1   **A comparison of patterns of microbial C:N:P stoichiometry between**

2   **topsoil and subsoil along an aridity gradient**

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22 **Abstract**

23 Microbial stoichiometry and its potential driving factors play crucial roles in

24 understanding the balance of chemical elements in ecological interactions and nutrient

25 limitations along aridity gradient. However, little is known about the variation in these

26 features along aridity gradient due to the lack of comprehensive field investigations.

27 Additionally, previous studies focused on the topsoil (0-10 or 0-20 cm); however, the

28 minimum sampling depth for topsoil could impact the results of the vertical distribution

29 of microbial stoichiometry. In the present study, we measured the variation in microbial

30 stoichiometry, examined the major influential factors (climatic, edaphic and biotic

31 factors) along an aridity gradient and determined whether the sampling depth affected

32 microbial C:N:P stoichiometry. From the topsoil to the subsoil, the microbial C:N, C:P

33 and N:P ratios varied from 6.59 to 6.83, from 60.2 to 60.5 and from 9.29 to 8.91,

34 respectively. Only the microbial C:N ratio significantly increased with soil depth. The

35 microbial C:N ratio significantly increased with increasing aridity in both topsoil and

36 subsoil, while the microbial N:P ratio decreased along the aridity gradient only for the

37 topsoil. This result implied that drought stimulated microbes tend to be more N

38 conservative, especially those in topsoil. Among all the factors, the soil organic carbon

39 (SOC) content and the fungi to bacteria ratio exerted the largest influence on the

40 microbial C:N, C:P and N:P ratios at both soil depths, implying that the substrate supply

41 and microbial structure together controlled the microbial stoichiometry. The results also

42 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 in topsoil.

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

84   soil (Nielsen et al., 2009). Moreover, edaphic variables, such as SOC (Maria et al., 2014;

85   Chen et al., 2016) and soil texture (Li et al., 2015), could be associated with nutrient

86   mineralization and availability, thus influencing the C:N:P stoichiometry in microbial

87   biomass (Griffiths et al., 2012). A labeled incubation experiment showed that the

88   mineralization of organic P was mainly driven by the microbial C demands in P-poor

89   soils (Aponte et al., 2010; Heuck et al., 2015). In addition, microbial C:N, C:P and N:P

90   ratios were also affected by biotic factors such as plant productivity and the composition

91   of the microbial community (Fanin et al., 2013; Chen et al., 2016). Generally, fungi

92   exhibit a higher C:N ratio than bacteria (Strickland and Rousk, 2010); thus, a shift in

93   the fungi to bacteria ratio is expected to result in microbial stoichiometry changes (Li

94   et al., 2012; Heuck et al., 2015). However, those findings were based on literature

95   analyses or small-scale experiments, and the variations in microbial C:N, C:P and N:P

96   ratios at the regional scale have rarely been assessed systematically, and the drivers of

97   these variations need to be addressed more specifically with appropriate experimental

98   designs. Moreover, most research has focused on the top 10 cm of soil, which often has

99   high C availability and nutrient contents. It can be assumed that the effects of potential

100   driving factors exhibit minimal differentiation at deeper soil depths. However, soil at a

101   deeper depth might contain microbial communities that are specialized for their

102   environment, and their functions may differ from the functions of the communities in

103   the topsoil (Fritze et al., 2000; Blume et al., 2002). Certainly, the drivers that are

104   responsible for the variations in microbial C:N, C:P and N:P ratios in deeper soil remain

105 poorly understood. Such knowledge of the nature of soil microbial stoichiometry is

106 fundamental for understanding ecosystem function, especially at the 10-20 cm soil

107 depth, which remains highly uncertain in the published studies.

108 Substrates for microorganisms, such as available nutrients and water, decline

109 exponentially with depth, and the top 20 cm of soil accumulates the greatest amount of

110 microbial biomass, thereby attracting the attention of most researchers (Fierer et al.,

111 2003; Xu et al., 2013). Soil at a 0-20 cm depth was regarded as the topsoil in some

112 studies, while other researchers divided the soil from 0-20 cm into different soil depths

113 to explore the vertical differences between these depths (Aponte et al., 2010; Peng and

114 Wang, 2016). However, most studies used 0-10 cm as the topsoil to facilitate sampling

115 and comparative research (Li and Chen, 2004; Cleveland and Liptzin, 2007; Chen et

116 al., 2016). The depth of topsoil varies among studies, and sampling depth can therefore

117 have impacts on the study of the vertical patterns in soil microbial stoichiometry

118 (Tischer et al., 2014). Given that soil represents a highly heterogeneous environment,

119 especially in terms of site-specific soil development history, it is difficult to draw

120 general conclusions (Xu et al., 2013; Camenzind et al., 2018). In addition, if a large

121 difference existed between the soil at 0-10 cm and that at 10-20 cm, microbial

122 stoichiometry would be underestimated due to the ambiguous limitation of topsoil

123 (Tischer et al., 2014). To identify the soil depth that is appropriate for sampling and to

124 improve the understanding of topsoil research at a global scale, we designed a study

125 that divided the topsoil into 0-10 cm and 10-20 cm depths to compare the differences

126   in microbial stoichiometry at the regional scale.

127   In Inner Mongolia grasslands, the aridity exhibits a gradient that increases from

128   northeast to southwest (aridity index ranges from 0.16 to 0.54), and the vegetation types

129   vary from meadows to typical and desert steppes, thus providing an ideal platform to

130   better estimate the patterns and drivers of microbial C:N:P stoichiometry along an

131   aridity gradient (Chen et al., 2014; Li et al., 2017). Moreover, we aim to compare the

132   differences in microbial stoichiometry at two soil depths, especially in the topsoil, to

133   identify the optimal soil depth for vertical studies on microbial stoichiometry. In

134   particular, we tested the following hypotheses: (i) the microbial C:N and C:P ratios

135   increase and the microbial N:P ratio decreases with increasing aridity index. (ii) due to

136   variations in resource supply between topsoil and subsoil, the effects of potential

137   driving factors on microbial C:N, C:P and N:P ratios might decrease with soil depth.

138   Finally, (iii) the microbial C:N, C:P and N:P ratios vary between topsoil and subsoil,

139   and then depth of 10 cm as topsoil, which could influence the research on the vertical

140   patterns of microbial stoichiometry.

141   **2   Materials and methods**

142   **2.1   Study area**

143   This study was performed across the Inner Mongolian temperate grassland, which is a

144   central part of the Eurasian steppe. The study area is located at 39.2-49.6°N latitude and

145   107.8-120.1°E longitude and covers an area of 440,000 km^2. From northeast to

146   southwest, the mean annual temperature increases from -1.7 to 7.7°C, and the mean

147   annual precipitation decreases from 402 mm to 154 mm, approximately 80% of which

148   falls in the growing season from May to August (Chen et al., 2013). Three grassland

149   types, meadow steppe, typical steppe and desert steppe, are distributed along the

150   northeastern to southwestern gradient and are dominated by *Stipa baicalensis* and

151   *Leymus chinensis*, *S. grandis*, and *S. klemenzii*, respectively. The soil types

152   corresponding to the three grassland types are categorized as chernozems, kastanozems,

153   and calcisols, respectively, according to the soil classification system of the Food and

154   Agriculture Organization of the United Nations.

155   **2.2   Sampling and data collection**

156   Along this transect, a total of 58 sites that were slightly disturbed by humans and

157   domestic animals were sampled, including 10 in the meadow steppe, 28 in the typical

158   steppe, and 20 in the desert steppe (Fig. A1). Five 1×1 m^2 subplots were established,

159   one at each corner and one in the center of 10×10 m^2 plot. The plant community in the

160   subplots was identified, and the above-ground biomass (AGB) was harvested. At each

161   site, three replicate soil samples at depths of 0-10 cm and 10-20 cm were collected from

162   three 1×1 m subplots arranged diagonally in a 10×10 m plot. The samples were mixed

163   to form one composite sample. After gentle homogenization and removal of roots, the

164   soil was sieved through a 2-mm mesh and stored to conduct the further experiments.

165   The total carbon concentrations were measured using an elemental analyzer (Vario EL

166   Ⅲ, Elementar, Germany). The soil inorganic carbon content was determined with a

167   carbonate content analyzer (Eijelkamp 08.53, Netherlands). The SOC content was

168   calculated by subtracting the soil inorganic carbon from the total carbon. The soil

169   elemental contents were reported in mmol·kg^{-1}. Soil pH was measured in a suspension

170   with a soil:water ratio of 1:2.5. After the removal of organic matter and carbonates, the

171   soil texture was determined using a particle size analyzer (Malvern Masterizer 2000,

172   UK).

173   **2.3   Aridity index**

174   The aridity index was extracted from the Global Aridity Index (Global-Aridity) dataset,

175   which provides high-resolution (30 arc-seconds or ~ 1km at the equator) global raster

176   climate data for the period 1950-2000 (http://www.cgiarcsi.org) (Zomer, Trabucco,

177   Bossio, & Verchot, 2008). The specific calculation formula is as follows:

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

179   $$\text{PET} = 0.0023 \cdot \text{RA} \cdot (\text{Tmean} + 17.8) \cdot \text{TD}0.5 (\text{mm/month})$$

180   where MAP represents the mean annual precipitation, obtained from the WorldClim

181   Global Climate Data (Hijmans et al. 2005); MAE represents the mean annual potential

182   evapo-transpiration (PET); Tmean represents the monthly mean temperature, TD is

183   calculated as the difference between the monthly maximum and minimum temperatures;

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

185   **2.4   Soil microbial analyses**

186 Microbial biomass carbon (MBC) and microbial biomass nitrogen (MBN) were

187 determined following the chloroform fumigation-K_2SO_4 extraction method, according

188 to Vance et al. (1987) and Wu et al. (1990). The soil was preincubated at 25°C for two

189 weeks at a field water capacity of 40%. Then, the soil was fumigated with chloroform

190 for 24 h in a vacuum. The fumigated and nonfumigated samples were extracted using

191 0.5 M K_2SO_4 with a soil:solution mass ratio of 1:4. The C and N contents were

192 measured with a multi N/C analyzer (Anaytik Jena, Germany). Using a universal

193 conversion factor of 0.45 (Jenkinson et al., 1976), the amounts of MBC and MBN were

194 calculated by subtracting the amounts of extractable C and N in the nonfumigated

195 samples from those in the fumigated samples (Vance et al., 1987; Wu and Joergensen

196 et al., 1990; Joergensen et al., 1996). Microbial biomass phosphorus (MBP) was

197 estimated according to the method described in Hedley and Stewart (1982) and

198 modified by Wu et al. (1990). The fumigation procedure was the same as that for MBC

199 and MBN. The fumigated and nonfumigated samples were extracted using 0.5 mol·L^{-1}

200 NaHCO$_3$ and were analyzed to determine the total phosphorus concentration using a

201 colorimetric method. Using a universal conversion factor of 0.40, the amount of MBP

202 was calculated by subtracting the amount of extractable P in the nonfumigated samples

203 from that in the fumigated samples (Hedley and Stewart.,1982). Phospholipid fatty

204 acids (PLFAs) were extracted from the soil using the method described by Bossio and

205 Scow (1998). Briefly, 8 g of soil (dry weight) was used for PLFA analysis. The resultant

206 fatty acid methyl esters were separated, quantified, and identified using capillary gas

207 chromatography.The following PLFAs were used as markers for each of the specific

208 groups: for fungi, 18:1ω9c, 18:2ω6c, 18:3ω6c; for bacteria, i13:0, a13:0, i14:0, i15:0,

209 a15:0, 15:1ω6, 2OH16:0, i16:0, 16:1ω7c, 16:1ω9c, a17:0, i17:0, 17:1ω8c, cy17:0, i18:0,

210 18:1ω7, 18:1ω5 and cy19:0.

211 **2.5   Statistical analyses**

212 The C:N, C:P and N:P ratios in the soil microbial biomass were log10 transformed

213 before analysis to improve their normality (Fig. A3). Paired samples t-tests were used

214 to determine the differences in the soil microbial biomass C, N and P between the

215 topsoil and subsoil and the differences in the C:N:P stoichiometry ratios in the soil

216 microbial biomass. Ordinary least squares regression analyses were conducted to

217 evaluate the relationship between the C:N:P ratios in the soil microbial biomass and

218 latitude, aridity index, AGB, SOC, sand percentage and fungi to bacteria ratio (F:B

219 ratio). The analyses were performed with SPSS 19.0 software (IBM Corporation,

220 Armonk, NY, USA). A structural equation model (SEM) was used to test the

221 multivariate effects (direct and indirect) on the C:N:P ratios in the microbial biomass

222 through hypothetical factor pathways (Fig. A4). The SEM was constructed using the

223 Amos 17.0 software package (Smallwaters Corporation, Chicago, IL, USA).

224 **3   Results**

225 **3.1   The variation in microbial C:N:P stoichiometry between soil depths along**

226 **the environmental gradient**

227   The results indicate well-constrained relationships among C, N and P in soil microbial

228   biomass (Fig. A2). The soil microbial biomass C:N, C:P and N:P ratios varied by an

229   order of magnitude. Significantly different water content, soil bulk density, sand

230   percentages and SOC content were found between soil depths ($P < 0.05$, Fig. 1a, 1b, 1c,

231   1f). The microbial biomass C, N and P concentrations in the topsoil were significantly

232   higher than that in the subsoil ($P < 0.05$, Table. 2). The C:N, C:P and N:P ratios in the

233   microbial biomass of the topsoil were 6.59, 60.2, and 9.29, respectively, while those

234   values in the subsoil were 6.83, 60.5 and 8.91, respectively (Table. 2). Moreover, the

235   microbial C:N ratio in the subsoil was significantly higher than that those in the topsoil

236   (Table. 2).

237   Significant positive relationships were found between the microbial C:N ratio and

238   latitude (Subsoil, $R^2 = 0.12$, $P < 0.05$, Fig. 2a), while a negative relationship was found

239   between the microbial N:P ratio and latitude (Topsoil, $R^2 = 0.18$, $P < 0.001$; Fig. 2c). In

240   addition, the decreasing trend was found between the microbial C:N ratio and MAT

241   (Topsoil, $R^2 = 0.14$, $P < 0.01$; Subsoil, $R^2 = 0.10$, $P < 0.01$, Fig. 2d), while a significant

242   negative relationship was found between the microbial N:P ratio and MAT (Topsoil,

243    $R^2 = 0.19$, $P < 0.001$; Fig. 2f). The results revealed a significant positive relationship

244   between the AI and the microbial C:N ratio (Topsoil, $R^2 = 0.10$, $P < 0.05$; Subsoil, R^2

245    $= 0.09$, $P < 0.05$, Fig. 2d) and significant negative relationships between the AI and the

246   microbial N:P ratio (Topsoil, $R^2 = 0.10$, $P < 0.05$; Fig. 2f). The microbial C:N ratio was

247   positively related to AGB (Topsoil, $R^2 = 0.06$, $P < 0.05$, Fig. 3a), SOC (Topsoil, $R^2 = 0.12$,

248   *P*< 0.01; Subsoil, R^2 =0.09, *P*< 0.05, Fig. 3d) and was negatively related to the sand

249   percentage (Topsoil, R^2 =0.11, *P*< 0.01; Subsoil, R^2 =0.11, *P*< 0.01, Fig. 3g). A

250   significant positive relationship was found between the microbial C:P ratio and the

251   content of soil organic matter (Subsoil, R^2 =0.08, *P*< 0.05, Fig. 3e). No or only weak

252   association was found between the microbial C:N, C:P and N:P ratios and the AGB and

253   F:B ratio in the subsoil (Fig. 3).

254   **3.2 Effects of potential driving factors on the microbial C:N:P stoichiometry at**

255   **topsoil and subsoil**

256   The final SEM adequately fit the data, as shown by several robust goodness-of-fit

257   measures (P value and minimum discrepancy). The model explained 38% (topsoil) and

258   27% (subsoil) of the variation in the microbial C:N ratio, 17% and 19% of that in the

259   microbial C:P ratio, and 29% and 16% of that in the microbial N:P ratio (Fig. 4a, b, c,

260   d, e, f). Effects of AI, AGB and SOC contenton the microbial C:N ratio were found at

261   both soil depths (Fig. 4a, 4b). The SOC content made the largest positive contribution

262   to the variation in the microbial C:N ratio in the topsoil (Fig. 4a, 4b). We found direct

263   effects of the sand percentage, SOC content and F:B ratio on the microbial C:P ratio at

264   both soil depths, and the SOC content made the largest contribution to the variation in

265   the microbial C:P ratio in the topsoil, which was higher than that in the subsoil (Fig. 4e,

266   4f). Influences of sand% and the SOC content on the microbial N:P ratio were found in

267   the topsoil, while the F:B ratio and the SOC content explained most of the variation in

268   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 latitude and the aridity gradient

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 Syers, 1976; Vitousek et al., 2010). 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).

289   As stated in our hypothesis, we observed that the microbial C:N ratio significantly

290   increased with increasing aridity index, while the microbial N:P ratio decreased with

291   increasing aridity index, indicating that drought (decreasing aridity index) affects

292   ecological stoichiometry by mediating the growth rate of microorganisms in semiarid

293   regions (Elser et al., 2000b; Peng and Wang, 2016). Dan and Wang noted that

294   increasing aridity reduced the soil microbial abundance in drylands, and a decreased

295   growth rate in dry areas might result in decreased allocation to P-rich ribosomal RNA

296   (and thus higher C:P and N:P ratios) (Wang et al., 2014; Maestre et al., 2015).

297   Additionally, microbial C:N ratio decreased with the decreasing aridity index, which

298   serves as a protective mechanism as microbes decrease their nitrogen use efficiency

299   (NUE, the ratio of N invested in growth over total N uptake) and tend to be more N

300   conservative under drier climatic conditions (Mooshammer et al., 2014; Delgado-

301   Baquerizo et al., 2017). Moreover, under drier climate conditions, the soil microbial

302   communities shift from acting as r-strategists (fast-growing copiotrophs) to acting as

303   K-strategists (slow-growing oligotrophs), as microorganisms with K-strategies have

304   lower nutrient demands (N and P) and growth rates, invest more nutrients into

305   extracellular enzymes to gain limited nutrients and thus have higher cellular C:N:P

306   ratios than r-strategists (Fierer et al., 2007; Fierer et al., 2010). Our study further

307   illustrated the latitudinal pattern of microbial stoichiometry and first attempted to

308   examine the variation in microbial stoichiometry along an aridity gradient at the

309   regional scale.

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

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

SEM also illustrated that the microbial community structure is an important feature in determining microbial stoichiometry. The F:B ratio has recently been found to have a 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

331   deeper soil depths (Tischer et al., 2014). Overall, the SEM highlighted the important

332   role of the C availability and microbial community structure in driving the variations in

333   microbial C:N, C:P and N:P ratios at both soil depths.

334   Moreover, AGB and AI also exerted direct influences on microbial C:N or C:P ratios,

335   and those impacts mainly acted in the topsoil but were weaker in the subsoil, supporting

336   our hypothesis. The climate imposes important controls on both the plant community

337   and the microbial taxa along with their interactions with soil nutrients (Chadwick et al.

338   1999; Vitousek 2004; Reich and Oleksyn, 2004). In particular, drier weather condition,

339   and the decreasing aridity index, could affect the growth and productivity of plants, and

340   then shape a shift in vegetation types along this grassland transect (Jaleel et al., 2009;

341   Cherwin & Knapp, 2012). At the same time, the meadow steppe ecosystem with high

342   productivity maintained relatively high soil C and N contents, which resulted in high

343   C:P and N:P ratios in these regions; thus, plant productivity exerted a positive influence

344   on microbial C:N (Aponte et al., 2010; Manzoni et al., 2010). Because of the vertical

345   distribution of the influence of the AI, the effect decreased with soil depth.

346   Interestingly, our results revealed that the microbial N:P ratio was mainly impacted by

347   the F:B ratio and SOC, while the sand percentage and SOC had direct negative effects

348   on the ratio in the subsoil, suggesting the flexibility of microbial stoichiometry in

349   response to distinct resource supplies between topsoil and subsoil (Peng and Wang,

350   2016). The soil depth affected the microbial biomass N and P, which decreased nearly

351   twofold from the topsoil to the subsoil (Table 2). However, the results showed that the

352   N and P cycles responded asymmetrically to soil depth, which might be attributed to

353   the high variability in P availability (Li et al., 2015; Zechmeister-Boltenstern et al.,

354   2016). Generally, P is mostly derived from parent material, while N is mainly a

355   biological element (Vitousek and Farrington, 1997; Vitousek et al., 2010). Therefore,

356   it is believed that P variations regulate large-scale patterns in microbial N:P

357   stoichiometry and nutrient-use strategies (Heuck et al., 2015; Camenzind et al., 2018).

358   With a high proportion of sand, the soil becomes porous, which may lead to increased

359   leaching of available P to deeper soil depths (Otten et al., 1999; Achbergerová and

360   Nahálka, 2011). Similarly, P leaching caused by weathering led to a shift in the N:P

361   ratio in the soil, and a vertical study found a high variation in the N:P ratio between soil

362   depths across a large scale (Tian et al., 2010). The high variability of the N:P ratio in

363   soil and soil microbial biomass therefore indicates that the N:P ratio could be an

364   indicator of the ecosystem nutrient status at deeper soil depths (Cleveland et al., 2007;

365   Chen et al. 2013; Li, et al. 2015).”.

366   **4.3   How deep should we dig to evaluate the topsoil the microbial stoichiometry**

367   **in vertical study?**

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

369   between topsoil and subsoil, suggesting that the resource supplies between topsoil and

370   subsoil were significantly different. We also observed that the microbial C:N, C:P and

371   N:P ratios varied between topsoil and subsoil, and significant difference was found in

372   microbial C:N ratio. Those results indicated that the flexibility of the microbial

373   stoichiometry responds to different resource supplies between soil depths (Tian et al.,

374   2010; Peng and Wang, 2016). Similar findings were found in the top 16 cm of soil in a

375   Mediterranean oak forest (0-8 cm and 8-16 cm), where the microbial nutrient ratios

376   (C:N, C:P and N:P) varied between soil depths (Aponte et al., 2010). Tischer et al.

377   (2014) sampled the top 20 cm of soil (0-5 cm, 5-10 cm, 10-20 cm) and observed that

378   the microbial C:N ratio changed with soil depth. Moreover, sampling to a depth of 10

379   cm showed a significant difference in the microbial N:P ratio (Tischer et al., 2014). The

380   detection of the differences in the microbial N:P ratio in our study depended strongly

381   on the sampling depth, suggesting that the microbial N:P ratio might provide insight

382   into the nature of ecosystem nutrient limitations in a vertical study (Cleveland and

383   Liptzin, 2007; Fierer et al., 2010). In addition, SEM also showed that the microbial N:P

384   ratio was controlled by multiple potential driving factors at different soil depths,

385   indicating that a 0-10 cm or shallower sampling interval should be used when studying

386   the vertical patterns in the microbial N:P ratio.

387   **5   Uncertainties and perspectives**

388   The first uncertainty was related to the determination of fungal and bacterial biomasses

389   by PLFA markers, which have limited targets for fungi and bacteria. This uncertainty

390   should be noted when interpreting the results in the present study. Methodological

391   advances in sequencing approaches might be used to more accurately index the

392   microbial community and reveal insights into the regulation of microbial C:N:P

393   stoichiometry in distinct soil microbial taxa or functional groups. Second, the theory

394 used to construct the model is another source of uncertainty; the theory related to the

395 drivers of microbial stoichiometry used in this study was mostly derived from a

396 literature review and summarized data. In future research, more control experiments

397 with the manipulation of C availability, especially at deeper soil depths, would further

398 improve our understanding of the changes in microbial stoichiometry and nutrient

399 limitations under the impacts of global change.

400 **6 Conclusion**

401 The ratios of C, N, and P in the microbial biomass were 6.59:60.2:9.29 in the topsoil,

402 which deviated from the 6.83:60.5:8.91 ratio in the subsoil. Moreover, significant

403 differences were found in the microbial C:N and N:P ratios between topsoil and subsoil,

404 indicating that the flexibility of microbial stoichiometry should be considered for

405 vertical study. In addition, The trend of microbial C:N ratio increasing with aridity

406 index, consistent with the perspective that microbes mediate their nitrogen use

407 efficiency and tend to be more N conservative under drier climatic conditions. The

408 microbial N:P ratio trend along the aridity gradient was consistent with the growth rate

409 hypothesis that a decreased growth rate in dry areas results in decreased allocation to

410 P-rich ribosomal RNA and thus a higher N:P ratio. These findings confirmed the

411 importance of SOC, the microbial structure and soil texture in shaping the pattern of

412 microbial stoichiometry in semiarid grassland systems. The influence of ecological

413 factors decreased from topsoil to subsoil, as well as the decline in climatic, edaphic and

414 biotic factors. Overall, these results illustrated N and P limitation in microbial biomass

415   at deeper soil depths along aridity gradient and limited responses to ecological factors

416   in the subsoil.

417   *Author contributions*. HH. WM and YY devised the study. YL carried out the

418   experiment and data analyses. DK, YC and DC assisted with the data analyses and

419   interpretation. XN, TW, XZ, MZ and HB assisted with the experiment. All authors

420   contributed to the preparation of the paper.

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

422   *Acknowledgments*. We thank the sampling team members of Institute of Botany,

423   Chinese Academy of Science, for assistance with the field data collection.

424   *Financial support*. This work was supported by the National Basic Research Program

425   of China (2015CB954201 and 2014CB954303) and National Natural Science

426   Foundation of China (31470498)

427 **Table 1.** Basic information of study sites

Biome	Latitude (°N)	Longitude (°E)	MAP (mm)	MAT (°C)	Aridity Index	AGB (g·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*

428 Note: Data represent the means, with minimum and maximum values in parentheses. MAT, mean annual temperature; MAP, mean annual

429 precipitation; AGB, above ground biomass.

430

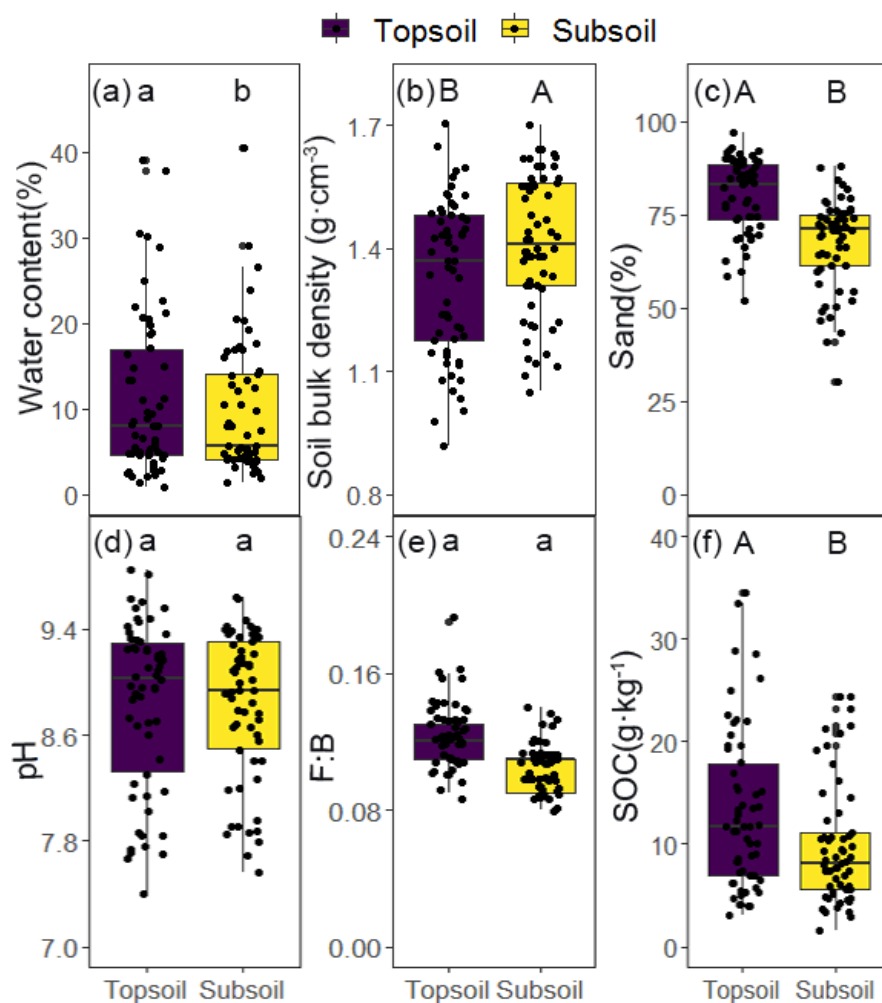



Figure 1. Basic characteristics of study sites across the Inner Mongolia grassland at different soil depths. Different letters indicate significant differences betwen soil depths on log10-transformed data (paired t-test, lowercase letter, *P*<0.05; uppercase letter, *P*<0.001)

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

436 different soil depths.

Depth	MBC (mmol·kg^{-1})	MBN (mmol·kg^{-1})	MBP (mmol·kg^{-1})	Microbial biomass		
				C:N	C:P	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

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

440 uppercase letter, $P<0.001$).

441

442

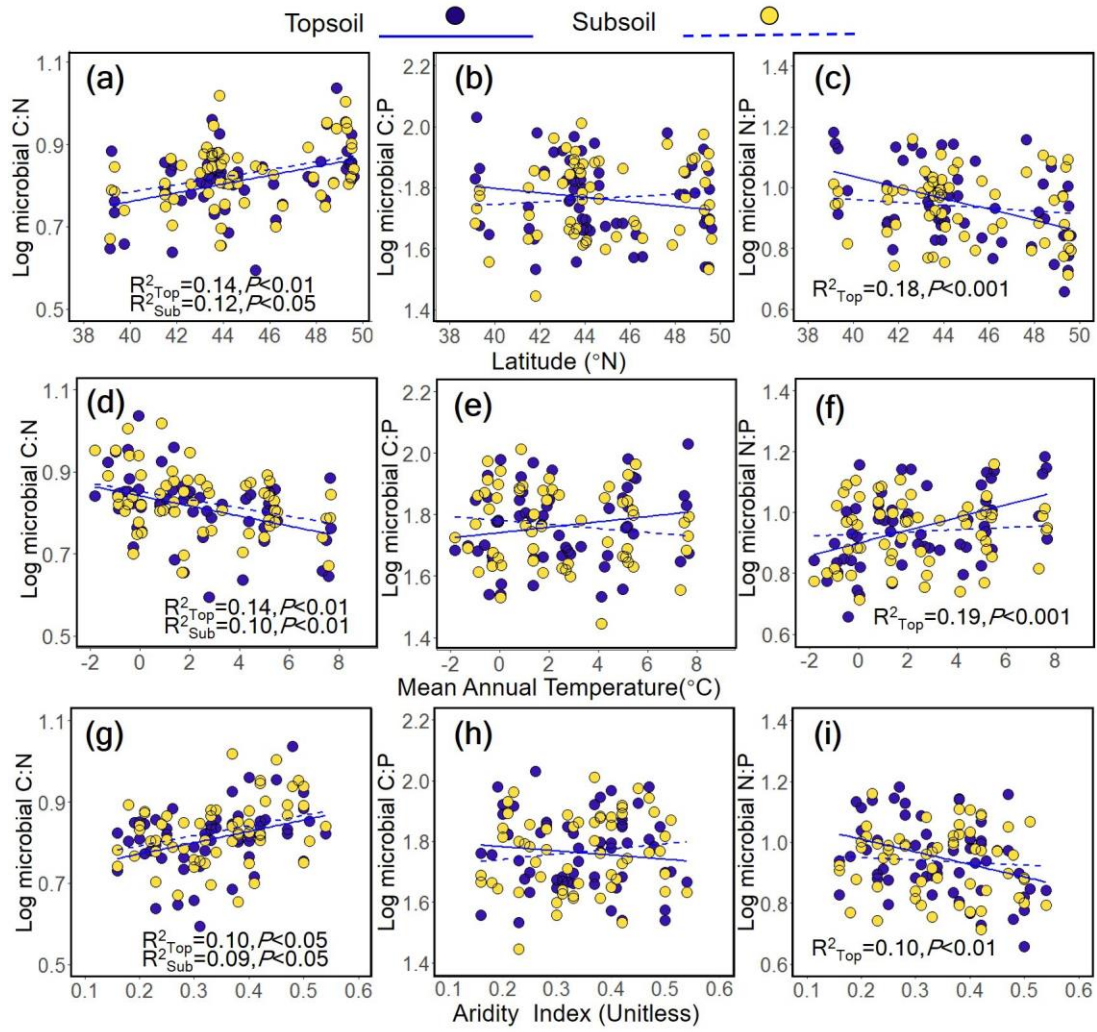



Figure 2. Relationships between the C:N, C:P and N:P ratios in soil microbial

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

the Inner Mongolian grassland.

446

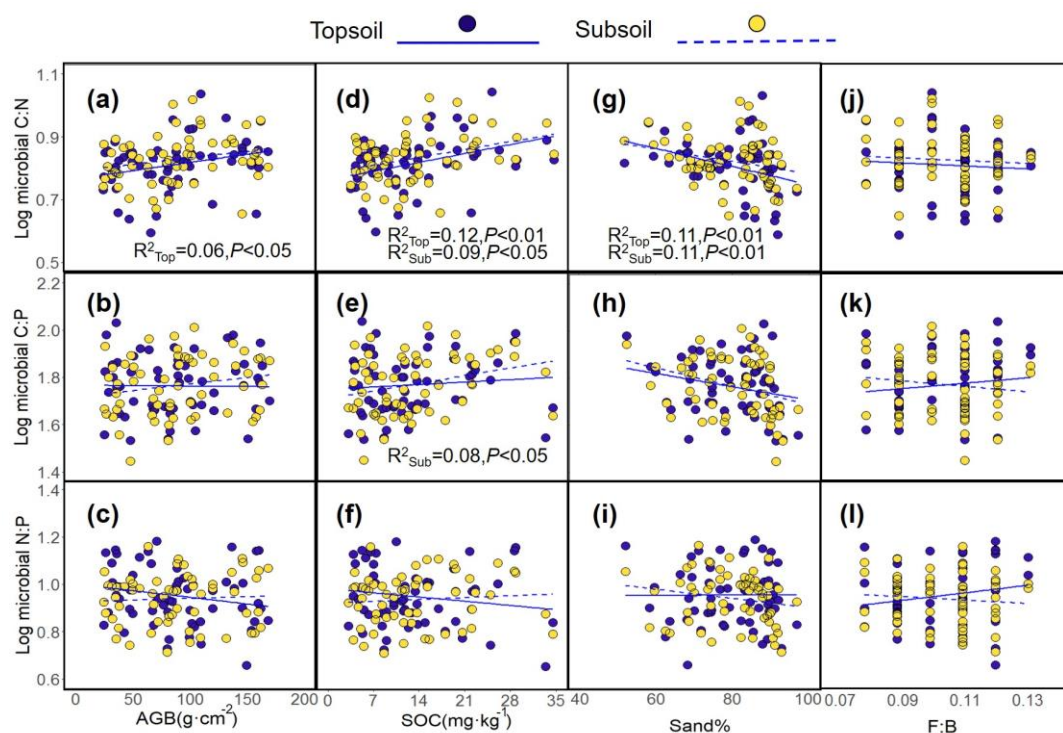



Figure 3. Relationships between the C:N, C:P and N:P ratios in the soil microbial

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

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

450

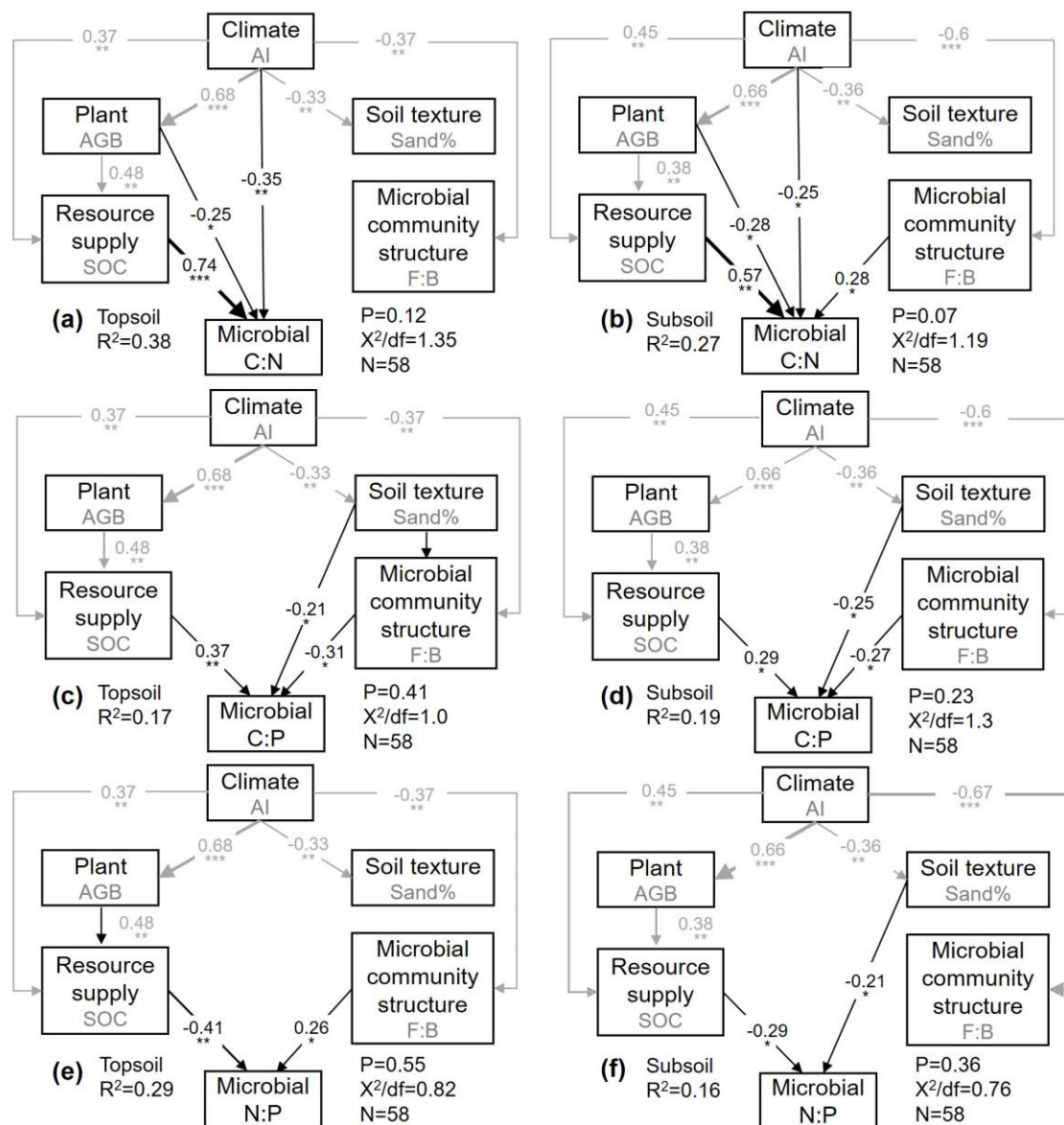
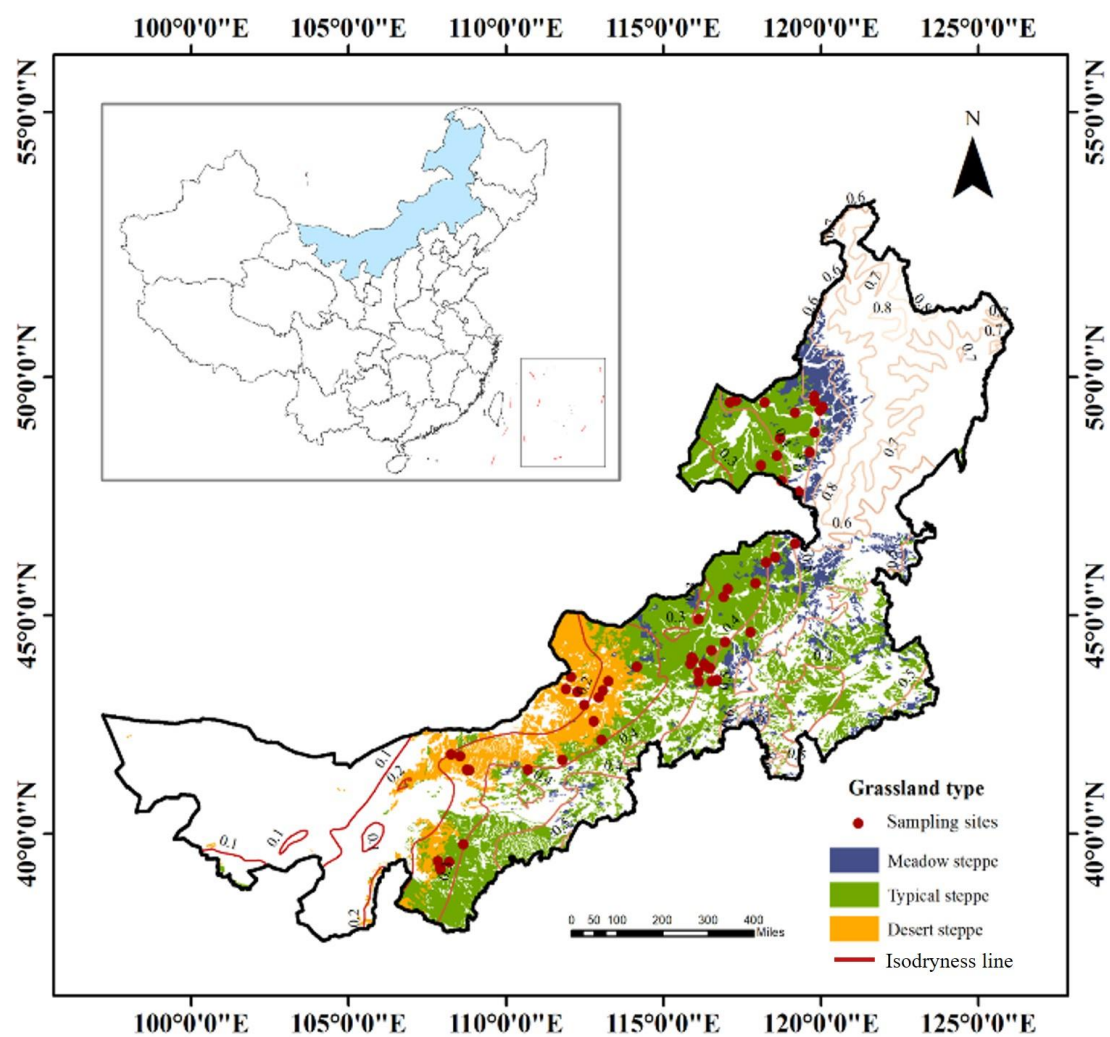
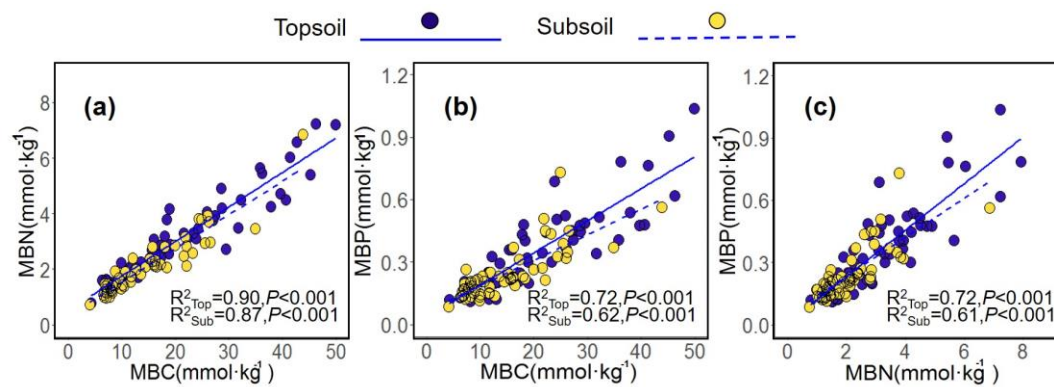
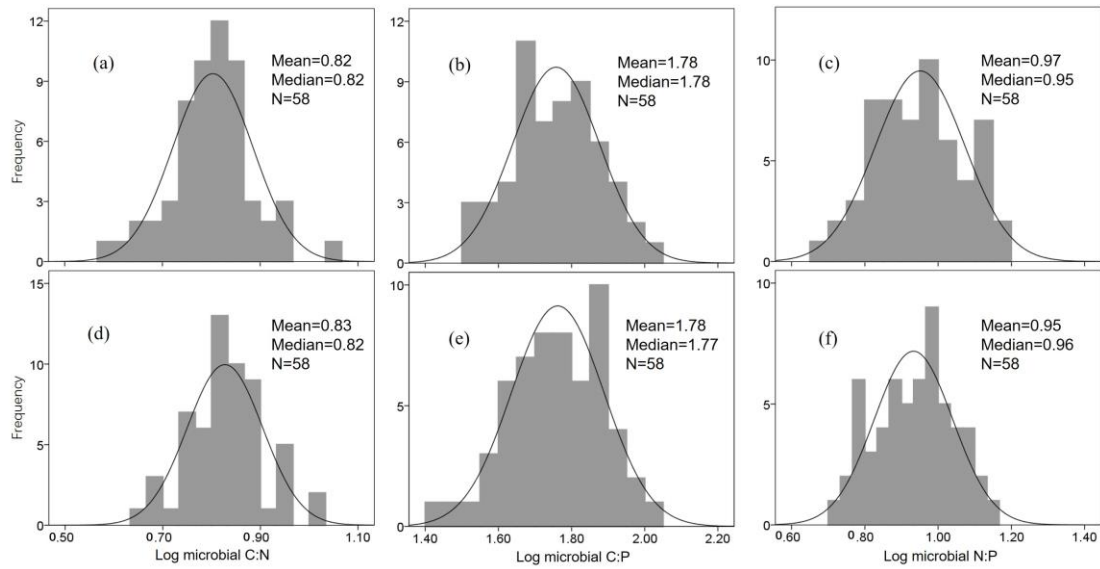



Figure 4. The structural equation model (SEM) shows the direct and indirect influences of various ecological factors on the microbial C:N (a,b), C:P (c,d) and N:P (e-f) ratios in the topsoil and subsoil. Black and gray arrows indicate direct and indirect pathways, respectively. Numbers on the arrows 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. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

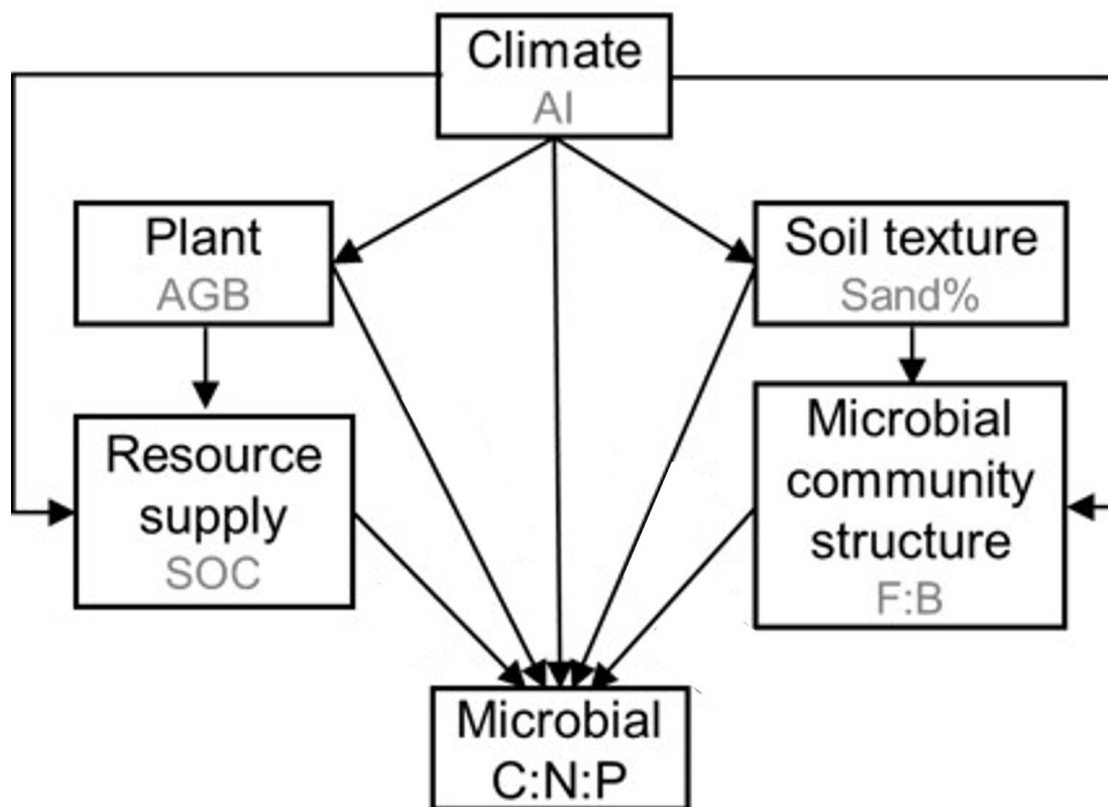
457 **Appendix**

458

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

460

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

462

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

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

465

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

467 stoichiometry

468 **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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