Improvement of subsoil physicochemical and microbial properties by short-term fallow practices

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Abstract

Fallow management can improve the soil nutrients in the topsoil and upper subsoil. However, little is

known about the effects of short-term (one year) fallowing with different treatments, such as vegetation

and fertilization, on subsoil (20-40 cm) properties. We conducted field trials to explore the changes in

subsoil properties in response to such treatments in the Yellow River Delta region in China. Different

vegetation and fertilization treatments were applied, and we measured the carbon and nitrogen contents,

microbial biomass and microbial community structure in the subsoil.

Fallowing without manure resulted in the storage of more total nitrogen (16.38%) than fallowing with

manure, and meadow vegetation improved the ammonium nitrogen content (45.71%) relative to

spontaneous vegetation. Spontaneous vegetation with manure improved the microbial biomass nitrogen

($P < 0.05$). Although the impact of short-term fallowing on microbial community structure was low, an

effect of management was observed for some genera. *Blastopirellula*, *Lysobacter*, and Acidobacteria *Gp6*

showed significant differences among fallow treatments by the end of the year ($P < 0.05$). *Blastopirellula*

33   abundance was related to the microbial biomass nitrogen and nitrogen mineralization rate in the subsoil.

34   Manure retained a high abundance of *Lysobacter*, which may strengthen soil-borne disease resistance.

35   The response of Acidobacteria *Gp6* showed that meadow vegetation without manure may not benefit

36   future crops. Although the treatments did not significantly improve microbial community structure in the

37   one-year period, annual fallowing improved certain subsoil properties and increased the number of

38   functional genera, which may enhance crop productivity in the future.

39   Introduction

40   Agricultural fallowing is an effective method for restoring degenerated farmland (Akobundu et al. 1999;

41   Burgers et al. 2005; Manalil & Flower 2014). However, long-term fallowing is usually used to increase

42   biodiversity protection and microclimate modulation rather than improve farmland fertility (Qiu et al.

43   2016). For example, the Chinese "Grain for Green" program has resulted in the extensive addition of

44   green cover in the past 20 years (Chen et al. 2019). However, an energy evaluation revealed increasing

45   chemical fertilizer input and agricultural pollution in northwestern China because the farmlands that did

46   not participate in the "Grain for Green" project were more profitable (Feng et al. 2005; Wang et al. 2014).

47   Based on these issues, an arable land fallow system (henceforth, the fallow system) was initiated in pilot

48   regions in 2016 (Reuters 2018; Wang et al. 2018). The purpose of the fallow system is to restore farmland

49   and improve agricultural productivity (Du 2018). The participating farmland will be used for food

50   production again after the fallow period.

51   Nutrient input is still necessary under fallow management (Gregory et al. 2016). Improved fallow systems

52   in Africa have also involved the addition of organic amendments to improve soil fertility, and maize

53   yields increased after the fallow period (Manyanga et al. 2014). According to some studies, plants

54   belonging to Fabales can improve soil quality and fallow efficiency by promoting nitrogen fixation

55   (Akobundu et al. 1999; Sileshi & Mafongoya 2006; Wick et al. 1998). Existing studies lack a comparison

56   of fertilization and vegetation effects under fallow management, especially the possible interactions

57   between these factors. We attempted to establish different fallow practices based on vegetation and

58   fertilization. Mixed grass can support diverse soil microbial functions through the reciprocal action of

59   residue and soil heterotrophic microbial communities (Eisenhauer et al. 2010; Zak et al. 2003). A

60   previous study indicated that plant roots are the main substrates of subsoil microorganisms (Börjesson et

61   al. 2012).

62   Topsoil is more sensitive than other soil layers to biogeochemical processes (Ma et al. 2016);

63   consequently, studies of the topsoil (up to 20 cm) in fallow areas are prevalent. A study of topsoil showed

64   that spontaneous fallow vegetation with fertilization improved soil microbial biomass nitrogen (MBN)

65   and some members of functional microbial communities, such as *Nitrospira*, *Steroidobacter*, and

66   *Lysobacter* (Li et al. 2018). In addition, subsoil also plays a crucial role in agroecosystems. Subsoil

67   (below 20 cm) can provide nutrient elements to crop roots and can be primed by the addition of fertilizer,

68   which causes N and C loss (Heitkötter et al. 2017; Kautz et al. 2013). In fallow studies, subsoil is

69   considered only in the case of subsoil remediation and when comparing soil layers (Chintu et al. 2004;

70   Gill et al. 2012; Gregory et al. 2016). Subsoiling, which is a general tillage method in northern China (He

71   et al. 2019), will be applied in our study farmlands. Therefore, it is necessary to study the subsoil to

72   improve the soil quality.

73   Compared with topsoil, which is more sensitive, subsoil is often ignored in the fallow practices. In fact, a

74   long-term study (fifteen years) showed that subsoil could retain 20% of total carbon from amendments

75   (Kätterer et al. 2014). Fallow vegetation impacts nitrification in subsoil over a short time period (15

76   months). The nitrate nitrogen content under Sesbania fallowing was greater than that under natural

77   fallowing because of the nitrogen mineralization of Sesbania (Mekonnen et al. 1997). Moreover, natural

78   fallowing and fallowing with maize litter (one year) resulted in more extractable nitrogen in the examined

79   soil layers (0-40 cm) than planted maize because of the high nitrogen utilization via enzymes in fallow

80   farmlands (Loeppmann et al. 2016). Soil carbon seems to be more stable than soil nitrogen. Therefore, the

81   impact of short-term fallowing may be reflected in upper subsoil nitrogen.

82   Organic amendments have little impact on microbial composition (Kätterer et al. 2014). A similar result was

83   also found in an exotic plant invasion study, in which microbial community structure was not significantly

84   affected by current vegetation and instead was largely determined by previous vegetation (Elgersma et al.

85   2011). However, another study found that microbial biomass can be significantly improved by no-tillage and

86   residue in short-term conservation management practices (1 to 2 years) (Guo et al. 2015). A study on

87   fertilization showed that *Bacillus* sp. was promoted by organic manure in one year (Zhang et al. 2015). A study

88   on the use of organic fertilizer for soil remediation found that the fertilizer altered some soil microbial

89   communities in a short period (2 months) by altering the characteristics and improving the water-holding

90   capacity of the soil which could be a benefit for shaping microbial community composition (Chessa et al.

91   2016; Wang et al. 2018).

92   In a long-term fallowing study, fallow farmlands maintained microbial biomass carbon (MBC) in

93   different soil layers (0-30 cm), and MBN in the topsoil (0-20 cm) because fallowing improves soil

94   structure and protects the integrity of fungal hyphae. Simultaneously, microbial biomass depends on the

95   available nutrients (Qiu et al. 2016). Based on the above findings, plants and farmyard manure can

96   promote the availability of soil nutrients. The impact of organic manure on microbial compositions occurs

97   over a short period of time. However, the impact may be high in the initial period and decrease with fertilizer

98   consumption. The soil microbial diversity and community structure may not change significantly in a short

99   period of time. Regardless, some microbial communities, such as heterotrophic bacteria, will present

100   continuous alterations. We hypothesize that the application of mixed plants and farmyard manure will

101   accelerate changes in subsoil nitrogen, microbial biomass nutrients and some bacterial communities under

102   short-term fallow management. To determine the impact of short-term fallowing on subsoil, we planned a

103   five-year field trial in a Chinese agricultural region and intended to analyze changes in the soil

104   environment and microbial community in the first, third and fifth years. In this study, the results from the

105   first year are reported. To accurately study the changes in microbiota, we considered separate seasons,

106   including the initial stage, flourishing stage, and resting stage (June, August, and October, respectively).

107   Based on relevant studies, we believe that microbial composition is generally used to assess the carbon-

108   nitrogen cycle, soil-borne disease resistance and crop productivity in agricultural management (Filip

109   2002; Lammel et al. 2015). In terms of long-term fallow management, analysis of soil microbial

110   community structure and physicochemical properties has been used as a reliable method for explaining

111   the ecological processes in agroecosystems (Hamer & Makeschin 2009; Hamer et al. 2008; Kätterer et al.

112   2014). Therefore, DNA analysis can be used as a precise taxonomical method to analyze soil microbial

113   community structure.

Materials & Methods

115   2.1. Study Site

116   The research area is located at the Shandong Wudi Field Scientific Observation & Research Base for

117   Land Use in Binzhou, China [E 117°43', N 37°48', at an altitude of 5 m above sea level (a.s.l)]. The

118   rainfall ranges from 0 mm to 421.8 mm, with a 10-year average of 55.3 mm. The average temperature is

119   13.9 °C, varying between −6.1 °C and 28.4 °C (Figure S1). Having a silty loam texture (approximately

120   3% clay and 78% silt), the study soil stemmed from diluvial sediments and is classified as a typical saline

121   alluvial soil (Fluvisols, FAO). An investigation by local agricultural institutions revealed that sorghum

122   [*Sorghum bicolor* (L.) *Moench*] monoculture was practiced for more than 5 years in our study area, and

123   soil fertility has decreased markedly in the study area according to a relevant study (Lv 2018). The survey

124   revealed that some farmlands (45%) around the study sites were already unmanaged, planted with other

125   crops or rebuilt as artificial lakes, and these could be found on the map (Liu 2014). Before the

126   experiment, the farmlands were compacted by a four-wheeled tractor which is a common practice after

127   harvest. From 2010 to 2015, the same arable land management scheme was applied to experimental plots.

128   2.2. Experimental Design

129   The experiment started in May 2016, and the study fields were flat and consisted of uniform features. The

130   chemical properties of the subsoil (20-40 cm layer) were as follows: electrical conductivity of 2.0 μS m^{-1},

131   total nitrogen (TN) content of 0.52 mg g^{-1}, soil organic carbon content (SOC) of 3.39 mg g^{-1}, available

132   nitrogen content of 20.59$\times$10^{-3} mg g^{-1}, available potassium (AK) content of 0.16$\times$10^{-3} mg g^{-1}, available

133   phosphorus (AP) content of 2.11$\times$10^{-3} mg g^{-1}, and a pH of 8.90. The data were provided by local

134   agricultural sectors.

135   We established four treatments in the study area. The total area of our experimental zone was 2 ha. There

136   were four parallel plots per treatment, and three were selected for statistical analysis. The size of each plot

137   was 5×6 m, and the plots were randomly distributed (spaced more than 1 m apart). We selected alfalfa

138   (*Medicago sativa* L.) and Dahurian wild rye (*Elymus dahuricus* Turcz.), which are found in the

139   indigenous meadow in the study area, to create plant mixtures to build meadows for fallowing without

140   manure fallow (NM) and with manure fallow (MM). We also examined the effects of fallowing with

141   natural regrowth of the spontaneous vegetation without manure fallow (NS) and with manure (MS). Then,

142   we performed fertilization with manure from local livestock; water must be mixed with cattle manure to

143   prevent hardening, and fertilization was accompanied by irrigation. Finally, no pesticides were used in

144   our study region.

145   Four fallow treatments were detailed as follows:

146   (i)   NS, no manure input, no-tillage, and spontaneous vegetation fallowing, referred to as natural

147   fallowing;

148   (ii)   NM, no manure input, minimum tillage (manual weeding) but sown with alfalfa (*M. sativa*) and

149   Dahurian wild rye (*E. dahuricus*), referred to as meadow fallowing;

150   (iii)   MS, no-tillage, natural fallowing with composted cattle manure (400 g kg^{-1} organic C, 7.0 g kg^{-1}

151   TN, 11.5 g kg^{-1} total P, and 9.8 g kg^{-1} total K) applied at 1500 kg ha^{-1}; and

152   (iv)   MM, minimum tillage, meadow fallowing (the plant cover same as in NM) with composted

153   cattle manure (same as in MS) applied at 1500 kg ha^{-1}.

154   2.3. Preparation of Soil Samples

155   The first soil sampling occurred in June 2016. The second and third samplings were performed in August

156   and October 2016, respectively. We selected 3 plots from each treatment. Based on the "S" sampling

157   technique, subsoil samples were collected from 6 points at a depth of 20-40 cm in each plot; a foil

158   sampler was employed. The samples were then mixed and homogenized. Each soil sample was passed

159   through a < 2 mm sieve to remove plant roots and stones and was then divided into three parts. The first

160   part was stored at -20 °C prior to DNA analysis, and another part was stored at 4 °C for microbial

161   biomass analysis. The remainder was air dried to assay the soil physicochemical properties.

162   2.4. Soil Physical and Chemical Properties

163   The soil pH was determined using a pH meter. The SOC content was determined using air-dried, finely

164   ground soil aliquots. Subsamples of 10 mg each were weighed in a tin cap containing phosphoric acid and

165   analyzed with a CN analyzer (SOC-L Analyzer and SSM-5000A unit, Shimadzu, Japan). The TN was

166   assayed by the Kjeldahl method (Bremner et al. 1996). The carbon to nitrogen ratio (C/N) was calculated

167   based on the SOC and TN. Soil ammonium concentrations (NH_4^+-N) in each sample were analyzed via a

168   continuous flow analyzer (Skalar San Plus automated wet chemistry analyzer, Breda, the Netherlands).

169   2.5. Biological Analysis

170   2.5.1. Soil Microbial Biomass

171   The soil MBC and MBN were determined in accordance with the fumigation-extraction method (Brookes

172   et al. 1985; Vance et al. 1987). For each study plot, three of six subsamples (each consisting of 10.0 g of

173   fresh soil stored at 4 ℃; the remaining three were blank controls) were fumigated with ethanol-free

174   chloroform for 24 h at 25 °C in an evacuated extractor (a vessel). The remaining samples were considered

175   controls. All fumigated and non-fumigated soil samples were extracted with 40 mL of 0.5 M K_2SO_4 (soil:

176   K_2SO_4 solution = 1:4) and shaken for 1 h on a reciprocal shaker. The extracts were filtered using Whatman

177   No.42 filter papers with a 7 cm diameter and stored frozen at -15 °C prior to analysis. The carbon and

178   nitrogen in the extracts were measured by a Multi N/C 3000 Analyzer (Elementar Analysensysteme

179   GmbH, Langenselbold, Germany).

180   The MBC was calculated as follows:

181    $MBC = EC / k_{EC}$

182   where EC = (organic carbon extracted from fumigated soils) - (organic carbon extracted from

183   nonfumigated soils) and k_{EC} = 0.3 (Joergensen 1996).

184   The MBN was calculated as follows:

185    $MBN = EN / k_{EN}$;

186   where EN = (total nitrogen extracted from fumigated soils) - (total nitrogen extracted from nonfumigated

187   soils) and k_{EN} = 0.45 (Joergensen 1996).

188   Finally, the microbial biomass carbon to nitrogen ratio (MBC/MBN) was calculated based on the MBC

189   and MBN.

190   2.5.2. DNA Extraction and MiSeq Illumina Sequencing

191   The total soil genomic DNA was extracted from 0.25 g of each soil sample using a FastDNA kit (MoBio

192   Labs, Solana Beach, USA). The DNA concentration and quality were checked using a NanoDrop

193   Spectrophotometer (NanoDrop Technologies Inc., Wilmington, USA). The extracted DNA was diluted to

194   10 ng μL^{-1} and stored at -20 °C for downstream analysis.

195   The primer pair 515F (5'- GTGCCAGCMGCCGCGG -3') and reverse primer 907R (5'-

196   CCGTCAATTCMTTTRAGTT-3') with a unique 6-nt barcode were used to amplify the hypervariable V4

197   region of the 16S rRNA gene (Angenent et al. 2005). The PCR mixture (25 μL) contained 1x PCR buffer,

198   1.5 mM MgCl$_2$, each deoxynucleoside triphosphate at 0.4 mM, each primer at 1.0 mM, 0.5 U of Ex Taq

199   (TaKaRa, Dalian) and 10 ng of soil genomic DNA. The PCR amplification program included an initial

200   denaturation at 94 °C for 3 min, followed by 30 cycles of 94 °C for 40 s, 56 °C for 60 s, and 72 °C for 60

201   s, and a final extension at 72 °C for 10 min. Three replicate PCR products for each sample were combined

202   and loaded in 1.0% agarose gel electrophoresis. The band with the correct size was excised and purified

203   using the TaKaRa MiniBEST Agarose Gel DNA Extraction Kit (TaKaRa, Dalian) and quantified with a

204   NanoDrop. All samples were pooled together, with equal molar amounts from each sample. The

205   sequencing samples were prepared using the TruSeq DNA Kit according to the manufacturer's

206   instructions. The purified library was diluted, denatured, rediluted, mixed with PhiX (equal to 30% of the

207   final DNA amount) as described in the Illumina library preparation protocols, and then applied to an

208   Illumina Miseq system for sequencing with the Reagent Kit v2 2×250 bp as described in the

209   manufacturer's manual.

210   2.5.3. Sequencing Data Processing

211   The processing of the raw sequences obtained through Illumina sequencing was performed using the

212   Quantitative Insights into Microbial Ecology (QIIME) pipeline (Caporaso et al. 2010). We assembled

213   paired-end reads using FLASH (Magoč & Salzberg 2011). Reads with quality score lower than 20,

214 ambiguous bases and improper primers were discarded before clustering. The resultant high-quality

215 sequences were subsequently clustered into operational taxonomic units (OTUs) at 97% similarity using

216 the UPARSE algorithm (Edgar 2013). Simultaneously, chimeras were checked and eliminated during

217 clustering. Taxonomic classification of representative sequences from individual OTUs was performed

218 using the Ribosomal Database Project (RDP version 2.12) Classifier (Wang et al. 2007). To compare the

219 relative difference between samples, a randomly selected subset of 8260 sequences per sample was used

220 for downstream analyses.

221 2.6 Statistical Analysis

222 Analysis of variance (ANOVA), principal component analysis (PCA), and Spearman's rank test were

223 performed with SPSS software (version 13.0). Repeated-measures ANOVA (RMANOVA) was employed

224 to determine the effects of sampling time and fallowing management. Then, PCA was used to analyze the

225 soil physicochemical properties for each time. There were 6 soil variables in the PCA, and the C/N and

226 MBC/MBN were not included in the PCA. Two-way ANOVA was used to identify the effect of

227 fertilization and vegetation at each sampling date. Downstream statistical analysis of soil DNA was

228 performed using QIIME and R (R Core Team 2013). Alpha diversity indices were calculated in QIIME.

229 Bray-Curtis distance metrics were used to implement nonmetric multidimensional scaling (NMDS) with

230 *monoMDS* in the *vegan* package in R. Permutational multivariate ANOVA (PERMANOVA) was

231 employed to test for dissimilarity among fallow treatments with *adonis* in the *vegan* package in R (Frost

232 et al. 2018). The contribution of fallow treatments was graphed via a Venn diagram with *systemPipeR* in

233 R. The effects of fallow practices on dominant genera in different months were also determined by two-

234 way ANOVA. The correlations between soil characteristics and the predominant genera were estimated

235 via Spearman's rank test. The distribution of all soil physicochemical data at time level was non-normal;

236 therefore, we used the Spearman's rank test to ensure the reliability of the results.

237 **Results**

238 3.1. Physiochemical Characteristics and Microbial Biomass in Subsoil

239   The RMANOVA showed that the impacts of sampling date were always significant and stronger than the

240   impacts of fallowing management (Table S1). Therefore, it was necessary to analyze the impact of

241   fallowing managements at each sampling time. The soil physiochemical properties were impacted by

242   different fallow practices. According to the PCA, the impact of treatment was low in June (Figure 1).

243   Beginning in August, differences between MM and spontaneous vegetation fallowing (MS and NS) were

244   observed. In October, only NS and NM overlap, and MS and MM do not overlap with other treatments.,

245   which indicated that the differences became larger.

246   Effects of the interaction between fertilization and vegetation appeared in August (Figure 2 and Table

247   S2). In August, the interaction had significant effects on TN, C/N, MBN, and MBC/MBN (two-way

248   ANOVA, $P < 0.05$). In October, cattle manure significantly increased the pH and C/N, while TN

249   decreased significantly (two-way ANOVA, $F = 8.367$, $F = 8.867$, and $F = 11.024$, respectively, $P < 0.05$).

250   Meadow vegetation significantly promoted NH_4^+-N (two-way ANOVA, $F = 8.797$, $P < 0.05$). The

251   interaction between fertilization and vegetation had significant effects on MBN and MBC/MBN (two-way

252   ANOVA, $F = 17.742$ and $F = 28.149$, respectively, $P < 0.01$).

253   3.2. Diversity of the Microbial Community in Subsoil

254   The alpha diversity indicators did not show any significant differences; therefore, we focused on the beta

255   diversity. NMDS revealed that the stress values increased from 0.0459 to 0.1065, which indicated that the

256   differences became smaller (Figure 3). The changes in the microbial community structure in August were

257   different from those in June and October, which caused an increase in the stress value (Table S3). The

258   NMDS stress value of the combination of June and October microbial community structure was 0.074,

259   which indicated that the NMDS was efficient.

260   In June, microbial community structure was significantly influenced by the interactions between

261   fertilization and vegetation (PERMANOVA, $F = 2.979$, $P < 0.01$). However, the microbial community

262   was not affected by fertilizer or vegetation in August and October. Therefore, the influence of manure

263   gradually diminished under short-term fallowing (Table S4). Moreover, the Venn diagram showed that

264   the manure strongly impacted microbial community structure in June. However, the differences became

265   smaller in October (Figure S2).

266   3.3. Microbial Communities in Subsoil

267   Each sample contained 34 bacterial phyla. October was the time period with the smallest difference at the

268   phylum level (Figure 4C). Acidobacteria *Gp6*, Acidobacteria *Gp4*, *Gemmatimonas*, *Luteimonas*, and

269   *Lysobacter* increased in abundance in October (Figure 4A). According to Figure 4B and Figure S3,

270   Acidobacteria *Gp10*, *Gaiella*, *Sphaerobacter*, *Nitrospira*, *Blastopirellula*, *Geminicoccus*, *Ramlibacter*,

271   and *Lysobacter* were all impacted by the interaction between fertilization and vegetation in June (two-way

272   ANOVA, P < 0.05). In October, the impact of manure on *Lysobacter* reappeared again (two-way

273   ANOVA, F = 10.904, P < 0.05). In addition, Acidobacteria *Gp6* was impacted by the interaction, and

274   *Blastopirellula* was still impacted by vegetation (two-way ANOVA, F = 8.573 and F = 6.084,

275   respectively, P < 0.05).

276   3.4. The Relationship Between Physicochemical Properties and Microbial Communities

277   We selected Acidobacteria *Gp6*, *Blastopirellula,* and *Lysobacter* for analysis because their abundances

278   were significantly impacted by the treatments after annual fallowing. Acidobacteria *Gp6* showed a

279   negative correlation with soil pH (Spearman's rank test, rho = -0.632, P < 0.001) (Table 1).

280   *Blastopirellula* showed a positive correlation with MBN and a negative correlation with MBC/MBN

281   (Spearman's rank test, rho = 0.485 and rho = -0.357, respectively, P < 0.05). *Lysobacter* showed a

282   negative correlation with pH and SOC (Spearman's rank test, rho = -0.483 and rho = -0.359, respectively,

283   P < 0.05).

Discussion

285   According to previous studies, short-term fallow management impacts extractable nitrogen but does not

286   significantly alter the soil carbon and microbial community structure. In our study, TN, NH$_4^+$-N, and

287   MBN were impacted by vegetation and fertilization. Microbial community structure was stable, consistent

288   with the findings of previous studies. In addition, some bacterial genera changed in response to vegetation

289   and fertilization. The differences in soil properties among the sampling dates were significant. Therefore,

290   we discussed the impact of fallowing treatments at different sampling times.

291   Based on the PCA, the impact of fertilization and vegetation on subsoil physicochemical properties lasted

292   from the growth season (August) to the end of the fallow period. Cattle manure increased the pH in

293   October, in accordance with previous studies (Li et al. 2018), which may limit the growth of some

294   microorganisms. The impact of fallow conditions on carbon was nonsignificant, in accordance with a

295   previous study (Loeppmann et al. 2016). The effects of spontaneous grass with manure on TN were

296   significant beginning in August. Based on a previous study, cattle manure can increase the diversity and

297   abundance of spontaneous grasses (Materechera & Modiakgotla 2006). Competition among spontaneous

298   grasses occurred because of manure input (Rajaniemi 2002). Some grasses that were present before

299   manure input died and became a source of nutrients at the flourishing stage. The flourishing plant roots in

300   the topsoil and subsoil resulted in high total nitrogen in MS (Figure S4). In October, the dominant grasses

301   were already present and continued to grow; consequently, they required more total nitrogen, resulting in

302   decreased total nitrogen in MS. The changes in C/N were caused by TN in June and August. In June, the

303   C/N was sensitive to vegetation because alfalfa planting can reduce the growth of deep roots of weeds,

304   which consume nitrogen in the subsoil (Forney et al. 1985; Ominski et al. 1999). However, the effects of

305   fertilization replaced the vegetation present in October. The amount of TN and C/N indicated that

306   fallowing without manure could save more TN. This finding is different from that of a previous study, in

307   which manure increased the TN content (Hao et al. 2003). This result may be due to the insufficient

308   application of fertilizer and a decrease in the subsoil TN content because of vegetation assimilation

309   (Mooleki et al. 2004). Based on the changes in ammonium nitrogen, our result is similar to a previous

310   study reported by Schroth et al. (1999). The mineral nitrogen also decreased in response to spontaneous

311   fallow vegetation, whereas the fallow treatments with alfalfa retained ammonium nitrogen.

312   MBC did not differ among treatments. In terms of MBN and MBC/MBN, the interaction significantly

313   impacted the MBN. The NM and MS showed that meadow vegetation without manure and spontaneous

314   vegetation with manure maintained more MBN. According to nitrogen consumption and a previous study,

315   MBN is impacted by available nitrogen content and plant growth (Bell et al. 2010; Yao et al. 2011).

316   Therefore, manure allowed the spontaneous vegetation to maintain vigorous growth in October. As a

317   result, MS retained more MBN than NS. Alfalfa usually uptake more nitrogen than grass and the manure

318   can enhance the nitrogen assimilation (Jefferson et al. 2013). Thus, NM retained more nitrogen for the

319   soil microorganisms than MM.

320   The alpha diversity results indicated that the impact of treatments and time was not significant (Table S1).

321   The short-term fallow treatment did not significantly disturb the microbial community structure in the

322   subsoil, similar to the findings of a previous study (Bossio et al. 1998). Even though the beta diversity

323   also was nonsignificant, it indicated that the effects of fallow conditions on microbial composition

324   decreased with time, in contrast to the alteration of soil nutrients. Therefore, we conducted a more in-

325   depth analysis of the microbial communities.

326   The differences in bacterial phyla composition were also nonsignificant. However, the effect of

327   treatments on genera persisted. In terms of taxonomy, high-throughput sequencing can complement

328   previous studies that used the phospholipid fatty acid method (Orwin et al. 2018). More genera showed

329   significant differences in June than in August and October. Therefore, we found that fertilization and

330   vegetation can change some microbial communities in a short period of time (Chessa et al. 2016; Wang et

331   al. 2018). Then, a legacy effect can adjust the microbial composition (Elgersma et al. 2011). However,

332   some genera continued to change after fallow management. We focused on these genera.

333   The proportion of Acidobacteria *Gp6* increased in all treatments by the end of the year, and the highest

334   abundance was observed in NS in October. A previous study showed that Acidobacteria *Gp6* was

335   negatively related to pH under saline-alkaline conditions (Cui et al. 2018), which was confirmed in our

336   study. Therefore, the abundance of Acidobacteria *Gp6* was relatively low under fallow conditions with

337   manure (MS and MM). However, Acidobacteria *Gp6* presented a significantly lower abundance in NM

338   than in NS. We assume that the lower carbon and nitrogen contents may have caused the decrease in

339   Acidobacteria *Gp6* abundance in NM (Sul et al. 2013). Notably, Acidobacteria *Gp6* plays an important

340   role in plant growth (Tao et al. 2018). The lower Acidobacteria *Gp6* abundance implied that the NM may

341   not promote crop yields in the future.

342   *Blastopirellula* was consistently impacted by fallow management throughout the experiment.

343   *Blastopirellula* is a heterotrophic bacterium that originates from seawater, requires an alkaline

344   environment and uses *N*-acetylglucosamine as a carbon and nitrogen source (Schlesner 2015). Based on

345   the correlation analysis, we believe that the nitrogen source strongly impacted the genus. According to the

346   fate of chitin degradation, chitin can be transformed to *N*-acetylglucosamine and then to MBN (Beier &

347   Bertilsson 2013). The proportion of *Blastopirellula* may reflect the nitrogen content and mineralization in

348   different fallow treatments. The genus was impacted by the meadow vegetation in October. Based on the

349   changes in ammonium nitrogen, we concluded that the meadow vegetation increased the efficiency of

350   nitrogen mineralization, which produced a nitrogen source for *Blastopirellula* (Sun et al. 2017). The

351   abundance in MS was relatively higher than that in NS, which indicated that manure alleviated the

352   deficiency of indigenous grass. Cow manure provided chitin as a nitrogen source, as mentioned in a

353   previous study (Labrie et al. 2001).

354   *Lysobacter* increased in response to manure addition by the end of the fallow period. Some strains of

355   *Lysobacter* are related to plant disease resistance (Ji et al. 2008; Kilic-Ekici & Yuen 2004). *Lysobacter*

356   can survive in saline soil, and it was negatively associated with pH in our study (Ma & Gong 2013;

357   Siddiqi & Im 2016). The reason for this association may be that the pH in our study area exceeded the

358   optimum value allowing survival of *Lysobacter*, which is 8.0 or 7.5 (Park et al. 2008; Weon et al. 2007).

359   However, our results indicated that fertilization simultaneously increased pH and the proportion of

360   *Lysobacter* in October because the pH of all treatments slightly decreased in October. The pH limitation

361   was alleviated. We believe that cattle manure can have long-term effects on *Lysobacter*, similar to the

362   findings of previous studies (Li et al. 2018; Soman et al. 2017). Therefore, fallow conditions with manure

363   may increase disease suppression in the subsoil.

364   In summary, the results support our hypothesis. The short-term fallow management practices impacted

365   subsoil nitrogen, MBN and microbial communities. In addition, the microbial community structure did

366   not change significantly. The effects of fallow practices on subsoil are as follows:

367   1) Manure had significant effects on soil characteristics (pH, TN content, and C/N) and *Lysobacter*,

368   which are related to disease resistance. Manure addition increased subsoil C/N and pH. Without

369   manure, more nitrogen was stored in the subsoil, decreasing the pH.

370   2) Meadow vegetation (alfalfa and Dahurian wild rye) mainly affected nitrogen transformation.

371   Compared with spontaneous vegetation, meadow vegetation improved nitrogen mineralization, which

372   was also shown by the changes in *Blastopirellula*.

373   3) The combination of manure and meadow vegetation did not provide more benefits than either of these

374   treatments applied individually. Spontaneous vegetation without manure increased the abundance of

375   Acidobacteria *Gp6*, and spontaneous grass with manure increased MBN. Meadow vegetation with

376   manure stored ammonium nitrogen. Meadow vegetation without manure decreased the abundance of

377   Acidobacteria *Gp6*, which may not benefit crop yields. In general, spontaneous vegetation with

378   manure was the most beneficial treatment.

Conclusions

380   Although this study showed that fallow practices did not significantly disturb the subsoil microbiota in the

381   first year, the different management practices still affected the soil physicochemical properties, especially

382   nitrogen. Fallowing without manure is a good method for improving total nitrogen accumulation in the

383   subsoil; however, the addition of manure improves the soil disease resistance. Additionally, fallowing

384   with meadow vegetation promoted nitrogen mineralization. Nevertheless, the combination of meadow

385   vegetation and manure was not optimal. If we wish to improve crop productivity via soil fertility and

386   superiority in the next year, NS and MS may be helpful methods. If the quality of target farmland is not

387   significantly degraded, MM can be used to promote soil sustainability and will result in some pasture

388   harvest. In the future, we will expand the temporal scale in our studies to explain the changes in the

389   subsoil environment in more detail.

Acknowledgments

391   We are grateful for the support of the Binzhou Land Improvement and Remediation Engineering

392   Technology Research Center and to all colleagues who provided assistance.

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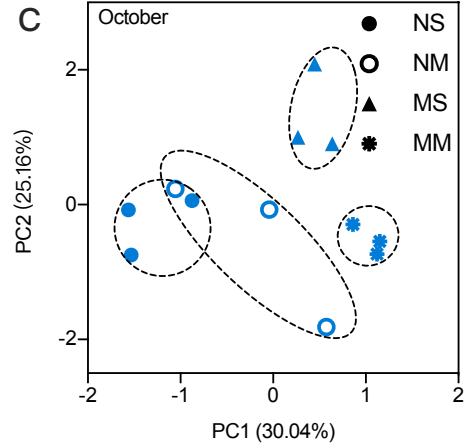
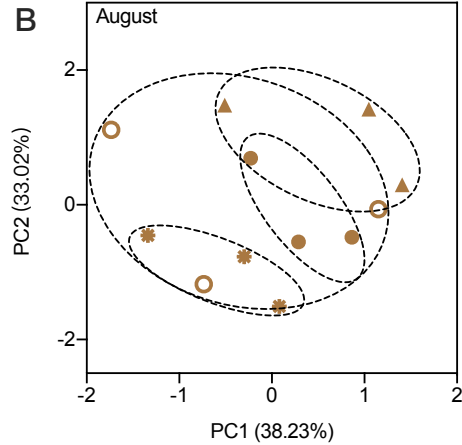
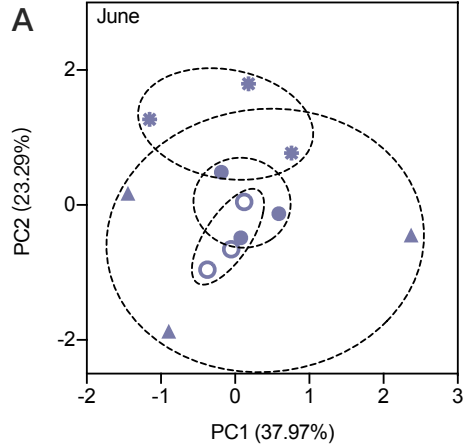
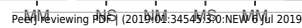
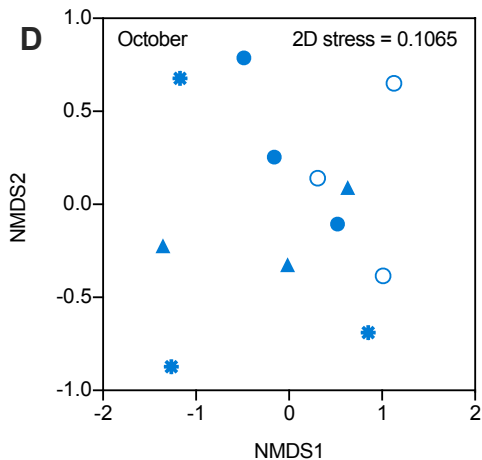
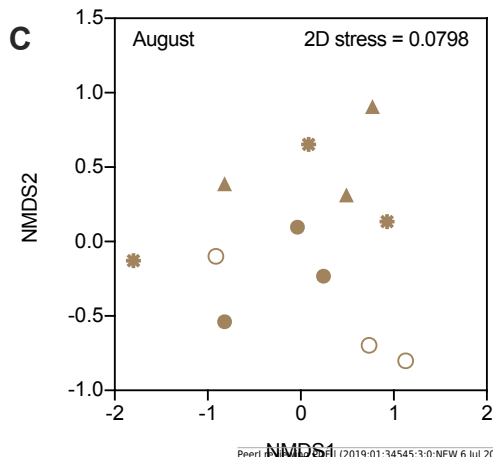
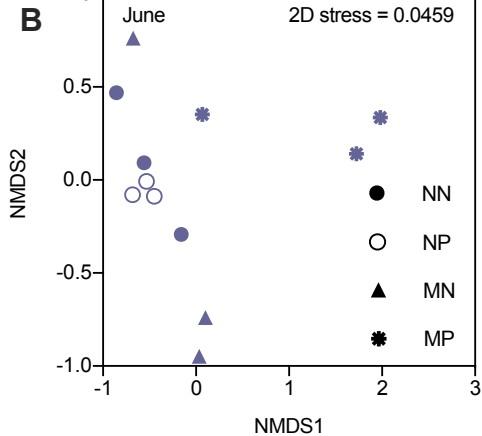
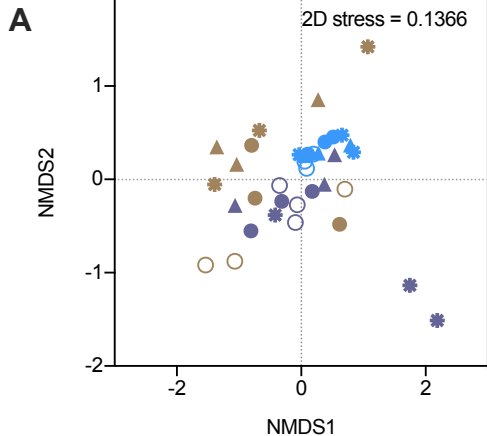
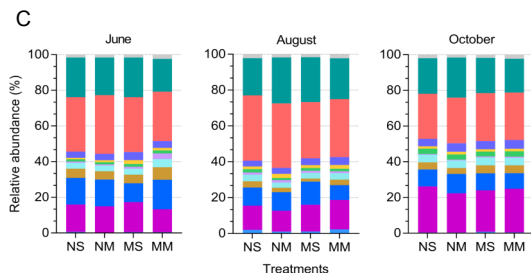
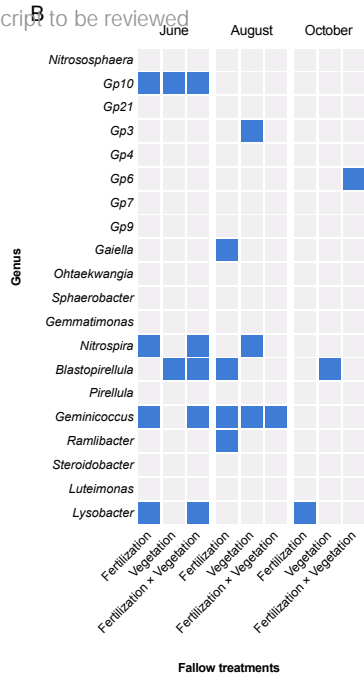
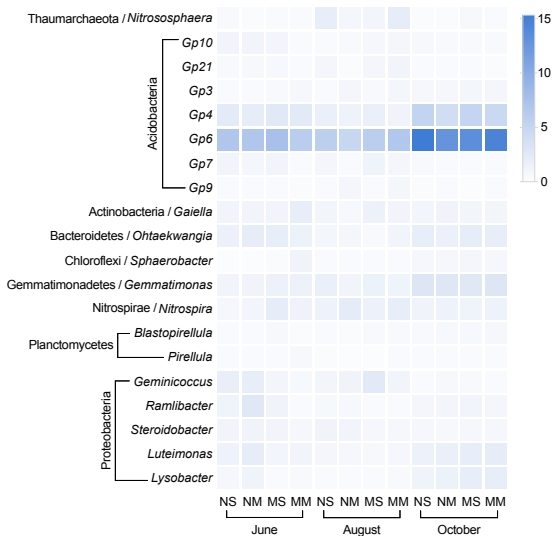
The principal component analysis for soil physicochemical properties in different months.

The comparison of differences among fallow management on soil properties, based on two-way ANOVA.

The plots, which were marked upper- and lowercase, represented the interaction factor's impact was significant. And the uppercase means the fertilization impacts and the lowercase means the vegetation impact (the simple effect test was used to analyze the significance of the difference). The plots which were only impacted by a principal factor, the name of the factor was marked in the upper right corner of each plot. *, ** and *** are used to show statistical significance at the 0.05, 0.01, and 0.001 level, respectively. SOC means soil organic carbon; TN means total nitrogen; C/N means the ratio of organic carbon to total nitrogen; MBC means microbial biomass carbon; MBN means microbial biomass nitrogen; MBC/MBN means the ratio of microbial biomass carbon to microbial biomass nitrogen; NH_4^+-N represents ammonia nitrogen.

Nonmetric multidimensional scaling ordination (NMDS) of soil bacteria.

Different colors represent seasons and the shapes represent different fallow managements.

The relative abundance of dominant bacteria in different groups and seasons, and a mosaic site in relation to treatments in different months, based on ANOVA.

The relative abundance of dominant genera in different groups and seasons (a) and a mosaic site in relation to treatments in different months and time factor (b), and significant responses of dominated genera based on two-way ANOVA ($P < 0.05$, blue color). Relative abundance stacked barplot (c) of microbial taxa (Phylum level).

The correlation between soil properties and selected genera.

Soil variables	Gp6	Blastopirellula	Lysobacter
pH	-0.632***	-0.304	-0.483**
SOC	0.016	-0.033	-0.359*
TN	-0.090	-0.280	-0.281
C/N	0.027	0.081	-0.148
MBC	-0.021	0.041	0.100
MBN	0.237	0.485**	0.293
MBC/MBN	-0.181	-0.357*	-0.152
NH_4^+-N	-0.265	-0.314	-0.257

[†] *, ** and *** are used to show statistical significance at the 0.05, 0.01, and 0.001 level, respectively. SOC means soil organic carbon; TN means total nitrogen; C/N means the ratio of organic carbon to total nitrogen; MBC means microbial biomass carbon; MBN means microbial biomass nitrogen; MBC/MBN means the ratio of microbial biomass carbon to microbial biomass nitrogen; NH_4^+-N represents ammonia nitrogen.