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

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

3 Yuqing Liu[1,2,a], Wenhong Ma[3,a], Dan Kou[2,4], Xiaxia Niu[2,3], Tian Wang[2,4], Yongliang

4 Chen[5], Dima Chen[2], Xiaoqin Zhu[2], Mengying Zhao[2,4], Baihui Hao[2,3], Jinbo Zhang[1],

5 Yuanhe Yang[2], Huifeng Hu[2] [*]

6

7 [1] School of Geography Science, Nanjing Normal University, Nanjing 210023, China

8

9 [2] State Key Laboratory of Vegetation and Environmental Change, Institute of Botany,

10 Chinese Academy of Sciences, Beijing 100093, China

11

12 [3] Department of Ecology, School of Ecology and Environment, Inner Mongolia

13 University, Hohhot 010021, China

14

15 [4] University of Chinese Academy of Sciences, Beijing 100049, China

16

17 [5] China Agricultural University, Beijing, 100083, China.

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19 [a]These authors contributed equally to this paper.

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21

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

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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

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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, calculated as precipitation/potential

129   evapotranspiration, ranges from 0.16 to 0.54), thus providing an ideal platform to better

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

131   gradient (Chen et al., 2014; Li et al., 2017). In this study, we aim to access the effect of

132   soil depth on soil microbial C:N:P stoichiometry along aridity gradient. We

133   hypothesized that the microbial C:N and C:P ratios decrease and the microbial N:P ratio

134   increases with temperature (Cleveland and Liptzin, 2007; Li et al., 2015; Xu et al.,

135   2013), and the microbial C:N and C:P ratios decrease and the microbial N:P ratio

136   increases with decreasing aridity index (Wang et al., 2014; Li et al., 2017). In addition,

137   the identification of soil depth for vertical study is differernt in some published

138   literature (Li and Chen, 2004; Aponte et al., 2010; Tischer et al., 2014; Peng and Wang,

139   2016). We predicted that variation of bacterial and fungal taxa between soil depths

140   might contribute to the shifts in C:N:P stoichiometry, especially in the N:P ratio

141   (Mouginot et al., 2014; Camenzind et al., 2018). Therefore, we focus on (i) the effects

142   of potential driving factors on microbial C:N, C:P and N:P ratios in topsoil and subsoil

143   (ii) the response of the microbial C:N, C:P and N:P ratios to soil depth.

144   **2   Materials and methods**

145   **2.1   Study area**

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

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

148   107.8-120.1°E longitude and covers an area of 440,000 km^2. From northeast to

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

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

151   falls in the growing season from May to August . Three grassland types, meadow steppe,

152   typical steppe and desert steppe, are distributed along the northeastern to southwestern

153   gradient and are dominated by *Stipa baicalensis* and *Leymus chinensis*, *S. grandis*, and

154   *S. klemenzii*, respectively. The soil types corresponding to the three grassland types are

155   categorized as chernozems, kastanozems, and calcisols, respectively, according to the

156   soil classification system of the Food and Agriculture Organization of the United

157   Nations.

158   **2.2   Sampling and data collection**

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

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

161   steppe, and 20 in the desert steppe (Fig. S1). Five 1×1 m^2 subplots were established,

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

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

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

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

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

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

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

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

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

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

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

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

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

175 UK).

176 **2.3   Aridity index**

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

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

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

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

181 $$\text{Aridity Index (AI)} = \text{MAP} / \text{MAE}$$

182 $$\text{PET} = 0.0023 \cdot \text{RA} \cdot (\text{Tmean}+17.8) \cdot \text{TD}^{0.5}(\text{mm/month})$$

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

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

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

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

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

188   **2.4 Soil microbial analyses**

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

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

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

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

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

194   0.5 M K_2SO_4 with a soil:solution mass ratio of 1:4. The C and N contents were

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

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

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

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

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

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

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

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

203    $NaHCO_3$ and were analyzed to determine the total phosphorus concentration using a

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

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

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

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

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

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

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

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

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

213   18:1ω7, 18:1ω5 and cy19:0.

214   **2.5   Statistical analyses**

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

216   before analysis to improve their normality (Fig. S3). Paired samples t-tests were used

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

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

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

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

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

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

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

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

225   through hypothetical factor pathways (Fig. S4). The SEM was constructed using the

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

3   Results

228   **3.1   The variation in microbial C:N:P stoichiometry between soil depths along**

229   **the environmental gradient**

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

231   biomass (Fig. S2). The soil microbial biomass C:N, C:P and N:P ratios varied by an

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

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

234   1f). The microbial biomass C, N and P concentrations in the topsoil were significantly

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

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

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

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

239   (Table. 2).

240   The results revealed a significant positive relationship between the AI and the microbial

241   C:N ratio (Topsoil, $R^2 =0.10$, $P< 0.05$; Subsoil, $R^2 =0.09$, $P< 0.05$, Fig. 2a) and the

242   decreasing trend between the AI and the microbial N:P ratio (Topsoil, $R^2 = 0.10$, $P<$

243   0.01; Fig. 2c). In addition, the decreasing trend was found between the microbial C:N

244   ratio and MAT (Topsoil, $R^2 =0.14$, $P< 0.01$; Subsoil, $R^2 =0.10$, $P< 0.01$, Fig. 2d), while

245   a significant negative relationship was found between the microbial N:P ratio and MAT

246   ($Topsoil, R^2 =0.19$, $P< 0.001$; Fig. 2f). The increasing trend between the microbial C:N

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247   ratio and latitude was found in topsoil and significant positive relationships were found

248   in subsoil (Topsoil, R^2 =0.14, $P<$ 0.01; Subsoil, R^2 =0.12, $P<$ 0.05, Fig. 2g), while a

249   negative relationship was found between the microbial N:P ratio and latitude (Topsoil,

250   R^2 =0.18, $P<$ 0.001; Fig. 2i). The microbial C:N ratio was positively related to AGB

251   (Topsoil, R^2 =0.06, $P<$ 0.05, Fig. 3a), SOC (Topsoil, R^2 =0.12, $P<$ 0.01; Subsoil, R^2

252   =0.09, $P<$ 0.05, Fig. 3d) and was negatively related to the sand percentage (Topsoil, R^2

253   =0.11, $P<$ 0.01; Subsoil, R^2 =0.11, $P<$ 0.01, Fig. 3g). A significant positive relationship

254   was found between the microbial C:P ratio and the content of soil organic matter

255   (Subsoil, R^2 =0.08, $P<$ 0.05, Fig. 3e). No or only weak association was found between

256   the microbial C:N, C:P and N:P ratios and the AGB and F:B ratio in the subsoil (Fig.

257   3).

258   **3.2 Effects of potential driving factors on the microbial C:N:P stoichiometry at**

259   **topsoil and subsoil**

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

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

262   27% (subsoil) of the variation in the microbial C:N ratio, 17% and 19% of that in the

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

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

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

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

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

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

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

270   4f). Influences of sand% and the SOC content on the microbial N:P ratio were found in

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

272   the microbial N:P ratio in the subsoil (Fig. 4e, 4f).

273   **4   Discussion**

274   **4.1   The pattern of microbial C:N, C:P and N:P ratios along aridity and the**

275   **latitude gradient**

276   As stated in our hypothesis, the increase in the microbial C:N ratio and the decrease in

277   the microbial N:P ratio that were found along a temperature gradient in this study are

278   in accordance with the findings of Li et al. (2015) and Chen et al. (2016), who reported

279   similar variations in microbial stoichiometry along latitudinal gradient. Temperature

280   drives the variation in the growth of the microbial community, as high growth rates at

281   low latitudes require high RNA contents, causing the N:P ratio to decline (Chadwick et

282   al., 1999; Kooijman et al., 2009; Xu et al., 2013). In addition that, we observed that the

283   microbial C:N ratio significantly increased with increasing aridity index, while the

284   microbial N:P ratio decreased with increasing aridity index, indicating that drought

285   (decreasing aridity index) affects ecological stoichiometry by mediating the growth rate

286   of microorganisms in semiarid regions (Elser et al., 2000b; Peng and Wang, 2016). Dan

287   and Wang noted that increasing aridity reduced the soil microbial abundance in

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288   drylands, and a decreased growth rate in dry areas might result in decreased allocation

289   to P-rich ribosomal RNA (and thus higher C:P and N:P ratios) (Wang et al., 2014;

290   Maestre et al., 2015). Additionally, microbial C:N ratio decreased with the decreasing

291   aridity index, which serves as a protective mechanism as microbes decrease their

292   nitrogen use efficiency (NUE, the ratio of N invested in growth over total N uptake)

293   and tend to be more N conservative under drier climatic conditions (Mooshammer et

294   al., 2014; Delgado-Baquerizo et al., 2017). Moreover, under drier climate conditions,

295   the soil microbial communities shift from acting as r-strategists (fast-growing

296   copiotrophs) to acting as K-strategists (slow-growing oligotrophs), as microorganisms

297   with K-strategies have lower nutrient demands (N and P) and growth rates, invest more

298   nutrients into extracellular enzymes to gain limited nutrients and thus have higher

299   cellular C:N:P ratios than r-strategists (Fierer et al., 2007; Fierer et al., 2010).

300   The microbial C:N ratio demonstrated an increasing trend with increasing latitude, in

301   contrast to the decreasing trend that was demonstrated for the microbial N:P ratio. Such

302   results paralleled the results of studies on ecological stoichiometry, which revealed that

303   the C:N ratio of microorganisms increased with latitude, while the N:P ratio decreased

304   with latitude, suggesting increasing N limitations in microorganism ecosystems in high-

305   latitude areas (Li et al., 2015; Chen et al., 2016). The regional scale microbial

306   stoichiometry followed the global-scale stoichiometry patterns that were observed for

307   plant leaves (Reich and Oleksyn, 2004; Yuan et al., 2011), litter (McGroddy et al.,

308   2004), and soil (Sardans et al., 2012), conforming to the substrate age hypothesis, which

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309   predicts young soils to be N-limited, whereas old soils tend to be P limited (Walker and

310   Syers, 1976; Vitousek et al., 2010). Our study further illustrated the latitudinal pattern

311   of microbial stoichiometry and first attempted to examine the variation in microbial

312   stoichiometry along an aridity gradient at the regional scale.

313   **4.2   Direct effects of ecological factors on controlling microbial C:N, C:P and N:P**

314   **ratios at topsoil and subsoil**

315   Among the ecological factors examined, our study found that the patterns of microbial

316   C:N and C:P ratios were associated with SOC content and the F:B ratio, suggesting that

317   the available C and microbial community structure together regulated the shift in

318   microbial stoichiometry. If the environmental parameters were considered individually,

319   SOC content was found to be significantly positively related to the microbial C:N and

320   C:P ratios, which is consistent with the results observed from global data analysis,

321   suggesting that SOC content may control microbial stoichiometry by mediating the

322   substrate stoichiometry, e.g., the soil C:N and C:P ratios (Hartman and Richardson,

323   2013; Maria et al., 2014; Mooshammer et al., 2014). In deeper soil, microbial metabolic

324   processes are limited by C availability and energy (C), such as denitrification and P

325   mineralization, thus resulting in the effect of SOC on miccrobial C:N:P stoichiometry

326   in subsoil (Fierer et al., 2003; Peng and Wang, 2016; Camenzind et al., 2018).

327   SEM also illustrated that the microbial community structure is an important feature in

328   determining microbial stoichiometry. The F:B ratio has recently been found to have a

329   vital influence on the patterns of microbial C:N and N:P ratios in soil at a large scale

330   (Chen et al., 2016). An experiment indicated that fungi have lower resource

331   requirements and higher C:N and C:P ratios than bacteria; thus, the shift in the F:B ratio

332   impacted microbial C:N:P stoichiometry (Mouginot et al., 2014). In our study, the

333   lower F:B ratio might have led to a shift in the microbial nutrient stoichiometry at

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

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

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

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

338   and those impacts mainly acted in the topsoil but were weaker in the subsoil. The

339   climate imposes important controls on both the plant community and the microbial taxa

340   along with their interactions with soil nutrients (Chadwick et al. 1999; Vitousek 2004;

341   Reich and Oleksyn, 2004). In particular, drier weather condition, and the decreasing

342   aridity index, could affect the growth and productivity of plants, and then shape a shift

343   in vegetation types along this grassland transect (Jaleel et al., 2009; Cherwin & Knapp,

344   2012). At the same time, the meadow steppe ecosystem with high productivity

345   maintained relatively high soil C and N contents, which resulted in high C:P and N:P

346   ratios in these regions; thus, plant productivity exerted a positive influence on microbial

347   C:N (Aponte et al., 2010; Manzoni et al., 2010). Because of the vertical distribution of

348   the influence of the AI, the effect decreased with soil depth.

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

the F:B ratio and SOC content, while the sand percentage and SOC content 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?

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

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

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

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

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

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

377 2010; Peng and Wang, 2016). Similar findings were found in the top 16 cm of soil in a

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

379 (C:N, C:P and N:P) varied with soil depths (Aponte et al., 2010). Tischer et al. (2014)

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

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

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

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

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

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

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

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

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

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

390 **5 Uncertainties and perspectives**

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

19

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

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

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

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

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

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

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

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

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

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

402 limitations under the impacts of global change.

403 **6 Conclusion**

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

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

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

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

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

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

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

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

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

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

414   importance of SOC content, the microbial structure and soil texture in shaping the

415   pattern of microbial stoichiometry in semiarid grassland systems. The influence of

416   ecological factors decreased from topsoil to subsoil, as well as the decline in climatic,

417   edaphic and biotic factors. Overall, these results illustrated N and P limitation in

418   microbial biomass at deeper soil depths along aridity gradient and limited responses to

419   ecological factors in the subsoil.

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

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

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

423   contributed to the preparation of the paper.

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

Biome	Latitude (° N)	Longitude (° E)	MAP (mm)	MAT (℃)	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*

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

432   precipitation; AGB, above ground biomass.

433
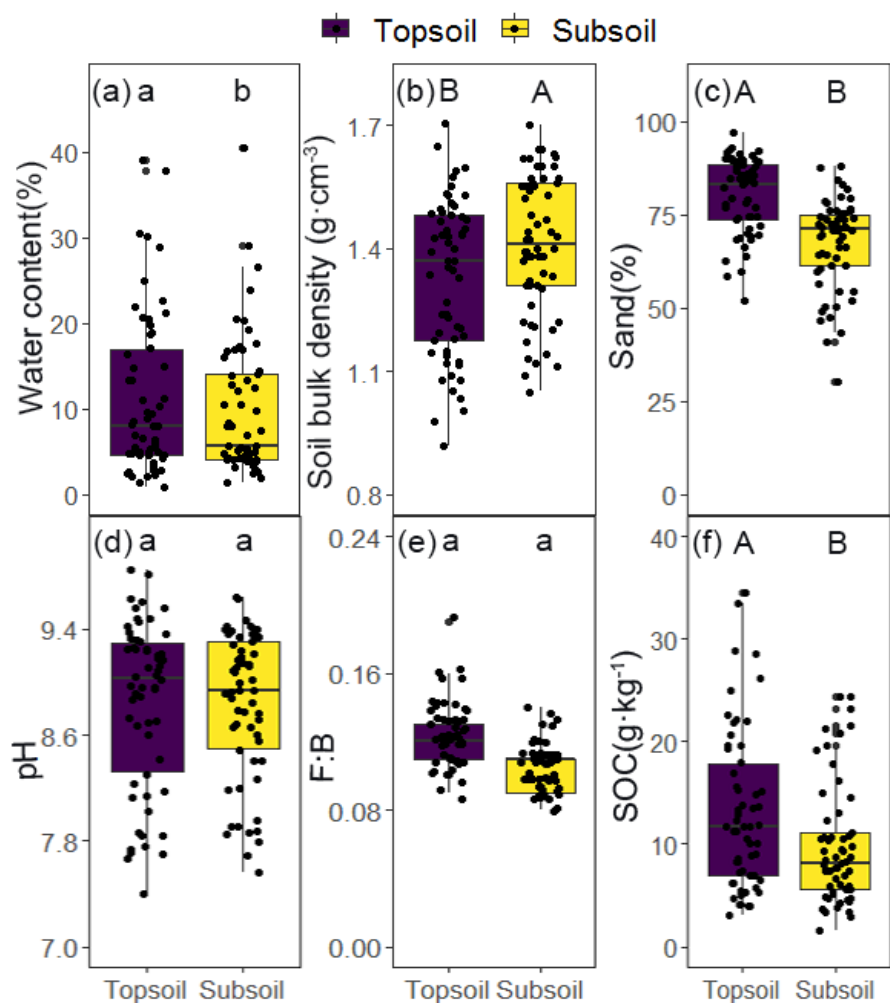
Figure 1. Basic characteristics of study sites across the Inner Mongolia grassland at different soil depths. Different letters indicate significant differences betwen soil depths on log10-transformed data (paired t-test, lowercase letter, $P<0.05$; uppercase letter, $P<0.001$)

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

439 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

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

443 uppercase letter, $P<0.001$).

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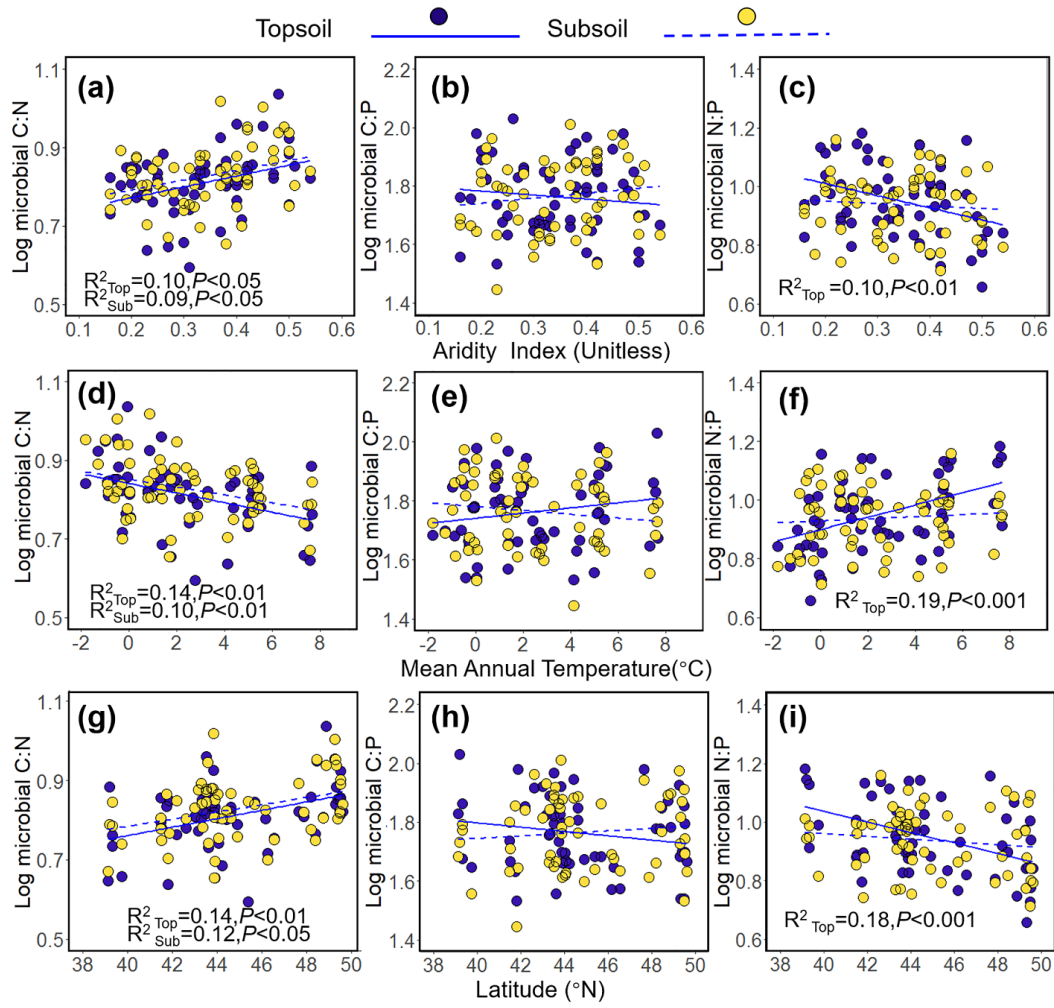
Figure 2. Relationships between the C:N, C:P and N:P ratios in soil microbial

biomass and aridity index (a-c), mean annual temperature (d-e) and latitude (g-i) in
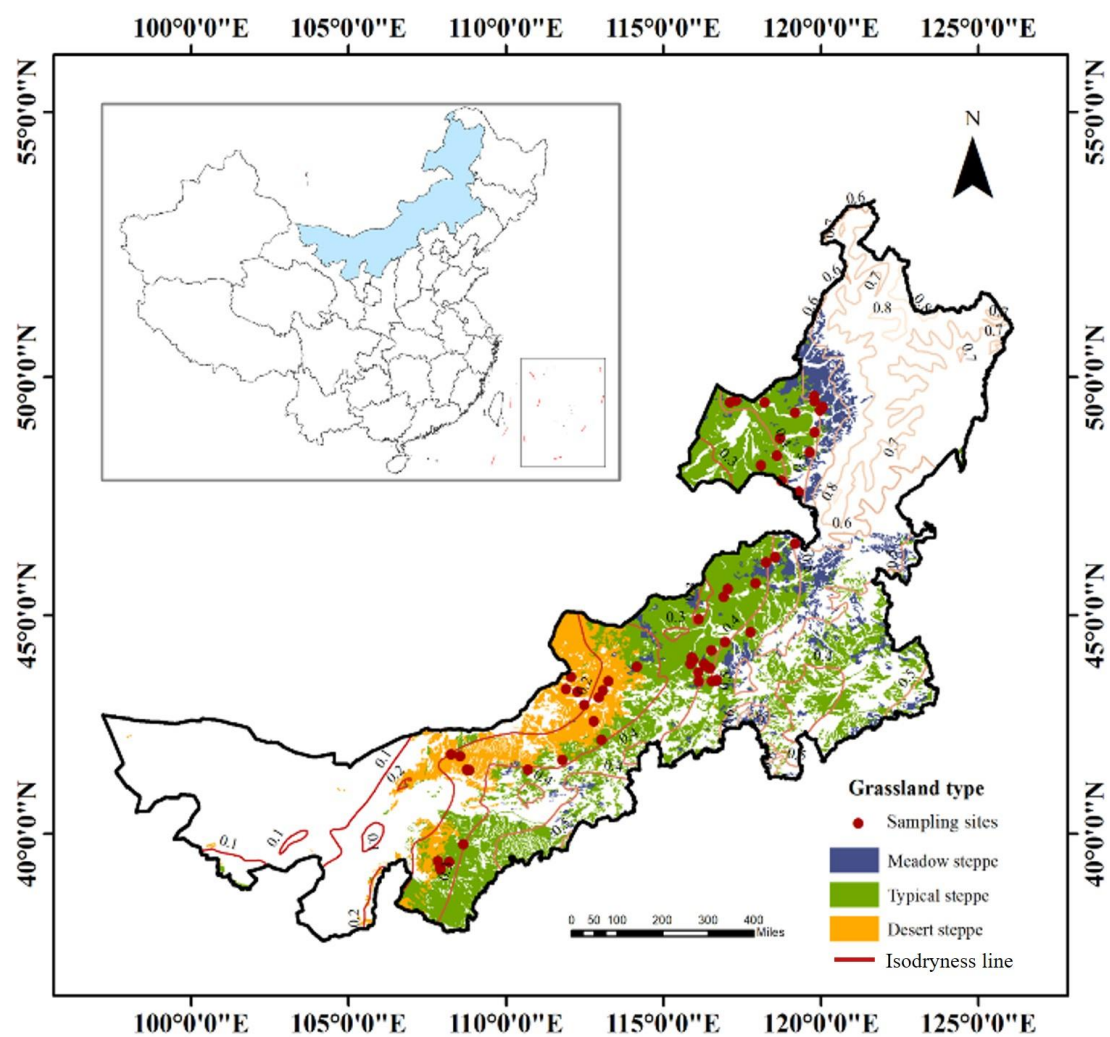
the Inner Mongolian grassland.

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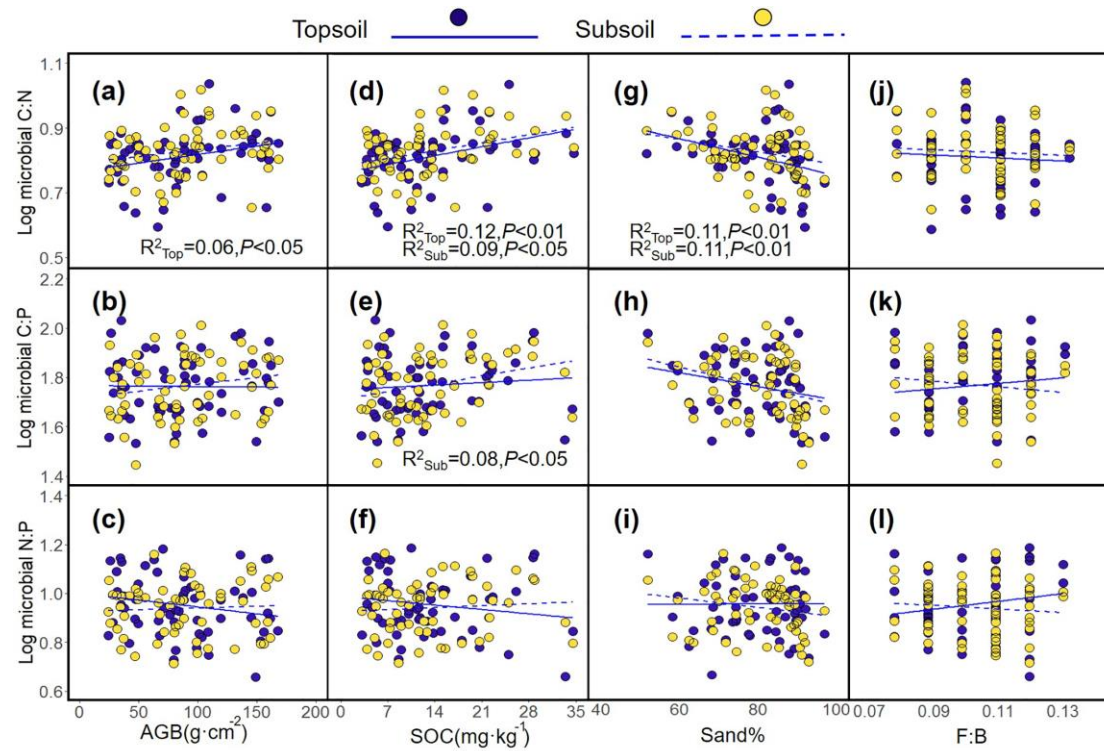
Figure 3. Relationships between the C:N, C:P and N:P ratios in the soil microbial

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.

453
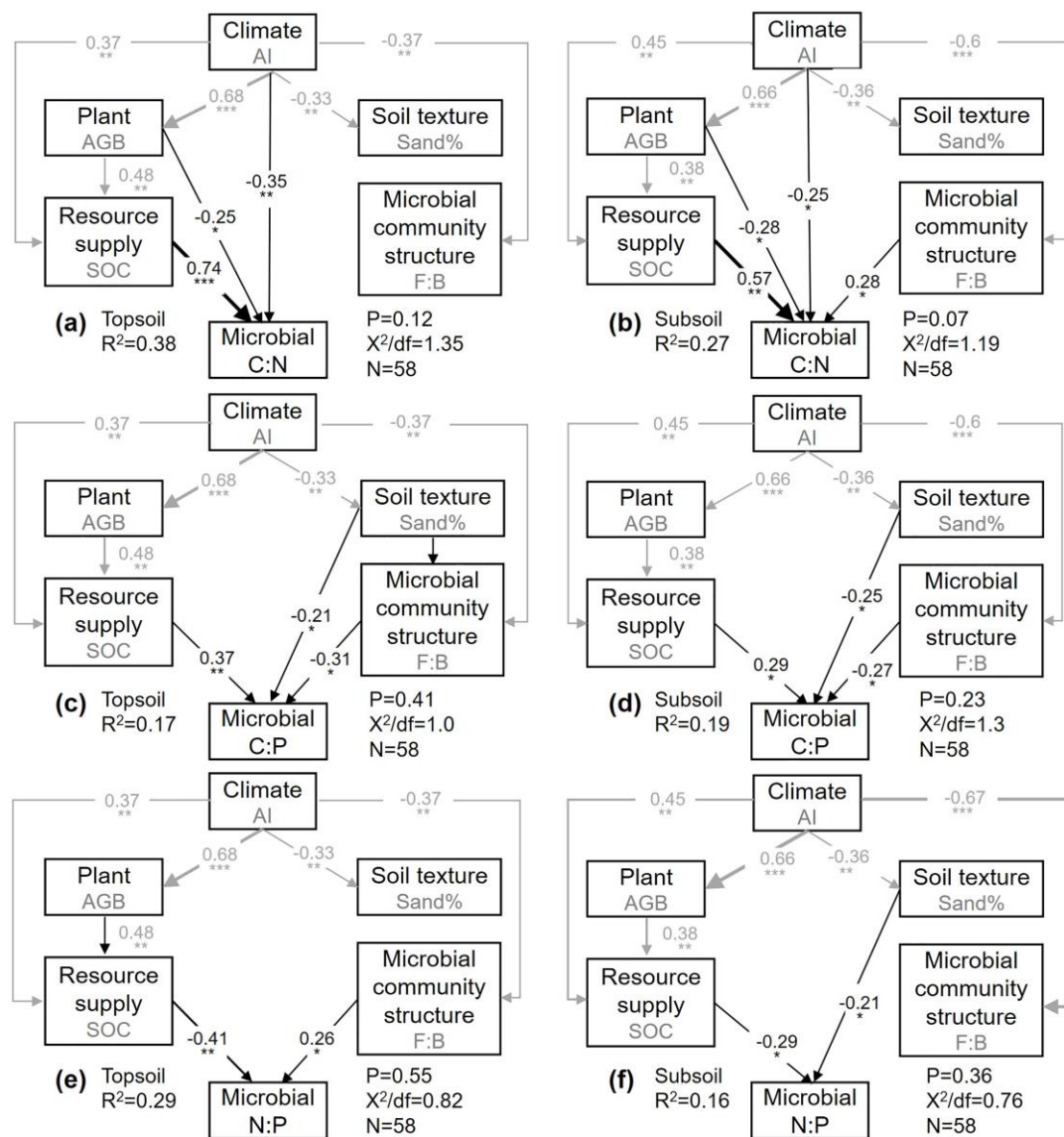
Figure 4. The structural equation model (SEM) shows the direct and indirect influences of various ecological factors on the microbial C:N (a,b), C:P (c,d) and N:P (e-f) ratios in the topsoil and subsoil. Black and gray arrows indicate direct and indirect pathways, respectively. Numbers on the arrows 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. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.
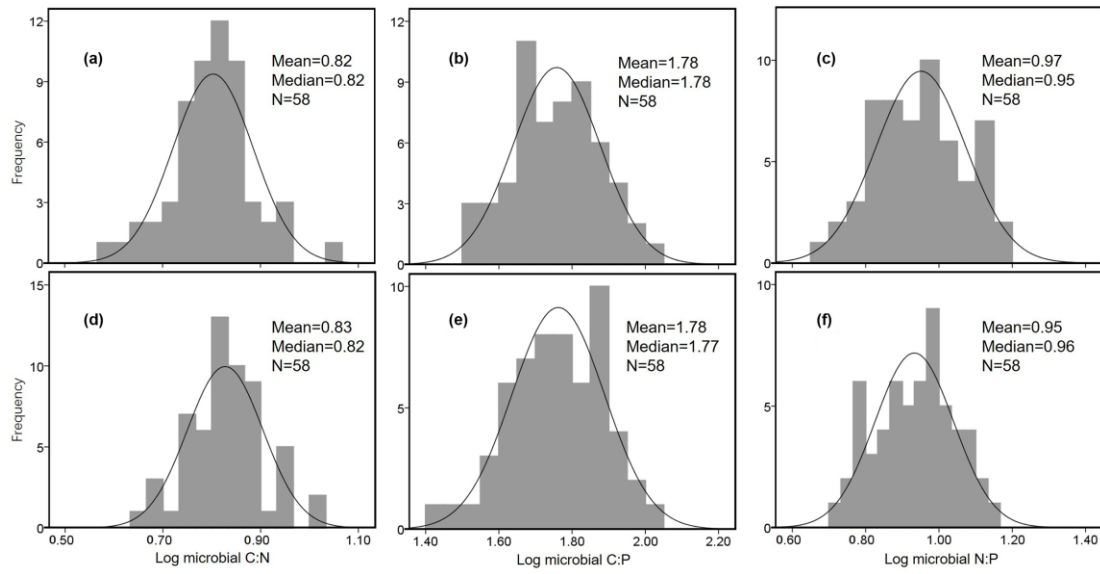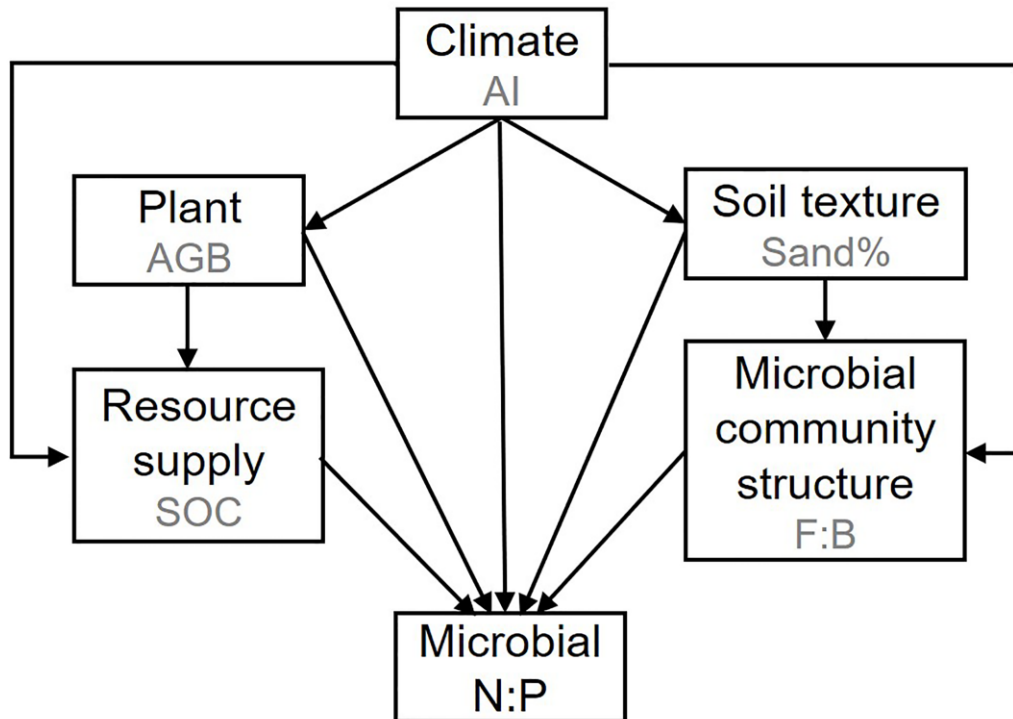
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Supplementary material

462 **Figure S1.** Geographic locations of the sampling sites in the Inner Mongolian grassland

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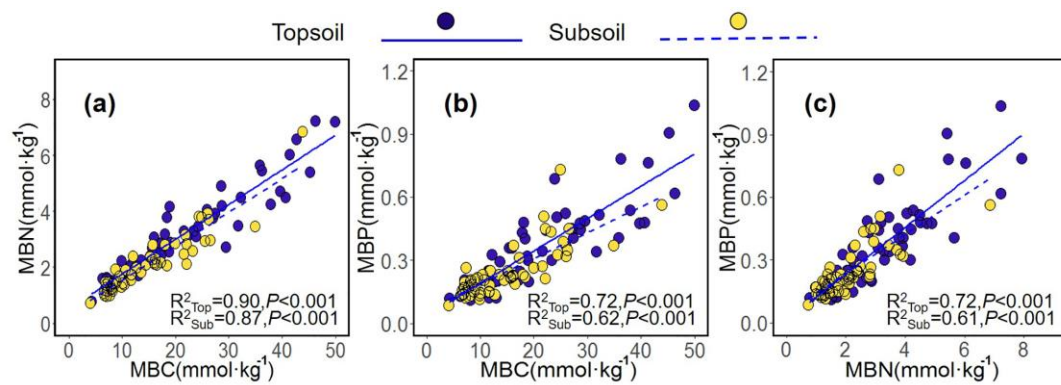
Figure S2. Relationships between the soil microbial biomass C, N and P concentrations

465

Figure S3. Histograms showing the frequency distributions of the soil microbial C:N, C:P and N:P ratios in the topsoil (a-c) and the subsoil (d-f)

468

469   **Figure S4.** Hypothetical model showing how ecological factors affect microbial C:N:P

470   stoichiometry

471 **Table S1.** 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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