



Deep soil microbial carbon use efficiency responds stronger to nitrogen deposition than top soil in tropical forests, southern China

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Abstract

Background and aims Soil microbial carbon use efficiency (CUE), a key parameter determining soil C fate, has been associated with soil nitrogen (N) availability. However, the responses of forest soil CUE to aggregated N deposition are not clear. Moreover, it remains unclear whether responses may be mediated by soil depth. It is crucial to investigate the patterns and drivers of CUE with N deposition in forest top and deep soil.

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Methods This study examined the responses of microbial CUE to short-term N addition treatments in the top (0–10 cm) and deep (60–80 cm) soils from two tropical forests in Hainan, southern China. Additionally, a meta-analysis was conducted to reveal a global pattern of forest soil microbial CUE response to N deposition.

Results The addition of N in the two tropical forests did not change topsoil CUE, whereas deep soil CUE increased by 25.5% (0.44 and 0.55 without and with N addition) on average. In the two soil depths, changes in CUE to N addition were negatively correlated with Fungi: Bacteria ratio (F: B). The negative relationship between resource limitation and CUE was found to be present with C limitation in top soil and N limitation in deep soil. N cycling enzyme activities played a vital

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role in regulating microbial resource limitation and CUE. The decrease in F: B and C: N ratios with the addition of N partly explained the stronger response of CUE in deep soil. At the global scale, the addition of N had no effect on the forest top soil CUE. However, the significant decline in pH and microbial biomass carbon with N deposition could reduce microbial CUE.

Conclusion The addition of N facilitated carbon storage in deep soil but had no effects on topsoil carbon storage in a tropical forest. Our results highlighted that the soil depth-driven variation in soil C: N and F: B can regulate microbial CUE, which could provide a basis for understanding the soil C cycle in tropical forest ecosystems.

Keywords CUE · Nitrogen addition · Labeled substrate · Phospholipid fatty acids · SIP-PLFA · Eco-enzymatic stoichiometric model

Introduction

Soil is the largest reservoir of terrestrial organic carbon (C) in the biosphere, and more than half of soil C is stored in forest ecosystems (Schlesinger and Bernhardt 2013). Tropical forest C accounts for 55% of the total forest C stock, 32% of which is stored in soil (Pan et al. 2011). Microbial carbon use efficiency (hereafter, CUE), defined as the fraction of growth to total assimilation C (Geyer et al. 2016), is the key parameter determining the fate of C in soil. Nitrogen (N) is one of the essential nutrients for soil microbial growth and metabolism. Therefore, the association between microbial CUE and soil N availability has garnered increasing attention with augmented N deposition during the last decade (Feng et al. 2022; Liu et al. 2018; Spohn et al. 2016a; Yuan et al. 2019). Even after extensive research, it is still challenging to identify general trends in forest microbial CUE response to N deposition and it remains uncertain whether responses may be mediated by soil depth.

Current research on the effects of nitrogen addition on forest microbial CUE mainly focuses on temperate and subtropical areas. Besides, as the responses of CUE to N addition are diverse, it limits our prediction of soil C storage under future N deposition in forest regions. For example, studies in temperate forests found long-term (> 6 years) N addition to decrease top soil (0–10 cm) microbial respiration and increase

microbial CUE. The N addition decreased soil pH and caused a microbial stoichiometric imbalance, resulting in significantly reduced soil microbial respiration rates (Li et al. 2021; Yuan et al. 2019). On the contrary, Kuske et al. (2019) studied a temperate pine forest in North Carolina, where N had been applied for 8 years. They found a reduction of CUE in litter, Oa, and mineral A horizons. CUE decreased as the ratio of C: N decreased. Another study found that 8 years of N addition did not change 0–20 cm soil microbial CUE as the energy and nutrients at the studied site are not limiting factors of microbe (Guo et al. 2021). Short-term N addition in subtropical forests was associated with an overall increase in topsoil CUE (Dai et al. 2022; Yang et al. 2023). Moreover, higher levels of N availability in the 30–60 cm soil also resulted in significantly higher CUE (Liao et al. 2021). However, forest soils from high-latitude regions show a general reduction of CUE with N addition. In addition, pH is an important regulating factor (Schroeder et al. 2022; Silva-Sánchez et al. 2019). Despite extensive research, it is still difficult to identify how N addition changes forest soil microbial CUE. The underlying drivers/mechanisms are also not clear.

Overall, the effect of N addition on microbial CUE is achieved by changing the availability of soil nutrients, altering microbial community, and influencing soil pH value. Microbial CUE increases with the increase in N availability, especially in N-limited ecosystems, as they decrease the investment in secreting enzymes for nutrient mining and reduce respiring C (Chen and Yu 2020; Manzoni et al. 2012). However, substrate stoichiometric imbalance i.e., a higher C: N ratio could decrease microbial CUE by overflow respiration and investment of C into enzymes for nutrient acquisition (Feng et al. 2021). The decomposition and uptake rates of microbial communities vary based on their composition, resulting in different adaptation strategies to changes in the nutrient environment. Recent research has indicated a negative association between CUE and the ratio of Fungi: bacteria (F: B), suggesting that fungi might exhibit lower CUE compared to bacteria due to their increased investment in the secretion of extracellular enzymes (Soares and Rousk 2019; Ullah et al. 2021). Similarly, Pold et al. (2020) reported that CUE of the slow-growing bacterial population was inferior to that of the fast-growing bacterial population. Soil pH is an important factor affecting microbial CUE (Hu et al. 2022). Lower pH

values may cause environmental stress for microbes, resulting in lower CUE (Rousk et al. 2009). On the other hand, lower pH values could influence iron reduction (Hall et al. 2016; Lipson et al. 2010; Schulz et al. 2016) and its related mineral protection for organic matter and hence for C availability (Ye et al. 2018; Chen et al. 2020) and CUE. Furthermore, pH has a significant impact on the composition of microbial communities. Higher microbial diversity allows for the utilization of various carbon sources, thereby increasing CUE (Domeignoz-Horta et al. 2020). The driving factors of N addition on topsoil microbial CUE have been well documented, however, the factors on deep soil are yet to be established.

Considering the responses of microbial CUE to N addition, subsoil is a large and persistent component of soil C pool, but it receives less attention compared to top soil. The subsoil which lies beneath the 30 cm depth, accumulates more than 50% of SOC and retains it for an extended duration (Chen et al. 2023a, b; Jobbágy and Jackson 2000; Rumpel and Kögel-Knabner 2011). C and nutrient cycling in deep soil are different from top soil which determines the response variation to environmental change (Fontaine et al. 2007; Mobley et al. 2015). Soil biotic and abiotic factors change with soil depth and hence CUE. In deep soil, lower substrate availability and oxygen content could determine the specific microorganism community and function (Dijkstra et al. 2015). Higher pH and mineral protection could regulate microbial metabolic activities and C allocation (Schmidt et al. 2011) while controlling the response of microbial CUE to N addition. The response of CUE to N addition could significantly influence forest future C predicting. It was found that forest microbial CUE increased/decreased or did not change with N addition in topsoil (0–10 cm), while in deeper soils (10–30 cm/30–60 cm), it increased with N availability, which was called ‘flexible CUE’ (Liao et al. 2021; Manzoni 2017). However, a recent global-scale study unveiled a correlation between the increase in soil depth (100 cm) and a corresponding decrease in soil microbial biomass as well as bacterial diversity (He et al. 2023). Therefore, it is not clear whether microbial CUE in deep soil decreases with the decrease in C input or the increase in microbial death in an anaerobic environment.

Hainan Island preserves the largest continuous area and most typical tropical forest, accounting for 1/3 of Chinese tropical forest (Yang et al. 2011). Tropical

forests in Hainan possess abundant species diversity, high productivity and ecosystem service capabilities (Zang et al. 2010). Hainan tropical forests carbon storage, its spatial and temporal features (Ren et al. 2014; Gao et al. 2023) and the driving factors i.e., forest succession (Xing et al. 2023), land use dynamics (Liu et al. 2022; Ma et al. 2022) and soil pH (Zhao et al. 2023) are widely studied. Nitrogen addition in Hainan affects soil C accumulation by changing soil microbial community structure and enzyme activities (Ma et al. 2020). However, studies on microbial CUE to N addition are not well understood, especially in the deep soil. How microbes change their resource use in response to substrate stoichiometry could be evaluated using assays of extracellular enzymes based on community-level resource capture. Compared to the labelled approach, the stoichiometrically defined CUE encompasses the biochemical CUE within it (Schimel et al. 2022). To indicate the factors and mechanisms of microbial CUE response in the top and deep soil layer to N addition, we conducted a short-term N addition experiment with soils collected from two tropical forests on Hainan Island. It consisted of both topsoil (0–10 cm) and deep soil (60–80 cm). To investigate the overall reactions of microbial CUE in forest soil to N addition, we additionally gathered a comprehensive global dataset and employed a meta-analysis approach to quantitatively evaluate the findings. We aimed to assess (1) the pattern of N addition on forest soil microbial CUE in different depths, and (2) the potential similarities or differences in the underlying mechanisms of microbial CUE response to N addition in different soil depths. The hypothesis was based on the fact that (1) N addition could have stronger effects on forest deep soil microbial CUE because the present microbial are comparatively more nutrient deficient than topsoil and (2) SOC could be the determining factor when considering deep soil as it is considerably less abundant in the deeper soil layer.

Materials and methods

Sites and soil sampling

Sites

Tropical forests in Hainan, located at the northern edge of the tropics, are one of the typical tropical

forest regions of China. The best tropical forest in Hainan is mainly distributed in the central mountainous region (Yang et al. 2021). The mean annual temperature ranges from 19.7 °C to 25.2 °C with a mean annual precipitation of 1500–3500 mm. The tropical forests cover a broad elevation range (278 to 1867 m) (Yang et al. 2018; Xu et al. 2015). The soil sampling plots were located in the Jianfengling National Nature Reserve (18°20′~18°57′N, 108°41′~109°12′E) and Diaoluoshan National Nature Reserve (18.44° N, 109.54° E) in the central mountainous region of Hainan Island (Fig. 1). The Jianfengling reserve contains classical tropical montane rainforest. The average yearly precipitation amounts to around 2400 mm, with the majority (80–90%) occurring between May and October. The mean annual temperature is 24.5 °C. According to the USDA taxonomy, the soil type is Oxisol, and the dominant plant families belong

to the *Lauraceae*, *Fagaceae*, and *Rubiaceae* (Yang et al. 2018). The Diaoluoshan reserve is categorized as a lowland tropical forest and the average yearly precipitation amounts to around 2160 mm, with the majority (80–90%) occurring between April and November. The mean annual temperature is 24.6 °C. The soil type is krasnozem, developed from granite or igneous rock and the dominant species is *Vatica mangachapoi*, which belongs to Dipterocarpaceae family (Zhao et al. 2019). The top and deep soil properties of the two studied forests are listed in the supplement materials (Table S1).

Soil sample collection

Soils were collected in May 2018, as microbial activity is predicted to be higher in the rainy season. In each tropical forest reserve, a 20 m× 20 m plot was

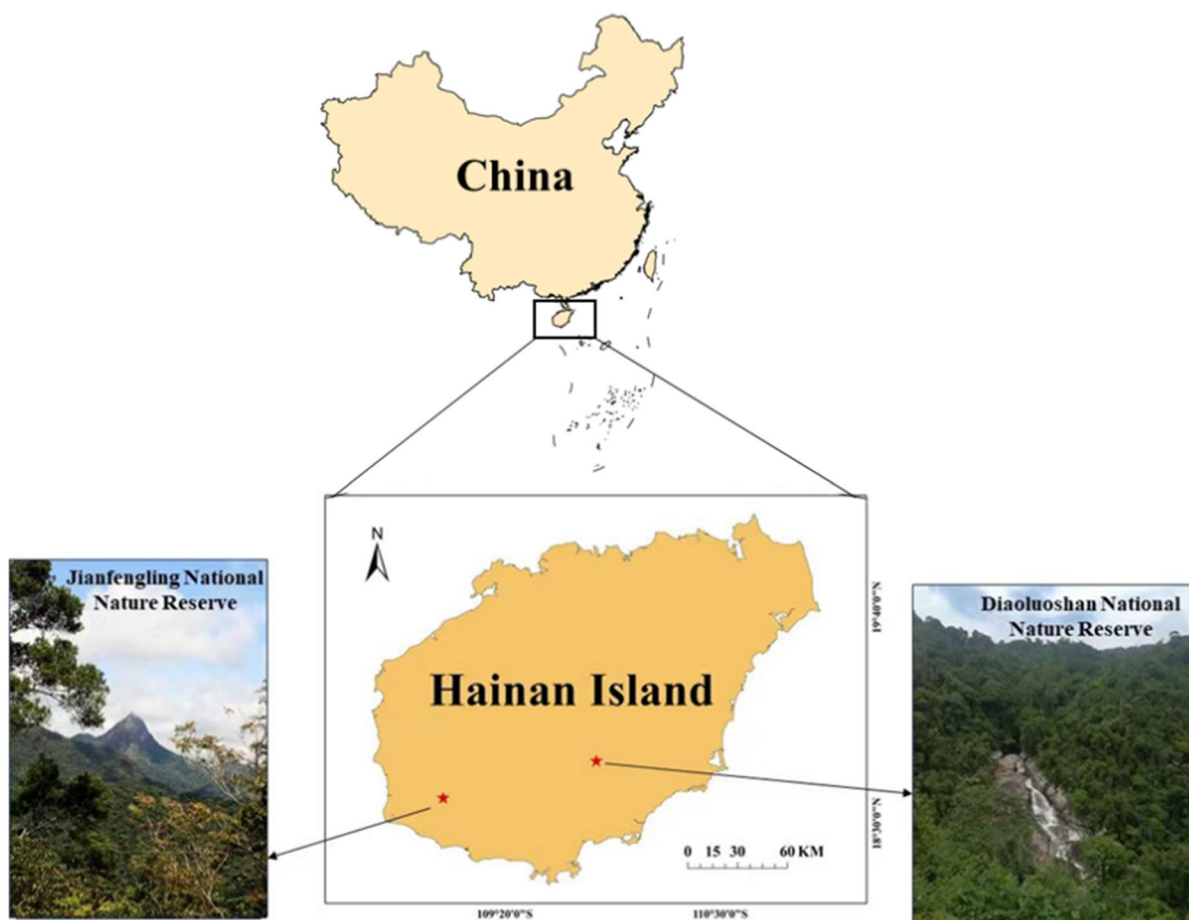


Fig. 1 The research sites

set and then 8 subplots on the diagonal, each 5 m × 5 m in size were selected as the sampling sites. At each sampling site, the top 10 cm soils were collected using a 5 cm diameter soil corner after removing surface litter and sand, while the 60–80 cm soils were collected using a 5 cm diameter soil corner on the soil profile. At each sampling site, three soil cores were collected and mixed as one subsample. In total, eight subsamples were collected. Soils were stored in polyethene bags and transported to the laboratory in ice coolers. The subsamples were finally mixed for further analysis. Soils were sieved to 2 mm to remove roots and stones and then were divided into three parts: 1) incubation (stored at 5 °C for 10 days before incubation) (Ramirez et al. 2012), (2) verification of background soil microbial properties (stored at –80 °C for < 1 week) and 3) assessment of background soil physicochemical properties (air dried).

Nitrogen addition experiment design

To determine the microbial CUE responses to N addition, fresh soils (200 g dry equivalent) of the top and deep layers from the two forests were weighed into culture flasks with an 11.6 cm internal diameter. All samples were adjusted to a 35% water-holding capacity and incubated at 20 °C in a dark environment for 160 days while keeping all conditions constant (Ramirez et al. 2012). Four different N treatments in the form of NH_4NO_3 were applied to the soils to create four treatment groups: 0 kg $\text{N}\cdot\text{ha}^{-1}\cdot\text{r}^{-1}$ (no-N), 25 kg $\text{N}\cdot\text{ha}^{-1}\cdot\text{r}^{-1}$ (low-N, LN), 50 kg $\text{N}\cdot\text{ha}^{-1}\cdot\text{r}^{-1}$ (medium-N, MN) and 100 kg $\text{N}\cdot\text{ha}^{-1}\cdot\text{r}^{-1}$ (high-N, HN). The mean density of the top 10 cm and the deep 20 cm soils are $1.21 \pm 0.01 \text{ g/cm}^3$ and $1.40 \pm 0.01 \text{ g/cm}^3$, respectively. Therefore, the final concentration of added N into the soil was 0, 0.021, 0.042 and 0.083 g N for the top 10 cm soil, and 0, 0.009, 0.018 and 0.036 g N for the 20 cm soils in the deep, respectively. The amount of N was consistent with those carried out in Jianfengling and Nutrient Enrichment Experiments in Chinese Forests (Zhou 2013). In addition, ^{13}C -labelled glucose (1 mg g^{-1} soil) was added into the samples with and without N addition in a single-use manner to capture the microbial carbon utilization change. The constant weight method was used to hold soil moisture constant throughout the 160-day incubation period. The amount of water sprayed into each sample was determined based on

the weight difference between the two-time points. The corresponding N load and water were mixed and sprayed each time onto the corresponding treatment soil. Each treatment was performed in triplicate and received N load every 16 days. Soil samples were collected for analysis at the end of 160 days. Since the respiration samples were broken and the data were lost, microbial CUE in this study was determined by the stoichiometric modeling methods.

Meta-analysis data collection

To understand the global pattern of forest soil microbial CUE response to N addition, a comprehensive literature search was conducted using the Web of Science (<http://apps.webofknowledge.com>) to identify peer-reviewed publications from 1990 to March 1, 2023. The used search terms were: ‘CUE’ OR ‘carbon use efficiency’ OR ‘growth efficiency’ OR ‘growth yield’ OR ‘microbial yield’, ‘soil microbial’ OR ‘soil microbe’, ‘nitrogen addition’ OR ‘nitrogen enrichment’ OR ‘nitrogen deposition’ OR ‘nitrogen fertilization’ OR ‘nitrogen input’ OR ‘nitrogen application’ and ‘forest’. The collected data for this meta-analysis had to meet specific criteria, including (1) availability of means, standard deviations or standard errors, and sample sizes of the variables in the papers; (2) establishment of treatment plots under identical abiotic and biotic conditions; and (3) selection of only one representative publication if multiple publications presented results from the same field plots. Ultimately, a total of 26 paired observations of microbial CUE that met the specified criteria are presented in Table S2.

The average values and standard deviations of microbial respiration rate, growth rate, and CUE were directly obtained from the tables, figures, and supporting information of each study for both soil samples with and without N addition. Data from the figures were obtained using Web Plot Digitiser (a free online tool). Microbial CUE measured with an ^{18}O -labelled water approach, $^{13/14}\text{C}$ -labelled substrate approach, and stoichiometric modeling were all collected. The $^{13/14}\text{C}$ -labelled substrate approaches use the amount of ^{13}C in the microbial biomass divided by the incubation time to measure the growth. We considered the amount of ^{18}O labelled water in DNA as the growth rate when measuring the CUE by the ^{18}O labeled water approach. The SOC, total nitrogen (TN), total phosphorus (TP)

and pH data of both soil samples with and without nitrogen (N) addition were collected. N addition rates and addition durations were extracted directly from the literature. The current discussion solely pertains to the fact of N addition. The detailed information on the sites is presented in Table S2.

Soil physiochemical properties and microbial properties analysis

Soil physiochemical properties

SOC content was measured using the concentrated sulphuric acid-potassium dichromate oxidation method according to Bao (2000). TN and TP were autoclaved with H₂O₂ and H₂SO₄-HClO₄, respectively. They were measured using the semi-micro Kelvin method and analyzed using an automatic flow analyzer (PROXIMA 1022/1/1, ALLIANCE) as described by Zhang et al. (2019). The soil pH was determined using the potentiometric method with a soil-to-water ratio of 1:2.5.

Soil extracellular enzyme activities

The potential activity of five extracellular enzymes—acid phosphatase (AP), β-1,4-glucosidase (BG), β-1,4-N-acetylglucosaminidase (NAG), leucine aminopeptidase (LAP), and phenol oxidase (POX)—was assessed to better understand how microbial C and nutrient acquisition activity responded to N addition over time. The activity of extracellular enzymes involved in C mining (BG), N-mining (NAG+LAP), P mining (AP), and organic matter degradation (POX) was assayed according to the method of Saiya-Cork et al. (2002). A 96-well fluorometric microplate was used for the enzyme assay, with eight replicates performed for each enzyme from each soil. For hydrolytic enzymes (i.e., AP, BG, NAG, and LAP), 1.0 g of fresh soil was weighed, suspended in 50 mM acetate buffer (pH=5.0), and stirred to obtain homogeneous soil suspensions. 200 μL of soil suspension and 50 μL of the corresponding substrate solution (0.2 mM 4-methylumbelliferyl [MUB], labelled substrate) were added to each sample. Blank wells received 200 μL of soil suspension and 50 μL of acetate buffer. Negative control wells received 200 μL of acetate buffer plus 20 μL of substrate solution. Quench standard wells were treated with 50 μL of standard substrate solution (0.01 mM

MUB) plus 200 μL of soil suspension. Reference standard wells received 50 μL of standard substrate solution plus 200 μL of acetate buffer. For the preparation of POX, 1.0 g of fresh soil was weighed and suspended in 50 mM acetate buffer (pH=5.0) and 25 mM L-3,4-dihydroxyphenylalanine (DOPA). Sample wells received 50 μL DOPA plus 200 μL of soil suspension. Negative controls received 200 μL of acetate buffer and 50 μL DOPA. Blank wells received 200 μL of soil suspension and 50 μL of acetate buffer. Hydrolytic enzymes were stored for <4 h in darkness at 20 °C and POX for 18 h in darkness at 20 °C before analysis. Hydrolytic enzyme activity was assessed by evaluating fluorescence at 360 nm excitation and 460 nm emission. POX activity was assessed at 450 nm using a microplate reader (Epoch™ 2, BioTek Instruments, Inc, USA).

CUE analysis with an ecoenzymatic stoichiometric modelling

The CUE was estimated in this study while following Sinsabaugh et al. (2016) and the following equations:

$$CUE = CUE_{max} * S_{C/N} / [K_{C/N} + S_{C/N}] \quad (1)$$

$$S_{C/N} = \frac{B_{C/N}}{L_{C/N}} * \frac{1}{EEA_{C/N}} \quad (2)$$

$$EEA_{C/N} = BG / (LAP + NAG) \quad (3)$$

where CUE_{max} is approximately 0.6 and $K_{C/N}$ is half-saturation constant for CUE based on the stoichiometry of C: N availabilities, which is approximately 0.5. $S_{C/N}$ is the scalar for the relative availability of hydrolysed N in relation to microbial community composition. $B_{C/N}$ is the ratio of microbial biomass carbon (MBC) and nitrogen (we used 8.6). $L_{C/N}$ is the C:N ratio of labile organic matter in the environment.

Microbial community and carbon utilization rate

The soil microbial community in samples was assessed using phospholipid fatty acid analysis (PLFA) (Bossio and Scow 1998; Frostegård et al. 2011). In summary, the lipids present in 5 g of freeze-dried soil were extracted and subsequently separated using a solid-phase extraction column from

Agilent Technologies, Inc. (USA). Following methylation of the separated fatty acids, the MIDI peaks (version 4.5; MIDI, Inc., USA) were identified, and gas chromatographic analysis was conducted using an Agilent 6850 Series Gas Chromatograph. The separation and quantification procedures for PLFA were followed according to Guan et al. (2018). The abundance of PLFAs, expressed in units of nmol per gram of dry soil, was then converted to biomass in grams of carbon (g C) using the same method.

Microbial carbon utilization was indicated by the amount of the labelled glucose that was absorbed by a microorganism into the fatty acid methylesters. This was determined by measuring the $\delta^{13}\text{C}$ values of individual fatty acid methylesters using a gas chromatograph combustion isotope ratio mass spectrometer Delta plus (Thermo Finnigan, San Jose, CA, USA) at Tsinghua University. To ensure accurate detection of $\delta^{13}\text{C}$, each sample was analyzed in triplicate. Isotope ratios were measured for 15 fatty acid methylesters. The C atom introduced during the methylation process was appropriately adjusted using the mass balance equation (Dungait et al. 2011):

$$n_{cd}\delta^{13}\text{C}_{cd} = n_c\delta^{13}\text{C}_c + n_d\delta^{13}\text{C}_d \quad (4)$$

where n is the number of C atoms, and c , d and cd are the $\delta^{13}\text{C}$ values of the underivatized compound, derivatizing agent (BF_3MeOH , $\delta^{13}\text{C} = -42.5\%$) and derivatized compound, respectively. The incorporation of ^{13}C into the PLFAs was calculated as:

$$\text{Incorporated } ^{13}\text{C} = (F_c - F_e) \times \text{PLFA} \quad (5)$$

where F_c is the fraction of ^{13}C in the control soil, F_e is the fraction of ^{13}C in the ^{13}C -glucose spiked soil, and [PLFA] is the concentration of PLFAs in the ^{13}C -glucose spiked soil.

F was calculated as follows:

$$F = R/(R + 1) \quad (6)$$

$$\text{Where } R = (\delta^{13}\text{C}/1000 + 1) \times R_{\text{PDB}} \quad (7)$$

Where R_{PDB} is the $^{13}\text{C}/^{12}\text{C}$ ratio of the Pee Dee Belemnite (PDB) standard.

Calculations and statistical analysis

In this study, a natural log response ratio was applied to assess the effects sizes of N addition on microbial

CUE and other parameters (Hedges et al. 1999). The software Metawin (version 3) was employed to calculate the 95% confidence intervals (CIs). In cases where the 95% CIs did not overlap with zero, it was determined that nitrogen (N) addition had a significant impact on the target variables.

Vector analysis (length and angle) of coenzymatic stoichiometry was used to identify microbial resource limitation according to Eqs. 8 and 9 (Moorhead et al. 2013).

$$\text{Vector Length} = \sqrt{(\ln \text{BG} / \ln(\text{NAG} + \text{LAP}))^2 + \left(\frac{\ln \text{BG}}{\ln \text{AP}}\right)^2} \quad (8)$$

$$\text{Vector Angle} = \text{Degrees}(\text{ATAN2}((\ln \text{BG} / \text{AP}), (\ln \text{BG} / \ln(\text{NAG} + \text{LAP})))) \quad (9)$$

A longer vector length indicates greater C limitation, while angles $< 45^\circ$ and $> 45^\circ$ indicate the relative degrees of N and P limitation, respectively.

To gain a mechanistic understanding of how soil nutrients and soil microbial properties followed by N addition mediated alterations in microbial CUE in two soil depths. The structural equation model (SEM) was performed with R software (version 4.2.0) using the lavaan package. The model fit was evaluated based on Schermelleh-Engel et al. (2003). To examine the effects of N addition, soil depth and their interaction effects on target variables, a Two-way variance analysis was used. A T-test was used to verify the N addition effects on target variables. Pearson's correlation was used to assess the relationships between CUE and other properties. All these analyses were conducted using SPSS 20 (IBM SPSS Predictive Analytics Community, USA) after normal test and variance homogeneity test. All figures were prepared in Graph-Pad Prism 7 (Graph Pad Software, USA).

Results

The response of forest soil microbial CUE to N addition

The results showed that N addition did not affect the microbial CUE in the top soil, which ranged from 0.26 to 0.52. CUE in the deep soil varied from 0.39 to 0.58. It increased by 25.5% (on average) with N addition. Microbial CUE in the deep soil was 25.3% and 46.4% higher than that in the topsoil without and with

N addition. It was 38.6% higher (an average) in the deep soil than in the topsoil (Fig. 2a). At the global scale, the forest soil microbial CUE varied from 0.31 to 0.33 without and with N addition. It had no significant response to N addition. Soil microbial CUE in the two tropical forests of Hainan was significantly higher than that of global forests without or with N addition (Fig. 2b).

Responses of soil biotic and abiotic properties to N addition

As presented in Table 1, soil SOC did not change while TN content increased with N addition, resulting in a significant decrease in C:N ratios. N addition did not change vector length at all depths but increased vector angle of the topsoil (Fig. 3b). Vector angle was less than 45° , which indicated microbial N limitation in the two tropical forests (Fig. 3b). In addition, no soil acidification was observed with the short-term addition of N as pH did not change. Hydrolase enzyme activities (including AP and BG) did not show any significant response to N addition, whereas, enzymes for acquiring N were significantly depressed (Fig. 3a). Oxidase enzyme activities also declined significantly with the addition of N on the two depths. N addition had no effect on total microbial PLFA, gram negative and fungi abundance

(Table 1). However, gram-positive bacteria abundance increased with the rate of addition of N, resulting in a significant reduction in fungi: bacteria (F: B) in the deep soil layer (Fig. 3c, d). Besides, N addition also increased gram-positive bacteria C utilization (Fig. 3e). At the global scale, N addition considerably decreased forest soil pH and MBC but did not change other properties (Fig. S1).

Factors regulating the response of forest microbial CUE to N addition

The decline in soil F: B with N addition was significantly correlated with the corresponding increase in microbial CUE (Fig. 4d, h) across soil depths. The increase in microbial CUE was negatively correlated with the decline in C: N in the deep soil (-0.99), while there was no clear relationship with changes in soil C: N at the top soil (Fig. 4a, e). There were no clear relationships between changes in soil microbial CUE and changes in vector angle as well as N-cycling enzymes in the top soil (Fig. 4b, c). On the contrary, changes in vector angle and N-cycling enzymes in the deep soil were negatively and positively correlated with the changes in microbial CUE, respectively (Fig. 4f, g). The regression function showed that microbial CUE was significantly related to F: B, which was -0.90 and -0.85 for the top and deep soil, respectively. The

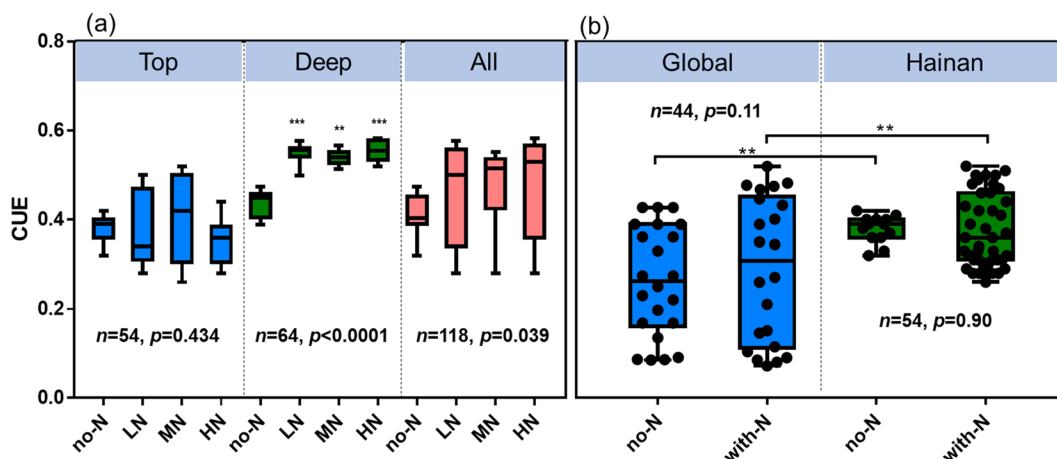


Fig. 2 The response of soil microbial carbon use efficiency to nitrogen addition in Hainan and global forest. no-N, LN, MN and HN was 0, 25, 50 and 100 Kg N ha⁻²yr⁻¹ nitrogen addition rate, respectively. with-N was soils with nitrogen addition. **and *** were the significance level of t-test with $p < 0.01$

and $p < 0.001$, respectively. Top, Deep and All was soils from 0–10 cm, 60–80 cm and the two of them, respectively. n is sampling size. The global datum for soil layers > 10 cm only including two sites, thus we selected soils of 0–10 cm of the global datum to compare with that in Hainan

Table 1 The response of soil properties in Hainan tropical forest to N addition

Variable	Soil Layer(L)		N addition (N)		Variation analysis			
	Top (n=24)	Deep (n=24)	No-N (n=12)	With-N (n=36)	L	N	L*N	
PLFA _{total} is total microbial abundance.	SOC	21.53±0.44	4.88±0.30	12.99±2.74	13.27±1.41	***(-)	ns	ns
AP, BG and POX are acid phosphatase, β-1,4-glucosidase and phenol oxidase, respectively. G ⁻ is gram negative bacteria.	TN	1.51±0.09	1.24±0.11	0.86±0.08	1.55±0.08	ns	**(+)	ns
Values are shown as mean ± standard error.	TP	0.15±0.02	0.07±0.01	0.11±0.02	0.11±0.01	ns	ns	ns
*, **, ***, ns are the significant level of the two-way variance analysis which indicates $p < 0.05$, $p < 0.01$, $p < 0.001$, and no significant difference, respectively. The - and + in the brackets represent the negative and positive effect direction, respectively	C: N	15.56±1.01	4.39±0.36	13.24±2.13	8.89±6.25	***(-)	***(-)	***(-)
	pH	4.44±0.15	4.59±0.11	4.97±0.74	4.37±0.08	ns	ns	ns
	G ⁻	1.40±0.11	2.06±0.08	0.90±0.07	1.21±0.09	ns	ns	ns
	Fungi	0.43±0.02	1.12±0.07	0.68±0.09	0.80±0.08	**(+)	ns	ns
	PLFA _{total}	4.60±0.30	4.83±0.18	3.86±0.48	4.99±0.21	ns	ns	ns
	Variable	Top (n=54)	Deep (n=64)	No-N (n=29)	With-N (n=89)	L	N	L*N
	AP	81.75±2.92	85.75±4.67	84.11±7.22	83.93±3.41	ns	ns	ns
	BG	79.55±3.62	82.34±3.88	83.14±6.39	80.40±2.89	ns	ns	ns
	POX	1.77±0.09	3.16±0.20	2.84±0.30	2.25±0.12	***(+)	**(-)	**(-)
	Vector length	1.31±0.01	1.31±0.01	1.31±0.02	1.31±0.01	ns	ns	ns
	Vector angle	41.30±0.24	39.86±0.28	39.99±0.35	40.71±0.24	ns	*(+)	*(+)

microbial CUE had negative correlations with vector angle (-0.90) and positive correlations with N-cycling enzymes (0.93) in deep soil.

Potential mechanism underlying the response of forest microbial CUE to N addition

The SEM indicated that factors influencing microbial CUE were the same (excluding pH and resource limitation) in the two depths. Meanwhile, the factors affecting the direction were also the same. However, factors affecting size were different. In specific, SOC content exerted the strongest negative influence on microbial CUE and the effect of size was higher in the deep soil. N addition rate and N-cycling enzyme activities positively influenced microbial CUE in the two depths. Their effect of size was higher in the topsoil than in the deep soil (Fig. 5c, d). In the topsoil, N-cycling enzyme activities directly influenced microbial CUE and got indirectly affected by altering microbial C limitation (Fig. 5a). Other than topsoil, N-cycling enzyme activities could only indirectly affect microbial CUE by altering the microbial N limitation in the deep soil (Fig. 5b). N addition rate was positively associated with microbial CUE in the two depths. Notably, there was a difference that N addition rate could influence CUE by direct and indirect ways in

the topsoil, while only a direct effect existed in the deep soil (Fig. 5a, b). Another interesting point was that the negative influences of resource limitation on CUE varied between soil depth, as C-limitation (vector L) for the topsoil, N/P-limitation for the deep soil (Fig. 5).

At the global scale, microbial growth rate, rather than respiration rate, was the determinant factor that positively regulated forest soil microbial CUE (Fig. 6). Soil pH indirectly exerted a positive influence on microbial CUE by regulating microbial growth rate (Fig. 6a). The effects direction of N addition rate on microbial CUE was consistent with that of the short-term N addition experiment in Hainan (Fig. 6b). SOC exerted positive influence on microbial CUE by indirectly regulating microbial growth rate and MBC (Fig. 6).

Discussion

Topsoil microbial CUE did not response to nitrogen addition

In this study, the topsoil microbial CUE did not change with N addition in the two tropical forests, which was consistent with the global results (Fig. 2). The non-significant response of CUE to N addition

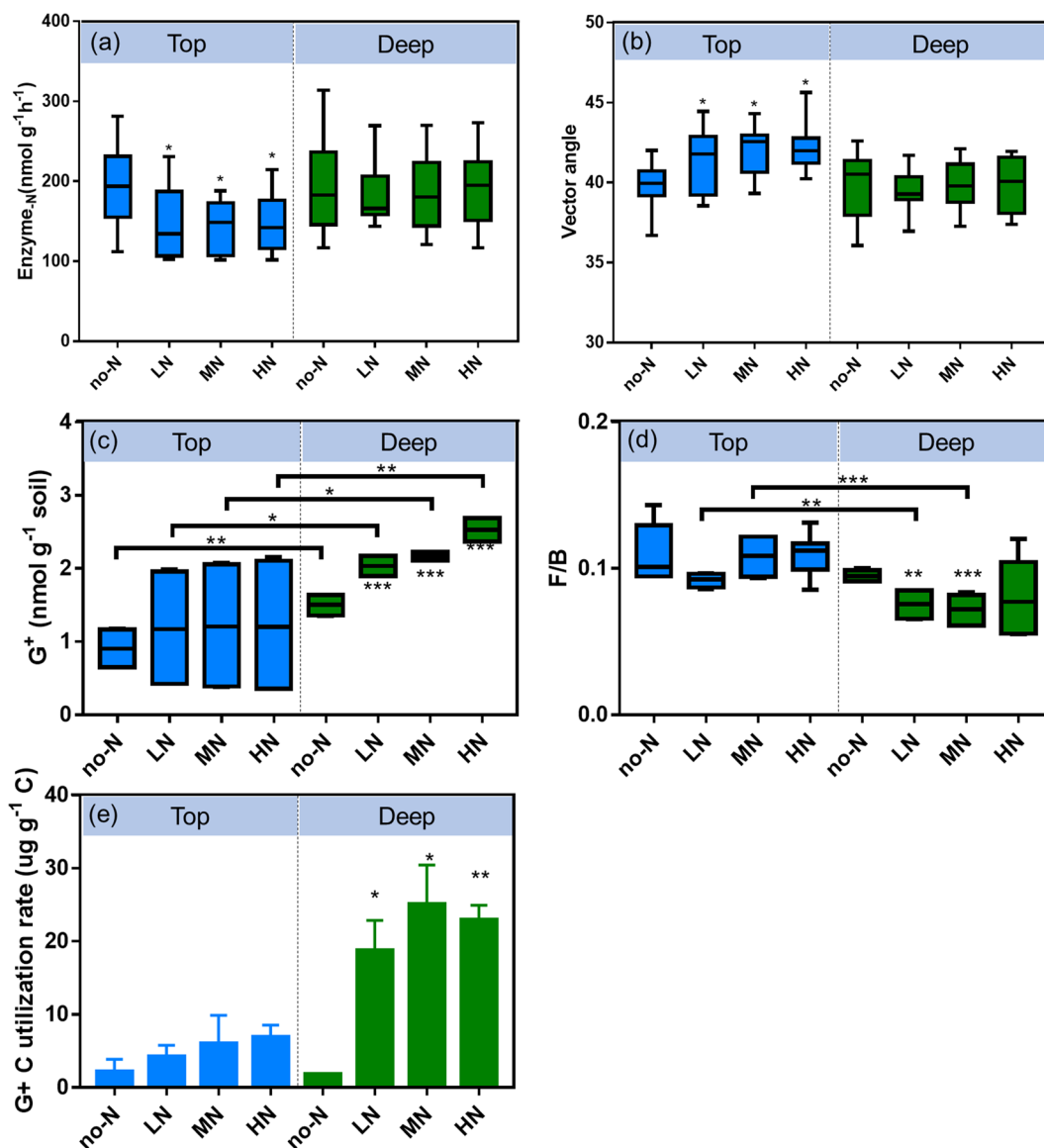


Fig. 3 The response of soil biotic parameters to nitrogen addition in the two tropical forests of Hainan. *, ** and *** represented that p for t-test was $p < 0.05$, $p < 0.01$, $p < 0.001$. ns was no significant difference. no-N, LN, MN and HN was 0, 25,

50 and 100 Kg N ha⁻²yr⁻¹ nitrogen addition rate, respectively. Enzyme_N was the sum of β -1,4-N-acetyl-glucosaminidase and leucine aminopeptidase for N miming. G⁺ is gram positive bacteria. F/B is the fungi: bacteria ratio

was also found in previous studies (Hu et al. 2022; Riggs and Hobbie 2016; Widdig et al. 2020). In the two tropical forests, SOC and N addition rates were the determinant factors that negatively and positively affect the response of soil microbial CUE to N addition (Fig. 5c, d). The findings validated the second hypothesis. It was also confirmed from previous studies that reported a negative association between

C: N and microbial CUE (Schroeder et al. 2022). A higher soil C: N ratio broke microorganism homeostatic balance while resulting in overflow respiration. It also led to the investment of C into enzymes for nutrients acquisition, thereby decreasing microbial growth rate and CUE (Manzoni et al. 2012; Feng et al. 2021). Therefore, N addition increased soil N availability and decreased soil C: N ratio (Table 1).

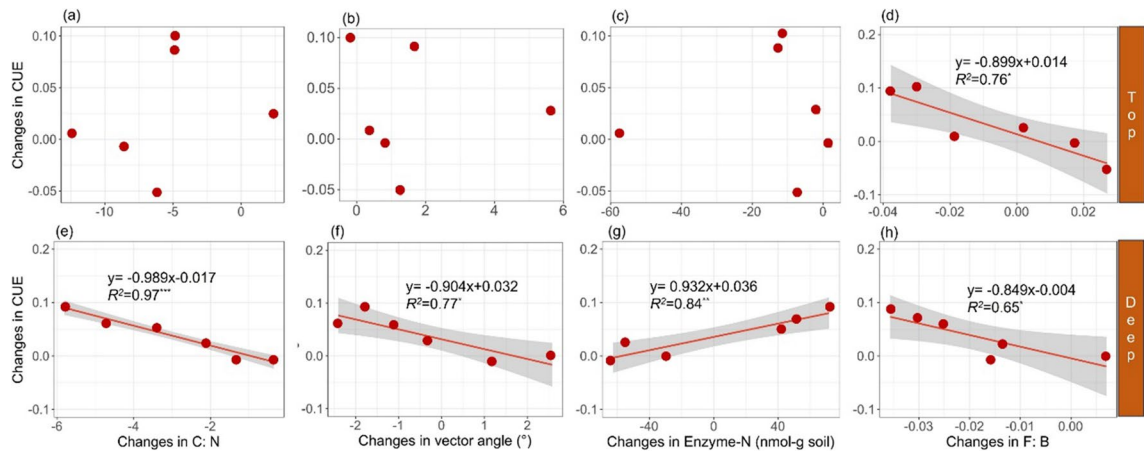


Fig. 4 Relationships between changes in microbial CUE and changes in soil biotic and abiotic factors at different depths across the three nitrogen addition rates. The changes were calculated as the proportional difference between the nitrogen addition treatment and control treatment. For example, changes

in C: N= [nitrogen addition samples (C: N)- control samples (C: N)]. The grey shaded area represents the 95% confidence intervals for the slope. Enzyme-N, enzymes for nitrogen cycling; F: B, fungi: bacteria

It was positively correlated with soil microbial CUE (Fig. 5). Besides, microorganisms in this study are limited by N as vector angle $< 45^\circ$ and the limitation was released with N addition (Fig. 3b). Numerous studies have observed an increase in CUE with higher N availability (Poepflau et al. 2019; Spohn et al. 2016b). This is attributed to microorganisms allocating less C towards N acquisition and extraction from soil organic matter (Cui et al. 2020), while allocating more C towards their growth (Manzoni et al. 2017). Alternatively, addition of N to N-limited soils can impede the loss of C through microbial respiration, thereby facilitating a higher incorporation of C into newly formed microbial biomass (Chen et al. 2019). This, in turn, leads to an augmentation in microbial carbon use efficiency (CUE) (Liu et al. 2018; Manzoni et al. 2012, 2017; Spohn et al. 2016b).

At the global scale, the positive effect of N addition rate on CUE was found to be the same as in Hainan. However, the effect of SOC on CUE was opposite to that in Hainan (Figs. 5 and 6). The positive influence of SOC on CUE was achieved by altering MBC (Fig. 6a). SOC functioned as a source of nutrients for microbes and exerted a positive influence on the abundance of microbial biomass. Thus, it is common to observe a positive correlation between MBC and SOC (Xu et al. 2012; Crowther et al. 2019). In forest soil, studies have demonstrated a positive association between pH levels and microbial CUE

(Li et al. 2021; Silva-Sánchez et al. 2019). The positive association of soil pH on microbial CUE was also found in forests at the global scale (Fig. 6b). The positive association between pH and CUE was achieved by mediating microbial growth rate (Fig. 6a). Studies found that soil acidification caused by mineral N fertilization may force microbes to allocate C for energy production, thus inhibiting microbial growth and lowering microbial CUE (Malik et al. 2018; Jones et al. 2019). At the global scale, the decreased MBC and soil pH induced by N addition (Fig. S1) could consequently result in a lower microbial CUE.

In addition, the global topsoil forest microbial CUE was considerably lower than that of the two tropical forests in southern China (Fig. 3), which could be attributed to the disparity in the assessment approach. According to Geyer et al. (2019), stoichiometric modelling consistently yields significantly higher estimates of microbial CUE compared to isotopic approaches. This lower CUE of the global forest is the result of many CUE estimates in this meta-analysis originating from the ^{13}C -substrate and ^{18}O - H_2O approaches (Table S2).

Deep soil microbial CUE increased with N addition

Microbial CUE in deep soil was higher than that of topsoil, which increased with N addition (Fig. 2a), indicating that adaptation of microbial CUE occurred

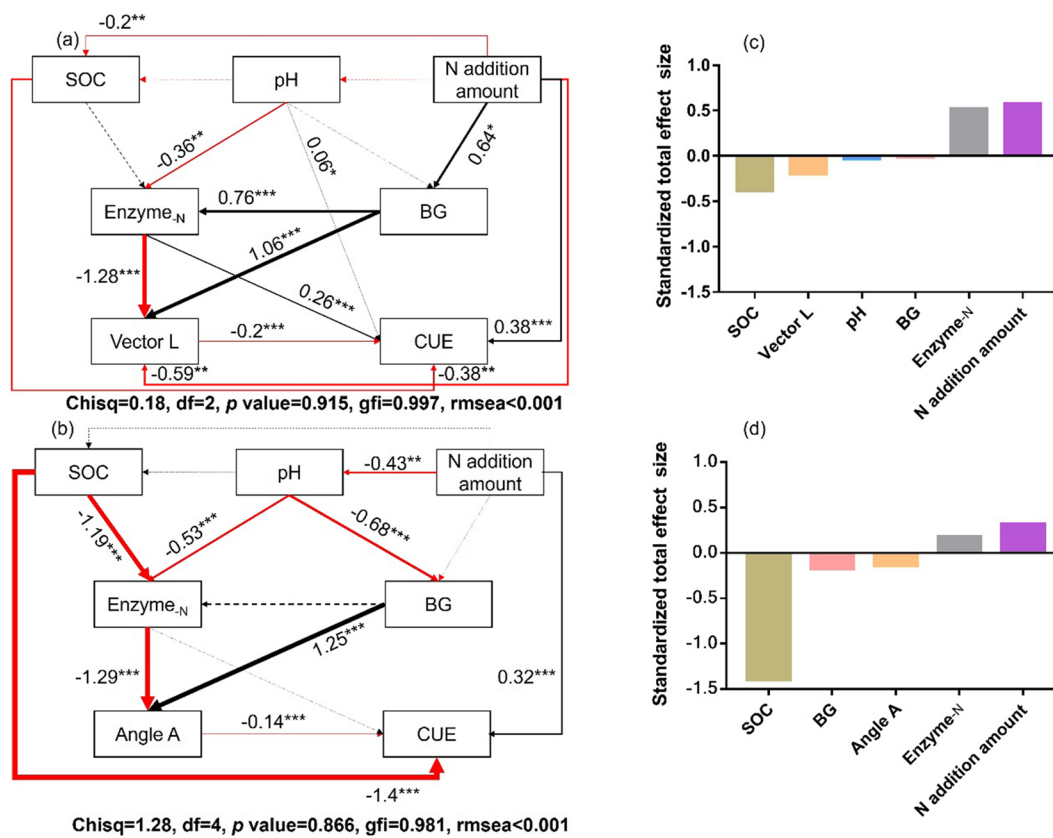


Fig. 5 The SEM model and variable effect size for the two tropical forests in the top (**a, c**) and deep soil (**b, d**). Enzyme_N represents the sum of β -1,4-N-acetyl-glucosaminidase and leucine aminopeptidase. BG represents β -1,4-glucosidase. SOC represents soil organic carbon. Vector L and Angle A are the indices for indicating microbial carbon and nitrogen/ phosphorus limitation, respectively. Black and red lines represent

in the deep soil. The above results validated our first hypothesis that N addition would have stronger effects on forest deep soil microbial CUE. Thus, N addition in the tropical forest promoted C storage of deep soil as higher CUE usually relates to higher C sequestration (Tao et al. 2023). Manzoni et al. (2017) reported that microbial CUE increased in 10–30 cm soil layers with N availability, which was called ‘flexible CUE’. This ‘flexible CUE’ could occur even in the 60–80 cm depth. Previous studies also found that the CUE in deep soil was higher than in topsoil due to microbial adaptation to the long-term substrate limited environment (Fierer et al. 2003; Blagodatskaya et al. 2007). Soil microbial community composition has different adaptation strategies to the nutrient environment, which determine the organic matter

positive and negative effect directions, respectively. Solid and dashed lines represent that the effect is significant and non-significant, respectively. The thicker line, the larger effect size of the variable. The number next to the line represents standardization coefficient. *, ** and *** represent that $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively

decomposition, absorption rates and then CUE. As demonstrated by Domeignoz-Horta et al. (2020), the change in CUE was positively correlated with bacteria diversity which restrained by resource limitation and availability. Furthermore, Maynard et al. (2017) observed that an increase in nitrogen availability led to a decline in F: B ratio and resulted in a higher CUE. It could be attributed to bacteria exhibiting higher CUE values than fungi. In our study, N addition decreased the F: B ratio of the deep soil layer and shifted the community to a bacterial-dominated composition (Fig. 3d) with a higher carbon utilization rate (Fig. 3e). The changes of soil F: B were correlated with an enhancement of microbial CUE (Fig. 4h). However, the reasons for the stronger response of deep soil microbial CUE to N addition was not

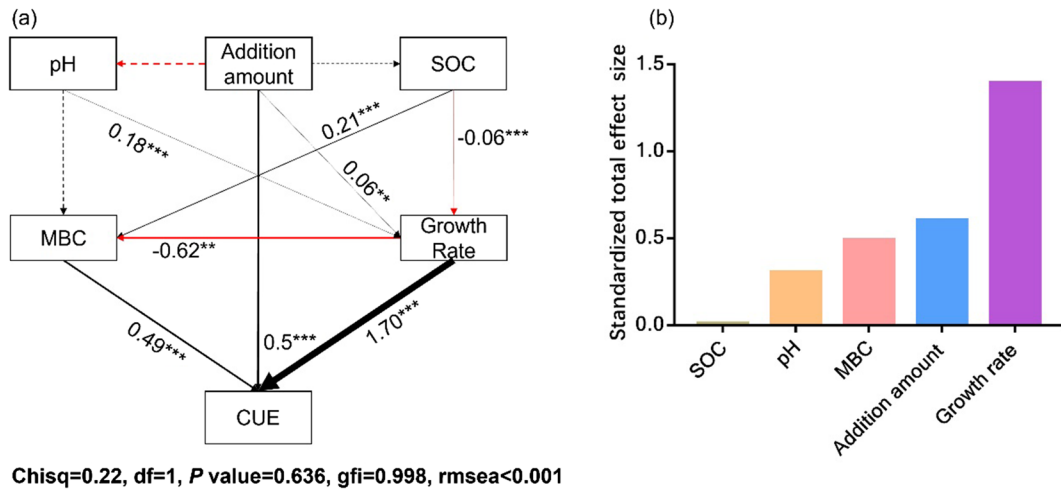


Fig. 6 The SEM model (a) and variable effect size (b) for the global forest ecosystem. MBC represents microorganism biomass carbon. SOC represents soil organic carbon. Black and red lines represent positive and negative effect direction, respectively. Solid and dashed lines represent that the effect

is significant and non-significant respectively. The thicker the line, the larger effect size of the variable. The number next to the line represents standardization coefficient. *, ** and *** represent that $p < 0.05$, $p < 0.01$ and $p < 0.001$

simply attributed to nutrients deficiency. Another reason for the higher CUE in deep soil could be the negative relationship between soil pH and CUE (Fig. S3). Thus, a significant reduction of soil pH with N addition in deep soil promoted soil CUE. The result was consistent with Schroeder et al. (2022). The relationship between CUE and pH was found to be negative for $\text{pH} < 5.5$ and positive for $\text{pH} \geq 6.5$. Soil pH was associated with microbial CUE through mediating N cycling enzyme activities (Fig. 5b). pH was negatively correlated with N cycling enzymes (Fig. S3). A reduction of soil pH with N addition in deep soil could stimulate the activities of N cycling enzymes (Fig. 5b), which exhibit optimal efficiency at lower pH levels (Chen et al. 2018). An augmentation in N-cycling enzyme activities consequently alleviated N limitation of microbes and enhances their CUE (Manzoni et al. 2017; Fig. 4g). Furthermore, the addition of N depressed the activity of oxidative enzymes responsible for the breakdown of complex compounds (Table 1), consequently reducing the energy demands for microbial N acquisition and enhancing microbial CUE (Gallo et al. 2004; Manzoni et al. 2010; Spohn et al. 2016b). Additionally, the negative effect of SOC on microbial CUE was found in the deep soil (Fig. 5). The significant decline in soil C: N in deep soil could reduce microorganism overflow respiration according

to the theory of stoichiometric homeostasis while resulting in a relatively higher microbial CUE (Manzoni et al. 2012; Feng et al. 2021; Fig. 4e).

Unneglectable role of enzymes in influencing microbial CUE

N cycling enzymes played a positive role in influencing microbial CUE in the two depths (Fig. 5). Changes in N cycling enzymes were related to a higher CUE with N addition (Fig. 4g). This positive relationship was different from the findings of the previous study, which indicated that a decline in microbial catabolic activity could increase microbial CUE, as less energy and effort are required for N extraction from organic matter (Craine et al. 2007). Soil microbes' growth and activity are often limited by resources (e.g. C, N or P), which is called microbial resources limitation (Ekblad and Nordgren 2002; Hill et al. 2014). As suggested by the resource allocation theory for enzyme production, microorganisms preferentially allocate their resources to secrete enzymes that require the most of the nutrients (Sinsabaugh and Moorhead 1994; Sinsabaugh and Follstad Shah 2012). Therefore, the positive association between N cycling enzyme and microbial CUE in this study can be partially attributed to the microorganism in the two tropical forests which were

limited by N and did not change with N addition, even though the limitation was released with N addition in the top soil (Fig. 3b). Thus, N cycling enzyme activities were inspired for acquiring N to meet the metabolism demand. The secretion of N-cycling enzymes could facilitate the acquisition of not only N but also C from organic sources (Fujita et al. 2018; Sinsabaugh and Follstad Shah 2012). Therefore, the inspired N cycling enzyme activities increased microbial C availability, released microbial C limitation and therefore CUE (Fig. 5a). A negative relationship was found to be present between soil pH and N cycling enzyme activities (Fig. 5a, b, S3b). Sinsabaugh et al. (2008) discovered a notable inverse correlation between soil pH and soil N-cycling enzyme activity across 40 ecosystems. The optimal pH for cellulase activity was considerably lower (5 ± 1) (Chen et al. 2018). In our study area, the soil pH was found to be significantly lower than that in the global forest (Fig. S2). Therefore, higher enzyme activities in suitable soil conditions could bring out more resources for microorganisms and thus could increase CUE. The behavior of microbial secreting enzymes is a cost-benefit balance, while consuming C, it also acquires the necessary resources for life. The effects of soil N-cycling enzyme activities should be paid more attention to while predicting the soil C response to N deposition in the future.

Limitations and prospect

By conducting an N addition experiment in the lab with the two soil layers having same amount of N, we demonstrated in this study that deep soil microbial CUE was more sensitive to N addition. However, the situations are completely different in the field. In the field, the N deposition may get partially consumed by plants and get leached with water. Therefore, in-situ N addition experiment may retain less relative to the top soil layer. Thus, the results here might have overestimated the deep soil microbial CUE response to N addition. However, the amount of N addition in this study had little effect on the change in microbial N-limited condition especially in the deep soil (Fig. 3b). Therefore, it can be concluded that low N addition is less likely to change the N-limited situation. On the contrary, topsoil microbial N-limitation was alleviated with N addition (Fig. 3b). Taken together, N addition is still expected to increase the deep soil microbial CUE even though the intensity of addition may differ. Notably, the nutrient limitation we were able to

calculate by enzyme vector analysis can only inform the relative nutrient limitation information and it was a short-term results. Therefore, upscaling the laboratory results to ecosystem should be performed carefully (Chen et al. 2023a, b). We have conducted a N addition experiment in a tropical forest in Hainan for 2 years. Therefore, further data from the in situ could be compared to the results from the laboratory to obtain a more comprehensive understanding of microbial CUE response to N addition.

The microbial CUE varied with the assessment approach and resulted in uncertainty. Geyer et al. (2019) illustrated that the microbial CUE measured using the labelled method was lower than the stoichiometry modelling method. It was also confirmed in this study (Fig. 2b). As the microbial CUE in the two depths was measured by the same method which making it comparable. For the global scale, the non-significant response of CUE to N addition might originate from the variations brought on by different evaluating methods. Based on this, we separated the analysis into regional and global to minimize the uncertainties brought by measuring methods in this study. In the future, different approaches should be compared as they have different underlying microbial responses at the metabolic, cell, community and ecosystem scales (Hu et al. 2022). As the in-situ N addition experiment with labeled substrate addition had been conducted in this study, the results of CUE could be compared with other regions to obtain a comprehensive understanding of the microbial CUE to N addition.

The factors of microbial CUE in the topsoil of global forest to those in the soil of tropical forests in Hainan were compared. However, the observed variables were completely inconsistent (Figs. 5 and 6). In the SEM model of tropical forests in Hainan, important factors such as the rate of microbial growth and total biomass at the global level were not selected. The regionally important factor enzymes were not represented in the global SEM model. As demonstrated by this study, microbial enzyme activities played a bidirectional effect on microbial CUE, indicating that more attention should be given to these processes in future.

Conclusions

In this study, N deposition was found to promote deep soil C storage in tropical forests but had no

effects on topsoil C storage. At the global scale, the association of microbial growth on soil microbial CUE was higher than that of respiration. N addition was found to regulate microbial CUE by changing soil C: N ratio and microbial composition. N addition was found to increase the proportion of bacterial which had higher C utilization in the community while resulting in higher CUE in tropical forests. The N deposition might have increased soil microbial CUE, where the primary SOC content was relatively lower. These results illustrated soil depth-driven soil substrate availability and microbial properties can regulate microbial CUE, which could reduce the uncertainty in long-term soil C response modelling to climate change.

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Author contributions **Yamin Jiang**: Conceptualization, Methodology, Writing-Original draft, Writing-Review and editing **Tianyan Su**: Investigation, Writing-Review and editing **Huafeng Wang**: Writing- Review and editing, Project administration **Qiuyang**: Resources, Writing-Review and editing, Supervision, Funding acquisition **Jingli Lu**: Methodology, Writing- Review and editing **Qunyou Fu**: Investigation **Han Mao**: Investigation **Wenxian Xu**: Investigation **Yiqi Luo**: Software, Writing- Review and editing **Wenjie Liu**: Conceptualization, Resources, Writing-Review and editing, Project administration, Funding acquisition **Huai Yang**: Resources, Project administration **Mengyang Fang**: Resources.

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Data availability All authors agreed that the data supporting the findings of this study are available for request.

Declarations

The authors declare that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Competing interests The authors declare that they have no competing interests associated with the research conducted in this study. This research received no specific funding or financial support that could have influenced the outcomes. The authors have no financial relationships with any organizations or companies that could be viewed as having a potential conflict of interest with the research.

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