A comparison of patterns of microbial C:N:P stoichiometry between topsoil and subsoil along an aridity gradient

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Abstract

Microbial stoichiometry and its potential driving factors play crucial roles in understanding the balance of chemical elements in ecological interactions and nutrient limitations along aridity gradient. However, little is known about the variation in these features along aridity gradient due to the lack of comprehensive field investigations. Additionally, previous studies focused on the topsoil (0-10 or 0-20 cm); however, the minimum sampling depth for topsoil could impact the results of the vertical distribution of microbial stoichiometry. In the present study, we measured the variation in microbial stoichiometry, examined the major influential factors (climatic, edaphic and biotic factors) along an aridity gradient and determined whether the sampling depth affected microbial C:N:P stoichiometry. From the topsoil to the subsoil, the microbial C:N, C:P and N:P ratios varied from 6.59 to 6.83, from 60.2 to 60.5 and from 9.29 to 8.91, respectively. Only the microbial C:N ratio significantly increased with soil depth. The microbial C:N ratio significantly increased with increasing aridity in both topsoil and subsoil, while the microbial N:P ratio decreased along the aridity gradient only for the topsoil. This result implied that drought stimulated microbes tend to be more N conservative, especially those in topsoil. Among all the factors, the soil organic carbon (SOC) content and the fungi to bacteria ratio exerted the largest influence on the microbial C:N, C:P and N:P ratios at both soil depths, implying that the substrate supply and microbial structure together controlled the microbial stoichiometry. The results also revealed that the aridity index (AI) and plant aboveground biomass (AGB) exerted

43 negative impacts on the microbial C:N ratio at both soil depths, and the effects of AI
44 decreased in the subsoil. The results of this study suggested that the flexibility of the
45 microbial N:P ratio should be considered when establishing the sampling depth for
46 microbial stoichiometry study.

47 **Keywords**: grassland ecosystem, C:N:P stoichiometry, soil microbial biomass, aridity
48 gradient

49 1 Introduction

50 Ecological stoichiometry is a powerful tool for understanding the balance of chemical
51 elements required by organisms and the functions of ecosystems (Elser et al., 2000a;
52 Sterner and Elser, 2002). C, N and P are regarded as critical elements in global
53 biogeochemical cycling, and C:N:P stoichiometry in soil microorganisms offers
54 essential insight into the nutrient limitations of the organisms and communities in an
55 ecosystem (Manzoni et al., 2010; McGroddy et al., 2004).
56 A few studies have addressed the pattern of microbial stoichiometry along latitudinal
57 (Cleveland and Liptzin, 2007; Li et al., 2015; Xu et al., 2013) or environmental
58 gradients (Li and Chen, 2004; Li et al., 2012; Li et al., 2015). For example, Cleveland
59 and Liptzin (2007) analyzed microbial stoichiometry at the global scale and showed an
60 increasing trend in the microbial N:P ratio with higher latitude. However, Li et al. (2015)
61 summarized the data and found that the microbial N:P ratio decreased with latitude.
62 Undoubtedly, there is uncertainty in the values of microbial C:N, C:P and N:P ratios in

63 global studies, and the variations in these patterns might have been partially caused by
64 the different methods that were used in the various studies (Xu et al., 2013; Chen et al.,
65 2016). Furthermore, less exploration of soil microbial stoichiometry along an aridity
66 gradient at the regional scale impedes our ability to disentangle the trend of the changes
67 in microbial stoichiometry amid climate changes. Climate change, such as global
68 warming, is increasing the degree of aridity in drylands owing to the decreased
69 precipitation and increased evaporation (Wang et al., 2014; Li et al., 2017). Given this
70 background, key ecosystem processes that are regulated by soil microbes, such as soil
71 respiration and nutrient mineralization, may be dramatically impacted by the increased
72 degree of aridity, especially in fragile areas of arid and semiarid ecosystems
73 (Delgadobaquerizo et al., 2013; Chen et al., 2014). Therefore, we conducted a field
74 investigation across a 2100-km climatic transect in the Inner Mongolian grasslands to
75 determine how the microbial C:N, C:P and N:P ratios were affected by changing
76 environmental conditions.

77 Previous studies have also shown that a variety of abiotic factors impact microbial
78 C:N:P stoichiometry (Cleveland and Liptzin, 2007; Manzoni et al., 2010; Hartman and
79 Richardson, 2013). For instance, control experiments have found that warming can
80 indirectly affect the turnover of microbial biomass N by stimulating soil respiration
81 (Veraart et al., 2011; Butterbach-Bahl et al., 2013). Climate could exert an influence on
82 microbial stoichiometry through changes to the microenvironment, such as soil
83 moisture and temperature, and it could also impact the availability of substrates in the

soil (Nielsen et al., 2009). Moreover, edaphic variables, such as SOC (Maria et al., 2014; Chen et al., 2016) and soil texture (Li et al., 2015), could be associated with nutrient mineralization and availability, thus influencing the C:N:P stoichiometry in microbial biomass (Griffiths et al., 2012). A labeled incubation experiment showed that the mineralization of organic P was mainly driven by the microbial C demands in P-poor soils (Aponte et al., 2010; Heuck et al., 2015). In addition, microbial C:N, C:P and N:P ratios were also affected by biotic factors such as plant productivity and the composition of the microbial community (Fanin et al., 2013; Chen et al., 2016). Generally, fungi exhibit a higher C:N ratio than bacteria (Strickland and Rousk, 2010); thus, a shift in the fungi to bacteria ratio is expected to result in microbial stoichiometry changes (Li et al., 2012; Heuck et al., 2015). However, those findings were based on literature analyses or small-scale experiments, and the variations in microbial C:N, C:P and N:P ratios at the regional scale have rarely been assessed systematically, and the drivers of these variations need to be addressed more specifically with appropriate experimental designs. Moreover, most research has focused on the top 10 cm of soil, which often has high C availability and nutrient contents. It can be assumed that the effects of potential driving factors exhibit minimal differentiation at deeper soil depths. However, soil at a deeper depth might contain microbial communities that are specialized for their environment, and their functions may differ from the functions of the communities in the topsoil (Fritze et al., 2000; Blume et al., 2002). Certainly, the drivers that are responsible for the variations in microbial C:N, C:P and N:P ratios in deeper soil remain

poorly understood. Such knowledge of the nature of soil microbial stoichiometry is fundamental for understanding ecosystem function, especially at the 10-20 cm soil depth, which remains highly uncertain in the published studies.

Substrates for microorganisms, such as available nutrients and water, decline exponentially with depth, and the top 20 cm of soil accumulates the greatest amount of microbial biomass, thereby attracting the attention of most researchers (Fierer et al., 2003; Xu et al., 2013). Soil at a 0-20 cm depth was regarded as the topsoil in some studies, while other researchers divided the soil from 0-20 cm into different soil depths to explore the vertical differences between these depths (Aponte et al., 2010; Peng and Wang, 2016). However, most studies used 0-10 cm as the topsoil to facilitate sampling and comparative research (Li and Chen, 2004; Cleveland and Liptzin, 2007; Chen et al., 2016). The depth of topsoil varies among studies, and sampling depth can therefore have impacts on the study of the vertical patterns in soil microbial stoichiometry (Tischer et al., 2014). Given that soil represents a highly heterogeneous environment, especially in terms of site-specific soil development history, it is difficult to draw general conclusions (Xu et al., 2013; Camenzind et al., 2018). In addition, if a large difference existed between the soil at 0-10 cm and that at 10-20 cm, microbial stoichiometry would be underestimated due to the ambiguous limitation of topsoil (Tischer et al., 2014). To identify the soil depth that is appropriate for sampling and to improve the understanding of topsoil research at a global scale, we designed a study that divided the topsoil into 0-10 cm and 10-20 cm depths to compare the differences

in microbial stoichiometry at the regional scale.

In Inner Mongolia grasslands, the aridity exhibits a gradient that increases from northeast to southwest (aridity index, calculated as precipitation/potential evapotranspiration, ranges from 0.16 to 0.54), thus providing an ideal platform to better estimate the patterns and drivers of microbial C:N:P stoichiometry along an aridity gradient (Chen et al., 2014; Li et al., 2017). In this study, we aim to access the effect of soil depth on soil microbial C:N:P stoichiometry along aridity gradient. We hypothesized that the microbial C:N and C:P ratios decrease and the microbial N:P ratio increases with temperature (Cleveland and Liptzin, 2007; Li et al., 2015; Xu et al., 2013), and the microbial C:N and C:P ratios decrease and the microbial N:P ratio increases with decreasing aridity index (Wang et al., 2014; Li et al., 2017). In addition, the identification of soil depth for vertical study is differernt in some published literature (Li and Chen, 2004; Aponte et al., 2010; Tischer et al., 2014; Peng and Wang, 2016). We predicted that variation of bacterial and fungal taxa between soil depths might contribute to the shifts in C:N:P stoichiometry, especially in the N:P ratio (Mouginot et al., 2014; Camenzind et al., 2018). Therefore, we focus on (i) the effects of potential driving factors on microbial C:N, C:P and N:P ratios in topsoil and subsoil (ii) the response of the microbial C:N, C:P and N:P ratios to soil depth.

2 Materials and methods

2.1 Study area

This study was performed across the Inner Mongolian temperate grassland, which is a central part of the Eurasian steppe. The study area is located at 39.2-49.6°N latitude and 107.8-120.1°E longitude and covers an area of 440,000 km^2. From northeast to southwest, the mean annual temperature increases from -1.7 to 7.7°C, and the mean annual precipitation decreases from 402 mm to 154 mm, approximately 80% of which falls in the growing season from May to August . Three grassland types, meadow steppe, typical steppe and desert steppe, are distributed along the northeastern to southwestern gradient and are dominated by *Stipa baicalensis* and *Leymus chinensis*, *S. grandis*, and *S. klemenzii*, respectively. The soil types corresponding to the three grassland types are categorized as chernozems, kastanozems, and calcisols, respectively, according to the soil classification system of the Food and Agriculture Organization of the United Nations.

2.2 Sampling and data collection

Along this transect, a total of 58 sites that were slightly disturbed by humans and domestic animals were sampled, including 10 in the meadow steppe, 28 in the typical steppe, and 20 in the desert steppe (Fig. A1). Five 1×1 m^2 subplots were established, one at each corner and one in the center of 10×10 m^2 plot. The plant community in the subplots was identified, and the above-ground biomass (AGB) was harvested. At each site, three replicate soil samples at depths of 0-10 cm and 10-20 cm were collected from three 1 × 1 m subplots arranged diagonally in a 10 × 10 m plot. The samples were mixed to form one composite sample. After gentle homogenization and removal of roots, the

soil was sieved through a 2-mm mesh and stored to conduct the further experiments. The total carbon concentrations were measured using an elemental analyzer (Vario EL III, Elementar, Germany). The soil inorganic carbon content was determined with a carbonate content analyzer (Eijelkamp 08.53, Netherlands). The SOC content was calculated by subtracting the soil inorganic carbon from the total carbon. The soil elemental contents were reported in $mmol \cdot kg^{-1}$. Soil pH was measured in a suspension with a soil:water ratio of 1:2.5. After the removal of organic matter and carbonates, the soil texture was determined using a particle size analyzer (Malvern Masterizer 2000, UK).

2.3 Aridity index

The aridity index was extracted from the Global Aridity Index (Global-Aridity) dataset，which provides high-resolution (30 arc-seconds or ~ 1km at the equator) global raster climate data for the period 1950-2000 (http://www.cgiarcsi.org) (Zomer, Trabucco, Bossio, & Verchot, 2008). The specific calculation formula is as follows:

$$\text{Aridity Index (AI)} = MAP / MAE$$

$$PET=0.0023 \cdot RA \cdot (Tmean+17.8) \cdot TD0.5(mm/month)$$

where MAP represents the mean annual precipitation, obtained from the WorldClim Global Climate Data (Hijmans et al. 2005); MAE represents the mean annual potential evapo-transpiration (PET); Tmean represents the monthly mean temperature, TD is calculated as the difference between the monthly maximum and minimum temperatures;

and RA represents the extra-terrestrial radiation on above the atmosphere.

2.4 Soil microbial analyses

Microbial biomass carbon (MBC) and microbial biomass nitrogen (MBN) were determined following the chloroform fumigation-K_2SO_4 extraction method, according to Vance et al. (1987) and Wu et al. (1990). The soil was preincubated at 25°C for two weeks at a field water capacity of 40%. Then, the soil was fumigated with chloroform for 24 h in a vacuum. The fumigated and nonfumigated samples were extracted using 0.5 M K_2SO_4 with a soil:solution mass ratio of 1:4. The C and N contents were measured with a multi N/C analyzer (Anaytik Jena, Germany). Using a universal conversion factor of 0.45 (Jenkinson et al., 1976), the amounts of MBC and MBN were calculated by subtracting the amounts of extractable C and N in the nonfumigated samples from those in the fumigated samples (Vance et al., 1987; Wu and Joergensen et al., 1990; Joergensen et al., 1996). Microbial biomass phosphorus (MBP) was estimated according to the method described in Hedley and Stewart (1982) and modified by Wu et al. (1990). The fumigation procedure was the same as that for MBC and MBN. The fumigated and nonfumigated samples were extracted using 0.5 $mol \cdot L^{-1}$ $NaHCO_3$ and were analyzed to determine the total phosphorus concentration using a colorimetric method. Using a universal conversion factor of 0.40, the amount of MBP was calculated by subtracting the amount of extractable P in the nonfumigated samples from that in the fumigated samples (Hedley and Stewart.,1982). Phospholipid fatty

acids (PLFAs) were extracted from the soil using the method described by Bossio and Scow (1998). Briefly, 8 g of soil (dry weight) was used for PLFA analysis. The resultant fatty acid methyl esters were separated, quantified, and identified using capillary gas chromatography.The following PLFAs were used as markers for each of the specific groups: for fungi, 18:1ω9c, 18:2ω6c, 18:3ω6c; for bacteria, i13:0, a13:0, i14:0, i15:0, a15:0, 15:1ω6, 2OH16:0, i16:0, 16:1ω7c, 16:1ω9c, a17:0, i17:0, 17:1ω8c, cy17:0, i18:0, 18:1ω7, 18:1ω5 and cy19:0.

2.5 Statistical analyses

The C:N, C:P and N:P ratios in the soil microbial biomass were log10 transformed before analysis to improve their normality (Fig. A3). Paired samples t-tests were used to determine the differences in the soil microbial biomass C, N and P between the topsoil and subsoil and the differences in the C:N:P stoichiometry ratios in the soil microbial biomass. Ordinary least squares regression analyses were conducted to evaluate the relationship between the C:N:P ratios in the soil microbial biomass and latitude, aridity index, AGB, SOC, sand percentage and fungi to bacteria ratio (F:B ratio). The analyses were performed with SPSS 19.0 software (IBM Corporation, Armonk, NY, USA). A structural equation model (SEM) was used to test the multivariate effects (direct and indirect) on the C:N:P ratios in the microbial biomass through hypothetical factor pathways (Fig. A4). The SEM was constructed using the Amos 17.0 software package (Smallwaters Corporation, Chicago, IL, USA).

3 Results

3.1 The variation in microbial C:N:P stoichiometry between soil depths along the environmental gradient

The results indicate well-constrained relationships among C, N and P in soil microbial biomass (Fig. A2). The soil microbial biomass C:N, C:P and N:P ratios varied by an order of magnitude. Significantly different water content, soil bulk density, sand percentages and SOC content were found between soil depths (P <0.05, Fig. 1a, 1b, 1c, 1f). The microbial biomass C, N and P concentrations in the topsoil were significantly higher than that in the subsoil (P <0.05, Table. 2). The C:N, C:P and N:P ratios in the microbial biomass of the topsoil were 6.59, 60.2, and 9.29, respectively, while those values in the subsoil were 6.83, 60.5 and 8.91, respectively (Table. 2). Moreover, the microbial C:N ratio in the subsoil was significantly higher than that those in the topsoil (Table. 2).

The results revealed a significant positive relationship between the AI and the microbial C:N ratio (Topsoil, R^2 =0.10, P< 0.05; Subsoil, R^2 =0.09, P< 0.05, Fig. 2a) and significant negative relationships between the AI and the microbial N:P ratio (Topsoil, R^2 = 0.10, P< 0.05; Fig. 2c). In addition, the decreasing trend was found between the microbial C:N ratio and MAT (Topsoil, R^2 =0.14, P< 0.01; Subsoil, R^2 =0.10, P< 0.01, Fig. 2d), while a significant negative relationship was found between the microbial N:P ratio and MAT (Topsoil, R^2 =0.19, P< 0.001; Fig. 2f). Significant positive relationships

were found between the microbial C:N ratio and latitude (Subsoil, R^2 =0.12, P< 0.05, Fig. 2g), while a negative relationship was found between the microbial N:P ratio and latitude (Topsoil, R^2 =0.18, P< 0.001; Fig. 2i).The microbial C:N ratio was positively related to AGB (Topsoil, R^2 =0.06, P< 0.05, Fig. 3a), SOC (Topsoil, R^2 =0.12, P< 0.01; Subsoil, R^2 =0.09, P< 0.05, Fig. 3d) and was negatively related to the sand percentage (Topsoil, R^2 =0.11, P< 0.01; Subsoil, R^2 =0.11, P< 0.01, Fig. 3g). A significant positive relationship was found between the microbial C:P ratio and the content of soil organic matter (Subsoil, R^2 =0.08, P< 0.05, Fig. 3e). No or only weak association was found between the microbial C:N, C:P and N:P ratios and the AGB and F:B ratio in the subsoil (Fig. 3).

3.2 Effects of potential driving factors on the microbial C:N:P stoichiometry at topsoil and subsoil

The final SEM adequately fit the data, as shown by several robust goodness-of-fit measures (P value and minimum discrepancy). The model explained 38% (topsoil) and 27% (subsoil) of the variation in the microbial C:N ratio, 17% and 19% of that in the microbial C:P ratio, and 29% and 16% of that in the microbial N:P ratio (Fig. 4a, b, c, d, e, f). Effects of AI, AGB and SOC contenton the microbial C:N ratio were found at both soil depths (Fig. 4a, 4b). The SOC content made the largest positive contribution to the variation in the microbial C:N ratio in the topsoil (Fig. 4a, 4b). We found direct effects of the sand percentage, SOC content and F:B ratio on the microbial C:P ratio at both soil depths, and the SOC content made the largest contribution to the variation in

the microbial C:P ratio in the topsoil, which was higher than that in the subsoil (Fig. 4e, 4f). Influences of sand% and the SOC content on the microbial N:P ratio were found in the topsoil, while the F:B ratio and the SOC content explained most of the variation in the microbial N:P ratio in the subsoil (Fig. 4e, 4f).

4 Discussion

4.1 The pattern of microbial C:N, C:P and N:P ratios along aridity and the latitude gradient

As stated in our hypothesis, the increase in the microbial C:N ratio and the decrease in the microbial N:P ratio that were found along a temperature gradient in this study are in accordance with the findings of Li et al. (2015) and Chen et al. (2016), who reported similar variations in microbial stoichiometry along latitudinal gradient. Temperature drives the variation in the growth of the microbial community, as high growth rates at low latitudes require high RNA contents, causing the N:P ratio to decline (Chadwick et al., 1999; Kooijman et al., 2009; Xu et al., 2013). In addition that, we observed that the microbial C:N ratio significantly increased with increasing aridity index, while the microbial N:P ratio decreased with increasing aridity index, indicating that drought (decreasing aridity index) affects ecological stoichiometry by mediating the growth rate of microorganisms in semiarid regions (Elser et al., 2000b; Peng and Wang, 2016). Dan and Wang noted that increasing aridity reduced the soil microbial abundance in drylands, and a decreased growth rate in dry areas might result in decreased allocation

to P-rich ribosomal RNA (and thus higher C:P and N:P ratios) (Wang et al., 2014; Maestre et al., 2015). Additionally, microbial C:N ratio decreased with the decreasing aridity index, which serves as a protective mechanism as microbes decrease their nitrogen use efficiency (NUE, the ratio of N invested in growth over total N uptake) and tend to be more N conservative under drier climatic conditions (Mooshammer et al., 2014; Delgado-Baquerizo et al., 2017). Moreover, under drier climate conditions, the soil microbial communities shift from acting as r-strategists (fast-growing copiotrophs) to acting as K-strategists (slow-growing oligotrophs), as microorganisms with K-strategies have lower nutrient demands (N and P) and growth rates, invest more nutrients into extracellular enzymes to gain limited nutrients and thus have higher cellular C:N:P ratios than r-strategists (Fierer et al., 2007; Fierer et al., 2010).

The microbial C:N ratio demonstrated an increasing trend with increasing latitude, in contrast to the decreasing trend that was demonstrated for the microbial N:P ratio. Such results paralleled the results of studies on ecological stoichiometry, which revealed that the C:N ratio of microorganisms increased with latitude, while the N:P ratio decreased with latitude, suggesting increasing N limitations in microorganism ecosystems in high-latitude areas (Li et al., 2015; Chen et al., 2016). The regional scale microbial stoichiometry followed the global-scale stoichiometry patterns that were observed for plant leaves (Reich and Oleksyn, 2004; Yuan et al., 2011), litter (McGroddy et al., 2004), and soil (Sardans et al., 2012), conforming to the substrate age hypothesis, which predicts young soils to be N-limited, whereas old soils tend to be P limited (Walker and

309 Syers, 1976; Vitousek et al., 2010). Our study further illustrated the latitudinal pattern
310 of microbial stoichiometry and first attempted to examine the variation in microbial
311 stoichiometry along an aridity gradient at the regional scale.

312 **4.2 Direct effects of ecological factors on controlling microbial C:N, C:P and N:P**
313 **ratios at topsoil and subsoil**

314 Among the ecological factors examined, our study found that the patterns of microbial
315 C:N and C:P ratios were associated with SOC content and the F:B ratio, suggesting that
316 the available C and microbial community structure together regulated the shift in
317 microbial stoichiometry. If the environmental parameters were considered individually,
318 SOC content was found to be significantly positively related to the microbial C:N and
319 C:P ratios, which is consistent with the results observed from global data analysis,
320 suggesting that SOC content may control microbial stoichiometry by mediating the
321 substrate stoichiometry, e.g., the soil C:N and C:P ratios (Hartman and Richardson,
322 2013; Maria et al., 2014; Mooshammer et al., 2014). In deeper soil, microbial metabolic
323 processes are limited by C availability and energy (C), such as denitrification and P
324 mineralization, thus resulting in the effect of SOC on miccrobial C:N:P stoichiometry
325 in subsoil (Fierer et al., 2003; Peng and Wang, 2016; Camenzind et al., 2018).

326 SEM also illustrated that the microbial community structure is an important feature in
327 determining microbial stoichiometry. The F:B ratio has recently been found to have a
328 vital influence on the patterns of microbial C:N and N:P ratios in soil at a large scale

(Chen et al., 2016). An experiment indicated that fungi have lower resource requirements and higher C:N and C:P ratios than bacteria; thus, the shift in the F:B ratio impacted microbial C:N:P stoichiometry (Mouginot et al., 2014). In our study, the lower F:B ratio might have led to a shift in the microbial nutrient stoichiometry at deeper soil depths (Tischer et al., 2014). Overall, the SEM highlighted the important role of the C availability and microbial community structure in driving the variations in microbial C:N, C:P and N:P ratios at both soil depths.

Moreover, AGB and AI also exerted direct influences on microbial C:N or C:P ratios, and those impacts mainly acted in the topsoil but were weaker in the subsoil. The climate imposes important controls on both the plant community and the microbial taxa along with their interactions with soil nutrients (Chadwick et al. 1999; Vitousek 2004; Reich and Oleksyn, 2004). In particular, drier weather condition, and the decreasing aridity index, could affect the growth and productivity of plants, and then shape a shift in vegetation types along this grassland transect (Jaleel et al., 2009; Cherwin & Knapp, 2012). At the same time, the meadow steppe ecosystem with high productivity maintained relatively high soil C and N contents, which resulted in high C:P and N:P ratios in these regions; thus, plant productivity exerted a positive influence on microbial C:N (Aponte et al., 2010; Manzoni et al., 2010). Because of the vertical distribution of the influence of the AI, the effect decreased with soil depth.

Interestingly, our results revealed that the microbial N:P ratio was mainly impacted by the F:B ratio and SOC, while the sand percentage and SOC had direct negative effects

on the ratio in the subsoil, suggesting the flexibility of microbial stoichiometry in response to distinct resource supplies between topsoil and subsoil (Peng and Wang, 2016). The soil depth affected the microbial biomass N and P, which decreased nearly twofold from the topsoil to the subsoil (Table 2). However, the results showed that the N and P cycles responded asymmetrically to soil depth, which might be attributed to the high variability in P availability (Li et al., 2015; Zechmeister-Boltenstern et al., 2016). Generally, P is mostly derived from parent material, while N is mainly a biological element (Vitousek and Farrington, 1997; Vitousek et al., 2010). Therefore, it is believed that P variations regulate large-scale patterns in microbial N:P stoichiometry and nutrient-use strategies (Heuck et al., 2015; Camenzind et al., 2018). With a high proportion of sand, the soil becomes porous, which may lead to increased leaching of available P to deeper soil depths (Otten et al., 1999; Achbergerová and Nahálka, 2011). Similarly, P leaching caused by weathering led to a shift in the N:P ratio in the soil, and a vertical study found a high variation in the N:P ratio between soil depths across a large scale (Tian et al., 2010). The high variability of the N:P ratio in soil and soil microbial biomass therefore indicates that the N:P ratio could be an indicator of the ecosystem nutrient status at deeper soil depths (Cleveland et al., 2007; Chen et al. 2013; Li, et al. 2015).”.

4.3 How deep should we dig to evaluate the topsoil the microbial stoichiometry in vertical study?

The results showed significant differences in the water content and SOC content

between topsoil and subsoil, suggesting that the resource supplies between topsoil and subsoil were significantly different. We also observed that the microbial C:N, C:P and N:P ratios varied between topsoil and subsoil, and significant difference was found in microbial C:N ratio. Those results indicated that the flexibility of the microbial stoichiometry responds to different resource supplies between soil depths (Tian et al., 2010; Peng and Wang, 2016). Similar findings were found in the top 16 cm of soil in a Mediterranean oak forest (0-8 cm and 8-16 cm), where the microbial nutrient ratios (C:N, C:P and N:P) varied with soil depths (Aponte et al., 2010). Tischer et al. (2014) sampled the top 20 cm of soil (0-5 cm, 5-10 cm, 10-20 cm) and observed that the microbial C:N ratio changed with soil depth. Moreover, sampling to a depth of 10 cm showed a significant difference in the microbial N:P ratio (Tischer et al., 2014). The detection of the differences in the microbial N:P ratio in our study depended strongly on the sampling depth, suggesting that the microbial N:P ratio might provide insight into the nature of ecosystem nutrient limitations in a vertical study (Cleveland and Liptzin, 2007; Fierer et al., 2010). In addition, SEM also showed that the microbial N:P ratio was controlled by multiple potential driving factors at different soil depths, indicating that a 0-10 cm or shallower sampling interval should be used when studying the vertical patterns in the microbial N:P ratio.

5 Uncertainties and perspectives

The first uncertainty was related to the determination of fungal and bacterial biomasses by PLFA markers, which have limited targets for fungi and bacteria. This uncertainty

should be noted when interpreting the results in the present study. Methodological advances in sequencing approaches might be used to more accurately index the microbial community and reveal insights into the regulation of microbial C:N:P stoichiometry in distinct soil microbial taxa or functional groups. Second, the theory used to construct the model is another source of uncertainty; the theory related to the drivers of microbial stoichiometry used in this study was mostly derived from a literature review and summarized data. In future research, more control experiments with the manipulation of C availability, especially at deeper soil depths, would further improve our understanding of the changes in microbial stoichiometry and nutrient limitations under the impacts of global change.

6 Conclusion

The ratios of C, N, and P in the microbial biomass were 6.59:60.2:9.29 in the topsoil, which deviated from the 6.83:60.5:8.91 ratio in the subsoil. Moreover, significant differences were found in the microbial C:N and N:P ratios between topsoil and subsoil, indicating that the flexibility of microbial stoichiometry should be considered for vertical study. In addition, The trend of microbial C:N ratio increasing with aridity index, consistent with the perspective that microbes mediate their nitrogen use efficiency and tend to be more N conservative under drier climatic conditions. The microbial N:P ratio trend along the aridity gradient was consistent with the growth rate hypothesis that a decreased growth rate in dry areas results in decreased allocation to P-rich ribosomal RNA and thus a higher N:P ratio. These findings confirmed the

importance of SOC, the microbial structure and soil texture in shaping the pattern of microbial stoichiometry in semiarid grassland systems. The influence of ecological factors decreased from topsoil to subsoil, as well as the decline in climatic, edaphic and biotic factors. Overall, these results illustrated N and P limitation in microbial biomass at deeper soil depths along aridity gradient and limited responses to ecological factors in the subsoil.

Author contributions. HH. WM and YY devised the study. YL carried out the experiment and data analyses. DK, YC and DC assisted with the data analyses and interpretation. XN, TW, XZ, MZ and HB assisted with the experiment. All authors contributed to the preparation of the paper.

Competing interests. The authors declare that they have no conflict of interest.

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Table 1. Basic information of study sites

Biome	Latitude (°N)	Longitude (°E)	MAP (mm)	MAT (°C)	Aridity Index	AGB ($g \cdot cm^{-3}$)	Dominant species
Meadow steppe	48.1(43.9–49.6)	119(116-120)	353(262–381)	–0.45(–1.81–1.71)	0.48 (0.38-0.54)	136(88-168)	*Stipa baicalensis* *Leymus chinensis*
Typical steppe	45.6(43.5-49.5)	117(114-119)	304 (205–402)	1.11(-2.09-3.29)	0.37 (0.25–0.50)	102(49.4-159.8)	*Stipa grandis* *Stipa kryovii*
Desert steppe	41.7(39.2-43.6)	115(108-113)	223(154–293)	5.63(4.13-7.67)	0.23 (0.16-0.32)	43.4(24.6-76.5)	*Stipa klemenzii* *Stipa breviflora*

Note: Data represent the means, with minimum and maximum values in parentheses. MAT, mean annual temperature; MAP, mean annual precipitation; AGB, above ground biomass.

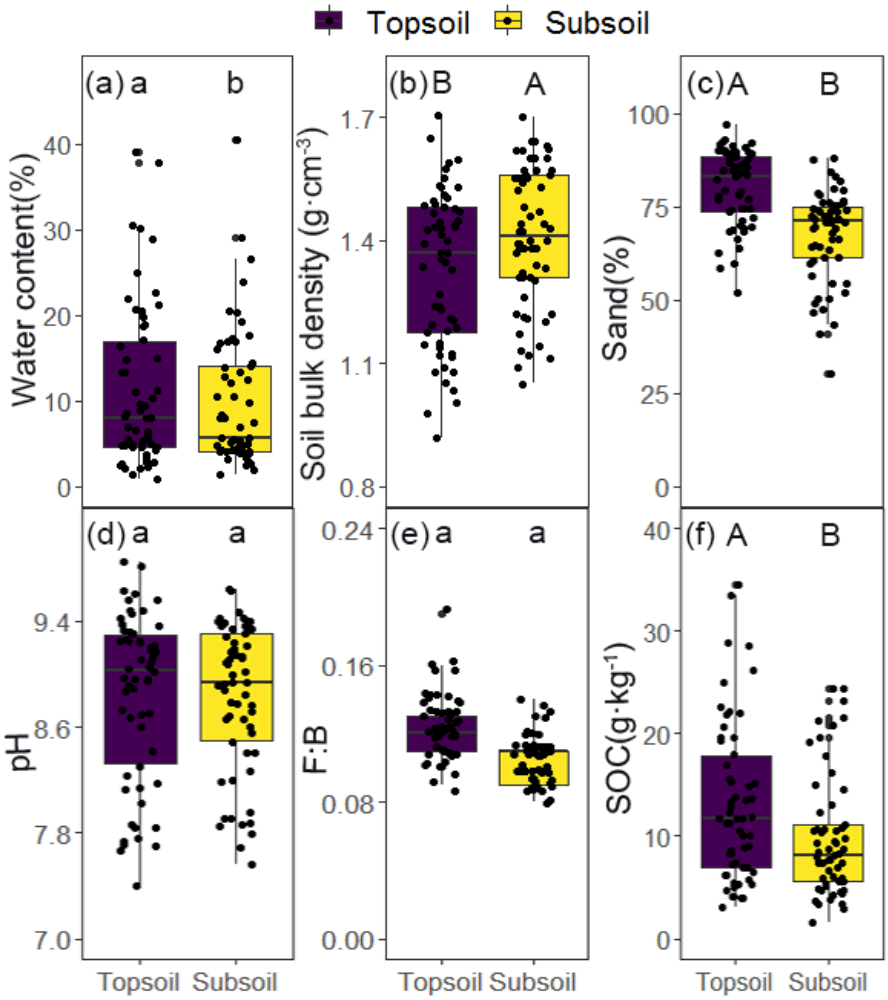
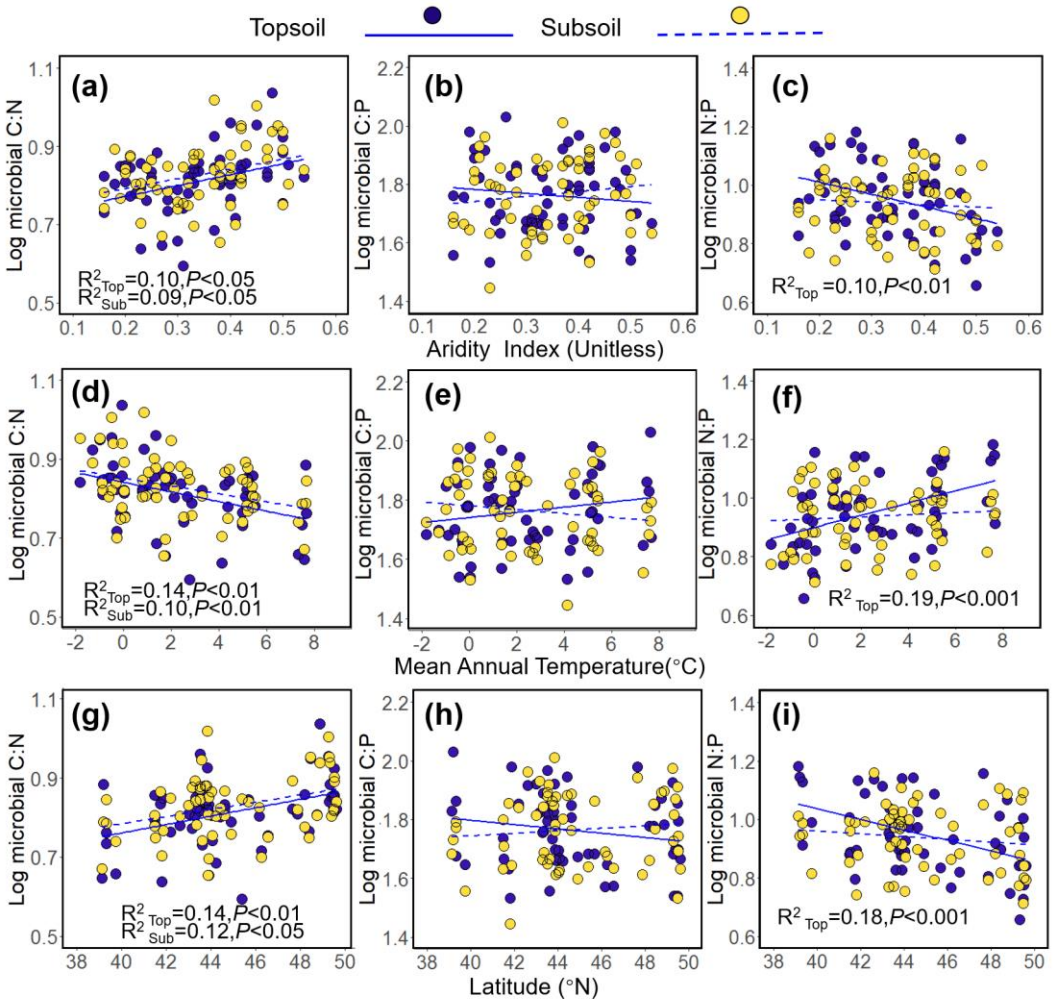


Figure 1. Basic characteristics of study sites across the Inner Mongolia grassland at different soil depths. Different letters indicate significant differences betwen soil depths on log10-transformed data (paired t-test, lowercase letter, $P<0.05$; uppercase letter, $P<0.001$)

Table 2. The microbial biomass C, N and P concentrations and microbial C:N:P stoichiometric ratios across the Inner Mongolian grassland at different soil depths.

Depth	MBC ($mmol \cdot kg^{-1}$)	MBN ($mmol \cdot kg^{-1}$)	MBP ($mmol \cdot kg^{-1}$)	Microbial biomass C:N	Microbial biomass C:P	Microbial biomass N:P
0-10 cm	21.8(18.5-25.1)A	3.23(2.80-3.67)A	0.38(0.32-0.44)A	6.59(6.26-6.91)a	60.2(55.6-64.8)a	9.29(8.0-9.97)a
10-20 cm	14.5(12.4-16.6)B	2.08(1.81-2.35)B	0.24(0.21-0.27)B	6.83(6.50-7.15)b	60.5(56.0-65.1)a	8.91(8.35-9.49)a

Note：Different letters indicate significant differences between soil depths based on log10-transformed data (paired t-test, lowercase letter, $P<0.05$; uppercase letter, $P<0.001$).

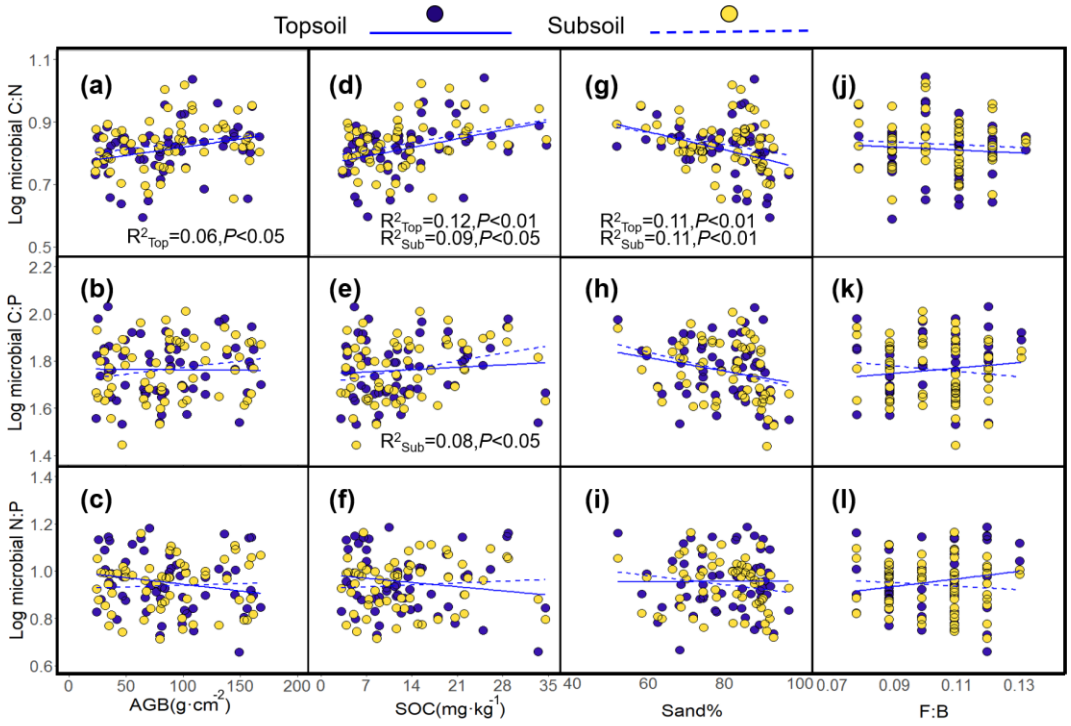


444

445 **Figure 2.** Relationships between the C:N, C:P and N:P ratios in soil microbial

446 biomass and aridity index (a-c), mean annual temperature (d-e) and latitude (g-i) in

447 the Inner Mongolian grassland.



448

449 **Figure 3.** Relationships between the C:N, C:P and N:P ratios in the soil microbial

450 biomass and AGB (a-c), SOC (d-f), sand percentage (g-i) and F:B ratio (j-l). AGB,

451 above ground biomass; SOC, soil organic carbon; F:B ratio, fungi to bacteria ratio.

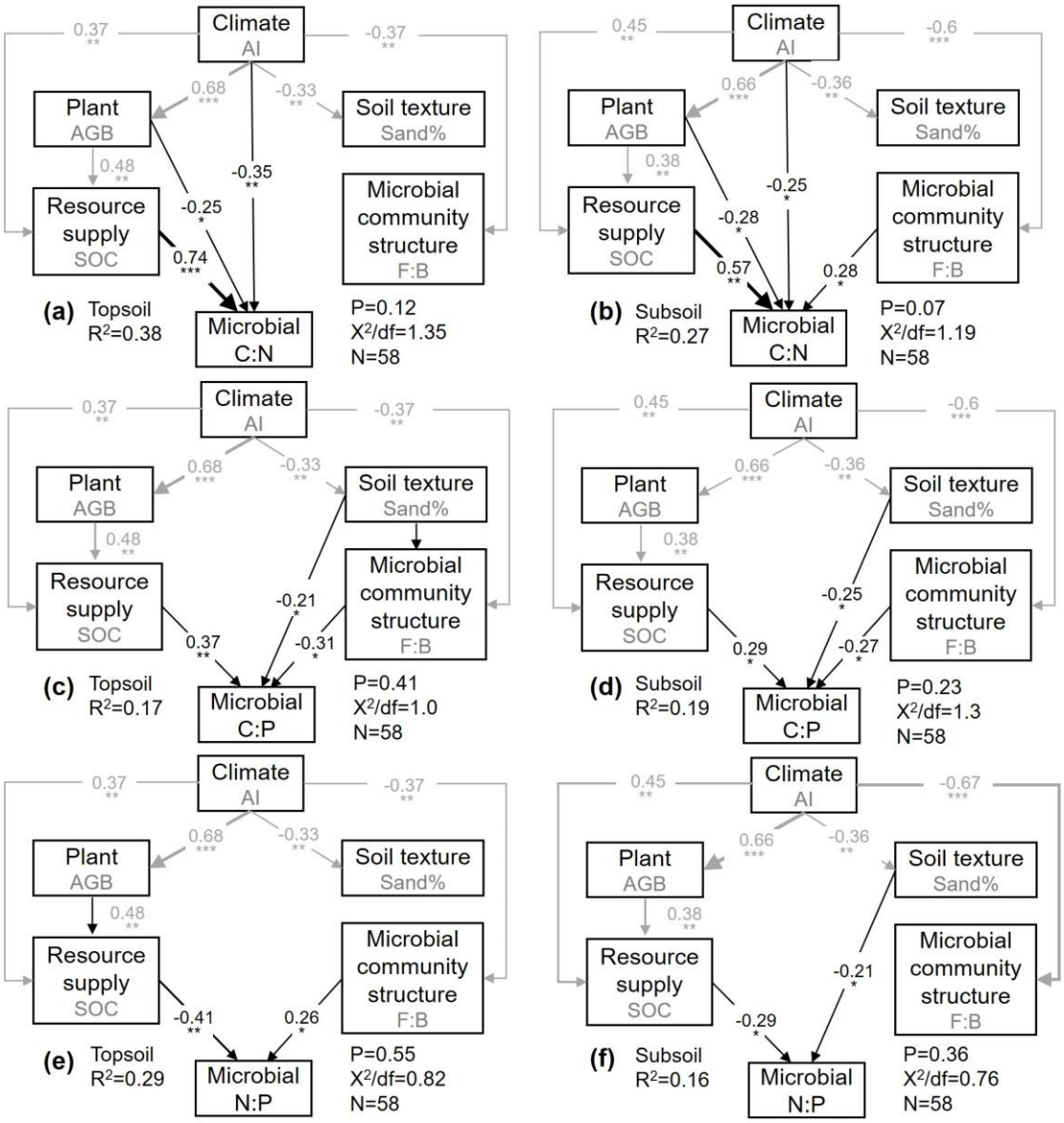
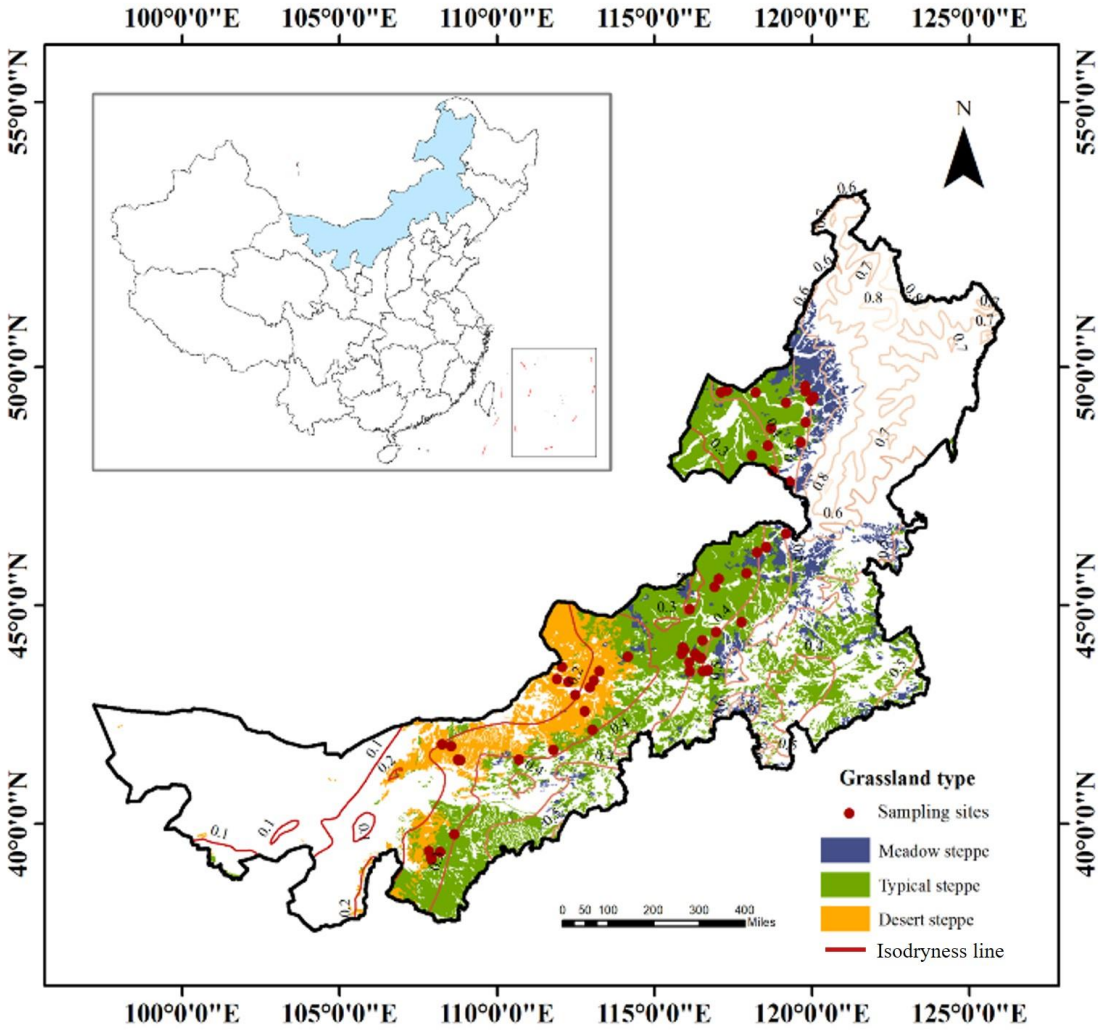


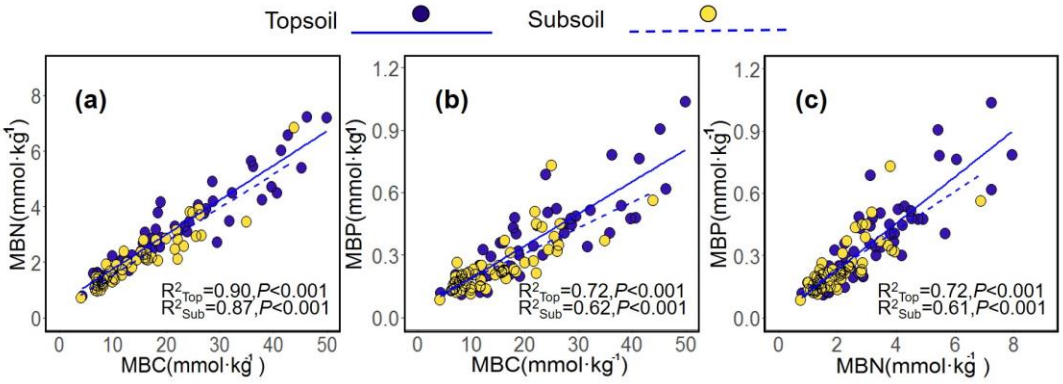
Figure 4. The structural equation model (SEM) shows the direct and indirect influences of various ecological factors on the microbial C:N (a,b), C:P (c,d) and N:P (e-f) ratios in the topsoil and subsoil. Black and gray arrows indicate direct and indirect pathways, respectively. Numbers on the arrows indicate standardized path coefficients, proportional to the arrow width. R^2 indicates the variation in the microbial C:N and C:P ratios explained by the model. *, P<0.05; **, P<0.01; ***, P<0.001.

459 **Appendix**



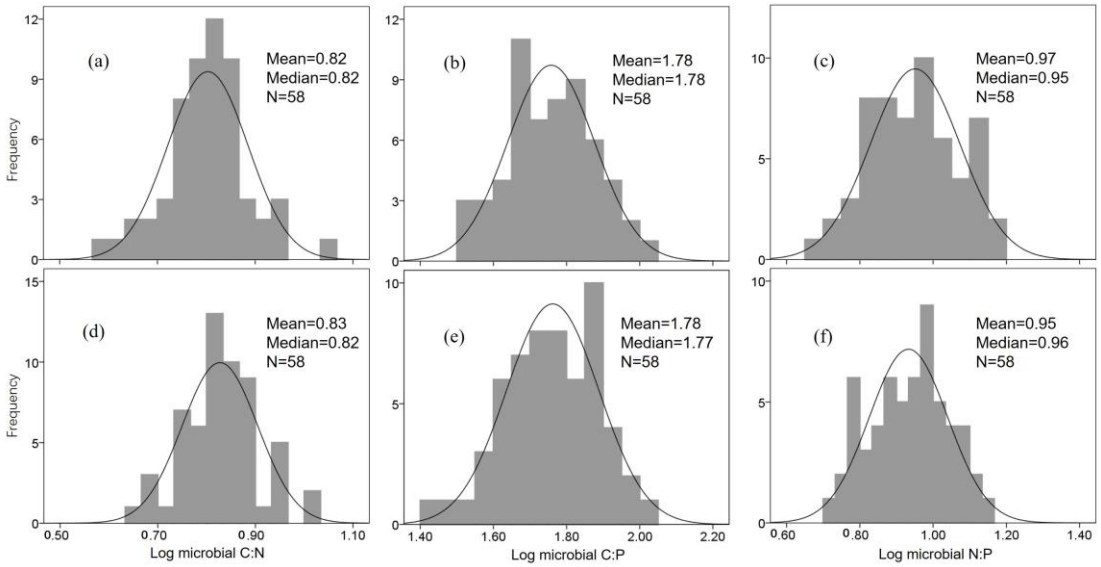
460

461 **Figure A1.** Geographic locations of the sampling sites in the Inner Mongolian grassland



462

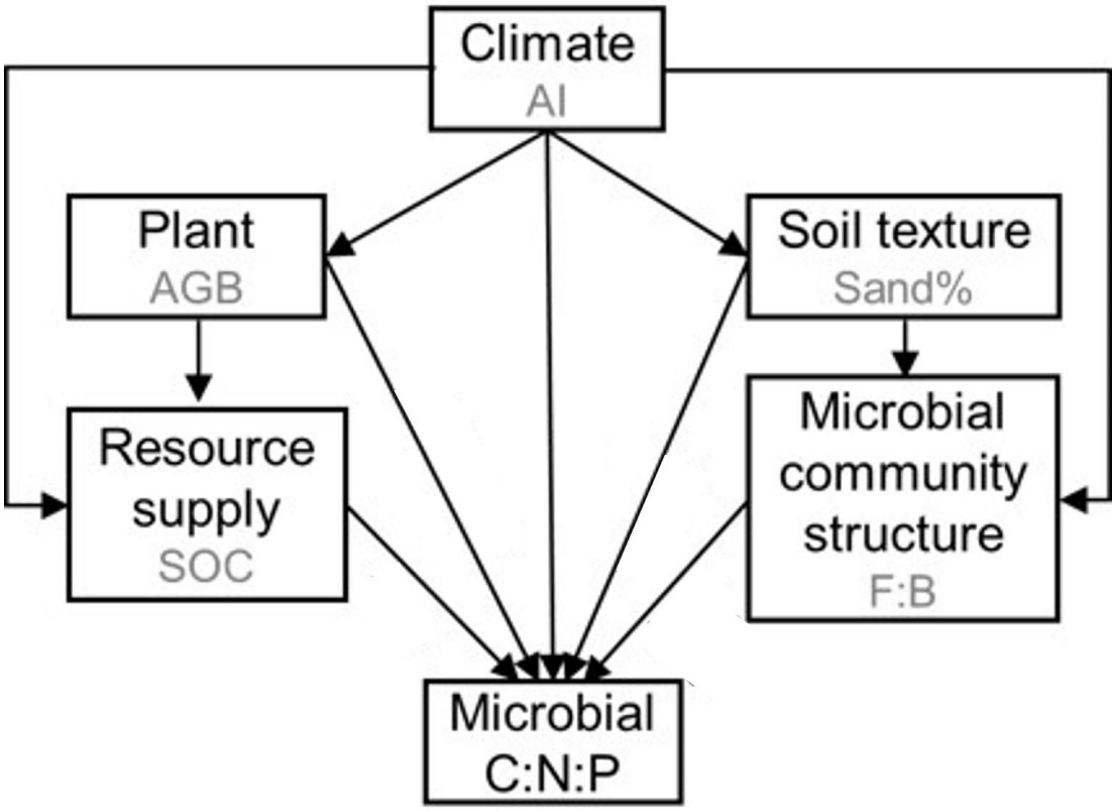
463 **Figure A2.** Relationships between the soil microbial biomass C, N and P concentrations



464

465 **Figure A3.** Histograms showing the frequency distributions of the soil microbial C:N,

466 C:P and N:P ratios in the topsoil (a-c) and the subsoil (d-f)



467

468 **Figure A4.** Hypothetical model showing how ecological factors affect microbial C:N:P

469 stoichiometry

470 **Table A1.** References to support the hypothetical models

Pathway	Interpretation	Reference
SOC → Microbial C:N:P	Influence of SOC on microbial C:N:P stoichiometry	(Hartman et al., 2013; Maria et al., 2014; Mooshammer et al.,2014)
AGB → Microbial C:N:P	Plant necromass represents the fundamental resource for microbes to maintain element balance	(Cleveland et al., 2007; Aponte et al., 2010; Manzoni et al., 2010; Li et al., 2012; Zechmeister-Boltenstern et al., 2016)
AI→Microbial C:N:P	Influence of increasing temperature on microbial C and N cycle	(Wang et al., 2014; Zechmeister-Boltenstern et al., 2016; Chen et al., 2016)

Sand percentage → Microbial C:N:P	Influence of soil texture associated water-holding capacity and nutrient availability on microbial C:N:P ratios	(Cleveland et al., 2007; Xu et al., 2013; Maria et al., 2014; Li et al., 2015; Zechmeister-Boltenstern et al., 2016)
F:B ratio→ Microbial C:N:P	Influence of a shift in the composition of microbial community on microbial C:N:P ratios	(Ross et al.,1993; Cleveland et al.,2007; Aponte et al., 2010; Tischer et al., 2014; Zechmeister-Boltenstern et al., 2016; Chen et al., 2016)

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