concentration, we used two-way ANOVA with a significance level of $P < 0.05$. One-way ANOVA was used to assess the effects of N addition on litter inputs with a significance level of $P < 0.05$. To determine relationships between soil physico-chemical properties and GRSP or MRC concentration, we used correlation analysis (*rcorr* function) in the Hmisc package (Harrell 2021) using individual values rather than plot means to account for high spatial variability within plots. Finally, to assess the direct and indirect links between N addition, soil properties or microbial community parameters, and GRSP, MRC, and SOC concentrations, we used structural equation modeling (SEM) in the lavaan package (Rosseel 2012). The prior conceptual model included all possible hypothesized paths between variables, and non-significant paths were removed from subsequent models until the best model fit was achieved. In the final model, we included N addition as an influencing factor, and nitrate-N, and pH as soil physico-chemical properties. As total PLFAs and F/B ratio were selected to represent microbial community composition, total GRSP and MRC were included in the SEM to represent C sources from the whole soil microbial community. The Chi-square (χ^2) test was used to evaluate the overall fit of the model, alongside root mean square error of approximation, comparative fit index (CFI), and the goodness of fit index (GFI; Schermelleh-Engel et al. 2003). All data analyses were carried out in R version 4.0.5 (R Core Team 2021).

Results

Soil physico-chemical properties

There were no differences in total nitrogen (TN) concentration, total phosphorus (TP) concentration, P_{extr} concentration, or soil pH between treatments or soil depths. However, total SOC concentration was more than twice as high in N-addition plots than in controls at 0-5 cm and 48% higher at 5-10 cm depth ($P < 0.01$, Table 1). Although there were no differences in SOC concentration between soil depths in the control plots, SOC concentration in N-addition plots was *c*. 49% higher at 0-5 cm than at 5-10 cm depth ($P = 0.04$). Although nitrate-N concentration did not differ between soil depths in either treatment, nitrate-N concentration was 66% higher in the N-addition plots than in the controls at 0-5 cm and 53% higher at 5-10 cm depth ($P < 0.01$, Table 1). Soil ammonium-N concentration did not differ between N-addition and control plots or between soil depths in the controls. However, in N-addition plots, soil ammonium-N concentration was 1.6-fold higher at 0-5 cm than at 5-10 cm depth ($P < 0.01$, Table 1). Finally, litter inputs did not differ between N-addition and control plots ($P > 0.05$, Table. S1). Therefore, soil physico-chemical properties and litter inputs did not differ markedly between soil depths, but SOC and nitrate-N concentrations were higher in long-term N-addition plots, particularly at the soil surface (0-5 cm).

Soil particle-size fractions

POC concentration was greater at 0-5 cm depth than at 5-10 cm depth in N-addition plots, but there was no difference in POC with soil depth in the controls. However, POC concentration was greater in N-addition plots than in control plots across soil depths ($P < 0.01$, Table. 2). MAOC concentrations were greater at 0-5 cm than 5-10 cm soil depth in both treatments ($P < 0.01$) and 1.16-fold greater in N-addition plots than control plots at 0-5 cm depth ($P < 0.01$, Table. 2) . Although both POC and MAOC concentrations were significantly higher in N-addition plots than in controls, the relative contributions of POC and MAOC to total SOC were similar between treatments and soil depths ($P > 0.05$, Table. 2).

Microbial community

Nitrogen addition only had a minor effect on PLFA biomarkers. The abundance of AMF biomarkers was c. 24% lower in the N-addition plots than in the controls at 0-5 cm soil depth ($P = 0.04$), but there were no differences between treatments at 5-10 cm depth ($P > 0.05$, Fig. 1c). By contrast, total PLFA biomass was 40% higher in the N-addition plots than in the controls, but the difference was only significant at 5-10 cm depth ($P = 0.02$, Fig. 1d). In general, PLFA biomarkers varied more between soil depths than between treatments. Bacterial biomarker abundance and the F/B ratio were significantly higher at 5-10 cm than at 0-5 cm soil depth in both treatments ($P = 0.02$ and $P = 0.04$ for control and N-addition plots, respectively, Fig. 1b, e). By

contrast, the abundances of fungal and AMF biomarkers were 25% and 9% greater, respectively, at 0-5 cm than at 5-10 cm depth in the control plots (P = 0.03 and P = 0.02, respectively), but there were no differences between depths in the N-addition plots (Fig. 1a, c). Hence, soil microbial biomarkers differed more with soil depth than between treatments, but the differences between soil depths tended to be more pronounced in the control plots.

Glomalin-related soil protein

The concentration of EE-GRSP did not differ between treatments or soil depths (P > 0.05, Fig. 2a). By contrast, the concentration of T-GRSP was 98% higher in N-addition plots than in the controls at 0-5 cm and 63% higher at 5-10 cm depth (P < 0.01, Fig. 2b). In the control plots, T-GRSP concentration was similar across both soil depths, whereas in N-addition plots, the T-GRSP concentration was 39% higher at 0-5 cm than at 5-10 cm depth (P = 0.03, Fig. 2b). Despite higher T-GRSP concentration under long-term N addition, the relative contributions of EE-GRSP and T-GRSP to total SOC were similar between treatments and soil depths (P > 0.05, Fig. 2c, d).

Soil amino sugars and microbial residual carbon

The soil concentrations of all three soil amino sugars (MurN, GalN, and GluN) were higher in the N-addition plots than in controls at 0-5 cm depth, but not at 5-10 cm depth. Total amino sugar concentration was 114% greater in N-addition plots at 0-5 cm depth than in controls (P < 0.01). In the control plots, the concentrations of amino sugars were similar between soil depths. However, in N-addition plots, the concentrations of GalN, GluN and total amino sugars were greater at 0-5 cm than at 5-10 cm depth (P < 0.01, Fig. S1). Hence, long-term N-addition increased the concentrations of amino sugars in surface soils (0-5 cm depth), resulting in marked differences in amino sugar concentrations between soil depths in N-addition plots.

Microbial residual carbon (MRC) showed the same pattern as amino sugars, with 140% greater fungal MRC, 53% greater bacterial MRC, and 120% greater total MRC concentration at 0-5 cm depth in the N-addition plots than in the controls (P < 0.01, Fig. 3a, b, c). As the increase in fungal MRC concentration with N addition was larger than the increase in bacterial MRC, the fungal MRC/bacterial MRC ratio was also higher in the N-addition plots (5.3 and 3.9 at 0-5 and 5-10 cm soil depth, respectively) than in the controls (3.3 and 3.2 at 0-5 and 5-10 cm soil depth, respectively; P < 0.01, Fig. 3d). Nonetheless, the contribution of fungal MRC to SOC did not differ between treatments or soil depths (P > 0.05, Fig. 3e), but bacterial MRC contributed 33% less to SOC in the N-addition plots than in controls at 0-5 cm, and 35% less at 5-10 cm depth (P < 0.05, Fig. 3f). The relative contribution of total MRC to SOC was similar between treatments at 0-5 cm depth, but at 5-10 cm depth the contribution of total MRC to SOC was 24% lower in the N-addition plots than in the controls (P

= 0.02, Fig. 3g). The contribution of plant-derived C to SOC, calculated by subtracting the total MRC from total SOC, was significantly higher in N-addition plots than in the control plot across soil depths ($P < 0.01$ and $P = 0.01$ at 0-5 and 5-10 cm soil depth, respectively), but there was no difference between soil depths ($P > 0.05$, Fig. 3h). Hence, despite increased MRC concentration in surface soils, the relative contribution of MRC to SOC was lower in N-addition plots than in controls.

Relationships among soil properties

Across soil depths, SOC increased with increasing nitrate-N concentration, total PLFA biomass, GRSP concentration, and MRC concentration, but declined with increasing AMF biomarker abundance and soil pH ($P < 0.05$, Fig. 4 and Table S1). There were strong positive correlations between nitrate-N concentration and SOC concentration, GRSP, and MRC ($P < 0.05$, Fig. S2). Moreover, MAOC and POC concentrations were positively correlated with SOC concentration ($P < 0.05$, Table. S1). Structural equation modeling (SEM) revealed that higher SOC concentration was directly associated with higher T-GRSP and total MRC concentrations (Fig. 5b). Moreover, the higher concentration of GRSP and MRC in soils under N-addition were mainly associated with greater nitrate-N concentration (Fig. S2 and Table S1). Moreover, a higher MAOC concentration was also correlated with greater GRSP and MRC concentrations. Contrary to the correlation results, the SEM model revealed a weak negative correlation between MAOC and SOC concentration, although this was not significant ($P > 0.05$, Fig. 5). Overall, T-GRSP and total MRC concentrations were closely correlated with soil nitrate-N concentration. By contrast, there were no significant direct links between T-GRSP or total MRC and total PLFAs or AMF abundance. Hence, the higher SOC concentration under N-addition was best explained by greater inputs of GRSP and MRC with enhanced soil N availability, rather than increased microbial biomass or changes in microbial community composition.

Discussion

Our study suggests that simulated N deposition increased SOC storage by enhancing the accumulation of soil microbial C inputs (GRSP and MRC) over a decade. As hypothesized, total SOC concentration was strongly correlated with the concentrations of GRSP and MRC in the soil, and both GRSP and MRC concentrations increased under N addition (H1). However, in contrast to our second hypothesis (H2), the relative contributions of GRSP and MRC to SOC in the N-addition plots was similar to that of the controls, suggesting that the increase in SOC can be attributed to proportional increases in plant- and microbially-derived C.

Glomalin-related soil protein

As hypothesized, total SOC was strongly correlated with MRC and GRSP concentrations, and all three

increased with N addition (H1). The increase in GRSP with N addition in our study is surprising, because our SEMs showed a direct link between AMF biomarker abundance and T-GRSP concentration in the soil (Fig. 5), but AMF biomarker abundance was lower in N-addition plots (Fig. 1c and Table S1). However, in partial contrast to our hypothesis, only T-GRSP concentration was higher in N-addition plots, whereas EE-GRSP concentration and the relative contributions of both EE-GRSP and T-GRSP to SOC was unchanged. Despite the correlation between AMF abundance and T-GRSP in our study, GRSP is not solely fungal origin (Cisse et al. 2021), as it also contains compounds of prokaryotic origin, as well as humic substances (Gillespie et al. 2011; Holátko et al. 2021). It is, therefore, possible that although AMF contribute substantially to GRSP at our study site, the EE-GRSP fraction was unrelated to mycorrhizal activity. Whether mycorrhizal fungi promote C accumulation is determined by the balance among three processes: i) deposition of mycorrhizal residues, ii) decomposition of those residues by microorganisms, and iii) increased plant growth through mycorrhizal associations (Treseder and Holden 2013). In our study, the balance between the first two processes may explain changes in fungal MRC and AMF-derived GRSP with N addition. Although the increase in fungal MRC with N addition may indicate greater deposition of mycorrhizal residues, EE-GRSP concentration, which represents faster-degrading GRSP, was highly variable in the N-addition plots (Fig. 2a). Although EE-GRSP has been linked to rapid turnover of AMF biomass (Lovelock et al. 2004), large temporal variation in EE-GRSP has been observed in tropical soils (Steinberg and Rillig 2003, Lovelock et al. 2004) which would not be captured by our single sampling time. EE-GRSP is also thought to be more sensitive to environmental changes than T-GRSP (Singh et al. 2013), and greater N availability may enhance the decomposition of labile substrates such as EE-GRSP (Neff et al. 2002).

Although T-GSRP includes numerous substances that do not originate directly from fungi, the strong correlation between T-GRSP and AMF abundance at our study site suggests that mycorrhizal processes likely contributed disproportionately to T-GRSP concentration. Nonetheless, T-GSRP was higher in the N-addition plots despite lower AMF abundance than in the controls. As the mean residence time of GRSP (7–42 years) is much longer than the residence time of AMF hyphae (days to months; Staddon et al. 2003; Treseder and Turner 2007; Treseder et al. 2007), the persistence of T-GSRP in soils over many years (Staddon et al. 2003) might explain higher T-GRSP concentration despite lower AMF abundance in the N-addition soils. However, it is possible that N addition stimulated the production of other microbial C sources, such as bacterial extracellular polymeric substances that are co-extracted with glomalin (Holatko et al. 2021). In addition, greater GRSP concentration enhances aggregate stability, preventing SOC from microbial degradation (Rillig 2004; Rillig et al. 2003; Wu et al. 2014) and thus reducing decomposition. Hence, based on our results and

previous studies, we propose that the large increase in T-GRSP with N addition can be attributed to higher initial T-GRSP production as N limitation was alleviated, coupled with slower turnover rates as soils became N-saturated (Yu et al. 2013). Given that various processes and organisms are involved in the production of GRSP and its persistence in soils, repeated sampling over multiple times and in-depth microbial analyses may elucidate the mechanisms by which GRSP concentration increases under N addition.

Soil microbial residual carbon

Soil fungal MRC, bacterial MRC, and total MRC concentrations were all greater in N-addition plots which supports our first hypothesis (H1). However, in contrast to our second hypothesis (H2), the relative contributions of bacterial MRC and total MRC to SOC were lower, and the relative contribution of fungal MRC to SOC was unchanged (Fig. 3). Overall, besides lower AMF abundance at 0-5 cm, there were no changes in soil microbial functional groups with N addition, and total PLFA biomass was only greater at 5-10 cm depth. Hence, the substantial increase in MRC with N addition cannot be attributed to changes in soil microbial biomass or community composition, similar to findings of some previous studies (Ding et al. 2013; Ma et al. 2017). However, others have observed a concurrent increase in both MRC and microbial biomass after N addition in temperate forests (Kindler et al. 2009; Liang et al. 2011). The accumulation of MRC depends on the balance between production and decomposition (Joergensen 2018; Six et al. 2006), and the relatively minor increase in microbial biomass in N-addition soils suggests that higher MRC concentration may not be the result of increased production. Instead, accumulation of MRC may indicate slower microbial decomposition of MRC under high N deposition (Fontaine et al. 2011). As N-acetyl-β- D-glucosidase (NAG) breaks down microbial residues such as fungal-derived chitin and bacterial-derived peptidoglycan (Sinsabaugh et al. 2008), the decline in NAG activity after N addition at our study site (Yuan et al. 2020) may have decreased decomposition of MRC. Alternatively, compared with control plots, the rapid turnover of the microbial biomass under N addition (Fisk and Fahey 2001) might also explain a higher MRC concentration in the soil without a concomitant increase in microbial biomass. As most of the MRC (> 90%) in soils is derived from dead microbial cells (Amelung 2001), increased turnover and death of microorganisms would contribute to higher MRC concentration with N addition (Dail et al. 2001).

The contribution of microbial residual C to SOC

Contrary to expectations and in contrast to our second hypothesis (H2), the relative contributions of GRSP and MRC to SOC were unchanged or lower in N-addition plots, respectively, even though MRC and T-GRSP concentrations were greater than in controls. Indeed, our results indicate that the increase in SOC concentration under N addition was greater than the increase in MRC or GRSP. Previous studies in tropical

forests have reported increased SOC under N deposition due to inhibited microbial activity, slower decomposition rates (Chen et al. 2013; Li et al. 2014) and reduced enzyme activities (Carreiro et al. 2000; Cusack 2013). Lu et al. (2021) proposed that soil C sequestration can be stimulated by N deposition in N-rich tropical forests through reduced microbial activity and slower decomposition. Our findings add to this by demonstrating that, although soil MRC and GRSP concentrations were higher under N addition, a large proportion of the additional SOC concentration in N-addition plots is likely plant-derived (Fig. 3h). Enhanced plant growth combined with reduced decomposition under N deposition also entails greater inputs of plant material, coupled with greater persistence of low molecular-weight plant C and microbial labile C to persist in soils. The higher MAOC and POC concentrations in N-addition plots support our assumption via three lines of evidence. First, MAOC comprised around 70% of total SOC, whereas MRC and GRSP together only accounted for around 35% of total SOC in this study. Therefore, much of the MAOC at our study site is likely derived from plant C, inconsistent with previous views that suggest the MAOC is largely of microbial origin (Koegel-Knabner et al. 2008). However, more recent work suggests that plant residual C contributes more to the formation of MAOC than previously thought (Angst et al. 2021). Thus, reduced decomposition under N deposition may result in greater plant residual C input into the stable C pool. Second, MRC can persist in a stable SOC pool (Liang et al. 2017) and the larger MAOC fraction in N-addition plots in our study can be partially attributed to the accumulation of MRC and T-GRSP. Moreover, T-GRSP promotes the formation and persistence of MAOC by facilitating physical protection via microaggregate formation (Koegel-Knabner et al. 2008; Rillig 2004; Rillig et al. 2003; Wu et al. 2014). Finally, POC is mainly derived from plants, and although it contains some recalcitrant components such as lignin, it still cycles much faster than MAOC (Cotrufo et al. 2019; Poeplau et al. 2018). The greater POC concentration in N-addition plots suggests greater inputs of plant-derived C into soils. Thus, although both GRSP and MRC concentrations were higher in N-addition plots, the relative contributions of POC and MAOC to total SOC (POC/SOC and MAOC/SOC, respectively) were similar to control plots, indicating similar greater contributions of both plant- and microbial-derived C to SOC under N addition. Consequently, we found no indication that N addition decreases SOC stability. However, as plant-derived C is thought to be less stable than microbial residual C (Cotrufo et al. 2019; Koegel-Knabner et al. 2008), a greater amount of plant-derived C in soils under N deposition might render the SOC pool more vulnerable to decomposition with rising temperatures which may diminish the N-enrichment effect and result in greater CO_2 emission.

Microbial C sources and greater SOC stocks under N deposition

Soil N availability can be used to estimate the N saturation of forest soils (Schimel and Bennett 2004), and

high N-deposition rates in natural ecosystems are often linked to increased soil nitrate-N concentrations (Killham 1990). Greater availability of soil N to plants has been linked to higher T-GRSP and SOC concentrations in temperate forest (Rotter et al. 2017; Sun et al. 2018) and subtropical forest (Zhang et al. 2015). Here, we also demonstrate a strong correlation between nitrate-N and MRC which suggests that soil N availability, especially nitrate-N, could promote the accumulation of MRC as well as GRSP and SOC concentrations in tropical forests.

Conclusions

Based on our findings and evidence from previous studies, we propose a conceptual framework (Fig. 6) to stimulate research into the potential mechanisms by which N deposition may promote SOC storage. We surmise that N deposition initially enhances soil C accumulation by increasing AMF abundance at the start of the N addition (Fontaine et al. 2011), resulting in a greater amount of T-GRSP (Fig. 6a). With continued N deposition, the long residence time of GRSP together with inhibited organic matter decomposition (Zhang et al. 2021) would favor the persistence and accumulation of GRSP (Fig. 6b). As there were no significant changes in fungal or bacterial PLFA abundances with N addition in our study (Fig. 1), enhanced MRC concentration can be attributed to either slower MRC decomposition (Yuan et al. 2020, Fig. 6c) or to accelerated microbial turnover. However, the greater accumulation of plant-derived C with N addition altered the relative contributions of GRSP or MRC to total SOC; the contributions of GRSP and MRC to SOC, therefore, remained unchanged or decreased, respectively, despite greater inputs (Fig. 6d). Greater MAOC and POC concentrations further indicate that reduced decomposition under N deposition may enhance labile and stable C pools by promoting both plant-derived C and microbial C inputs. Hence, considering the accumulation of GRSP and MRC, their long residence time in soil, and decreased SOM decomposition under N addition, we surmise that GRSP and MRC will accumulate over time in soils under N deposition, thereby enhancing SOC stocks. However, the greater contribution of plant-derived C to total SOC suggests that greater inputs of microbial C do not necessarily entail a more stable C pool. Consequently, a large proportion of extra SOC under N deposition might be sensitive to other climate change impacts such as rising temperature or atmospheric CO_2 level.

Statements & Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Authors' contributions:

Faming Wang designed the experiment. Jingfan Zhang, Jinge Zhou, Yingwen Li, and Yongxing Li performed the experiments. Jingfan Zhang analyzed the data. Jingfan Zhang, Emma Sayer, Hans Lambers and Faming Wang wrote this manuscript. All co-authors revised the manuscript.

Data Availability

Data are available under reasonable requests.

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Table 1. Soil physiochemical characteristics at 0-5 and 5-10 cm depth in long-term nitrogen (N)-deposition treatments in a secondary tropical forest, where CK is control, and +N is N-addition, TN is total N concentration, TP is total P concentration, TC is total carbon concentration, P_{extr} is extractable P concentration; values are means ±SE for *n* = 5 plots per treatment. Different capital letters within a column indicate significant differences between soil depths; different lowercase letters within a column indicate significant differences between treatments at $P < 0.05$ (after correction for multiple comparisons)

Soil depths	Treatment	TN ($g\ kg^{-1}$)	TP ($g\ kg^{-1}$)	SOC (%)	C/N	pH	nitrate-N ($mg\ kg^{-1}$)	ammonium-N ($mg\ kg^{-1}$)	P_{extr} ($mg\ kg^{-1}$)
0-5 cm	+N	2.5±0.3	0.24±0.01	5.5±0.6Aa	22.7±1.9a	3.5±0.1	17.0±1.7a	4.7±0.7A	2.7±0.4
	CK	2.1±0.5	0.22±0.02	2.4±0.2b	13.3±2.2b	3.8±0.1	10.2±1.1b	3.2±0.5	2.2±0.5
5-10 cm	+N	1.6±0.2	0.20±0.01	3.7±0.3Ba	25.1±2.0a	3.6±0.1	9.4±1.2a	1.8±0.4B	1.5±0.4
	CK	1.5±0.2	0.23±0.01	2.5±0.3b	16.6±1.2b	3.8±0.1	6.14±0.5b	2.0±0.4	1.1±0.1

Table. 2 Concentrations of soil organic carbon (SOC) particle size fraction and their contributions to Total SOC in long-term nitrogen (N)-addition treatments in a secondary tropical forest, where CK is control, and +N is N addition, POC particulate organic carbon (C), MAOC is mineral-associated organic C, POC/SOC is the ratio of POC to SOC and MAOC/SOC is the ratio of MAOC to SOC; values are means ±SE for *n* = 5 plots per treatment; different lowercase letters within a column indicate significant differences between treatments at $P < 0.05$.

Soil depths	Treatment	POC (g C kg^{-1}soil)	MAOC (g C kg^{-1}soil)	POC/SOC (%)	MAOC/SOC (%)
0-5 cm	+N	16.1±1.0Aa	38.7±1.8Aa	29.6±3.9	70.4±3.9
	CK	7.9±0.8b	16.0±0.3b	32.6±5.1	67.4±5.1
5-10 cm	+N	11.4±0.3Ba	25.7±0.7B	30.7±1.9	69.3±1.9
	CK	5.8±0.3b	18.9±0.8	23.5±2.0	76.5±2.0

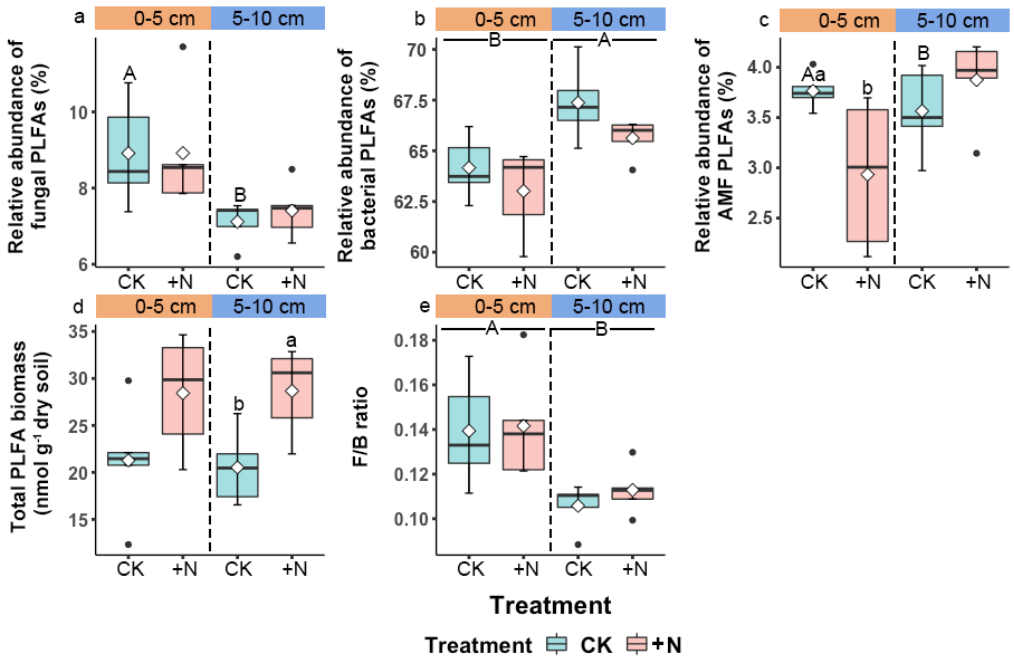


Figure 1. Phospholipid fatty acid (PLFA) biomarkers representing microbial functional groups in the soil at 0-5 cm and 5-10 cm depth, showing the relative abundances of (a) fungal PLFAs, (b) bacterial PLFAs, (c) arbuscular mycorrhizal fungi (AMF) PLFAs, (d) total PLFA biomass, and (e) the ratio of fungi to bacteria (F/B ratio), where CK is control, +N is N addition; capital letters indicate significant differences between soil depths under the same addition treatment and lowercase letters represent significant differences between addition treatments at the same soil depth at $P < 0.05$. Boxes denote the 25th and 75th percentiles and median lines are given for $n = 5$ plots per treatment and depth, diamonds represent mean values, whiskers indicate values up to 1.5x the interquartile range, and dots indicate outliers.

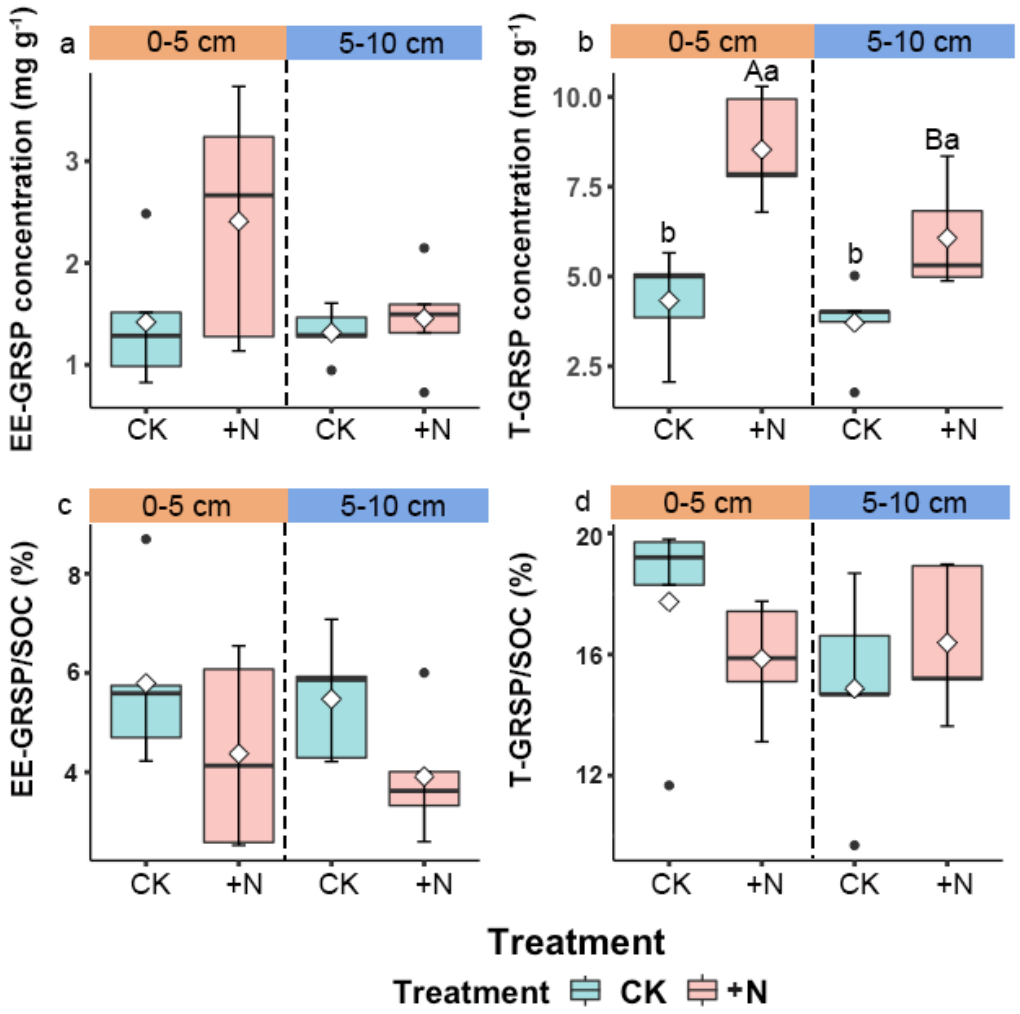


Figure 2. The concentration of glomalin related soil protein (GRSP) and its contribution to soil organic carbon (SOC) at 0-5 cm and 5-10 cm soil depth, showing the concentration of (a) easily-extracted GRSP (EE-GRSP), (b) total GRSP (T-GRSP), and the contribution of (c) EE-GRSP and (d) T-GRSP to SOC, where CK is control and +N is N addition; capital letters denote significant differences between soil depths under the same addition treatment and lowercase letters denote significant differences between addition treatments at the same soil depth at $P < 0.05$. Boxes denote the 25th and 75th percentiles and median lines are given for $n = 5$ plots per treatment and depth, diamonds represent mean values, whiskers indicate values up to 1.5x the interquartile range, and dots indicate outliers.

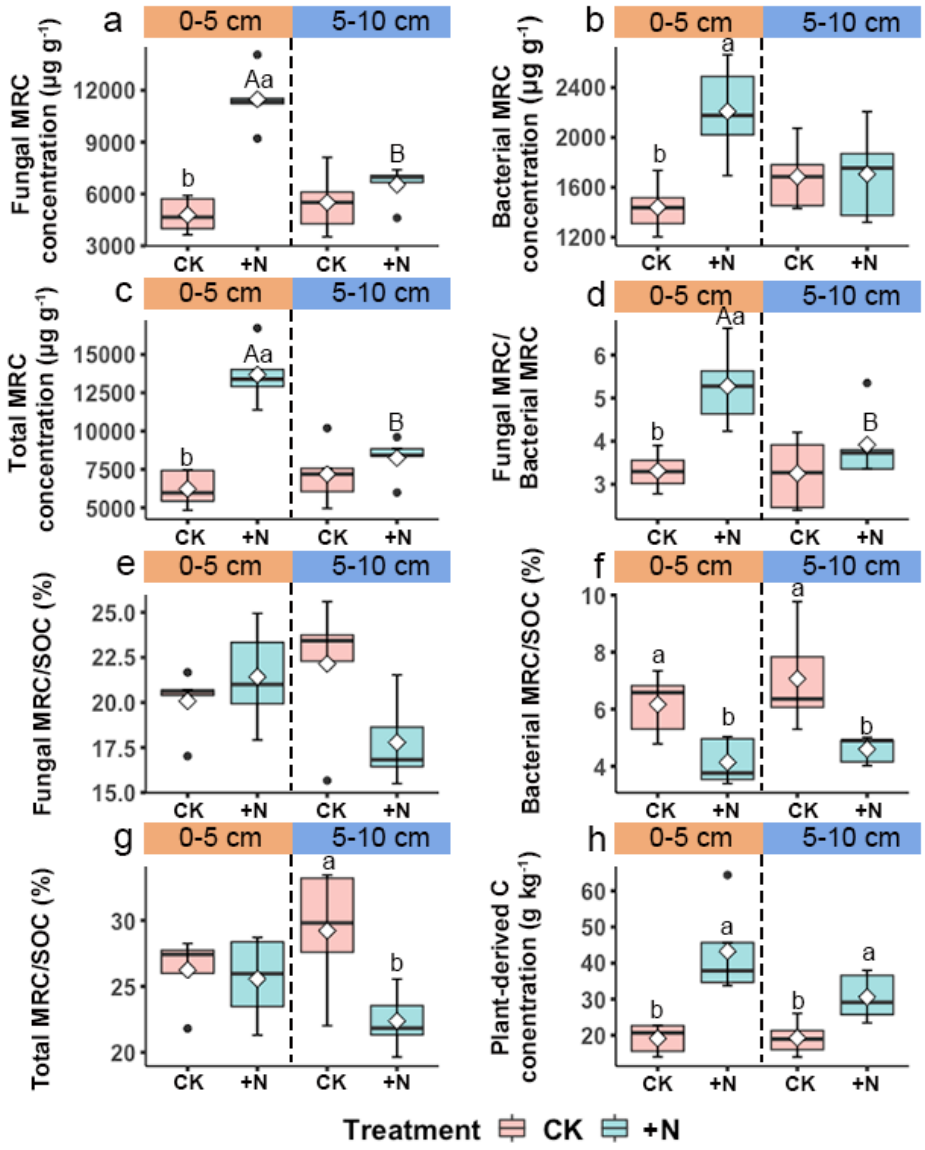


Figure 3. Microbial residual carbon (MRC) in soils and its contribution to soil organic carbon (SOC), showing the concentrations of (a) fungal MRC, (b) bacterial MRC, (c) total MRC, and (d) the ratio of fungal to bacterial MRC; and the contributions of (e) fungal MRC, (f) bacterial MRC, (g) total MRC to SOC, and (h) plant-input C concentration, where CK is control and +N is N addition; capital letters denote significant differences between soil depths under the same addition treatment and lowercase letters denote significant differences between addition treatments at the same soil depth at $P < 0.05$. Boxes denote the 25th and 75th percentiles and median lines are given for n = 5 plots per treatment and depth, diamonds represent mean values, whiskers indicate values up to 1.5x the interquartile range, and dots indicate outliers.

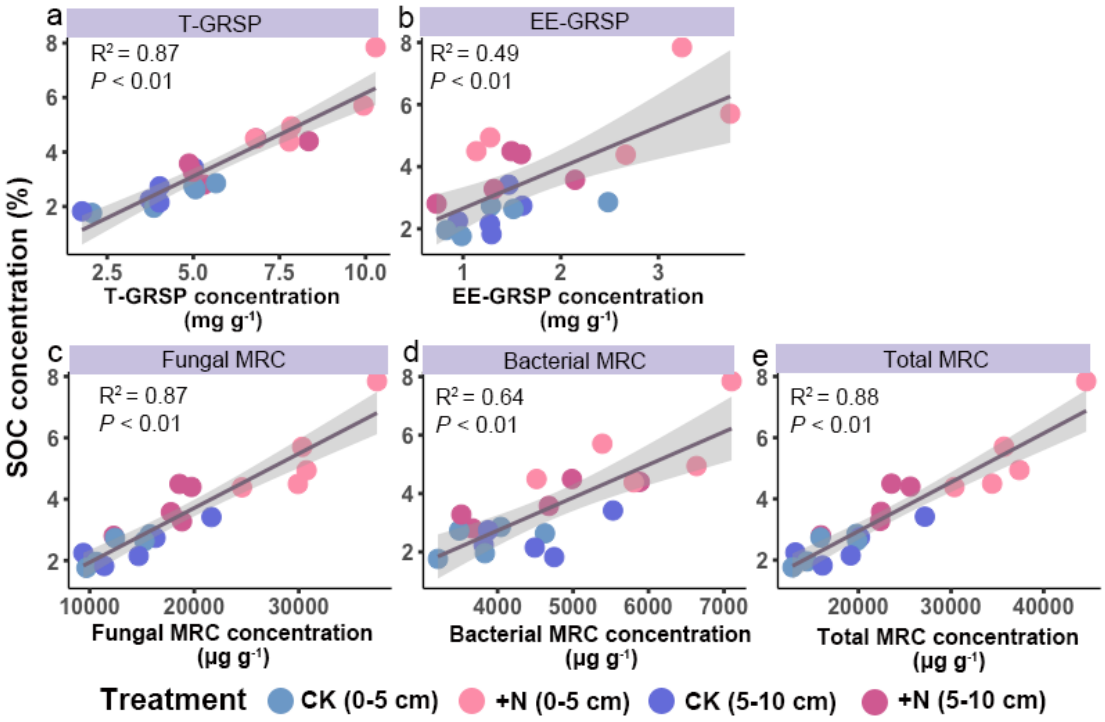
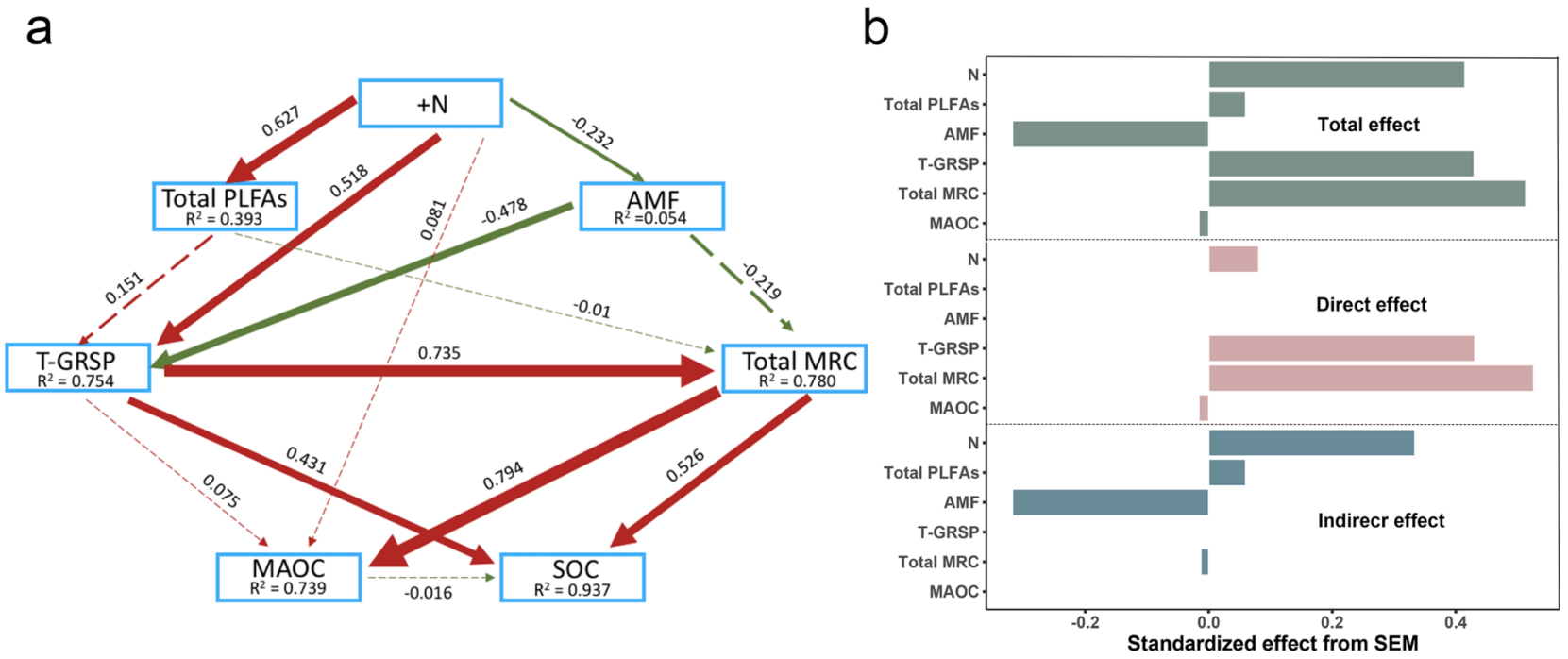


Figure 4. Correlations between soil organic carbon (SOC) concentration and (a) T-GRSP, (b) EE-GRSP, (c) fungal MRC, (d) bacterial MRC or (e) total MRC across soil depths, showing the determination coefficient (R^2) and *P*-value from linear regression analyses. CK (0-5 cm) and +N (0-5 cm) denote values from control plots and N-addition plots at 0-5 cm depth soil, respectively; CK (5-10 cm) and +N (5-10 cm) denote values from control plots and N-addition plots at 5-10 cm soil depths, respectively. Abbreviations follow the legends to Figs. 2, 3.



χ^2=4.7, *P*-value=0.69, df=7, CFI=1,GFI= 0.96, RMSEA=0, AIC=587

Figure 5. Final structural equation model (SEM) showing the links between soil properties, microbial community composition and soil organic carbon (SOC) concentrations in a nitrogen (N)-addition experiment. In panel **(a)** solid red and green arrows indicate significant positive and negative correlations, respectively ($P < 0.05$), and dashed red and green arrows indicate non-significant positive and negative correlations, respectively. Numbers parallel to arrows represent standardized path coefficients, the magnitudes of which are proportional to the thickness of arrows. In panel **(b)**, the total effect is the sum of direct and indirect effects. +N indicates N addition, Total PLFAs is total biomass of phospholipid fatty acids, AMF is arbuscular mycorrhizal fungi abundance, T-GRSP is total glomalin-related soil protein, and Total MRC is total microbial residual carbon, MAOC is mineral-associated organic carbon. Model fit statistics are shown, where χ^2 is the Chi-square statistic; df is degrees of freedom; CFI is the comparative fit index; GFI is the goodness-of-fit index; RMSEA is the root mean square error of approximation; and AIC is Akaike's information criteria.

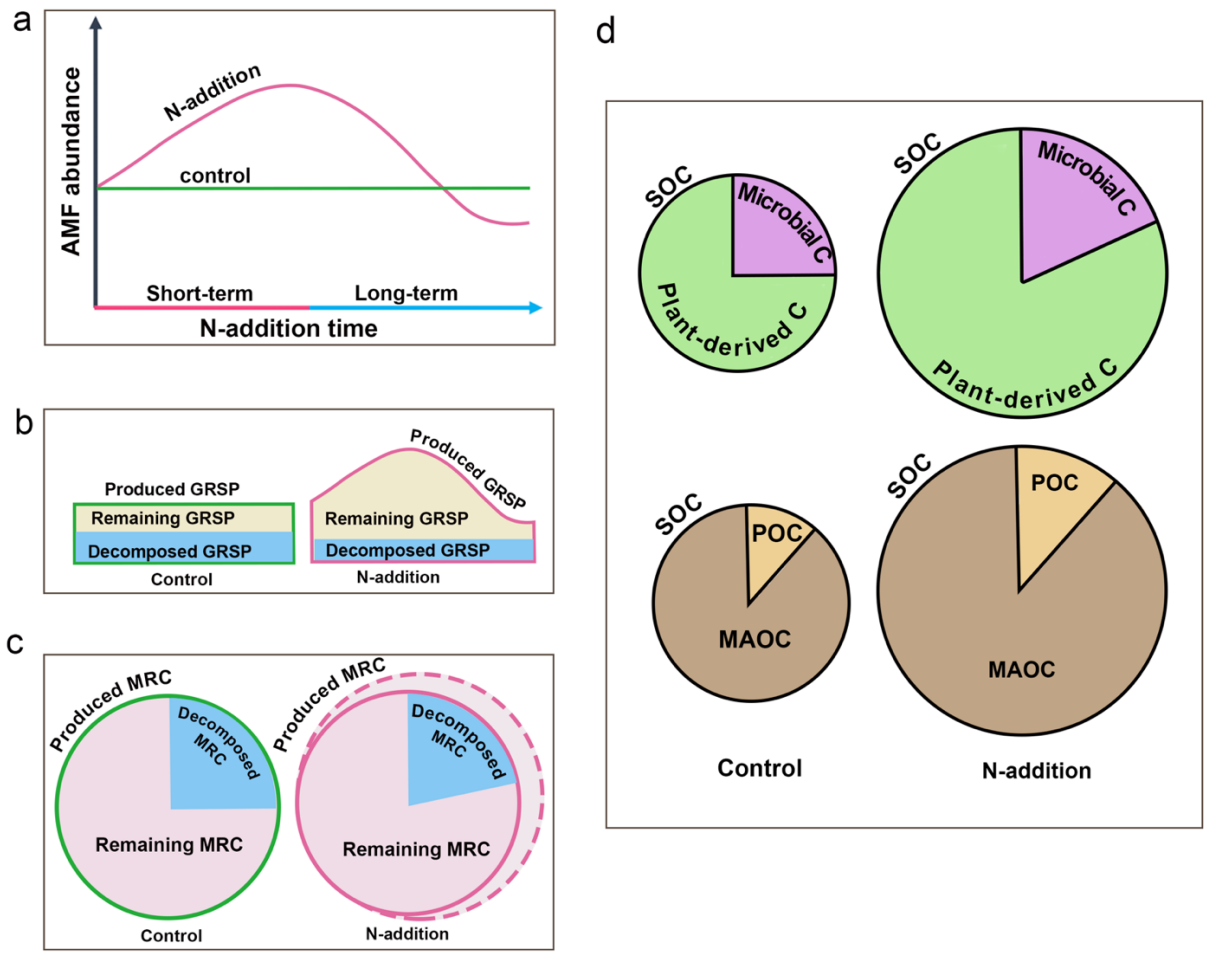


Figure 6. Conceptual diagrams showing how the accumulation of glomalin-related soil protein (GRSP) and microbial residual C (MRC) contribute to the soil organic carbon (SOC) contents under N addition, and the underlying microbial mechanisms. **(a)** The abundance of arbuscular mycorrhizal fungi (AMF) initially increases with the alleviation of N-limitation and then declines over time as soils become N-saturated. **(b)** Conceptual diagram illustrating how the production and decomposition of GRSP remains constant in the controls, whereas the production of GRSP is initially stimulated by N-addition and the decomposition of GRSP is suppressed, resulting in greater overall accumulation of GRSP with N-addition. **(c)** Conceptual diagram of MRC accumulation, where the production and decomposition of MRC remain constant in the controls, but N-addition remained or enhanced the production of MRC and suppresses the decomposition of MRC, resulting in higher MRC content in N-addition plots. **(d)** Conceptual diagram showing N addition induced greater accumulation of microbial C, while microbial C contributes less to the SOC due to the greater increase of plant-derived C; the increased content of microbial C and plant-derived C leads to greater amount of labile C and stable C with the ratio of labile/stable C unchanged.