Soil aggregate development and associated microbial metabolic limitations alter grassland carbon storage following livestock removal

Wenliang Ju [a, b, c], Daryl L. Moorhead [d], Guoting Shen [e], Yongxing Cui [f], Linchuan Fang [a, b, g, *]

[a] *School of Resources and Environmental Engineering, Wuhan University of Technology, Wuhan 430070, China*

[b] *Institute of Soil and Water Conservation, Chinese Academy of Sciences, Ministry of Water Resources, Yangling 712100, China*

[c] *School of Environment, Tsinghua University, Beijing 100084, China*

[d] *Department of Environmental Sciences, University of Toledo, 2801 W. Bancroft St, Toledo, OH 43606-3390, USA*

[e] *Department of Soil Ecology, Helmholtz Centre for Environmental Research – UFZ, Theodor-Lieser-Straße 4 06120 Halle (Saale), Germany*

[f] *Sino-French Institute for Earth System Science, College of Urban and Environmental Sciences, Peking University, Beijing, 100871, China*

[g] *CAS Center for Excellence in Quaternary Science and Global Change, Chinese Academy of Sciences, Xi'an 710061, China*

*** Corresponding author**

Linchuan Fang (Email: flinc629@hotmail.com)

School of Resources and Environmental Engineering, Wuhan University of Technology, 122 Luoshi Road, Wuhan, Hubei, 430070, China

Abstract

Natural restoration of degraded grasslands following livestock removal changed soil nutrient and carbon concentrations, soil aggregate distributions, and patterns of extracellular enzyme activities. Here, we used an ecoenzymatic stoichiometry model to quantify microbial resource limitations in semiarid grassland soil aggregates at 0, 11, 26, and 36 years after livestock removal and linked these limitations to microbial carbon use efficiency (CUE), which was also estimated from stoichiometry theory (CUE_{ST}). Overall, livestock removal altered the size distribution of soil aggregates, increased microbial resources and stimulated extracellular enzyme activities. Longer livestock removal increased the proportion of small aggregates. Microbial carbon (C) and phosphorus (P) limitations in soil aggregates declined to minimum levels at 26 years following livestock removal and then increased after 36 years, with an opposite trend for CUE_{ST}. Additionally, the greatest resource limitations to microorganisms were found in smaller aggregates under long-term livestock removal. Random forest and structural equation models revealed that soil abiotic factors, especially total nitrogen and pH, were key determinants of microbial resource limitation in soil aggregates. Moreover, microbial C and P limitations had significant direct effects on the estimated microbial CUE_{ST}. Thus, increasing microbial metabolic limitations after long-term livestock removal could reduce microbial C turnover, potentially reducing soil C sequestration. Overall, this study revealed that livestock removal altered soil aggregate development as well as the allocation of resources in aggregates and consequent microbial resource limitations, ultimately providing information that may be useful for developing grassland management strategies to enhance C sequestration.

Keywords: Ecoenzymatic stoichiometry; Livestock removal; Microbial carbon turnover; Resource limitation; Semiarid grasslands; Soil aggregates

1. Introduction

Grassland soils in semiarid regions store large amounts of carbon that can help mitigate climate change, but overgrazing by livestock has led to extensive degradation (Wiesmeier et al., 2015; Sattari et al., 2016; Prommer et al., 2020). Livestock removal (LR), as a "nature-based" eco-engineering measure, has been used globally to restore degraded grassland ecosystems (Wang et al., 2020; Liu et al., 2021a; Oliveira et al., 2021). This approach can improve vegetation diversity, which in turn stimulates microbial growth and turnover rates and increases soil organic carbon (SOC) stocks (Prommer et al., 2020). However, long-term livestock removal can reduce vegetation diversity and richness (Jing et al., 2014; Zhang et al., 2018) and inputs of organic matter to the soil (Wilson et al., 2018). A recent study reported that the total and particulate organic carbon contents of grasslands in Inner Mongolia, China, peaked 21 years after livestock removal but declined over 34 years (Dong et al., 2021). These patterns are consistent with the intermediate disturbance hypothesis as applied to grazing disturbance in grasslands (Fensham et al., 2011; Gao and Carmel, 2020). Moreover, it is likely that soil microbial metabolic limitations develop when declining inputs disrupt the balance between resource supply and microbial demand (Sinsabaugh and Shah, 2012; Mooshammer et al., 2014b; Roy and Bagchi, 2021). However, it is unclear whether and/or how the metabolism of soil microorganisms in semiarid grasslands is affected by long-term livestock removal.

Restoration also alters the physical structure of soils, especially soil aggregation, which is a key factor affecting soil microbial communities (Bach and Hofmockel, 2014; Wilpiszeski et al., 2019). Environmental conditions such as substrate availability and moisture in the pore spaces within soil aggregates determine the potential for biogeochemical reactions mediated by microorganisms (Schutter and Dick, 2002;

Wilpiszeski et al., 2019). A recent study found that enzyme activity, microbial carbon use efficiency (CUE) and respiration in soil macroaggregates are more sensitive to warming than in microaggregates (Liu et al., 2021b). Additionally, nitrogen and water inputs can redistribute microbial activity across different-sized soil aggregates in arid and semiarid grasslands, which in turn affects SOC stability and microbial nutrient limitation (Wang et al., 2015). Past studies have focused on the effects of livestock grazing and removal on soil aggregate size distribution, microbial diversity and activity (Prieto et al., 2011; Olivera et al., 2014; Goenster-Jordan et al., 2021; Liu et al., 2021a; Roy and Bagchi, 2021), but to our knowledge none have linked soil microbial metabolism to aggregate-scale microhabitats. Understanding the status of microbial metabolism in various soil aggregates is needed to summarize microscale biogeochemical reactions.

Microorganisms are key players in soil biogeochemical cycles, degrading organic matter and regulating soil nutrient cycling and carbon (C) balance (Bahram et al., 2018). These processes are partly driven by the activities of extracellular enzymes (Nannipieri et al., 2002; Sinsabaugh et al., 2009), and both soil microbial metabolism and extracellular enzyme activities (EEAs) are controlled by resource (e.g., C and nutrients) limitations (Sinsabaugh and Shah, 2012; Camenzind et al., 2018). Over the past two decades, an increasing number of global or regional studies have quantified the level of soil microbial metabolic limitations using the ecoenzymatic stoichiometry theory (EEST) (Sinsabaugh et al., 2009; Moorhead et al., 2016; DeForest and Moorhead, 2020; Wang et al., 2020; Feyissa et al., 2022). However, whether EEST can reveal the prevalence and consequences of microbial resource limitation within and between size categories of grassland soil aggregates has not been elucidated.

The EEST represents the theoretical intersection of ecological stoichiometry and

metabolism, predicting the metabolic limitations of microorganisms in soil ecosystems from patterns of extracellular enzyme activities (Sinsabaugh and Shah, 2012; Cui et al., 2021). This assumes that the soil community derives its resource requirements from the catalysis of polymeric substrates so that relative resource limitations can be quantified by the relative activities of EEAs associated with C, nitrogen (N) and phosphorus (P) acquisition (Sinsabaugh et al., 2009). The EEST also suggests that soil microbial communities maintain relative stoichiometric homeostasis, and when the stoichiometry of resource supply does not match the stoichiometry of microbial demand (i.e., the stoichiometric imbalance theory or SIT), microbial activity will be limited by specific nutrients (Sterner and Elser, 2002). The SIT suggests that microbial decomposers not only regulate enzyme production and stoichiometry but can also change biomass stoichiometry and element use efficiencies to maximize growth and maintain community homeostasis (Mooshammer et al., 2014b; Yuan et al., 2019). Moreover, microbial resource limitations are directly relevant to the fate of soil C (Camenzind et al., 2018).

Microorganisms can either use organic C as an energy source or convert it into biomass and enzymes, with carbon use efficiency (CUE) estimated as the proportion of total C uptake allocated to production (Sinsabaugh et al., 2013). Thus, CUE is a key parameter to assess microbial contributions to soil C turnover, with higher CUE possibly promoting C accumulation and stabilization in soils (Manzoni et al., 2012; Geyer et al., 2019). Sinsabaugh and Shah (2012) developed a stoichiometric model linking EEAs, microbial biomass and environmental resources with microbial CUE_{ST} that can serve as a model for microbial C turnover (Sinsabaugh et al., 2016; Gai et al., 2021; Feyissa et al., 2022; Schimel et al., 2022). Therefore, it is both possible and likely useful to identify how changing microbial resource limitations in developing soil

aggregates with long-term livestock removal affect CUE and soil C balance.

This study collected soils from 0-36 years after livestock removal from semiarid grasslands on the Loess Plateau of China to decipher microbial metabolic limitations in developing soil aggregates. We investigated possible microbial resource limitations in different sized aggregates using an ecoenzymatic vector model (Moorhead et al., 2016) and microbial CUE estimated from stoichiometry theory (CUE_{ST}) (Sinsabaugh et al., 2016; Schimel et al., 2022). Here, we propose three hypotheses: (i) Short-term livestock removal will generally increase microbial metabolism in soil aggregates as plant C inputs to soils increase with reduced grazing and soil aggregates develop in the absence of trampling. (ii) Long-term livestock removal will increase microbial resource limitation due to declining resource inputs and shifting allocations among different size classes of developing soil aggregates. (iii) An overall negative feedback on microbial CUE will result from increasing resource limitations and subsequently reduce C sequestration over the long term.

2. Materials and methods

2.1. Study area, site design, and sampling

The study area was selected in a semiarid steppe on the Loess Plateau located in the Yunwushan National Natural Grassland Protection Zone (106°21′–106°27′ E, 36°10′–36°17′ N) in Guyuan city, Ningxia Hui Autonomous Region, China (Jing et al., 2014; Liu et al., 2021a). This natural area has been protected since 1982 to monitor the long-term recovery of degraded grasslands. The area covers 6700 ha, and its elevation ranges between 1800 and 2100 m. This area has a typical continental monsoon climate, with an average annual precipitation of 425 mm and an average annual temperature of 7 °C. Over 60% of precipitation occurs in July-September, and the highest and lowest temperatures occur in July (25 °C) and January (-14 °C), respectively. The soil is

147 classified as a Haplic Calcisol in the FAO/UNESCO soil classification system.

148 In early September 2018, we studied four grassland sites along a chronosequence
149 of livestock removal times. Livestock was removed (stocking rate is 0) at three sites in
150 1982, 1992, and 2007 to allow natural restoration, corresponding to removal durations
151 of 36 years (LR36), 26 years (LR26), and 11 years (LR11), respectively. Meanwhile,
152 continued moderate grazing (8 sheep ha^{-1} y^{-1}) at the remaining site since 1982 was
153 considered as a reference (LR0). To minimize the effect of site conditions on the
154 experimental results, all sites were selected with similar soil types, elevations, slopes,
155 and management histories (Table S1). Three sampling plots (20 m × 10 m) were
156 randomly established at each LR site (> 200 ha), and separated by > 50 m. After
157 removing the litter layer, five soil cores were collected from the top 10 cm of the soil
158 profile in each plot along a sigmoidal transect using sterile aluminium containers (10
159 cm high and 15 cm diameter) to preserve the original physical and biochemical
160 properties of the samples. After removing litter, roots, stones, and debris, the bulk soil
161 samples at each site were passed through an ethanol-sterilized 8 mm sieve. All sieved
162 samples were mixed into a composite sample for each plot and stored at 4 °C for further
163 analysis.

164 *2.2. Soil aggregates and physiochemical analysis*

165 Pretreated soil samples were dried in a sterile environment at 4 °C and stored for
166 one week to achieve ~20% moisture before aggregates were isolated. This facilitated
167 the dry-sieving process and maintained the typical moisture status of the soils in the
168 area studied. Three aggregate size classes were isolated through a modified "dry sieving
169 of optimal moisture-fresh soil" method (Schutter and Dick, 2002; Bach and Hofmockel,
170 2014) as follows: large macroaggregates (> 2 mm; lma), small macroaggregates (0.25-2
171 mm; sma), and microaggregates (< 0.25 mm; mia). This procedure likely minimizes the

disturbance of drying and wetting to the soil microbial community and extracellular enzyme activity (Bach and Hofmockel, 2014). In brief, approximately 300 g of fresh soil at ~20% moisture was manually separated into three aggregate sizes by dry sieving on a series of 2 sterile sieves (2 mm and 0.25 mm). The resulting soil aggregates were divided into two subsamples of each size class. One subsample was stored at 4 °C for subsequent analysis of extracellular enzyme activity and microbial biomass. The other subsample was air-dried for physicochemical analysis. Soil pH was measured using a pH metre (Model 225, Denver Instrument, USA) in a 1:2.5 soil:water (w/v) mixture. The soil organic carbon (SOC) content was measured by the dichromate oxidation method. The total nitrogen (TN) content of the soil was measured using an automatic Kjeldahl analyser (Kjeltec 2300, FOSS Corporation, Denmark). The total phosphorus (TP) content was measured by the molybdenum blue method using an ultraviolet spectrophotometer (UV3200, Shimadzu Corporation, Japan).

2.3. Analysis of soil EEAs and microbial biomass

We quantified extracellular enzyme activities (EEAs) in each size class of soil aggregates using a fluorimetric microplate enzyme assay (Marx et al., 2001; Saiya-Cork et al., 2002), including C-acquiring enzymes (β-1,4-glucosidase (BG; EC 3.2.1.21) and β-D-cellobiosidase (CBH; EC 3.2.1.91)), N-acquiring enzymes (β-1,4-N-acetylglucosaminidase (NAG; EC 3.1.6.1) and L-leucine aminopeptidase (LAP; 3.4.11.1)), and P-acquiring enzymes (alkaline phosphatase (AP; EC 3.1.3.1)). These EEAs were measured fluorescently using substrate solutions labelled with highly fluorescent compounds, 4-methylumbelliferone (MUB) or 7-amino-4-methylcoumarin (AMC). Specifically, sample suspensions were prepared by adding 1 g of fresh soil to 125 ml of 50 mM acetate buffer. The resulting soil suspension was continuously stirred as 200 µl aliquots were dispensed into 96-well microplates that served as the sample

assay, blank, and quench standard. Twelve replicates were used for sample assay wells, and six replicates were used for blank and quench standard wells. In addition, 50 µl of 200 mM substrate solution was added to sample wells, 50 µl of acetate buffer was added to blank wells, and 50 µl of standard (10 mM MUB or AMC) was added to quench standard wells. Negative control wells received 200 µl of acetate buffer and 50 µl of substrate solution. Reference standard wells received 200 µl of acetate buffer and 50 µl of standard. There were four replicate wells for each negative control and reference standard. The microplates were incubated in the dark at 25 °C for up to 4 h, and the reaction was then stopped by adding 10 µl of 1.0 M NaOH to each well. Fluorescence was measured using a microplate reader with 365 nm excitation and 450 nm emission filters (M200Pro, Tecan Infinite, Switzerland).

The microbial biomass C (MBC), N (MBN), and P (MBP) contents in each size class of soil aggregates were determined using the chloroform fumigation–extraction method (Brookes et al., 1982; Vance et al., 1987). Briefly, 30 g of fresh soil was exposed to ethanol-free $CHCl_3$ at 25 °C for 24 h in a dark environment. For the MBC and MBN analyses, the sample was extracted using 100 ml of 0.5 M K_2SO_4 after removal of the fumigant. A nonfumigated control was extracted using the same method. The C and N contents of the extracts were measured using a TOC/TN analyser (Multi N/C 3100, Analytik Jena, Germany). For the MBP analyses, the sample was extracted using 100 ml of 0.5 M $NaHCO_3$ after removal of the fumigant. Unfumigated controls were extracted using the same method. The P content of the extracts spiked with molybdenum–antimony reagent was measured at 710 nm using an ultraviolet spectrophotometer. The conversion coefficients for MBC, MBN, and MBP used in this study were 0.45, 0.54, and 0.40, respectively (Joergensen and Mueller, 1996).

2.4. Calculating microbial stoichiometric imbalance, homeostasis, resource limitation,

and CUE

The ratio of soil resource stoichiometry to microbial resource stoichiometry was used to assess the relative stoichiometric element imbalance in the resource supply and demand of microbial communities (Mooshammer et al., 2014b). This imbalance (C:N, C:P, or N:P) was calculated as the ratio of $SOC:TN_{resource}$ (or SOC:TP or TN:TP) to $MBC:MBN_{microbes}$ (or MBC:MBP or MBN:MBP). Ecological stoichiometry theory suggests that the C:N:P ratio in microbial biomass is tightly constrained despite wide variation in C:N:P ratios in soils due to the stoichiometric homeostasis of microbial communities (Sterner and Elser, 2002). Accordingly, Equation (1) was used to calculate the degree of microbial element stoichiometric homeostasis.

$$H' = \mathrm{Ln}\,(x)/\mathrm{Ln}\,(y) \quad (1)$$

where x is the microbial resource stoichiometry (MBC:MBN, MBC:MBP or MBN:MBP) and y is the soil resource stoichiometry (SOC:TN, SOC:TP or TN:TP). An $H' \gg 1$ indicates strong stoichiometric homeostasis, whereas $H' \approx 1$ indicates no or very weak stoichiometric homeostasis.

Vector analysis (vector length and vector angle) of soil EEAs was used to quantify microbial resource limitations (Moorhead et al., 2016). Vector length (Equation (2)) represents microbial C limitation, quantifying relative C versus nutrient acquisition; vector angle (Equation (3)) quantifies relative P versus N acquisition.

$$\text{Vector length} = \mathrm{SQRT}(x^2+y^2) \quad (2)$$

$$\text{Vector angle } (°) = \mathrm{DEGREES}(\mathrm{ATAN2}(y,\ x)) \quad (3)$$

where x represents [Ln (BG)/Ln (NAG+LAP)] for the relative enzyme activities of C versus N acquisition and y represents [Ln (BG)/Ln (AP)] for the relative enzyme activities of C versus P acquisition. Vector angles > 45° suggest a relative microbial P limitation, whereas angles < 45° represent relative microbial N limitation. The strength

of microbial P limitation increases with an increasing vector angle, whereas microbial N limitation increases with a decreasing angle. Microbial C limitation increases with vector length.

Microbial CUE derived from the model of stoichiometry theory (CUE_{ST}) was calculated using Equations (4)-(6) (Sinsabaugh and Shah, 2012; Sinsabaugh et al., 2016; Schimel et al., 2022). CUE_{ST} is a fundamentally different way of viewing resource use efficiency than classical CUE estimates. Rather, it assesses how microbes shift C use among polymeric sources in response to substrate stoichiometry.

$$CUE_{ST} = CUE_{max} \times ((S_{C:N} \times S_{C:P})/[(K_{C:N} + S_{C:N}) \times (K_{C:P} + S_{C:P})])^{0.5} \quad (4)$$

$$S_{C:N} = B_{C:N}/L_{C:N} \times 1/EEA_{C:N} \quad (5)$$

$$S_{C:P} = B_{C:P}/L_{C:P} \times 1/EEA_{C:P} \quad (6)$$

where $K_{C:N}$ and $K_{C:P}$ are half-saturation constants for CUE_{ST} based on the stoichiometry of substrate C:N and C:P availabilities. We assumed that $K_{C:N}$ and $K_{C:P}$ were 0.5 for the model in this study and that CUE_{max} was 0.6 (Sinsabaugh et al., 2016). $B_{C:X}$ is the elemental C:N or C:P ratio of microbial biomass. $L_{C:X}$ is the elemental composition of the available substrate, and $EEA_{C:X}$ is the ratio of enzymatic activities directed towards acquiring C and nutrients (X) from the environment. $L_{C:N}$ and $L_{C:P}$ were estimated as SOC:TN and SOC:TP, respectively. $EEA_{C:N}$ was calculated as BG/(NAG+LAP), and $EEA_{C:P}$ was calculated as BG/AP.

2.5. Statistical analyses

All the following statistical analyses were performed in R software v.4.1.3. Prior to statistical analysis, all data were first tested for normality and homogeneity of variance using the "car" package. One-way analysis of variance (ANOVA) with Tukey's HSD test ($P < 0.05$) was used to determine the effects of livestock removal

duration and soil aggregate size on different variables (i.e., soil physicochemical properties, enzyme activities, and microbial biomass and resource metabolism). Linear regression was used to determine microbial stoichiometric homeostasis, the relationship between vector length and vector angle, and the relationship of CUE_{ST} with microbial resource limitation. Scatter and box plots were constructed using the "ggplot2" package, and linear regression plots were constructed using the "ggplot2" and "ggpmisc" packages. A correlation heatmap was generated to examine the Pearson correlation between different variables using the "ggcorrplot" package. We conducted a principal components analysis (PCA) for all variables using the "FactoMineR" package.

The main predictors (i.e., interference, abiotic/biotic factors, and metabolic limitation) for vector length, vector angle, and CUE_{ST} were identified by a classification random forest (RF) analysis using the "randomForest" package (Breiman, 2001). We estimated the importance of these predictors using the percentage increase in the MSE (mean squared error) of variables. The significance of RF models and predictors was tested using the "rfPermute" and "rfUtilities" packages, respectively. Variation partitioning analysis (VPA) in the "vegan" package was also used to determine the contribution of the main predictors to the variation in microbial resource limitation and CUE_{ST}. Furthermore, we used a structural equation model (SEM) to evaluate the strength of the direct and indirect relationships between selected variables and microbial resource limitations and CUE_{ST}. The selected variables were significant predictors identified by RF analysis. SEM was performed and fitted using the "lavaan" package, and the model paths were adjusted according to the "modificationIndices" function (Rosseel, 2012). A maximum likelihood estimation method was adopted for SEM. The best-fit model was assessed based on the chi-square test (x^2), degrees of freedom (*df*), *P value*, goodness-of-fit index (GFI), standardized root mean square

residual (SRMR), and root mean square error of approximation (RMSEA).

3. Results

3.1. Soil chemistry, EEAs, and microbial biomass

Overall, the analyses showed that the duration of LR had significant effects on soil nutrients and EEAs and their stoichiometry, whereas aggregate size affected moisture, TP and microbial P ($P < 0.05$, Table S2). The estimated bulk soil moisture, SOC, TN, and TP contents decreased slightly from LR0 to LR11, peaked at LR26 and then decreased slightly at LR36 (Table S3). The same pattern existed for MBN and MBP, whereas MBC was constant for LR0 and LR11. In general, individual enzyme activities increased from LR0 to LR26 and then declined to their lowest values at LR36. The total activity in bulk soils in which all enzymes were combined also increased from LR0 to LR26 and then declined to the lowest level at LR36. This pattern was also similar for the C-acquiring (BG+CBH), N-acquiring (NAG+LAP), and P-acquiring (AP) groups of enzymes.

The proportional allocations of the three soil aggregate sizes were approximately equal under current livestock grazing (Table S1). Livestock removal increased the maximum proportion of lma to 52% at LR11, which fell to 18-19% at LR26 and LR36. The proportion of sma fell to a low of 26% at LR11 but then increased to 43% and 62% at LR26 and LR36, respectively. The maximum for mia was 39% of the total aggregates at LR26 and then fell to 19% at LR36. Concentrations of soil C, N, and P and microbial C, N and P showed similar patterns of distribution between soil aggregate size classes over time after livestock removal (Table S4). The largest proportions of C, N and P were associated with mia aggregates at LR0, representing 36-46% of the totals. In contrast, the lma aggregates contained 49-71% of the soil and microbial nutrients at LR11. Allocations were comparable between the sma and mia classes at LR26, ranging

from 39-45% of the totals. Finally, the sma aggregates contained 62-70% of all nutrients at LR36.

The activities of C- and N-acquiring enzymes in soil aggregates were similar to bulk soils, generally peaking at LR26 and then decreasing at LR36 (Table 1). The activity of AP in lma and mia increased with the duration of LR, but the lowest AP was in sma of LR36. The patterns of relative enzyme activity among aggregate size classes were similar to those of soil and microbial C distributions. The greatest fraction of C-, N-, and P-acquiring and total enzyme activity was associated with lma aggregates at LR11 (48-58%). However, enzyme activities at LR26 were greatest for the sma fraction (48-52%) and much lower for the lma and mia classes. Additionally, in contrast to microbial and resource distributions, enzyme activities at LR36 were variable; total C-acquiring enzyme activities (BG+CBH) were highest for sma aggregates, although CBH was highest for the mia class. The activities of NAG were highest for sma, whereas LAP was highest for mia aggregates. Finally, AP was lowest for sma, and total enzyme activity was similar for all aggregate sizes.

3.2. Microbial stoichiometry, homeostasis and imbalances

Although microbial biomass in soil varied in bulk soil and between soil aggregate size classes, livestock removal did not have a significant effect on the stoichiometry of microbial biomass. Additionally, stoichiometric imbalances between resources and soil microbial communities were variable but did not show significant effects of LR duration or aggregate size on C:N or C:P (Fig. S1). Imbalances in the C:N ratio between soil resources and microbial biomass averaged approximately 10.2, indicating a much higher C vs. N content in soil than biomass. In contrast, the ratio between C:P elements was very close to 1, indicating a balanced relationship between soil and biomass C vs. P concentrations. The N:P imbalance was the only one affected by LR, averaging

346 approximately 0.09 overall but achieving a significantly higher peak value of
347 approximately 0.12 at LR26; thus, the relative P vs. N content of biomass was much
348 larger than that in soil (Table 1). Finally, linear regression analyses (*H'*) between Ln
349 (microbial stoichiometry) and Ln (soil resource stoichiometry) revealed no significant
350 relationships (Fig. S2).

351 *3.3. Microbial resource limitations and CUE*

352 Despite having no significant effects on the stoichiometry of microbial biomass,
353 both the duration of LR and aggregate size influenced enzyme stoichiometry and
354 estimates of microbial resource limitations, albeit not consistently (Fig. 1). The ratios
355 of Ln(BG):Ln(NAG+LAP), Ln(BG):Ln(AP) and Ln(NAG+LAP):Ln(AP) generally
356 decreased to LR26, suggesting declining C and N limitations. However,
357 Ln(BG):Ln(NAG+LAP) and Ln(BG):Ln(AP) achieved their maximum values for sma
358 at LR36. Also, microbial C limitation was generally lower at LR11 and LR26 than at
359 LR0 but showed little difference between aggregate sizes within sites, the exception
360 being that microbial C limitation was significantly higher in sma at LR36. The vector
361 angles for all sites and aggregates were > 50°, suggesting an overall P limit to microbial
362 metabolism. However, the strength of P limitation varied with both LR and aggregate
363 size, with the lowest values at LR0 and LR26, the highest values at LR36, and the
364 overall higher values in smaller aggregates at all sites except LR26. Thus, soil
365 development increased microbial C and P limitation in small aggregates over time after
366 livestock removal. Moreover, linear regression analysis showed a significant positive
367 relationship between vector angle and vector length when pooling all LRs and
368 aggregates, suggesting that microbial P limitation increased with C limitation (Fig. 1D).
369 However, this general relationship was not consistent between aggregate sizes, being
370 positive for sma and mia but negative for lma.

Estimates of stoichiometric CUE_{ST} were relatively low, generally below 0.2 for all aggregates at all sites (Fig. 2A). However, the duration of LR had a significant effect on the stoichiometric CUE_{ST}, reaching an overall peak at LR26 (0.17 ± 0.02) and then declining to LR36. The differences in CUE_{ST} with different aggregate sizes were inconsistent between sites, although it was interesting that the lowest overall estimate of CUE_{ST} corresponded to the highest estimate of C limitation in small aggregates at LR36. In contrast, the highest estimate of CUE_{ST} was in the lma aggregates at LR11, which did not correspond to any particular environmental or enzyme characteristic, although microbial biomass C was highest. Linear regression analysis showed a significant negative correlation between microbial CUE_{ST} and vector length ($P < 0.001$, Fig. 2B) as well as vector angle ($P < 0.01$, Fig. 2C). These general relationships between CUE_{ST} and resource limitations were consistent among the three aggregate sizes. These results suggested that increasing microbial C and especially P limitation during long-term LR decreased microbial CUE_{ST}.

3.4. Drivers of microbial resource limitation and CUE and their linkages

The first two axes of the PCA together explained 49.96% of the environmental variation (i.e., soil abiotic and biotic factors) across all LR sites (Fig. 3A). The abiotic and biotic factors at LR26 were significantly different from those at the other sites and between aggregate sizes at this site, indicating that 26 years of LR altered nutrients, enzyme activities, and microbial biomass in soil aggregates in ways that were inconsistent across time and aggregate size. PCA showed that vector length and CUE_{ST} were not strongly related to soil abiotic factors, whereas the vector angle was related to pH and SOC:TN. Microbial CUE_{ST} was negatively correlated with vector length and angle. These results were also confirmed by Pearson correlations (Fig. 3B). At the soil aggregate level, linear analysis showed weak correlations between both vector length

and vector angle and soil nutrients (C, N, and P) (Fig. S3).

We used RF analysis and VPA to reveal contributors to soil microbial metabolism (Fig. 4) and found that the duration of LR was the most important variable predicting vector length and angle (Fig. 4A and C). Key variables for predicting vector length also included TN, pH, and MBP; predicting vector angle also included pH, aggregate size, TN, MBP, and moisture. VPA showed that interference (i.e., between LR duration and aggregate size) and abiotic, and biotic factors accounted for 64.0% of the variation in vector length and 83.2% of the variation in vector angle (Fig. 4B and D). However, abiotic factors were the main contributors to variations in both vector length and angle. The majority of variation in vector angle (27.4%) was shared by interference and abiotic factors. Vector length was found to be the most important variable for predicting CUE_{ST}, followed by vector angle and LR (Fig. 4E). Interference, abiotic factors and microbial resource limitation accounted for 43.9% of the variation in microbial CUE_{ST}, with resource limitation (28.5%) being the major contributor (Fig. 4F).

The SEMs of soil microbial metabolism all yielded adequate fits (Fig. 5, $P > 0.05$, GFI > 0.9, and RMSEA < 0.08). The duration of LR indirectly affected vector length (microbial C limitation) through pH, TN, and MBP (Fig. 5A). TN (-0.67) and MBP (0.64) exerted significant direct effects on microbial C limitation. The duration of LR (0.33) directly affected vector angle (microbial P limitation), whereas aggregate size indirectly affected the angle through moisture, pH, TN, and MBP (Fig. 5B). Soil pH (0.89) was the most important direct factor affecting microbial P limitation, followed by MBP (0.47). The duration of LR indirectly controlled CUE_{ST} by affecting microbial C and P limitations (Fig. 5C). Moreover, microbial C limitation had a greater direct negative impact on CUE_{ST} than P limitation.

4. Discussion

4.1. Livestock removal alters microbial resource allocations

The current study showed that both microbial biomass and all EEAs were highest at LR11 and LR26 following livestock removal, and both SOC and TN were highest at LR26 (Table 1 and S3). These findings support our first hypothesis, in which livestock removal may increase soil microbial resources and stimulate extracellular enzyme activities. The most likely explanation for this is that livestock removal in these grasslands increased plant litter inputs and diversity, which in turn increased microbial biomass production and extracellular enzyme activities (Kooch et al., 2020; Wang et al., 2020; Oliveira et al., 2021). Indeed, livestock removal increased plant biomass and vegetation diversity by a factor of 3-8 after 25 years in this study area and greatly increased soil nutrients (Zhang et al., 2018). The theory of microbial resource allocation suggests that soil EEAs are closely related to resource availability such that an abundance of effective resources can increase enzyme production (Sinsabaugh and Shah, 2012). The positive correlation we found between nutrients and EEAs in soil aggregates following livestock removal is consistent with this theory (Fig. 3). However, the intensity of grazing and duration of livestock removal are also key factors affecting aboveground biomass and belowground ecology in grasslands and have both positive and negative effects on soil enzymes (Prieto et al., 2011; Olivera et al., 2014). As noted by Zhang et al. (2018), longer-term removal of livestock can reduce plant diversity, productivity, and soil resources. We also found that nutrients, microbial resources and enzyme activity in soil aggregates were greatly reduced after 36 years of livestock removal. This is consistent with the intermediate disturbance hypothesis (IDH) (Fensham et al., 2011; Gao and Carmel, 2020; Dong et al., 2021) in that moderate-term

livestock removal should produce higher microbial resource production than continued livestock grazing or longer-term removal.

The size distributions of soil aggregates also changed with livestock removal (Table S1), along with distributions of resources and microbial characteristics among aggregates (Table S4). Livestock removal generally increased the proportion of water-stable aggregates (> 0.25 mm), which is basically consistent with reports that grassland vegetation restoration on the Loess Plateau increases soil stability (An et al., 2013; Liu et al., 2021a). The longer duration of LR showed a shift in EEAs and microbial biomass from larger to smaller aggregates (Table S4). However, aggregate size did not significantly affect microbial resource allocation (Table S2). Indeed, the relationship between the internal resources of aggregates and the distribution of microbial resources appears complex, and the theory of resource allocation may not be applicable among aggregate size fractions.

4.2. Livestock removal increases microbial resource limitations

Vector analysis of enzyme stoichiometry revealed that the duration of LR affected apparent microbial resource limitation in soil aggregates (Fig. 1). The general decrease in overall vector length for all aggregate sizes combined indicated that livestock removal tended to reduce microbial C limitation from LR0 to LR26 (Fig. 1B), which is consistent with increasing plant production (Zhang et al., 2018) and soil nutrient concentrations (Table S3). However, vector length increased again by LR36, at which point plant production and soil nutrients had declined. Thus, the pattern of C limitation during livestock removal also appears to be consistent with the IDH. Reducing livestock grazing over intermediate time periods increases above- and belowground litter inputs as well as labile C inputs from plant roots (Panchal et al., 2022), which provide more energy to soil microbes, likely alleviating C limitation and reducing C-acquiring

enzyme synthesis (Wardle et al., 2004; Averill and Finzi, 2011; Goenster-Jordan et al., 2021).

Soil nutrients also affected microbial C limitations. In particular, TN was a key factor in microbial C limitation in our study, and its increase after livestock removal appeared to alleviate C limitation (Figs. 4A and 5A). An increase in soil nitrogen can provide a positive feedback for plant growth and reduce the C requirement for microbial respiration without altering microbial growth by increasing the availability of high-quality C substrates that can be readily assimilated (Spohn et al., 2016). Similarly, soil pH and MBP were also key factors affecting microbial C limitation because changes in relative P demand can in turn affect relative C demand. For example, additions of lime and/or phosphate fertilizers increased microbial C limitation in forests with acidic, P-limited soils, likely as a result of reducing P limitation (DeForest and Moorhead, 2020). The C limitation of microorganisms in this study was greatest in small aggregates (sma) at the longest duration, LR36 (Table S4), but was greater in sma than in mia aggregates and thus not clearly related to aggregate sizes or soil resources within aggregates (Fig. S3A) and contrary to expectations based on Cotrufo et al. (2013).

The vector angles for soil enzymatic stoichiometry at all sites and for all aggregate sizes were > 50° (Fig. 1C), suggesting that microorganisms remained P-limited in all soil aggregates with or without livestock removal. The pattern of change in microbial P limitation was generally consistent with that of C limitation; overall, it decreased at LR26 and increased at LR36. We suggest that reasons for this overall response may include the following: 1) P input from livestock manure declines after livestock removal (Sattari et al., 2016), although C input from plants increases, and 2) plant growth requirements increase competition with soil microbes for P (van der Heijden et al., 2008; Dijkstra et al., 2015). Direct evidence was provided by the reduction of P content in

soil aggregates and microbially available P following livestock removal (Table 1). Moreover, changes in other soil characteristics likely affected the availability of P. One of the main abiotic factors affecting microbial P limitation in soil aggregates was pH, followed by TN and moisture (Figs. 4B and 5B). These factors can affect P bioavailability through a range of P mineralization-related impacts on microbial communities (Zhang et al., 2018; Chen et al., 2021) and phosphatase activity (Ragot et al., 2016). However, the distribution of microbial P limitation among different aggregate sizes over time was not easily related to resource allocation (Fig. S3B). The vector angle only decreased at LR26 for the two smallest aggregate sizes (sma and mia) (Fig. 1C). Otherwise, the angle generally increased over the LR duration. Clearly, the reasons for changes in apparent microbial resource limitations (EEAs) among soil aggregate sizes are not simple and require further exploration.

Taken together, microbial C and P limitations in soil aggregates were lowest after 26 years and highest after 36 years of livestock removal, which is consistent with our second hypothesis, in which controls on resource limitation shift with progressive soil development, although we did not anticipate this nonlinear pattern. The selection of appropriate livestock stocking in semiarid grasslands may be key to controlling soil microbial resource limitation, and the positive relationship between EEA vector length and vector angle (Figs. 1 and 5) indicated that microbial C limitation and P limitation were coupled across soil aggregates (Sinsabaugh et al., 2009; Moorhead et al., 2016). However, some of the overall relationships do not appear to be consistent within and between aggregates. For example, the relationship between C and P limitations was positive for sma and mia but negative for lma across time. This inconsistency is not surprising because the form, quality, and availability of resources are likely to vary among the different sized aggregates, especially as soil aggregates develop over time.

Nonetheless, we believe that our study is the first to reveal that multiple resource allocations and constraints of microorganisms in semiarid grasslands following grazing exclusion vary among and within the smaller-scale microhabitats of soil aggregates, including size scale differences (Fig. 6).

4.3. Mechanisms of microbial C turnover and its implications

Patterns of soil microbial stoichiometry indicated relatively stable element homeostasis following long-term livestock removal (Figs. S1 and S2). This is in line with the fact that microorganisms typically exhibit a broad ability to respond to changes in the environment to maintain homeostasis (Sterner and Elser, 2002; Fanin et al., 2013; Mooshammer et al., 2014b). If the stoichiometry of resource supply does not match microbial demands (i.e., stoichiometric imbalance), microorganisms can adapt through multiple mechanisms, such as adjusting extracellular enzyme production and element use efficiency (Sinsabaugh et al., 2009; Mooshammer et al., 2014a; Yuan et al., 2019). The results of correlations between microbial stoichiometric imbalances with soil resource availabilities, vector length/angle, and CUE_{ST} indicated that imbalances within soil aggregates reflected estimated microbial resource limitations and carbon use efficiency (Fig. S4).

The microbial CUE_{ST} in the soil aggregates ranged from 0.10-0.20 (Fig. 2A), generally lower than the mean microbial CUE of 0.26 derived from a broad range of ecosystems (Sinsabaugh et al., 2016). Possible explanations for this are that stoichiometric calculations based on total nutrients may lead to an underestimation of CUE (Sinsabaugh et al., 2013; Schimel et al., 2022) or that soil microbial growth in semiarid regions is more susceptible to nutrient limitation and drought (Dijkstra et al., 2015; Yuan et al., 2019; Cui et al., 2021). Long-term LR reduced microbial CUE_{ST} compared to moderate LR in soil aggregates (Fig. 2), which is consistent with all three

of our hypotheses, likely inextricably linked to changing energy, nutrient and environmental conditions (Fig. 3).

Intriguingly, microbial resource limitations became the dominant control of variation in microbial CUE_{ST} among soil aggregates (Fig. 4), which is consistent with the notion that microbial metabolism can effectively control microbial CUE (Mooshammer et al., 2014a; Sinsabaugh et al., 2016; Geyer et al., 2019). Moreover, the pattern of microbial CUE_{ST} across aggregate size was opposite to that of microbial C and P limitations (Fig. 2B and C). For example, an increase in plant litter and root exudates from vegetation regrowth can provide energy to microorganisms and stimulate the priming effect, in turn enhancing the microbial conversion of C (Blagodatskaya and Kuzyakov, 2008; Shahzad et al., 2015). In contrast, our SEM results revealed that increasing microbial C and P limitations with long-term LR reduced microbial CUE_{ST} (Fig. 5C), which specifically supports our third hypothesis and has also been reported in several recent studies (Gai et al., 2021; Feyissa et al., 2022). Resources, such as C and P availability and their stoichiometry are important regulators of microbial CUE, such that microorganisms with limited access to resources may increase their investment in enzyme production sustaining growth, which in turn reduces carbon conversion efficiency (Manzoni et al., 2012; Sinsabaugh et al., 2013; Mooshammer et al., 2014b; Mehnaz et al., 2019). The relatively low CUE in small soil aggregates (sma) at LR36 may also be due to this reason, i.e., the lowest CUE_{ST} was coincident with the highest vector length and angle.

The pattern of C accumulation in soil aggregates during livestock removal was generally consistent with the changing pattern of microbial CUE_{ST} (Fig. 6 and Fig. S5). Higher microbial CUE leads to more C sequestration as microorganisms convert substrate C into microbial biomass (i.e., microbial metabolites, biomass, and necromass)

and deposit it in the soil via the 'microbial carbon pump', enhancing a stable soil carbon pool (Miltner et al., 2012; Cotrufo et al., 2013; Liang et al., 2017; Prommer et al., 2020). Moreover, soil development following livestock removal redistributes resources among aggregate size classes, which in turn influences microbial resource limitations in these small-scale microenvironments. In summary, the decline in microbial C conversion efficiency with longer-term livestock removal may reduce the microbial contribution to C storage, which is inextricably linked to increased resource limitation reducing microbial anabolism (Fig. 6). We highlight that microbial C turnover driven by resource limitation can reflect changes in the soil C balance in semiarid grasslands under livestock grazing or removal, which is critical for understanding and exploiting the C sink potential of grasslands in mitigating global climate change.

5. Conclusions

We found that a moderate duration (~26 years) of livestock removal maximally reduced soil microbial resource limitations in semiarid grasslands. Microbial C and P limitations were also coupled in this semiarid grassland, but microbial P limitation varied more significantly between soil aggregate sizes. A nonlinear relationship between resource allocation and microbial metabolic limitation within aggregates is likely complicated by the complexity of soil aggregate development. Microbial stoichiometric homeostasis and imbalances revealed that microorganisms in soil aggregates maintained relatively consistent element homeostasis between soil and microbial communities during natural grassland restoration, likely by regulating resource acquisition by altering enzyme activities and CUE_{ST}. However, increased microbial metabolic limitations can reduce microbial C turnover under longer-term (~36 years) livestock removal, potentially reducing soil C sequestration. Overall, we demonstrated that microbial ecological stoichiometry methods for estimating carbon

use efficiency were useful for assessing likely patterns in microbial metabolism and C balance and were applicable to evaluating smaller-scale microhabitats, i.e., soil aggregates. Additional studies are required to further verify the contribution of microbial metabolic limitation to C sequestration in soil aggregates using other methods such as functional communities and genes, microbial necromass quantification, stable isotope-labelled microcosm incubation assays, calorimetry and metabolic flux analysis. Further management practices are needed to realize the potential significance of semiarid grasslands to the global carbon balance.

Declaration of competing interest

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

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Data availability

Data will be made available on request.

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Figure and table captions:

Fig. 1 The ecoenzymatic vector model quantifies microbial resource limitation in soil aggregates.

Fig. 2 Microbial CUE in soil aggregates and their linear relationship with vector analysis.

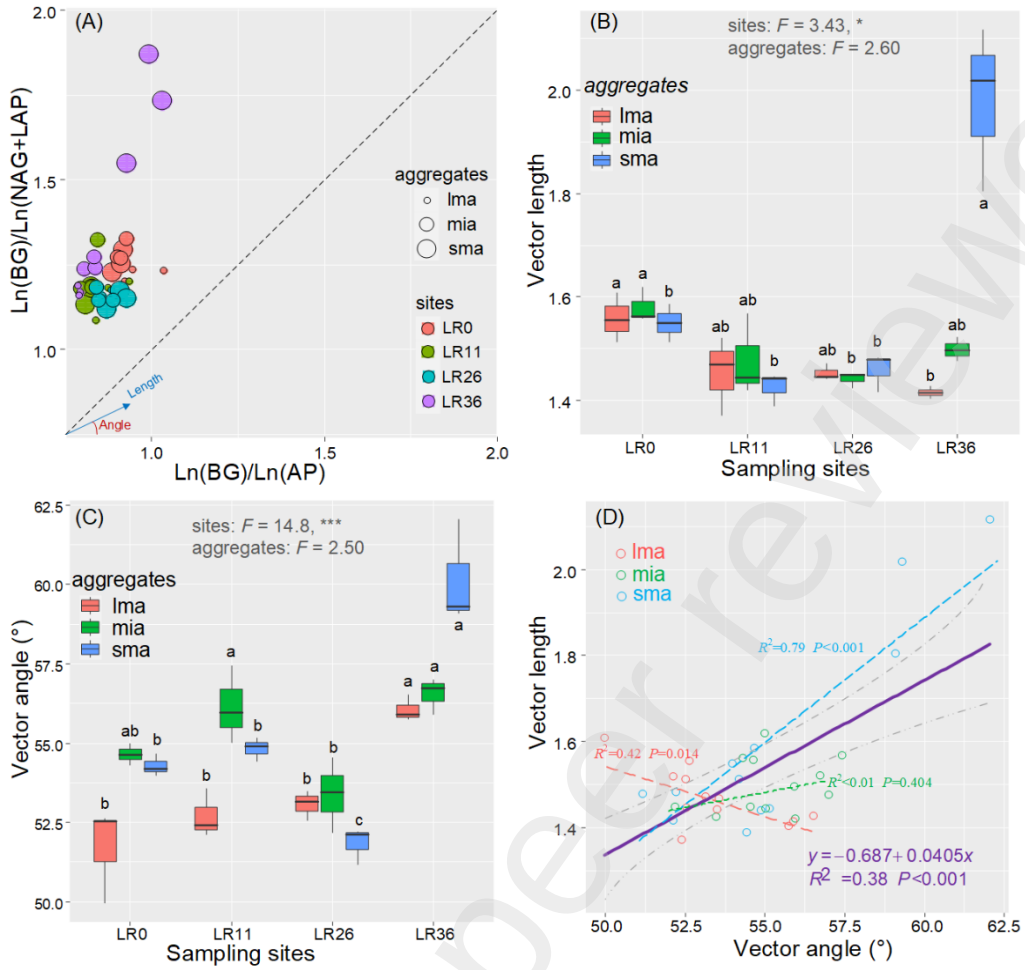
Fig. 3 The relationships between microbial resource limitation and CUE and soil properties, extracellular enzyme activities, and microbial biomass.

Fig. 4 Potential drivers of variation in microbial resource limitation and CUE in soil aggregates after livestock removal.

Fig. 5 Results from the structural equation model illustrating the major pathways of influencing factors on microbial resource limitation and CUE.

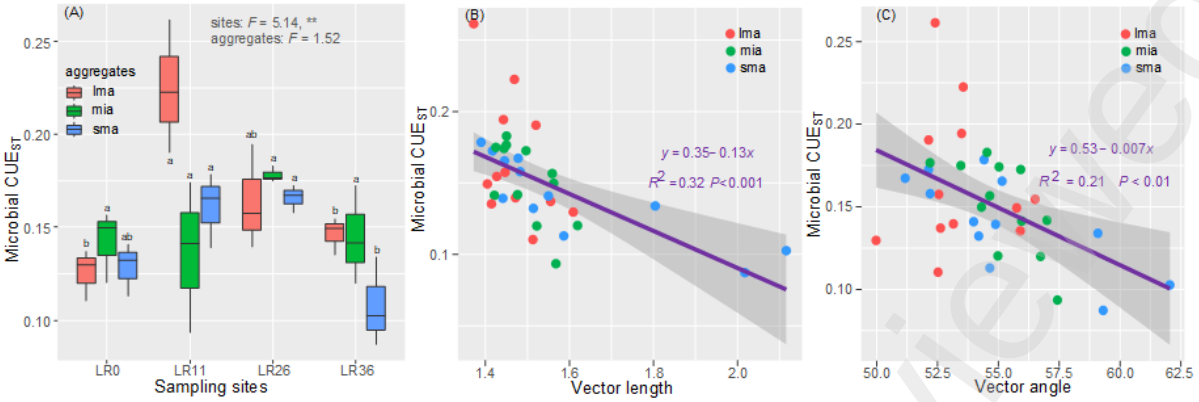
Fig. 6 Conceptual diagram of microbial resource limitation and carbon conversion patterns in semiarid grassland soil aggregates following long-term livestock removal.

Table 1 Soil chemistry, EEAs, and microbial biomass in soil aggregates during livestock removal.



828

829 **Fig. 1** The ecoenzymatic vector model quantifies microbial resource limitation in soil
830 aggregates. Relationships between Ln(BG)/Ln(NAG+LAP) versus Ln(BG)/Ln(AP) in
831 soil aggregates at all sites (A). Vector length represents microbial C limitation (B) and
832 vector angle represents microbial N or P nutrient limitation (C). Linear relationships
833 between vector length and vector angle (D). BG, β-1,4-glucosidase; NAG, β-1,4-N-
834 acetylglucosaminidase; LAP, L-leucine aminopeptidase; and AP, alkaline phosphatase.
835 lma, large macroaggregates, > 2 mm; sma, small macroaggregates, 2-0.25 mm; and
836 mia, microaggregates, < 0.25 mm. (B) and (C), Different lowercase letters indicate
837 statistical differences (Tukey's HSD test, $P < 0.05$) between different sites for the same
838 sized aggregates.



839

840 **Fig. 2** Microbial CUE in soil aggregates and their linear relationship with vector analysis. (A),
841 Box plots showing changes in microbial carbon use efficiency (CUE) from stoichiometry
842 theory (CUE_{ST}) in soil aggregates during long-term livestock removal. Different lowercase
843 letters indicate statistical differences (Tukey's HSD test, $P < 0.05$) between different sites for
844 the same sized aggregates. Linear regression showing the relationship between microbial
845 CUE_{ST} and vector length (B) and vector angle (C).

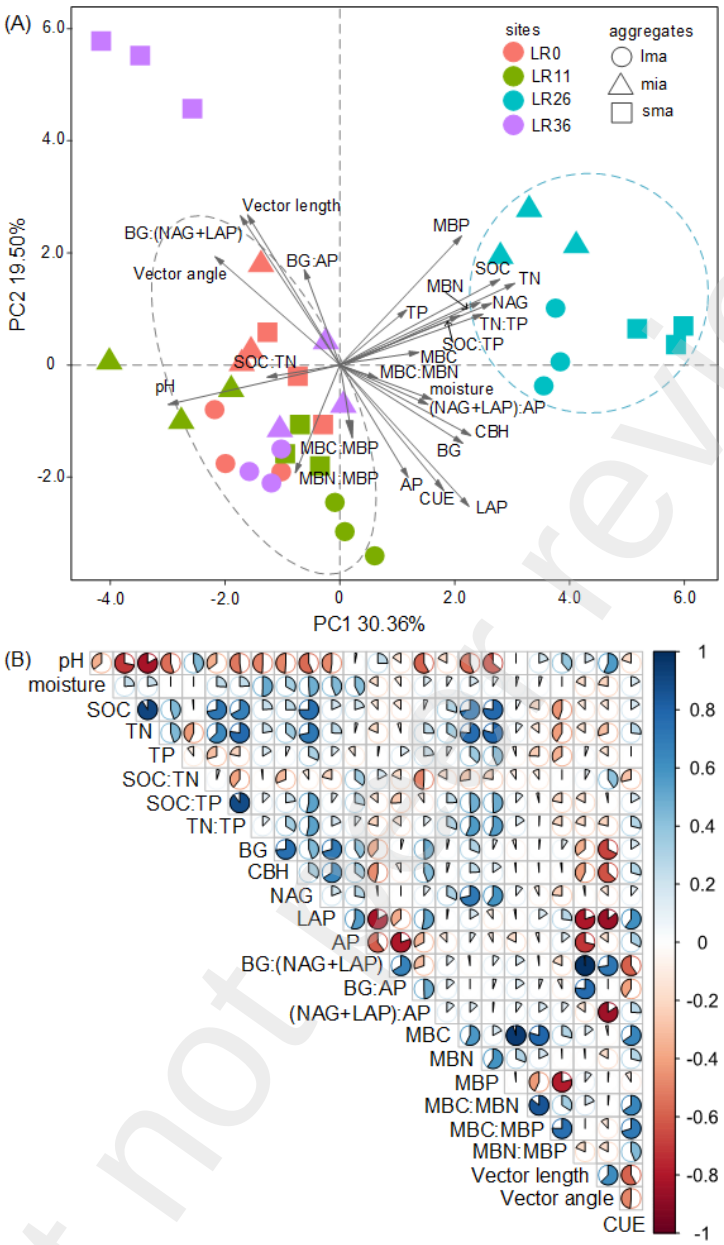
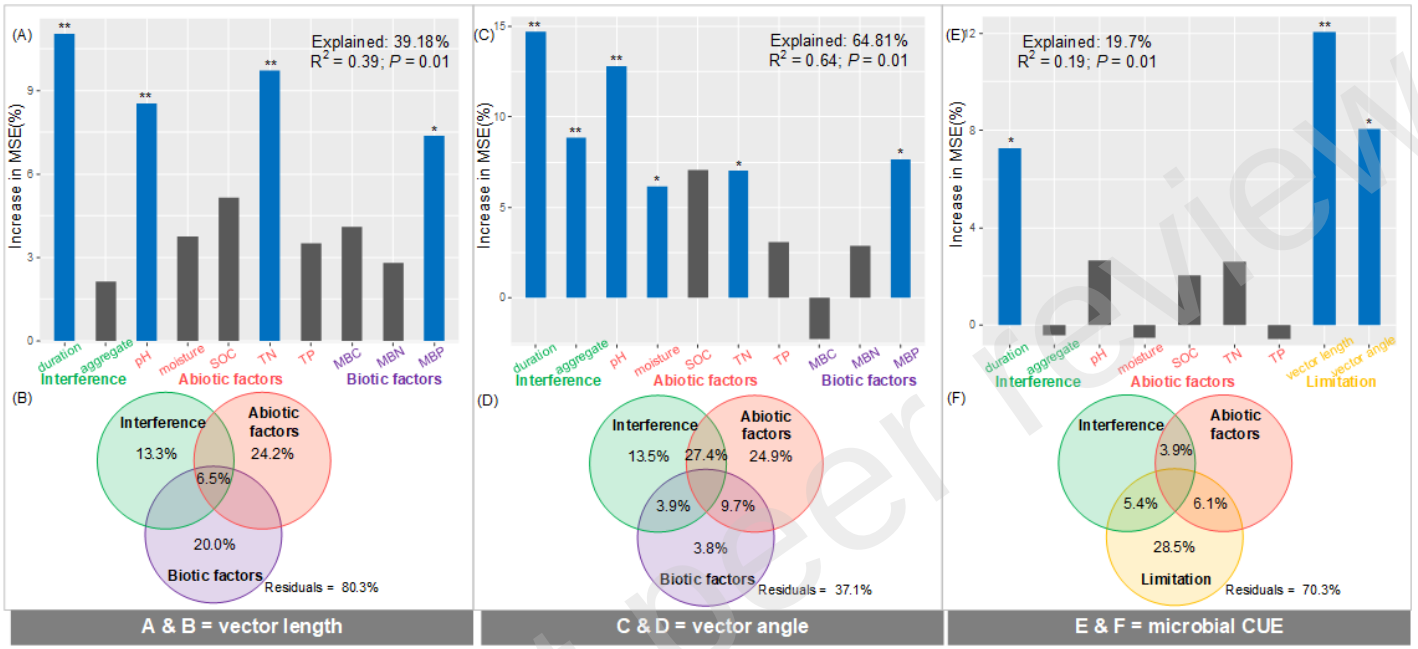
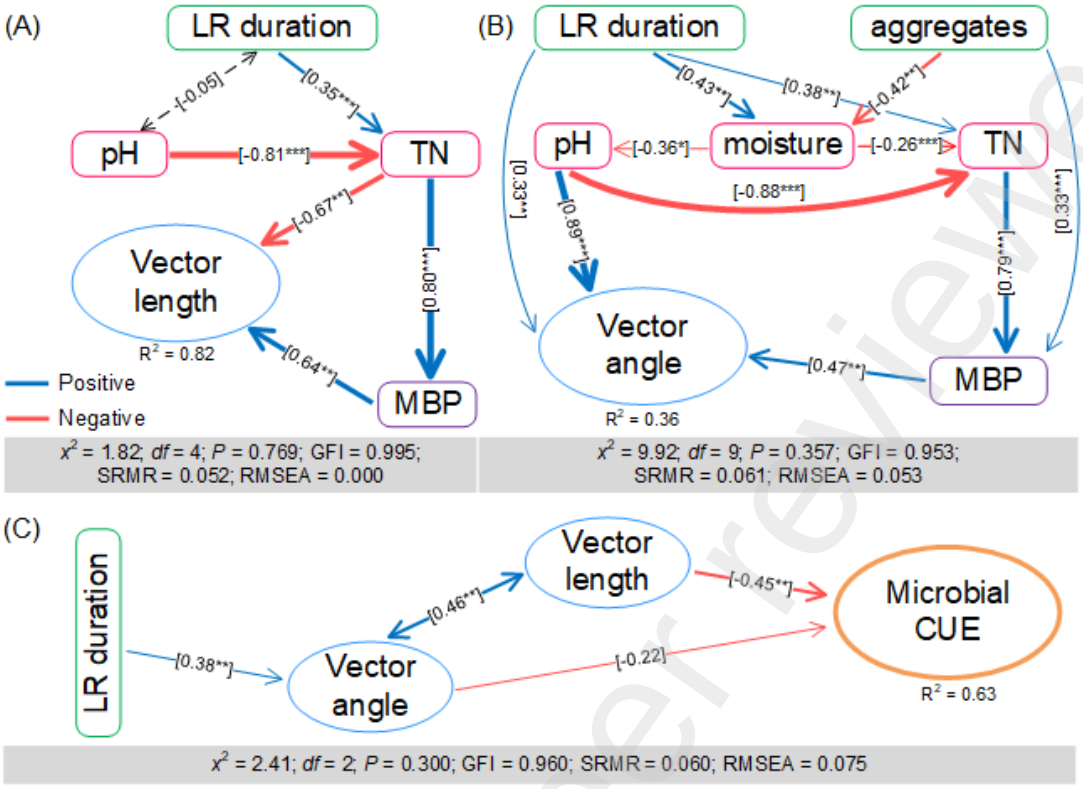


Fig. 3 The relationship between microbial resource limitation and CUE and soil properties, extracellular enzyme activities, and microbial biomass. (A), Principal component analysis (PCA); and (B), correlation heatmaps based on Pearson correlation analysis. Soil properties included pH, moisture, SOC, TN, and TP; extracellular enzyme activities included BG, CBH, NAG, LAP, and AP; and microbial biomass included MBC, MBN, and MBP. Vector length and vector angle represent microbial resource limitation; CUE represents microbial carbon use efficiency from stoichiometry theory (CUE_{ST}).



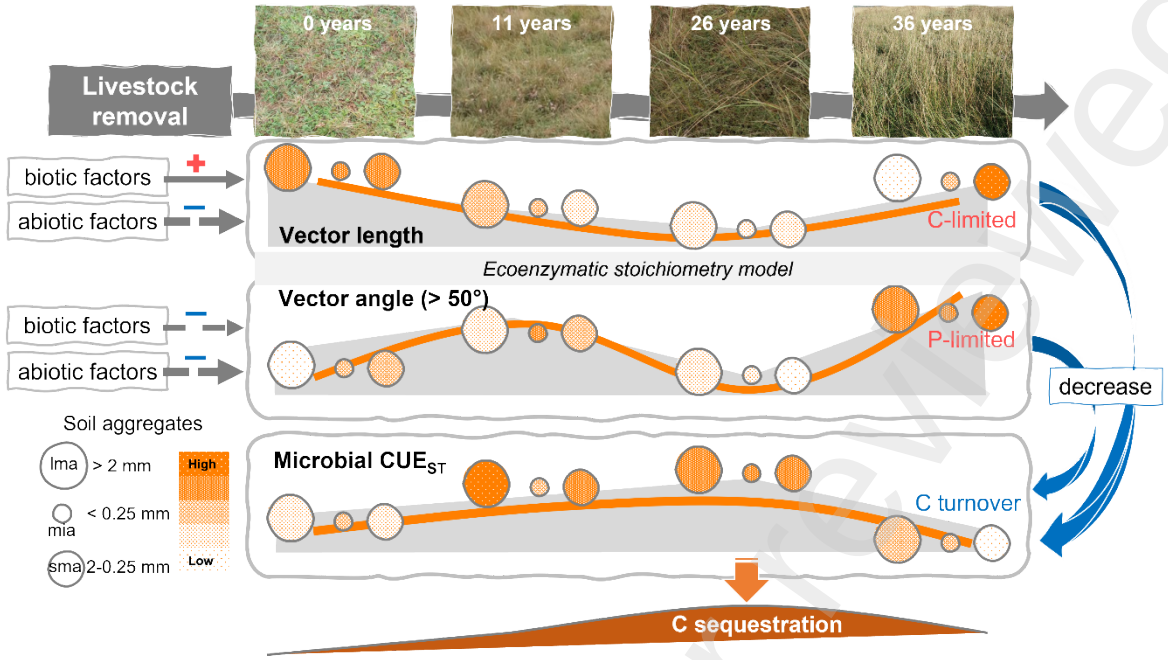
854

855 **Fig. 4** Potential drivers of variation in microbial resource limitation and CUE in soil aggregates after livestock removal. Random forest
856 (RF) analysis predicts the importance (percentage of increase in mean square error) of variables as drivers for vector length (A), vector
857 angle (C), and microbial CUE (E). Percentage increases in the MSE (mean squared error) of variables were used to estimate the importance
858 of these predictors, and higher MSE% values imply more important predictors. Significance levels are as follows: * $P < 0.05$ and ** $P <$
859 0.01. Variation partitioning analysis (VPA) showing the contribution of interference and soil abiotic and biotic factors to variation in vector
860 length (B), vector angle (D), and microbial CUE (F). Vector length represents microbial C limitation, and vector angle represents microbial
861 P vs. N limitation; microbial CUE represents microbial carbon use efficiency from stoichiometry theory (CUE_{ST}).



862

863 **Fig. 5** Results from the structural equation model illustrating the major pathways of influencing
864 factors on microbial resource limitation and CUE. Structural equation model (SEM) for vector
865 length (A), vector angle (B), and microbial CUE (C). Vector length represents microbial C
866 limitation, and vector angle represents microbial P vs. N limitation. Microbial CUE represents
867 microbial carbon use efficiency from stoichiometry theory (CUE_{ST}). The solid blue lines
868 represent direct positive impacts, and the solid red lines represent direct negative impacts. The
869 double-headed arrows indicate interactions between variables. Dotted lines represent indirect
870 effects. Please refer to the statistical analysis for the definitions of parameters represented by
871 the SEM fit.



872

873 **Fig. 6** Conceptual diagram of microbial resource limitation and carbon conversion patterns in
874 semiarid grassland soil aggregates following long-term livestock removal. Microbial resource
875 limitations affect microbial CUE and thus C sequestration. Vector analysis (vector length and
876 vector angle) of soil extracellular enzyme activities is used to quantify microbial resource
877 limitations. Microbial carbon use efficiency (CUE) is estimated from stoichiometry theory
878 (CUE_{ST}). The effects of interference, biotic factors, and abiotic factors on vector length and
879 vector angle are based on VPA, SEM and correlation analysis. Interference included the
880 duration of livestock removal and soil aggregate size; abiotic factors included pH, moisture,
881 SOC, TN, and TP; and biotic factors included MBC, MBN, and MBP. lma, large
882 macroaggregates, > 2 mm; sma, small macroaggregates, 2-0.25 mm; and mia, microaggregates,
883 < 0.25 mm.

884 **Table 1** Soil chemistry, EEAs, and microbial biomass in soil aggregates during livestock removal.

Characteristics	Sites (years since livestock removal)											
	LR0			LR11			LR26			LR36		
	lma	sma	mia	lma	sma	mia	lma	sma	mia	lma	sma	mia
pH	7.96 ± 0.06 bc	7.96 ± 0.03 a	8.00 ± 0.05 a	8.01 ± 0.04 ab	7.96 ± 0.02 a	8.00 ± 0.02 a	7.87 ± 0.02 c	7.77 ± 0.03 b	7.85 ± 0.01 b	8.06 ± 0.02 a	8.00 ± 0.01 a	8.03 ± 0.01 a
Moisture (%)	20.6 ± 0.40 b	19.8 ± 0.18 c	11.6 ± 0.44 c	21.0 ± 0.88 b	21.5 ± 0.56 b	17.7 ± 0.38 b	23.6 ± 0.45 a	24.0 ± 0.11 a	17.9 ± 0.10 b	18.5 ± 0.32 c	19.3 ± 0.32 c	23.2 ± 0.38 a
SOC (g kg^{-1})	28.7 ± 1.18 c	29.1 ± 0.74 c	32.6 ± 1.62 b	28.8 ± 0.04 c	31.9 ± 1.39 b	30.9 ± 0.72 b	37.1 ± 0.41 a	39.8 ± 0.47 a	38.7 ± 1.44 a	31.7 ± 1.06 b	32.7 ± 1.22 b	33.7 ± 1.34 b
TN (g kg^{-1})	2.69 ± 0.03 c	2.89 ± 0.03 c	3.02 ± 0.05 b	2.84 ± 0.03 b	2.91 ± 0.04 c	2.87 ± 0.09 c	3.65 ± 0.06 a	3.86 ± 0.06 a	3.85 ± 0.02 a	2.90 ± 0.04 b	3.08 ± 0.03 b	2.86 ± 0.03 c
TP (g kg^{-1})	0.93 ± 0.02 a	0.93 ± 0.03 b	0.93 ± 0.03 ab	0.85 ± 0.12 a	0.95 ± 0.02 b	0.93 ± 0.03 ab	0.90 ± 0.12 a	1.04 ± 0.02 a	0.99 ± 0.03 a	0.87 ± 0.04 a	0.95 ± 0.03 b	0.89 ± 0.06 b
SOC:TN	10.7 ± 0.36 ab	10.1 ± 0.17 a	10.8 ± 0.70 ab	10.1 ± 0.09 b	11.0 ± 0.49 a	10.8 ± 0.23 ab	10.2 ± 0.27 b	10.3 ± 0.26 a	10.1 ± 0.34 b	10.9 ± 0.25 a	10.6 ± 0.45 a	11.8 ± 0.50 a
SOC:TP	30.8 ± 1.28 b	31.3 ± 1.37 c	34.9 ± 2.21 ab	34.3 ± 5.13 ab	33.4 ± 1.74 bc	33.3 ± 1.97 b	41.9 ± 5.78 a	38.3 ± 0.52 a	39.1 ± 2.07 a	36.3 ± 1.06 ab	34.5 ± 0.67 b	38.1 ± 1.87 ab
TN:TP	2.89 ± 0.03 b	3.11 ± 0.14 b	3.24 ± 0.08 b	3.38 ± 0.50 ab	3.05 ± 0.02 b	3.09 ± 0.20 b	4.12 ± 0.58 a	3.72 ± 0.11 a	3.89 ± 0.12 a	3.32 ± 0.14 ab	3.26 ± 0.11 b	3.24 ± 0.23 b
BG (nmol g^{-1} h^{-1})	127 ± 13.8 a	136 ± 17.3 ab	107 ± 10.8 ab	117 ± 19.1 a	109 ± 1.86 b	76.9 ± 1.71 c	129 ± 10.4 a	157 ± 6.54 a	98.8 ± 9.00 bc	101 ± 7.30 a	68.6 ± 11.3 c	130 ± 14.3 a
CBH (nmol g^{-1} h^{-1})	36.5 ± 12.8 b	47.9 ± 12.1 a	41.4 ± 9.73 a	36.0 ± 4.84 b	35.9 ± 15.7 a	17.7 ± 9.29 a	64.9 ± 11.9 a	55.7 ± 7.57 a	30.1 ± 18.3 a	32.0 ± 3.35 b	7.95 ± 1.30 b	38.5 ± 6.14 a
NAG (nmol g^{-1} h^{-1})	4.97 ± 1.16 c	7.47 ± 1.36 b	5.23 ± 0.35 b	6.46 ± 2.32 bc	5.80 ± 0.90 b	3.32 ± 1.93 b	11.4 ± 0.37 a	20.2 ± 3.24 a	12.4 ± 2.77 a	10.1 ± 2.73 ab	11.1 ± 1.99 b	12.2 ± 1.17 a
LAP (nmol g^{-1} h^{-1})	47.3 ± 3.49 a	42.1 ± 3.54 b	32.2 ± 2.18 a	55.1 ± 9.56 a	50.4 ± 5.44 b	32.0 ± 6.02 a	54.3 ± 7.59 a	61.9 ± 4.33 a	40.7 ± 4.04 a	41.0 ± 2.88 a	0.88 ± 0.38 c	37.0 ± 3.51 a
AP (nmol g^{-1} h^{-1})	152 ± 28.6 c	227 ± 17.5 b	165 ± 6.37 b	222 ± 26.4 bc	302 ± 19.0 a	199 ± 38.2 b	261 ± 30.7 b	274 ± 38.9 ab	210 ± 20.3 b	344 ± 32.8 a	73.7 ± 7.25 c	362 ± 3.46 a
BG:(NAG+LAP)	2.42 ± 0.18 a	2.75 ± 0.32 b	2.86 ± 0.32 a	1.93 ± 0.43 a	1.95 ± 0.20 b	2.25 ± 0.53 ab	1.97 ± 0.08 a	1.92 ± 0.02 b	1.87 ± 0.13 b	1.98 ± 0.09 a	5.89 ± 1.45 a	2.65 ± 0.20 ab
BG:AP	0.87 ± 0.28 a	0.60 ± 0.05 b	0.65 ± 0.04 a	0.54 ± 0.15 ab	0.36 ± 0.03 b	0.39 ± 0.06 b	0.50 ± 0.04 ab	0.58 ± 0.10 b	0.48 ± 0.07 b	0.29 ± 0.01 b	0.94 ± 0.21 a	0.36 ± 0.04 b
(NAG+LAP):AP	0.36 ± 0.09 a	0.22 ± 0.01 b	0.23 ± 0.01 ab	0.28 ± 0.03 ab	0.19 ± 0.01 bc	0.18 ± 0.03 bc	0.25 ± 0.02 ab	0.30 ± 0.04 a	0.26 ± 0.05 a	0.15 ± 0.01 b	0.16 ± 0.02 c	0.14 ± 0.01 c
MBC (mg kg^{-1})	188 ± 50.0 b	223 ± 36.7 b	297 ± 25.7 ab	448 ± 50.2 a	228 ± 54.9 b	165 ± 56.7 b	290 ± 63.2 b	324 ± 19.7 ab	349 ± 42.7 a	205 ± 46.0 b	360 ± 50.0 a	294 ± 90.8 ab
MBN (mg kg^{-1})	219 ± 12.4 b	252 ± 8.54 bc	255 ± 12.8 b	259 ± 7.14 a	227 ± 14.4 c	205 ± 16.1 c	270 ± 11.9 a	300 ± 2.29 a	295 ± 15.9 a	254 ± 15.1 a	262 ± 8.22 b	251 ± 8.16 b
MBP (mg kg^{-1})	5.65 ± 0.62 b	7.33 ± 0.76 ab	6.92 ± 0.67 b	4.64 ± 0.16 b	6.66 ± 0.49 b	6.14 ± 0.31 b	8.11 ± 0.62 a	8.54 ± 0.45 a	9.93 ± 0.50 a	5.55 ± 0.19 b	7.90 ± 0.27 ab	7.09 ± 1.11 b
MBC:MBN	0.86 ± 0.21 b	0.88 ± 0.11 b	1.17 ± 0.12 a	1.73 ± 0.19 a	0.99 ± 0.20 ab	0.82 ± 0.32 a	1.08 ± 0.27 b	1.08 ± 0.07 ab	1.19 ± 0.16 a	0.80 ± 0.13 b	1.38 ± 0.20 a	1.16 ± 0.38 a
MBC:MBP	34.2 ± 11.8	31.0 ± 8.31	43.4 ± 7.94	96.9 ± 13.4	33.9 ± 6.11	26.6 ± 7.77	35.9 ± 7.95	38.1 ± 4.14	35.2 ± 4.10	36.7 ± 7.06	45.6 ± 6.31	41.9 ± 15.0

MBN:MBP	b 39.0 ± 4.88 bc	a 34.7 ± 4.66 a	a 37.1 ± 3.64 a	a 55.8 ± 1.81 a	a 34.2 ± 0.86 a	a 33.5 ± 3.88 a	b 33.5 ± 4.02 c	a 35.2 ± 1.64 a	a 29.8 ± 3.12 a	b 45.8 ± 1.26 b	a 33.1 ± 0.39 a	a 36.0 ± 5.49 a

Note: LR0, 0 years of livestock removal, control; LR11, 11 years of livestock removal; LR26, 26 years of livestock removal; and LR36, 36 years of livestock removal. lma, large macroaggregates, > 2 mm; sma, small macroaggregates, 2-0.25 mm; and mi, microaggregates, < 0.25 mm. SOC, soil organic carbon; TN, total nitrogen; TP, total phosphorus, BG, β-1,4-glucosidase; CBH, β-D-cellobiosidase; NAG, β-1,4-N-acetylglucosaminidase; LAP, L-leucine aminopeptidase; AP, alkaline phosphatase; MBC, microbial biomass C; MBN, microbial biomass N; and MBP, microbial biomass P. Different lowercase letters stand for statistical differences (Tukey's HSD test, $P < 0.05$) between different sites for similar size aggregates.