Enhanced carbon acquisition and use efficiency alleviate microbial carbon relative to nitrogen limitation under soil acidification

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Abstract

Background: Soil microbial communities cope with an imbalanced supply of resources by adjusting their element acquisition and utilization strategies. Although soil pH has long been considered an essential driver of microbial growth and community composition, little is known about how soil acidification affects microbial acquisition and utilization of carbon (C) and nitrogen (N). To close the knowledge gap, we simulated soil acidification and created a pH gradient by adding eight levels of elemental sulfur (S) to the soil in a meadow steppe.

Results: We found that S-induced soil acidification strongly enhanced the ratio of fungi to bacteria (F:B) and microbial biomass C to N (MBC:MBN) and subsequently decreased the C:N imbalance between microbial biomass and their resources. The linear decrease in the C:N imbalance with decreasing soil pH implied a conversion from N limitation to C limitation. To cope with enhanced C versus N limitation, soil microbial communities regulated the relative production of enzymes by increasing the ratio of β-glucosidase (BG, C-acquiring enzyme) to leucine aminopeptidase (LAP, N-acquiring enzyme), even though both enzymatic activities decreased with S addition. Structural equation modeling (SEM) suggested that higher C limitation and C:N-acquiring enzyme stimulated microbial carbon-use efficiency (CUE), which counteracted the negative effect of metal stress (i.e., aluminum and manganese) under soil acidification.

Conclusions: Overall, these results highlight the importance of stoichiometric controls in microbial adaption to soil acidification, which may help predict soil microbial responses to future acid deposition.

Keywords: Soil acidification, Stoichiometric imbalance, Metal stress, Ecoenzymatic stoichiometry, Element-use efficiency
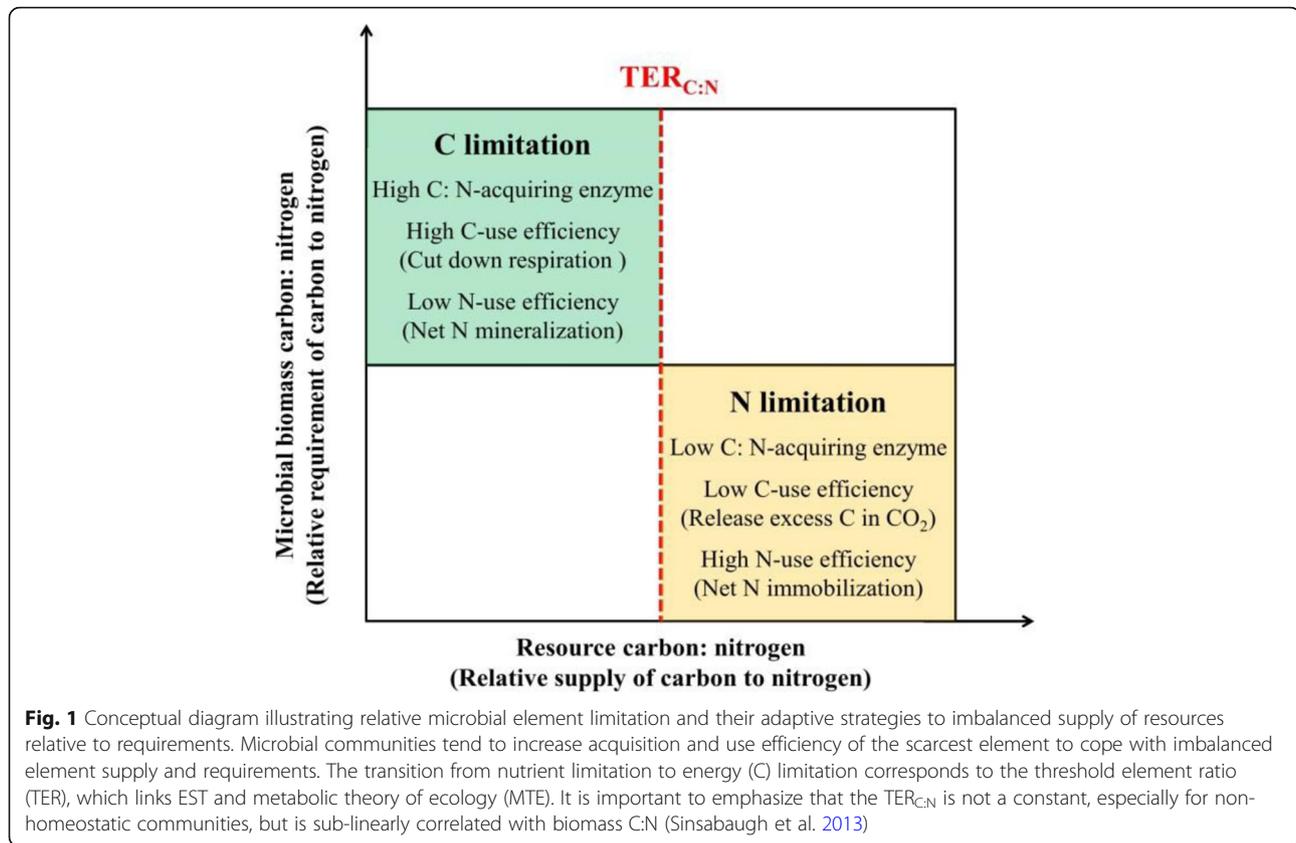
Introduction

Ecological stoichiometry theory (EST) suggests that microbial growth and metabolism are limited by the scarcest elements when the stoichiometry of microbial resources becomes mismatched from the optimal ratios of microbial demands (Mooshammer et al. 2014a;

Sterner and Elser 2002). Soil microbial communities, in turn, could cope with an imbalanced supply of resources by adjusting their element acquisition and utilization strategies (Fig. 1) (Spohn 2016). For instance, microbes secrete more C-acquiring enzymes in soils with narrow C:N ratios after long-term N enrichment (Schleuss et al. 2019), while N-acquiring enzymatic activities are higher in extremely barren ecosystems with wide soil C:N ratios (Cui et al. 2018; Tapia-Torres et al. 2015). On the other hand, microbes can adjust element utilization via

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Fig. 1 Conceptual diagram illustrating relative microbial element limitation and their adaptive strategies to imbalanced supply of resources relative to requirements. Microbial communities tend to increase acquisition and use efficiency of the scarcest element to cope with imbalanced element supply and requirements. The transition from nutrient limitation to energy (C) limitation corresponds to the threshold element ratio (TER), which links EST and metabolic theory of ecology (MTE). It is important to emphasize that the $TER_{C:N}$ is not a constant, especially for non-homeostatic communities, but is sub-linearly correlated with biomass C:N (Sinsabaugh et al. 2013)

regulating their physiological processes when facing resource imbalance (Spohn 2016). Net N immobilization by microbes occurs when N is limited (Mooshammer et al. 2014b), and excess C can be released to the atmosphere by increasing microbial respiration (Manzoni et al. 2010), corresponding to a low carbon-use efficiency (CUE). Conversely, microbes mineralize and release considerable N into the soil rather than retaining it in biomass (i.e., lower nitrogen-use efficiency, NUE) when N is sufficient in their substrates (Mooshammer et al. 2014b). The threshold elemental ratio (TER) was proposed to reflect the critical ratios of substrate C:N (or P), at which microbial growth and metabolism shifts from elemental mineralization (i.e., C:N or C:P lower than TER, C limitation) to immobilization (i.e., C:N or C:P higher than TER, nutrient limitation; Fig. 1) (Frost et al. 2006). Recent studies have linked microbial adaption processes with changing element limitations induced by climate change and anthropogenic disturbance (Guo et al. 2020; Yuan et al. 2019).

Terrestrial ecosystems worldwide are facing a growing risk of soil acidification (Lu et al. 2014; Schrijver et al. 2012; Yang et al. 2012) as a result of atmospheric nitrogen (N) and sulfur (S) deposition and inorganic fertilizer application (Bowman et al. 2008; Cui et al. 2014). Soil acidification has been shown to affect soil C and N

cycling, thereby changing ecosystem functions (Poschenrieder et al. 2008; Wang et al. 2006). Soil enzyme activities are sensitive to soil acidification (Kunito et al. 2016) due to the increasing toxic effects of protons (H^+) and aluminum ions (Al^{3+}) (Van Den Berg et al. 2005). Similarly, increasing manganese (Mn^{2+}) and exchangeable Al^{3+} contents with decreasing pH (Feng et al. 2019) would decrease soil CUE (Jones et al. 2019) because microbial resistance to toxic metals is energy-intensive (Bellion et al. 2006). Furthermore, given that the bacterial community is more sensitive to low pH and Al^{3+} stress than fungi (Rousk et al. 2010a), a shift towards fungal dominance is expected following soil acidification (Chen et al. 2013; Meng et al. 2019). In addition to direct detrimental effects, soil acidification increased soil N availability and decreased soil C:N ratios (i.e., increasing N supply to microorganisms) (Meng et al. 2019; Xiao et al. 2020), whereas the microbial biomass C:N ratio (reflecting the relative status of microbial C and N demands according to Zechmeister-Boltenstern et al. 2015) would vary following the ratio of fungi to bacteria (Strickland and Rousk 2010). We need to examine soil acidification impacts on C and N cycling from a stoichiometric standpoint, considering the potential variations in C:N ratios of both microbial biomass and resources under soil acidification. However, how soil

acidification affects microbial nutrient limitation and further regulates microbial nutrient acquisition and utilization is still an open question.

A variety of N addition experiments showed that stoichiometric controls play a key role in alleviating microbial C limitation by increasing C-acquiring enzymatic activity and CUE under N addition (Spohn et al. 2016; Yuan et al. 2019). However, concomitant changes in nitrogen availability and soil acidification could both contribute to the observed C processes in N addition studies, which is hard to disentangle (Yuan et al. 2019). Moreover, higher soil N availability tends to decrease soil fungi to bacteria (F:B) ratio due to bacterial preference of N-rich environments, while soil acidification increases it because of the higher proliferation of fungi relative to bacteria (Rousk et al. 2010a; Strickland and Rousk 2010). These conflicting responses of the F:B ratio to soil availability versus acidification would further add uncertainty to predicting microbial nutrient acquisition and utilization under N addition (Averill and Waring 2018). Therefore, direct evidence is needed to estimate whether stoichiometric adjustment processes help the microbial community cope with the expected higher N supply but lower N requirements under soil acidification without exogenous N input.

Over the last two decades, temperate semi-arid grassland, one of China's most important ecosystems, has experienced elevated soil acidification from atmospheric acid deposition (Yang et al. 2012). Our objectives were to investigate how S-induced soil acidification affects microbial nutrient limitation, acquisition, and utilization strategies, as revealed by enzymatic stoichiometry and element-use efficiency. We conducted an acidification gradient experiment by applying eight elemental S levels to the soil in a meadow steppe. We investigated soil acidification impacts on enzyme activities and the coupled relationships of microbial C:N ratios with their bioavailable resources. We hypothesized that (1) S-induced soil acidification could alleviate microbial N vs. C limitation by increasing N supply but reducing microbial N requirements by increasing the ratio of F:B; (2) to cope with decreasing N limitation but increasing C limitation, the microbial community would increase C:N-acquiring enzymatic ratios; and (3) increasing microbial CUE due to higher C requirements may counteract the negative effects of enhanced metal stress on CUE under soil acidification.

Materials and methods
Site description
This experiment was conducted in a semi-arid meadow fenced since 2013 at the Erguna Forest-Steppe Ecotone Research Station in Inner Mongolia, China (50° 10′ N, 119° 23′ E, 650 m a.s.l.). The long-term mean annual air temperature of this site is − 2.5 °C, and the mean annual precipitation is 374 mm (according to the data from 1957 to 2016). The meadow grassland in this study is dominated by *Leymus chinensis, Stipa baicalensis, Carex duriuscula*, and *Pulsatilla chinensis*. The soil is classified as chernozem (FAO) with a composition of 39% sand, 37% silt, and 24% clay. The soil bulk density is 1.21 g cm^{-3}, and the average soil pH (0–10 cm) is 6.8. No fertilizer was added before this experiment, and natural S deposition is lower than 0.4 g S m^{-2} year^{-1} in this grassland (Ge et al. 2014).

Experimental design and soil sampling
The S addition experiment was arranged in a randomized block design that was established in early 2017. Eight rates of S addition (0, 1, 2, 5, 10, 15, 20, and 50 g S m^{-2} year^{-1}) were assigned in each of five replicate blocks. Each block consisted of eight plots of size 6 × 6 m and a 2-m buffer zone between adjacent plots. Lower S addition rates (i.e., 1 and 2 g S m^{-2} year^{-1}) simulated increasing natural acid deposition in recent decades (Yu et al. 2017), and the higher rates mimicked aggravating soil acidification induced by accumulative acid deposition in the long term. Elemental S (> 99.9% purity), which has been widely used to modify soil pH in farmlands (Bole 1986) and grasslands (Owen et al. 1999), was added evenly in the form of powder on May 20th each year since 2017. Soil samples were collected in August 2018 (i.e., 3 months after the second-year S addition), where five cores of topsoil (0–10 cm) were taken randomly and then mixed thoroughly for each plot. The homogenized soil samples were passed through a 2-mm sieve to remove rocks and plant residuals immediately; the sieved soil samples were transferred to the laboratory within 2 h and divided into three subsamples. Subsamples for measuring microbial biomass, enzyme activities, and inorganic nitrogen were stored at 4 °C before analysis, and subsamples for determining PLFAs were held at − 20 °C. The remaining soil samples were air-dried at room temperature and used for the determination of the soil abiotic characteristics.

Measurement of soil biochemical properties
Soil abiotic characteristics
Soil pH was measured in a soil slurry using a soil to water ratio of 1:5 (w/v) by a pH meter (Precision and Scientific Corp., Shanghai, China). Soil dissolved carbon (DOC) and total dissolved nitrogen (TDN) concentrations were extracted from 10 g of fresh soil with 40 mL of 0.5 M K$_2$SO$_4$ solution and determined using a TOC analyzer (HT1300, Analytikjena, Jena, Germany). Other soil abiotic characteristics, including soil organic carbon (SOC), total nitrogen (TN), exchangeable Al^{3+}, and

available Mn^{2+}, were determined using air-dried soil as described in detail in Methods S1.

Soil microbial biomass

Soil microbial biomass carbon (MBC) and nitrogen (MBN) were measured using the chloroform ($CHCl_3$) fumigation-extraction method within 7 days of sampling (Vance et al. 1987). Microbial biomass was calculated as E/k, where E = (dissolved element extracted from a fumigated soil sample) – (dissolved element extracted from a non-fumigated soil sample) and conversion factor k was 0.45 and 0.54 for MBC (Joergensen 1996) and MBN (Brookes et al. 1985), respectively.

Soil enzyme activities

Enzyme measurements for β-glucosidase (BG), β-N-acetyl-glucosaminidase (NAG), and leucine aminopeptidase (LAP) were performed on fresh soil samples within 14 days after sampling with a colorimetric method using ρ-nitrophenyl-β-D-glucopyranoside, ρ-nitrophenyl-N-acetyl-β-D-glucosaminide and leucine ρ-nitroanilide (Sigma, St. Louis, USA) as the substrate under the optimal pH, temperature and substrate concentrations (Robertson et al. 1999; Sinsabaugh et al. 1999; Tabatabai 1994) (a detailed description is provided in Methods S1). Enzyme activities were expressed as μmol ρNP per gram soil per hour, and specific enzyme activities were calculated as mmol ρNP per gram MBC per hour.

Microbial community composition

The microbial community composition of the soil samples was determined using the phospholipid fatty acids (PLFAs) method. The PLFAs were extracted from frozen soil samples and then separated and methylated (Bossio and Scow 1998) before analysis. The methylated PLFAs were then analyzed with an Agilent 7890A gas chromatograph (Agilent Technologies, Palo Alto, California, USA) and identified with a MIDI Sherlock Microbial Identification System (MIDI Inc., Newark, DE, USA). The sum of the following PLFAs represents bacteria: i14: 0, 14:0, i15:0, a15:0, 15:0, i16:0, 16:0, 16:1ω7c, 16:1ω9c, 16:1ω11c, i17:0, a17:0, 17:0, cy17:0, 17:1ω8c, 18:0, 18: 1ω5c, 18:1ω7c, 18:1ω9c, 18:1ω8t, and cy19:0 (Bååth and Anderson 2003; Frostegård and Bååth 1996), and general fungi were 18:2ω6c (Bååth and Anderson 2003; Olsson et al. 1995). Here, we calculated the ratio of fungi to bacteria (F:B) using the concentrations of general fungi relative to the sum of bacterial PLFAs. We calculated microbial stress biomarkers using the ratio of saturated FAs (16:1 ω5c, 17:1 ω8c, and 18:1 ω7c) to monounsaturated FAs (14:00, 15:00, 16:00, 17:00 and 18:00) (sat: mono) and cyclopropyl FAs (cy17:0 + cy19:0) to their monoenoic precursor (16:1ω7c + 18:1ω7c) (cy:pre) (Kaur et al. 2005; Siles et al. 2015).

Calculations

Stoichiometric imbalance

We calculated the stoichiometric imbalance between microbes and their resources (i.e., total dissolved forms of C and N) by dividing stoichiometric ratios of resources by stoichiometric ratios of microbial biomass (Mooshammer et al. 2014a). We used DOC and TDN as resources for microorganisms rather than total organic elements because these labile forms were considered to be more accessible for microbes and thus better indicators of microbial resources (Mooshammer et al. 2014a; Zhang et al. 2019). Additionally, we also calculated the stoichiometric imbalance using SOC:TN as a resource stoichiometric ratio to compare with the labile forms.

Ecoenzymatic stoichiometry

Enzymatic stoichiometry was calculated as the ratio of nutrient-acquiring enzyme activity (i.e., BG as C-acquiring enzyme and NAG + LAP as N-acquiring enzyme). A type II standard major axis (SMA) regression analysis was conducted to detect the relationships between $\log_e$-transformed enzymes (in nmol ρNP g^{-1} soil h^{-1}) with the smatr package in R (Warton et al. 2012).

Threshold element ratios (TER)

We calculated the TER for C:N to understand the trade-off between energy (i.e., carbon) and nitrogen controls of microbial communities in response to soil acidification, which connects the EST with the Metabolic Theory of Ecology (MTE) using the following equations (Sinsabaugh et al. 2009):

$$TER_{C:N} = (E_{C:N} \times B_{C:N})/n_0 \quad (1)$$

where $TER_{C:N}$ is the threshold element ratio (dimensionless) for C:N; $E_{C:N}$ represents the ratio of C-acquiring enzyme (BG) to N-acquiring enzymes (NAG + LAP); $B_{C:N}$ represents the C:N ratio of soil microbial biomass; and n_0 is a dimensionless constant calculated by raising e to the intercept of the SMA regression relationship between $\log_e$ (BG) and $\log_e$ (NAG + LAP). We compared the estimated $TER_{C:N}$ with DOC:DON, with a higher TER indicating that microbial growth was limited by energy (net nitrogen mineralization), while a lower TER represented N limitation (net N immobilization) (Sinsabaugh et al. 2013).

Carbon and nitrogen use efficiency

We calculated the CUE and NUE based on the C:N stoichiometry of soil resources, microbial mass and enzymes (Sinsabaugh et al. 2016) using equations (2), (3), (4), and (5):

$$CUE = CUE_{max} [S_{C:N}/(S_{C:N} + K)] \quad (2)$$
$$S_{C:N} = (1/E_{C:N}) (B_{C:N}/L_{C:N}) \quad (3)$$
$$NUE = NUE_{max} [S_{N:C}/(S_{N:C} + K)] \quad (4)$$
$$S_{N:C} = (1 - 1/E_{C:N}) (L_{C:N}/ B_{C:N}) \quad (5)$$

where CUE_{max} is 0.6 and represents the upper limit for microbial growth efficiency based on thermodynamic constraints (Roels 1980; Sinsabaugh et al. 2013) and NUE_{max} is fixed to 1.0 (Sinsabaugh et al. 2016); $S_{C:N}$ represents the extent to which enzymatic allocations offset the differences between the elemental composition of soil resources and microbial biomass; K is the half-saturation constant (0.5); $L_{C:N}$ represents the C:N ratio of soil labile resources (i.e., DOC: TDN) (Sinsabaugh et al. 2016). The estimated microbial CUE and NUE were proven to be effective in predicting nutrient requirements and use efficiency (Geyer et al. 2019) and closely matched the physiological metabolism process, such as microbial respiration (Yuan et al. 2019) and ammonification rate (Zechmeister-Boltenstern et al. 2015). The reliability of estimated CUE to predict microbial carbon utilization was further proven by the negative correlation between estimated CUE and microbial metabolic quotient in our study (Appendix 3 in the Supporting Information).

Statistical analysis

The Kolmogorov-Smirnov test and Levene's test were performed to ensure the normality of data and homogeneity of variances. We used a linear mixed-effects model to test the effects of S on soil biochemistry indices, enzyme activity, and stoichiometry. S addition rates were designated as fixed effects with blocks as random effects. Duncan's multiple range test was conducted to detect the differences between each S addition rate ($P < 0.05$). We used a linear regression analysis to test the correlation between variables and decreasing soil pH.

We conducted a structural equation modeling (SEM) analysis in this study to examine the direct and indirect strength of soil acidification on microbial nutrient cycling. In this analysis, we assumed that a decrease in soil pH may first alter the stoichiometry of microbial biomass and its resources and toxic metal ions, thus affecting stoichiometric imbalance and microbial stress and further causing changes in enzyme stoichiometry and nutrient use efficiency (Table S1 and Fig. S1). Principal component analysis (PCA) was conducted to simplify our models using the extracted PC1 values of toxic metal concentrations (i.e., Al^{3+} and Mn^{2+}) and microbial stress biomarkers (including sat:mono and cy:pre), respectively (Fig. S2). The piecewise SEMs were finally established with Amos 24.0 (Amos Development Co., Greene, Maine, USA) using the maximum likelihood estimation method. The χ^2 test ($P > 0.05$), root square mean errors of approximation (RMSEA < 0.08), and Akaike information criteria (AIC) were used to evaluate the adequacy of the model.

Results

Responses of soil abiotic properties to S addition

Soil pH showed a significant decrease from 6.87 to 5.99 with the increasing rate of S addition ($P < 0.01$, Fig. 2), while the exchangeable H^+ and Al^{3+} increased by up to 82.62% and 52.71%, respectively ($P < 0.01$, Table S2). In addition, soil available Mn^{2+} concentrations increased significantly along the S addition gradient (Table S2). Sulfur addition increased the ratio of SOC:TN from 11.86 to 12.32 ($P = 0.04$, Fig. S3). Soil DOC and TDN increased ($P < 0.01$, Table S2) along the S addition gradient. We observed a significantly lower DOC:TDN in the S addition treatments than in the control plots, which was positively correlated with soil pH ($R^2 = 0.15$, $P = 0.03$, Fig. 2b). Sulfur addition significantly decreased the NO_3^--N concentration but increased DON ($P < 0.01$, Table S2).

Responses of microbial biomass, stoichiometric imbalance, and community structure to S addition
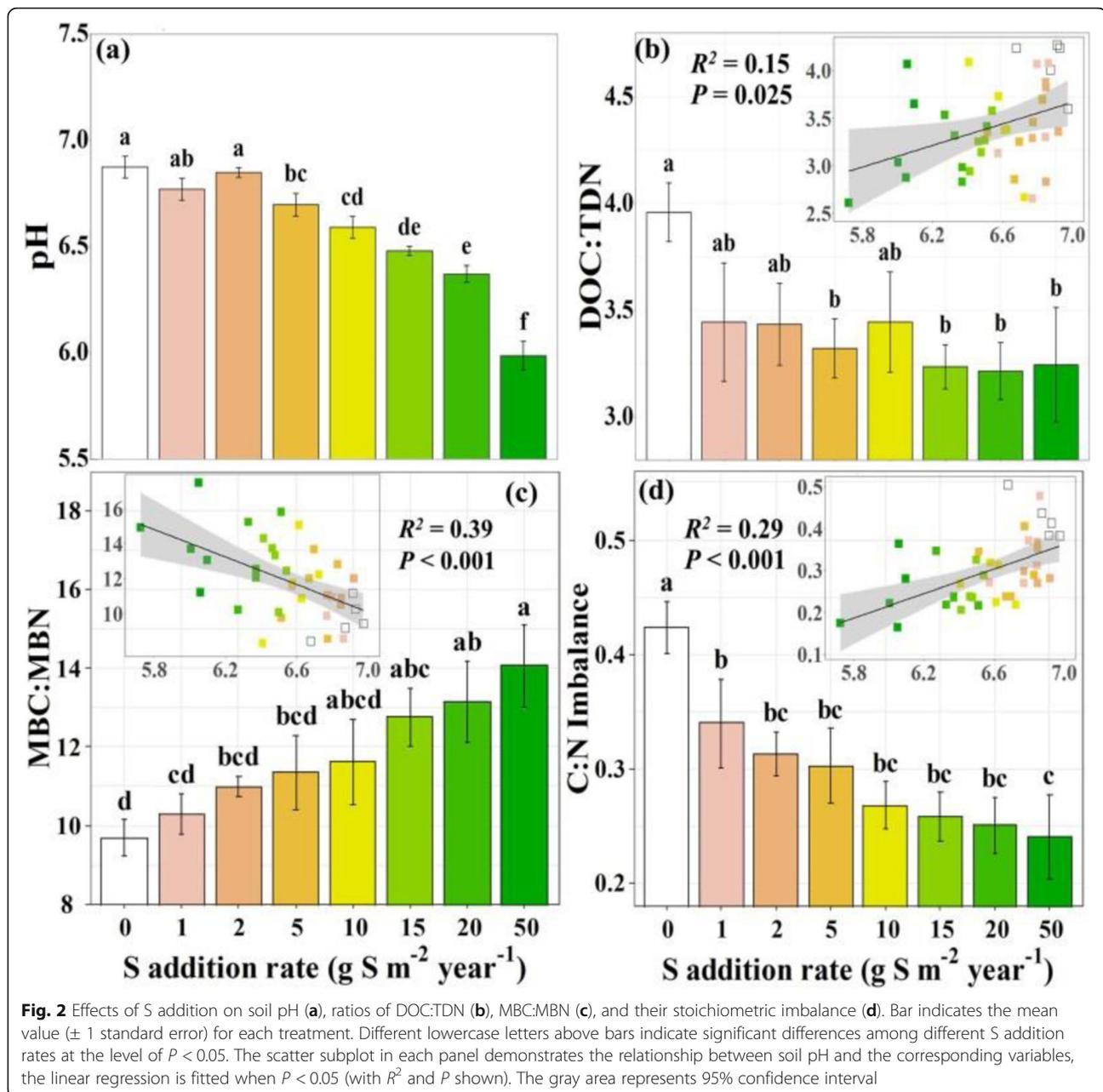
Sulfur addition decreased both soil MBC and MBN (Table S2) but increased MBC:MBN along with decreasing soil pH ($R^2 = 0.39$, $P < 0.01$, Fig. 2c), resulting in a dramatic decline in the C:N imbalance from 0.42 to 0.24 ($R^2 = 0.29$, $P < 0.01$, Fig. 2d). Sulfur addition significantly decreased the total PLFAs ($R^2 = 0.13$, $P = 0.03$, Fig. 3a) but increased the relative abundance of fungi ($R^2 = 0.43$, $P < 0.01$, Fig. 3c). However, the relative abundance of bacteria was the lowest at the highest level of S addition (Fig. 3b), causing a significant increase in the ratio of fungi to bacteria (F:B) with soil acidification. With respect to soil microbial stress indicators, S addition increased the ratios of saturated-to-monounsaturated PLFAs (sat:mono) and cyclopropyl FA-to-monoenoic precursor (cy:pre), with both ratios negatively relating to soil pH ($P < 0.01$, Fig. 3e, f and Fig. S2b).

Responses of ecoenzymatic stoichiometry and threshold element ratio to S addition

Soil BG and LAP activity decreased with S-induced acidification ($P < 0.01$, Fig. 4a, c). The NAG activity ($P = 0.19$, Fig. 4b) and BG: (NAG + LAP) ratio showed no response to S addition (Fig. 4d), while the BG:LAP ratio increased significantly with increasing S addition rate and decreasing soil pH (Fig. 4e). The significant correlation was observed between the BG:LAP ratio and the C:N imbalance (Fig. 4f). Specific NAG activity (per unit of MBC) increased with decreasing pH (Fig. S4). The type II regression showed that C- and N-acquiring enzyme activities were positively correlated ($R^2 = 0.65$, $P < 0.01$, Fig. S5). The TER increased with decreasing soil pH under S addition ($R^2 = 0.18$, $P < 0.01$, Fig. 5a).

Responses of microbial nutrient-use efficiency to S addition and linkages with the stoichiometric imbalance and community structure

Compared with the control plot, high rates of S addition significantly increased CUE (Fig. 5b) but decreased NUE (Fig. 5c). Significant correlations were observed between

Fig. 2 Effects of S addition on soil pH (**a**), ratios of DOC:TDN (**b**), MBC:MBN (**c**), and their stoichiometric imbalance (**d**). Bar indicates the mean value (± 1 standard error) for each treatment. Different lowercase letters above bars indicate significant differences among different S addition rates at the level of $P < 0.05$. The scatter subplot in each panel demonstrates the relationship between soil pH and the corresponding variables, the linear regression is fitted when $P < 0.05$ (with R^2 and P shown). The gray area represents 95% confidence interval

soil pH and CUE (negative, $R^2 = 0.24$, $P < 0.01$, Fig. 5b), and between soil pH and NUE (positive, $R^2 = 0.25$, $P < 0.01$, Fig. 5c).
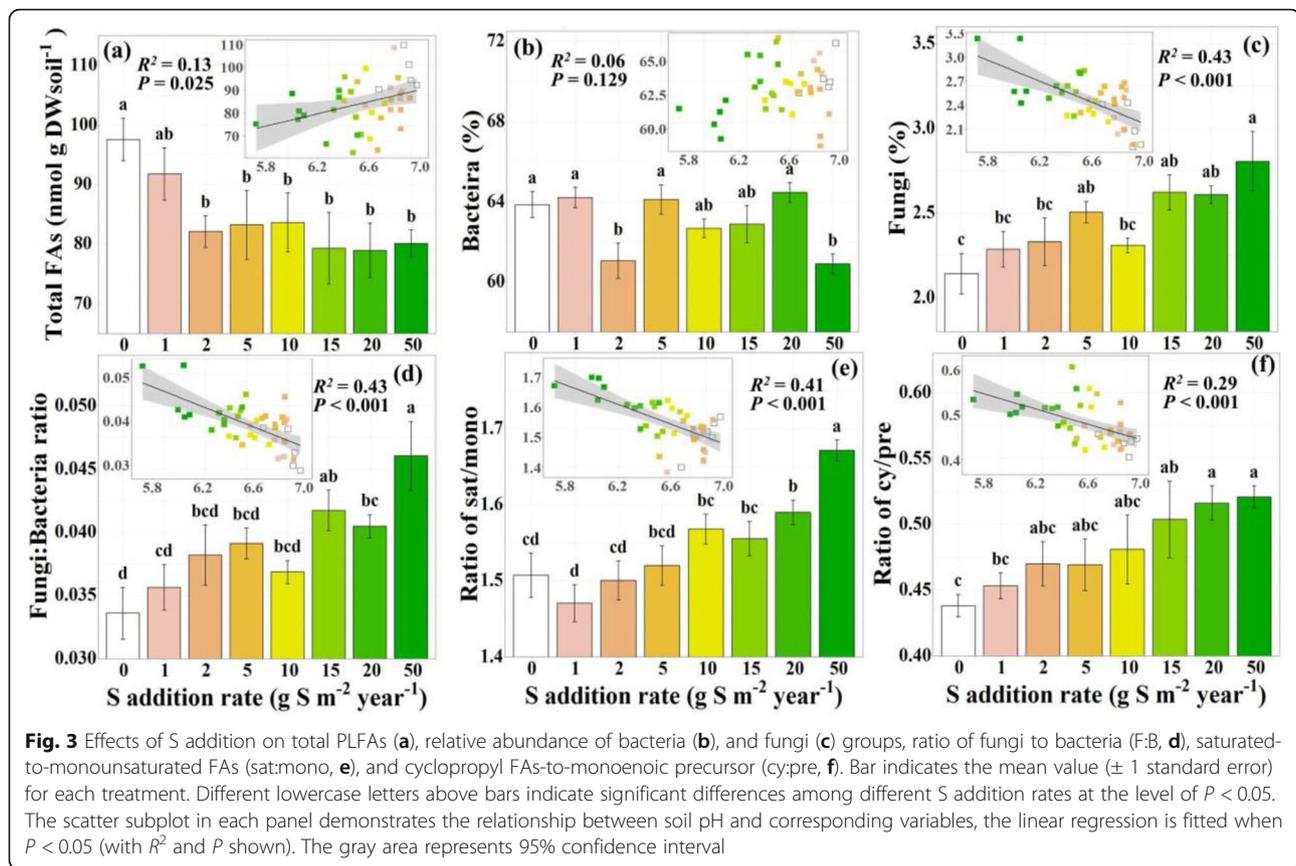
Our final SEMs fit the data well ($\chi^2_{CUE} = 30.54$, $P_{CUE} = 0.082$ and $\chi^2_{NUE} = 31.42$, $P_{NUE} = 0.088$, respectively) and explained 54% and 51% of the total variance in CUE and NUE, respectively (Fig. 6a, c). The piecewise SEM analyses showed that soil acidification (i.e., decreasing pH) under S addition increased CUE but decreased NUE by enhancing the F:B ratio and BG:LAP ratio and reducing the C:N imbalance (Fig. 6b, d). Additionally, soil acidification also

indirectly decreased NUE by increasing toxic metal concentrations and microbial stress (total standard effect size = − 0.16 and − 0.21, respectively, Fig. 6c).

Discussion

Sulfur-induced soil acidification enhanced microbial C relative to N limitation

Consistent with our first hypothesis, soil acidification alleviated N limitation but aggravated C limitation for the microbial community, as indicated by the increase in TER but the decrease in the C:N imbalance between microbial biomass and dissolved nutrients (Fig. 2d). The
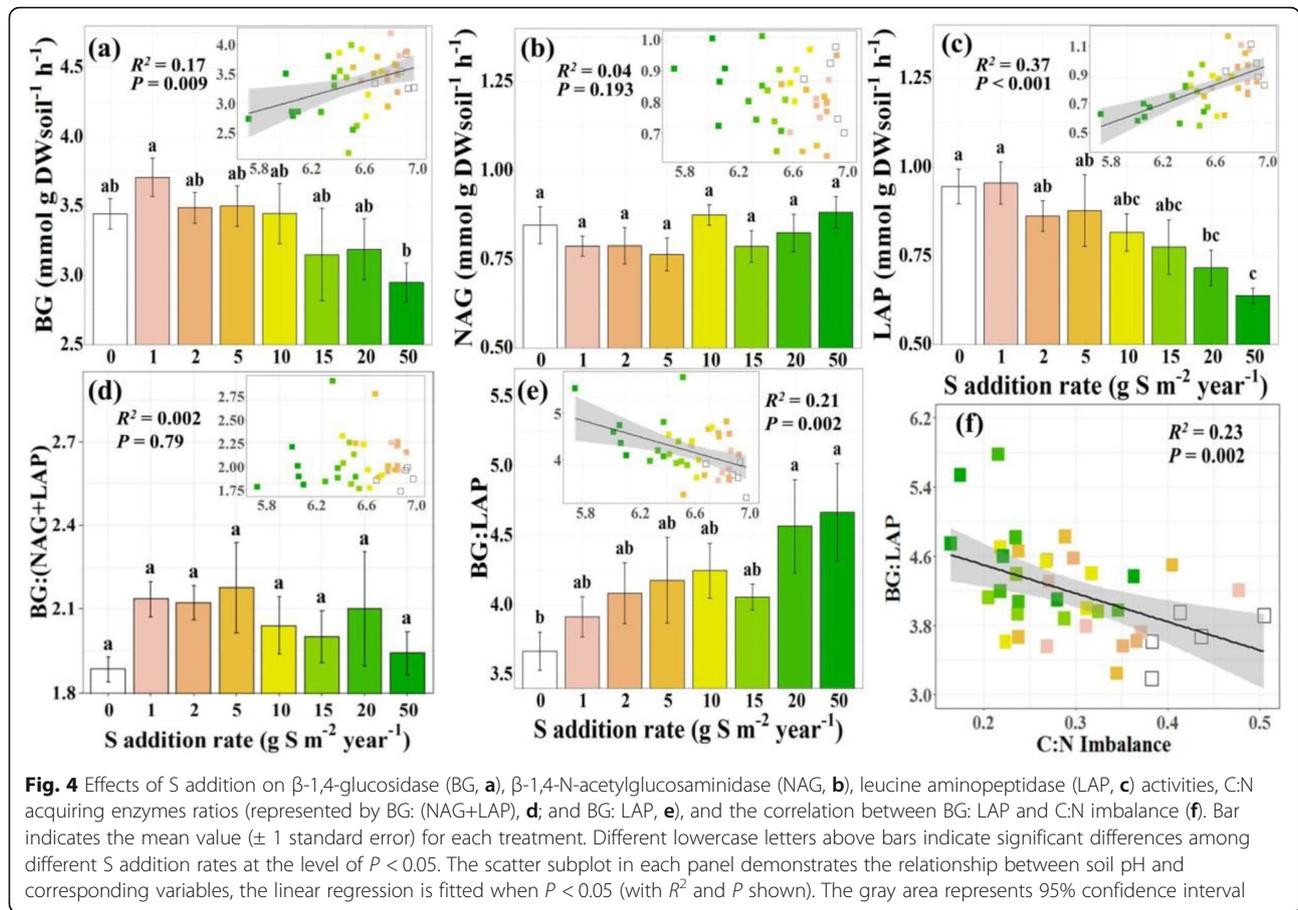
Fig. 3 Effects of S addition on total PLFAs (**a**), relative abundance of bacteria (**b**), and fungi (**c**) groups, ratio of fungi to bacteria (F:B, **d**), saturated-to-monounsaturated FAs (sat:mono, **e**), and cyclopropyl FAs-to-monoenoic precursor (cy:pre, **f**). Bar indicates the mean value (± 1 standard error) for each treatment. Different lowercase letters above bars indicate significant differences among different S addition rates at the level of $P < 0.05$. The scatter subplot in each panel demonstrates the relationship between soil pH and corresponding variables, the linear regression is fitted when $P < 0.05$ (with R^2 and P shown). The gray area represents 95% confidence interval

TER is a useful indicator for predicting microbial nutrient limitation compared with resource stoichiometry (Frost et al. 2006) and nitrogen limitation occurs when the C:N ratio is greater than the TER (Sinsabaugh et al. 2013). In this study, DOC:TDN was greater than the TER under high soil pH (i.e., higher than 6.6) but lower than the TER when the pH was lower than 6.6 (Fig. 2b and 5a), suggesting a conversion from N limitation to C limitation with soil acidification.

One crucial explanation for alleviating N limitation was the increasing N supply for the microbial community (Table S2) through inhibiting N leaching loss (Kemmitt et al. 2005; Van Den Berg et al. 2005) and plant N uptake (Vanguelova et al. 2007) under soil acidification. Additionally, increasing C vs. N limitation was potentially from the lower root exudation, one of the most critical microbial C resources, under soil acidification (Treseder 2008). However, the converse trends of DOC:TDN with SOC:TN in our study corroborated previous studies showing the differential responses of labile and total soil nutrient stoichiometry to changing soil pH (Guo et al. 2020; Yuan et al. 2019). Despite similar trends between the C:N imbalance calculated from DOC:TDN and SOC:TN, only dissolved nutrients contributed to the variation in stoichiometric imbalance

(Fig. 6 and Fig. S3c). These results suggested that dissolved nutrient stoichiometry could be a better indicator for nutrient limitation, also reported in regional-scale studies (Wild et al. 2015), field manipulation experiments (Guo et al. 2020; Yuan et al. 2019), and modeling studies (Kaiser et al. 2014).
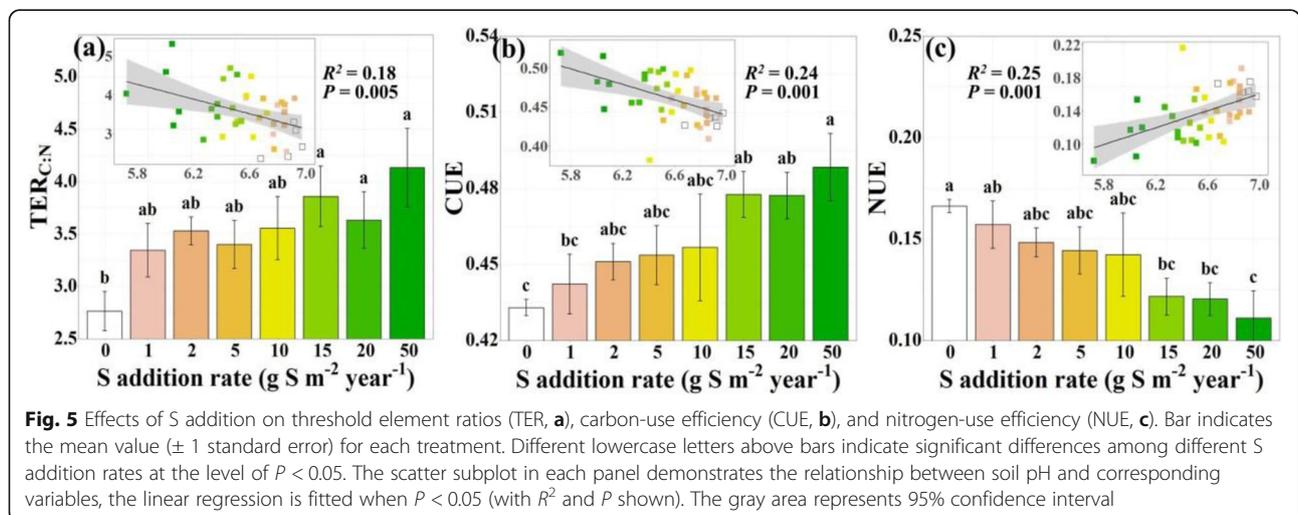
Another mechanism of increasing microbial C limitation was the relatively higher C requirement, as evidenced by the higher MBC:MBN ratio (Zechmeister-Boltenstern et al. 2015) under elemental S addition (Fig. 2), which was primarily attributed to the higher F:B ratio (Fig. 3). Bacteria have a relatively narrow pH range for growth and are vulnerable to soil acidification (Fig. S6a), whereas fungi tend to be more tolerant (Fig. S6b) (Rousk et al. 2010a; Rousk et al. 2009). Moreover, fungi have a higher biomass ratio of C:N than bacteria (Strickland and Rousk 2010), contributing to an increased C:N ratio and C requirement of the microbial community under soil acidification (Fig. 6a). Our results suggested that acidification-induced changes in soil microbial community structure played a considerable role in regulating biomass stoichiometry.
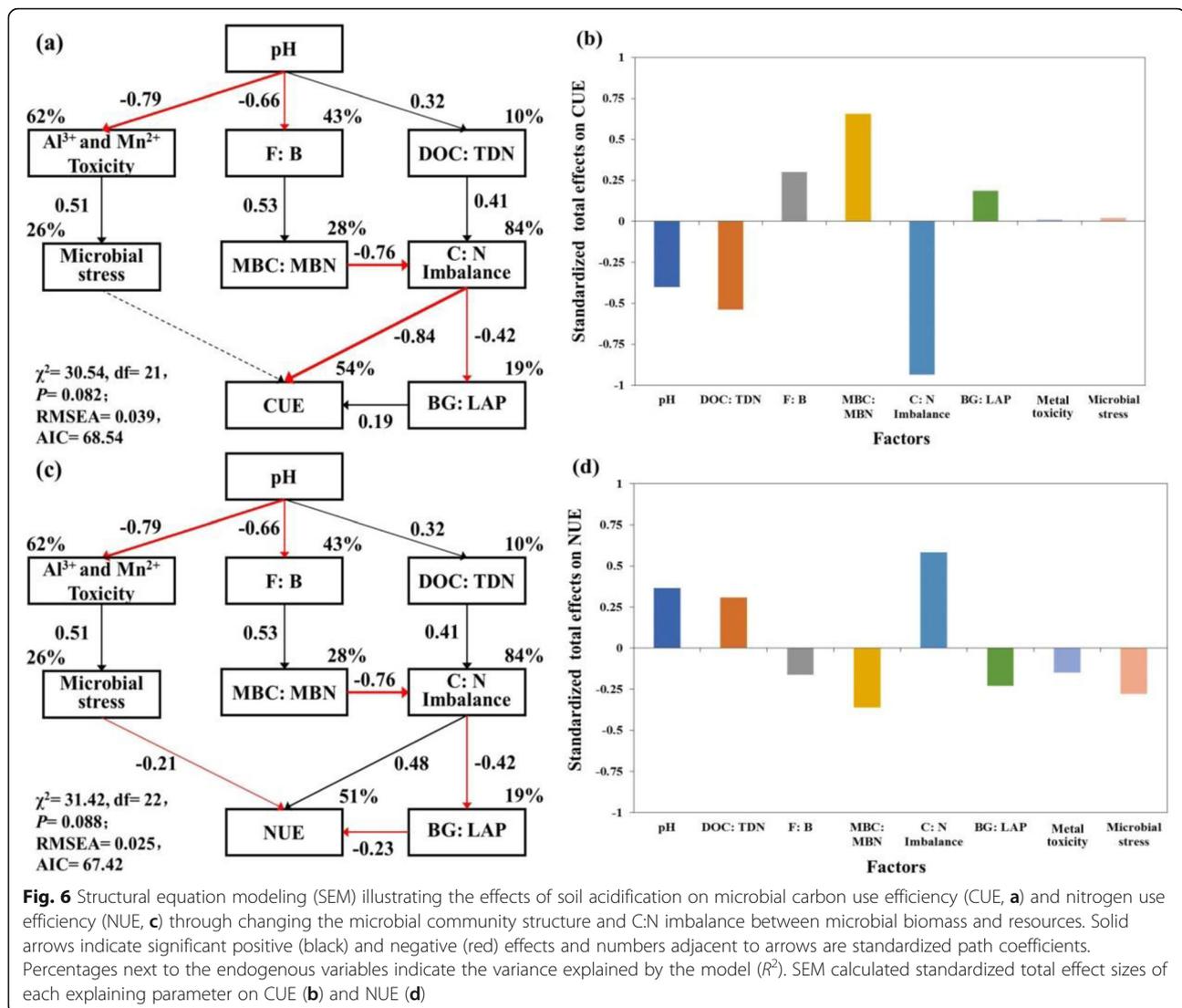
Decreasing N limitation and increasing C limitation were also found in N-addition experiments (Schleuss et al. 2019; Yuan et al. 2019), accompanied by soil acidification. This shift in C and N limitations with N

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Fig. 4 Effects of S addition on β-1,4-glucosidase (BG, **a**), β-1,4-N-acetylglucosaminidase (NAG, **b**), leucine aminopeptidase (LAP, **c**) activities, C:N acquiring enzymes ratios (represented by BG: (NAG+LAP), **d**; and BG: LAP, **e**), and the correlation between BG: LAP and C:N imbalance (**f**). Bar indicates the mean value (± 1 standard error) for each treatment. Different lowercase letters above bars indicate significant differences among different S addition rates at the level of $P < 0.05$. The scatter subplot in each panel demonstrates the relationship between soil pH and corresponding variables, the linear regression is fitted when $P < 0.05$ (with R^2 and P shown). The gray area represents 95% confidence interval

addition was attributed to changes in soil resource stoichiometry (i.e., higher N input with N addition rates) (Yuan et al. 2019). However, being different from N-addition studies, variations in stoichiometric imbalance (i.e., increasing C to N limitation here) were better explained by microbial biomass stoichiometry (i.e., nutrient demand, standard effect size = − 0.76) rather than resource stoichiometry (i.e., nutrient supply, standard effect size = 0.41) under S-induced soil acidification (Fig. 6). This calls for future N-addition studies to combine

Fig. 5 Effects of S addition on threshold element ratios (TER, **a**), carbon-use efficiency (CUE, **b**), and nitrogen-use efficiency (NUE, **c**). Bar indicates the mean value (± 1 standard error) for each treatment. Different lowercase letters above bars indicate significant differences among different S addition rates at the level of $P < 0.05$. The scatter subplot in each panel demonstrates the relationship between soil pH and corresponding variables, the linear regression is fitted when $P < 0.05$ (with R^2 and P shown). The gray area represents 95% confidence interval

Fig. 6 Structural equation modeling (SEM) illustrating the effects of soil acidification on microbial carbon use efficiency (CUE, **a**) and nitrogen use efficiency (NUE, **c**) through changing the microbial community structure and C:N imbalance between microbial biomass and resources. Solid arrows indicate significant positive (black) and negative (red) effects and numbers adjacent to arrows are standardized path coefficients. Percentages next to the endogenous variables indicate the variance explained by the model (R^2). SEM calculated standardized total effect sizes of each explaining parameter on CUE (**b**) and NUE (**d**)

acid and nutrient additions when evaluating the effects of soil acidification on microbial nutrient limitation (Averill and Waring 2018). Moreover, regarding the high turnover of dissolved nutrients and microbial biomass as affected by soil microclimate and plant growth (Bardgett et al., 2005), multiple samplings over the growing season are still needed.

Microbial community increased soil C- to N-acquiring enzyme ratio to cope with higher C limitation under soil acidification

Soil microorganisms secrete extracellular enzymes to acquire limited elements from organic matters (Waring et al. 2013). Although a substantial part of extracellular enzymes are stabilized in the soil matrix (Allison 2006) and lack association with active cells (Nannipieri et al. 2018), they can still reflect the catalytic history of a soil as continuously imprinted by soil microorganisms in

response to environmental changes (Dilly and Nannipieri 2001). Therefore, changes in enzymatic activities and stoichiometry are widely regarded as effective indicators of microbial nutrient status in long-term observations (Schleuss et al. 2019; Tapia-Torres et al. 2015; Yuan et al. 2019). Our results showed alteration of enzymatic activities to adapt to decreasing C:N imbalance (Fig. 4f), which partially supported our second hypothesis. Adjusting elemental acquisition through regulating enzyme activities is one of the most important strategies for microbial communities to maintain elemental balances and activities in terrestrial ecosystems (Sterner and Elser 2002; Waring et al. 2013), especially in nutrient-limited areas (Tapia-Torres et al. 2015). Here, we found that specific BG activity (per unit MBC) remained unchanged, while specific LAP decreased with soil acidification (Fig. S4), indicating that the microbial community tended to invest less energy in producing N-acquiring

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enzymes relative to C-acquiring enzymes to cope with increasing C to N limitation. However, specific NAG activity (per unit MBC) increased with soil acidification (Fig. S4), which could be attributed to several mechanisms: (i) NAG is also regarded as a C-acquiring enzyme when N is sufficient and usually increases with N addition in temperate grasslands (Schleuss et al. 2019; Wang et al. 2015) because NAG (i.e., chitinase) is responsive for hydrolyzing chitin (i.e., N-containing polysaccharide) (Sámi László et al. 2001). (ii) NAG production is expected to increase with higher fungal abundance (Fig. 3) concurrent with higher chitin production from fungal necromass (i.e., more substrate for NAG) under soil acidification. Similarly, experimentally manipulating microbial communities to decrease F:B ratio substantially reduces NAG activity even in the absence of changes in pH (Domeignoz-Horta et al. 2020). Therefore, we should be more cautious when treating NAG activity simply as an N-acquiring enzyme, especially in areas where chitin could be an essential C source (Mori 2020). Instead, we found that the BG:LAP ratio could better predict microbial C *vs.* N limitation, as suggested by the significant correlation between the BG:LAP ratio and the C:N imbalance (Fig. 4f). One possible reason is that bacteria depend more on proteins as a source of N (Hofmockel et al. 2010). The N-acquisition strategy of our bacteria-dominated community was largely correlated with LAP consequently. Our result was also supported by the resource allocation theory that microbial communities might increase enzyme production to mine the scarcest elements (Allison and Vitousek 2005). Moreover, here we used optimal pHs for enzymes to determine maximum potential activities (Tabatabai et al. 1994) and allow comparison with other studies (Nannipieri et al. 2018), but the pH optimum for some extracellular enzymes may shift under long-term pH manipulation due to changes in functional microbial communities (Puissant et al. 2019). Therefore, the role of changing pH in enzyme assay should be considered in further researches. Overall, our results showed that the microbial community could alter enzyme production to adapt to increasing C limitation induced by soil acidification.

Microbial community increased soil CUE but reduced NUE to cope with higher C limitation under soil acidification

Based on EST, microbial communities tend to regulate their element utilization strategies by adjusting the physiological process of element-use efficiencies to adapt to element-limiting environments (Mooshammer et al. 2014a; Sterner and Elser 2002). Consistent with our expectation, the C:N imbalance between microbial biomass and their resources was a key factor contributing to CUE and NUE variations under soil acidification (Figs. 6,

S7). The negative correlation between the C:N imbalance and CUE suggested that alleviated N limitation allowed the microbial community to allocate more C to building biomass rather than providing energy for N acquisition with soil acidification. This was in line with Spohn et al. (2016) who observed an increase in the CUE of microbial communities with increasing N supply for microbes. Analogously, the microbial community releases more ammonium (i.e., low NUE) when decomposing substrates with a high C:N ratio (Mooshammer et al. 2014b), suggesting that the changes in microbial N utilization largely depend on N in their resources (Keiblinger et al. 2010). Here, we also found a potential shift from net N immobilization to mineralization, as suggested by increasing TER with soil acidification, which was supported by Li et al. (2020), who found that N mineralization increased with decreasing soil pH. The increasing F:B ratio showed a positive effect on microbial CUE but negatively affected NUE (Fig. S7) because fungal communities usually have higher C requirements and CUE than bacterial communities (Riggs and Hobbie 2016). Additionally, both CUE and NUE showed significant correlations with BG:LAP, as we expected, supporting the principle of "return on investment" (Schimel and Weintraub 2003) that microbes invest more C in producing C-acquiring enzymes than N-acquiring enzymes (i.e., higher BG:LAP; Fig. 4e) to mineralize more C from substrates and increase the flow of C back to the microbes (i.e., higher CUE) to cope with increasing C limitation.

Our results clearly showed that microbes with high S addition rates were facing higher stresses, as suggested by PLFA stress indicators (i.e., ratios of sat:mono and cy:pre, Fig. 3d, e). Higher sat:mono and cy:pre ratios reflect lower fluidity of the cell membrane (Los and Murata 2004) and stationary growth phase of microbes (Bossio and Scow 1998), respectively, both of which were proven to be efficient in predicting acidity and metal stresses (Åkerblom et al. 2007; Garcia-Sanchez et al. 2015; Rousk et al. 2010b). Decreased microbial CUE and NUE might result from microbes overcoming metal stress through the C- and N-costly pathways of cation efflux pumps, reactive oxygen scavenging, secretion of detoxifying compounds and elevating genes for metal resistance (Auger et al. 2013; Malik et al. 2017; Silver et al. 1989). However, the 13% increase in CUE with soil acidification in our study suggested that the negative effect of microbial stress was offset by the pathway of stoichiometric controls on microbial CUE (Fig. 6a). The different responses of microbial CUE to acidification between our study and previous studies may largely be attributed to different ranges of soil pH. The pH range (from 6.9 to 5.9) in our study was much higher than the pH threshold (5.5) proposed by Jones et al. (2019) at which detrimental effects

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of exchangeable Al occurred. This suggests that the effect of changing pH on CUE can vary significantly depending on the initial soil pH and ecosystem type. Although our study proved a stronger regulation by stoichiometric adaption than metal toxicity in soil acidification, whether the negative effects of growing microbial stress under high atmospheric N and S deposition, especially when soil pH falls below 5.5, would exceed the capacity of microbial adaption to element limitation is still an open question. Consequently, more long-term observations are needed for a better understanding of microbial adaption to soil acidification.

Conclusions

Our study demonstrated that soil acidification resulted in conversion from microbial N limitation to C limitation, as suggested by a decrease in the C:N imbalance between microbial biomass and their resources in calcareous grassland soils. To cope with the increasing C *vs.* N limitation, the microbial community tended to alter enzyme production and increase CUE but decrease NUE under soil acidification. Our results revealed that changing the nutrient-use efficiencies of the microbial communities under soil acidification was not only a microbial adjustment to increasing N availability but, more importantly, an adaption to an altered community structure. Our results also highlighted the importance of stoichiometric controls on microbial elemental use efficiency relative to the detrimental effects of metal stress. However, long-term observation is needed because a continuous drop in pH may aggravate the adverse effects of metal stress on microbial nutrient acquisition and utilization processes. These findings may improve the understanding of soil microbe-driven nutrient cycling and help better simulation and projection of future dynamics of terrestrial biosphere under climate change.

Abbreviations
DOC: Dissolved organic carbon; TDN: Total dissolved nitrogen; MBC: Microbial biomass carbon; MBN: Microbial biomass nitrogen; BG: β-glucosidase; NAG: β-N-acetyl-glucosaminidase; LAP: Leucine aminopeptidase; TER: Threshold element ratio; CUE: Carbon-use efficiency; NUE: Nitrogen-use efficiency; F:B: The ratio of fungi to bacteria; sat:mono: The ratio of saturated-to-monounsaturated PLFAs; cy:pre: The ratio of cyclopropyl FA-to-monoenoic precursor; SMA: Standardized principal axis; PCA: Principal component analysis; SEM: Structural equation model

Supplementary Information
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Additional file 1 Appendix 1- Additional methods (Detailed priori hypothetical pathways and a *priori* structural equation model); **Appendix 2**- Additional results (Soil biochemical characteristics, SMA regression for enzymes and correlation between variables and element-use efficiency);

Appendix 3- Reliability test for estimated CUE (Soil respiration and its relationship with estimated CUE).

Authors' contributions
Y.J. designed the study. T.L. and H.L. set up the field experiment and applied fertilizer every year. T.L. and Z.W. performed soil sampling and completed laboratory analyses. T.L. and Y.M. performed statistical analyses and graphs. R.W., J.C. and R.F.T. contributed to the interpretation and discussion of the results. T.L. prepared the manuscript with suggestions from all the co-authors. All authors read and approved the final manuscript.

Availability of data and materials
The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors have no conflicts of interest to declare that are relevant to the content of this article.

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