Low-frequency nitrogen deposition addition affects CO_2 emission through regulating soil bacterial community composition

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The impacts of nitrogen deposition and earthworms on soil respiration and microbial communities in the Huang-Huai-Hai Plain have received increasing attention in the literature. However, research on the impact of earthworms and nitrogen deposition frequency on soil carbon dioxide (CO_2) emission and the microbial community is still limited. We conducted a field study in a farming ecosystemto investigate the effects of earthworm activity and nitrogen deposition frequency on soil respiration in the Huang-Huai-Hai Plain. Nitrogen deposition frequencyhad a significant effecton soil respiration, but neither earthworms nor their interaction with Nitrogen deposition frequencywere notsignificant. Low-frequency nitrogen addition (NL) significantly increased soil respiration by 25%, while high-frequency nitrogen addition (NH), earthworm addition (E), earthworm and high-frequency nitrogen addition (E*NH), and earthworm and low-frequency nitrogen addition (E*NL) also increased soil respiration by 21%, 21%, 12%, and 11%, respectively. The main reason for the rise in soil respiration was alterations in the bacterial community.

While soil pH, soil NO_3^--N, and fungal diversity were lower under the NL treatment than under the NH treatment, the bacterial richness was higher. The abundance of Corynebacteriales, Gammaproteobacteria, and keystone taxa (Myxococcales) were favorably connected with the CO_2 emissions, while Verrucomicrobia, Thermoleophilia, and Verrucomicrobia were negatively correlated. Our results demonstrate to the ecological importance of the bacterial community in mediating carbon cycling in the Huang-Huai-Hai Plain and show that the enhanced CO_2 emissions were affected by the diverse soil bacterial community composition deriving from different frequent nitrogen depositions.

1   **Low-frequency nitrogen deposition addition affects CO_2 emission through regulating soil**
2   **bacterial community composition**

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14   **Keywords:** Bacterial community; Earthworm; Nitrogen deposition; Soil respiration; Soybean

16   **Abstract**

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18   communities in the Huang-Huai-Hai Plain have received increasing attention in the literature.
19   However, research on the impact of earthworms and nitrogen deposition frequency on soil
20   carbon dioxide (CO_2) emission and the microbial community is still limited. We conducted a
21   field study in a farming ecosystem to investigate the effects of earthworm activity and nitrogen
22   deposition frequency on soil respiration in the Huang-Huai-Hai Plain. Nitrogen deposition
23   frequency had a significant effect on soil respiration, but neither earthworms nor their interaction
24   with Nitrogen deposition frequency were not significant. Low-frequency nitrogen addition (NL)
25   significantly increased soil respiration by 25%, while high-frequency nitrogen addition (NH),
26   earthworm addition (E), earthworm and high-frequency nitrogen addition (E*NH), and
27   earthworm and low-frequency nitrogen addition (E*NL) also increased soil respiration by 21%,
28   21%, 12%, and 11%, respectively. The main reason for the rise in soil respiration was alterations
29   in the bacterial community. While soil pH, soil NO_3^--N, and fungal diversity were lower under
30   the NL treatment than under the NH treatment, the bacterial richness was higher. The abundance
31   of Corynebacteriales, Gammaproteobacteria, and keystone taxa (Myxococcales) were favorably
32   connected with the CO_2 emissions, while Verrucomicrobia, Thermoleophilia, and
33   Verrucomicrobia were negatively correlated. Our results demonstrate to the ecological
34   importance of the bacterial community in mediating carbon cycling in the Huang-Huai-Hai Plain
35   and show that the enhanced CO_2 emissions were affected by the diverse soil bacterial community
36   composition deriving from different frequent nitrogen depositions.

37   **Introduction**

38   Large amounts of CO_2 are released into the environment by soil through respiration, which

39   raises atmospheric CO_2 concentrations and undermines ecological sustainability (Bond-Lamberty

40   et al., 2010). The carbon budget of terrestrial ecosystems can be seriously affected by even minor

41   changes in soil respiration (Heimann et al., 2008). One of the most active parts of the terrestrial

42   ecosystem, the agricultural system is crucial to the global carbon cycle (Crippa et al., 2021). Soil

43   carbon emissions from farmland must be understood in order to predict and manage soil carbon

44   stores (Wu et al., 2019). The main elements of soil respiration are soil microbial and plant root

45   respiration, both of which are regulated by biotic and abiotic factors (Chen et al., 2019; Lei et al.,

46   2021), such as root dynamics, microclimate, substrate availability, nutrition levels, and soil

47   microbial activity (Allison et al., 2010; Talmon et al., 2011; Wagai et al., 2013; Bolat et al.,

48   2016; Wang et al., 2019). These variables have been incorporated into parameterizing models

49   that forecast shifts in the global carbon cycle.; However, there is still remains uncertainty in

50   estimating soil respiration, which limits our capacity to forecast carbon cycling under scenarios

51   of climate change (Liu et al., 2020b). Microbial ecology is one of the most promising fields in

52   the hunt for novel indicators of soil carbon cycling (Liu et al., 2020b). Soil microorganisms play

53   significant roles in predicting CO_2 emission through microbial processes (Liu et al., 2018). Both

54   theoretical and practical evidences point to the possibility of predicting soil carbon fluxes using

55   the functional and taxonomic characteristics of soil microbial communities (Liu et al., 2019;

56   Allison et al., 2010; Liu et al., 2018). For instance, changes in the soil microbial community

57   composition have an impact on soil carbon respiration and fixation (Monteux et al., 2018; Müller

58   et al., 2018). Previous studies have revealed that the compositions of these microbial

59   communities may play a role in regulating CO_2 emissions (Chen et al., 2021; Wang et al., 2019)

60   since copiotrophs have a faster respiration rate than oligotrophs and that proteobacteria and

61   actinobacteria are positively connected with CO_2 emissions (Liu et al., 2018; Chen et al., 2021;

62   Liu et al., 2020). So far, the extent to which the structure and the composition of the microbial

63   community altered CO_2 emission in farming systems has not yet been thoroughly determined.

64   Fertilization is typically thought to be the primary method for increasing crop yield, it also

65   has a significant impact on the carbon pool and carbon flux in the soil. The structure and function

66   of the world's ecosystems are significantly impacted by changes brought on by anthropogenic

67   atmospheric nitrogen deposition (Cao et al., 2021). The influence of nitrogen deposition is still

68   being debated despite the fact that many nitrogen addition experiments have been carried out to

69   examine how ecosystem carbon exchange mechanisms respond to nitrogen deposition (Cao et

70   al., 2020; Yang et al., 2020). The frequency of nitrogen amendment is a key factor in methods

71   for simulating nitrogen deposition. In reality, nitrogen deposition occurs continuously, therefore

72   12 times applications each year rather than a single or two or three additions more nearly

73   resemble atmospheric nitrogen deposition (Cao et al., 2020). At present, there are few reports on

74   the effects of nitrogen deposition frequency on soil respiration, particularly when it comes to

75   microbial communities. Hence, nitrogen deposition frequency experiments that integrate plant,

76   soil and microbial components of ecosystems are needed to fill this gap in understanding (Cao et

77   al., 2020).

78   In soil formation and function processes, earthworms play a crucial role as keystone

79   detritivores and ecosystem engineers (Yang et al., 2019; Fahey et al., 2013). They can affect soil

80 carbon dynamics since they are ecosystem engineers living in the soil (Jennings et al., 2016).
81 Previous research has demonstrated that soil CO_2 emissions can rise as a result of earthworm
82 invasion (Lubbers et al., 2013). Through their interactions with microbes, macro,
83 and microfauna, earthworms greatly influence the decomposition process and increase
84 heterotrophic activity, which in turn affects soil carbon dioxide emissions (Fisk et al., 2004).
85 Earthworms directly or indirectly affect the nitrogen cycle and have the potential to alter
86 ecosystem functions and services in relation to the nitrogen cycle (Xue et al., 2022). However,
87 there is still limited research on the effects of simulated nitrogen deposition frequency and
88 earthworm addition on ecosystem carbon emissions. Therefore, disentangling how nitrogen
89 deposition frequency and earthworm addition affect CO_2 emission and its relationship with the
90 soil microbial community is of great significance for mediating C cycling in farmland.
91   Here, we aimed to explore how the microbial community regulates the response of CO_2
92 emission to different frequencies of nitrogen deposition and earthworm addition. In this study,
93 one growing season field experiment with six treatments was performed in the Huang-Huai-Hai
94 Plain. The specific questions of this study we tied to address: (1) How does CO_2 emission
95 respond to nitrogen deposition frequency and earthworms? (2) What were the underlying
96 mechanisms influencing CO_2 emission response to nitrogen deposition frequency and
97 earthworms?

98 **Materials and methods**

99 Research site

100 This research was performed at the farm of Jinming Campus of Henan University, Kaifeng
101 City, Henan Province, China (34°49′N, 114°18′E). A permanent 25×10 m² rainout shelters with
102 steel frames and covered with clear polyethylene roof was built in late summer 2021 to control
103 precipitation inputs each year, to avoid extreme rainfall that death of soybean and earthworms.
104 The appropriate rainfall amounts were selected to simulate natural precipitation in the local area
105 with the long-term means. The region belongs to the warm temperate continental monsoon
106 climate with an annual mean temperature is 14 °C and the annual mean precipitation is 650 mm
107 (80% occurring between July and August). The soil texture is sandy loam soil.

108 Experimental design

109 The experiment used a randomized block design involving two factors of nitrogen and
110 earthworm, including six treatments: C(control), E (earthworm addition), NH (high-frequency
111 nitrogen addition), NL (low-frequency nitrogen addition), E*NH (earthworm and high-frequency
112 nitrogen addition), and E*NL (earthworm and low-frequency nitrogen addition). Each treatment
113 was replicated five times with an area of 1m × 1 m per plot.

114 The total amount of earthworms (*Metaphire guillelmi*) added was controlled at 8.0-8.9g.
115 Each block was surrounded by glass to prevent earthworms from escaping. Nitrogen (urea) was
116 added by water dissolving and root topdressing. The total amount of nitrogen added was the
117 same as the conventional local field nitrogen application. Two nitrogen deposition frequencies

118   were set based on previous research 10132752: high-frequency nitrogen application (12N) and
119   low-frequency nitrogen application (2N). From seedling stage (VE) to drum stage (R6), high-
120   frequency nitrogen was uniformly added 12 times / 29 N kg·hm^{-2} and low-frequency nitrogen
121   was uniformly added twice / 174 N kg·hm^{-2}. Earthworms were added in July 15th and August
122   21st, 2022.

123   Measurement of soil respiration

124   Soil respiration and temperature were measured every seven days during the Soybean
125   growing season using a Li-8100 portable soil CO_2 flux system (Li-Cor, Inc. Lincoln, NE, USA)
126   and a thermocouple probe (Li-8100-201, Li-Cor, Inc. Lincoln, NE, USA) connected to the Li-
127   8100 in June 2022 to October 2022. Soil volumetric water content at 0-5cm soil depth was
128   determined adjacent to each collar using a soil detector (TR-6D). All measurements were
129   performed between 9 a.m. and 11:30 a.m. To avoid the respiration of aboveground parts of plants
130   and litter decomposition, all living plants and litter inside the collars were removed by hand two
131   days before soil respiration was measured. If it rains heavily, our measurement would be
132   postponed for two days.
133   Soil sampling and analysis and plant index measurements
134   Each plot was destructively sampled in September 2022. Three soil cores were randomly
135   collected and pooled by depth from each plot using a soil corer (inner diameter 5 cm) at 0-10 cm
136   depths, sieved through a 2 mm mesh to separate gravel and roots, and divided into three parts.
137   One subsample was stored at 4 °C for the analysis of the chemical properties of soil i.e. the N
138   content in the form of ammonium and nitrate using a colourimetric method (Smart Chem 200
139   Discrete Auto Analyser, Systea, Italy). Another subsample was air-dried and ground for analysis
140   of pH、total N (TN) and total carbon (TC). The soil pH was measured with a soil pH tester. The
141   soil TN and TC concentrations were measured by a Vario ELIII Elementar (Elementar
142   Analysensysteme GmbH, Germany) elemental analyzer. The third part was stored at -20 °C for
143   the analysis of the microbial community diversity composition spectrum.
144   After removing the whole plant from the soil, rinsed it slowly with running water to
145   separate the above and below-ground parts of the plant from the cotyledon nodes. The washed
146   roots were dried in an oven at 65℃ to a constant weight, and the root biomass was weighed.
147   Aboveground biomass was weighed after two weeks of natural air drying. Grain yield was
148   measured by removing the mature pods from the plants, placing them in paper bags, and leaving
149   them in a ventilated place for drying to constant weight. The plant height was measured by
150   selecting 3 plants from each plot and measuring them with a tape measure. The number of pods
151   per plant and number of grains per plant were also artificially measured by selecting 3 plants
152   from each plot. 100 grain weight was randomly selected from the grain yield of each plot and
153   weighed by precision scale.

154   DNA extraction, PCR amplification, and Illumina sequencing

155   Soil DNA was extracted from each sample using E.Z.N.A Soil DNA Kit (Omega Bio-tek,
156   Norcross, Georgia, USA) according to the manufacturer's protocol. The purity and concentration

157 of the extracted DNA were determined by a NanoDrop-2000 spectrophotometer (Thermo Fisher
158 Scientific, Waltham, MA, USA). Purified soil DNA was fully pooled together after quantitative
159 determination and then for downstream manipulations. The V3-V4 of bacterial 16S rRNA genes
160 were amplified with the following universal primer set: upstream primers 338F (5′-
161 ACTCCTACGGGAGGCAGCA-3′) and downstream primers 806R (5′-GGACTA
162 CHVGGGTWTCTAAT3′). For fungi, the primers ITS5 (5′-GGAAGTAAA
163 AGTCGTAACAAGG -3′) and ITS2 (5′-GCTGCG TTC TTCATCGATGC-3′)45 were used to
164 amplify the ITS_V1 region of the rDNA gene. PCR reactions were performed in 25 μL reaction
165 mixtures containing 5μL 5×reaction buffer, 5μL 5×GC buffer, 2μL 2.5mM dNTPs, 1μL
166 Forwardprimer (10uM), 1μL Reverseprimer (10uM), 2μL DNA Template, 8.75μL ddH2O,
167 0.25μL Q5 DNA Polymerase. The reaction conditions were programmed of an initial denaturing
168 step at 98°C for 2 min, denaturation 98°C 15s,annealing 55°C 30s,extension 72°C 30s,final
169 extension 72°C 5min and 10°C hold 25-30cycles. Samples were sequenced in an Illumina MiSeq
170 High-Throughput Sequencing (HTS) platform (Illumina, San Diego, CA, USA) at Personal
171 Biotechnology Co. Ltd Shanghai, China to determine soil microbial community composition.

172 Statistical analyses

173   The sequenced data was performed using QIIME 2 2019.4 with slight modification.Briefly,
174 raw sequence data were demultiplexed using the demux plugin followed by primers cutting with
175 cutadapt plugin. Sequences were then merged, filtered and dereplicated using functions of
176 fastq_mergepairs, fastq_filter, and derep_fulllength in Vsearch. All the unique sequences were
177 then clustered at 98% (via cluster_size) followed by chimera removing. At last, the non-chimera
178 sequences were re-clustered at 97% to generate OTU representative sequences and OTU table.
179 Representative sequences were aligned with mafft and used to construct a phylogeny with
180 fasttree. Alpha-diversity metrics (Observed_species, Simpson) were estimated using the diversity
181 plugin with samples were rarefied. Meanwhile, principal coordinates analysis (PCoA) was
182 selected to illustrate the clustering of different samples. ASVs were given a taxonomy using the
183 Silva v132 99% OTU reference sequences and the classify-sklearn nave Bayes taxonomy
184 classifier in the feature-classifier plugin (Liu et al., 2020).
185   Two-way ANOVAs ($p < 0.05$) was used to analyze the significant differences between
186 nitrogen deposition frequency and earthworms on CO_2 emission and soil properties. One-way
187 ANOVA with Duncan testing ($p < 0.05$) was used to evaluate the significant differences in soil
188 properties and CO_2 emission among the six treatments. Linear regression analysis was used to
189 study the relationship between soil respiration, soil property and soil microbial community under
190 six treatments. Spearman's correlation analyses were performed to assess the relationships
191 between soil properties, respiration, plant biomass and microbial community. Soil chemical
192 properties data were analyzed with SPSS software (version 26, IBM, Chicago, IL, USA). We
193 conducted a classification random forest analysis to identify the major statistically significant
194 microbial predictors of the composition (relative abundance: number of sequences of major
195 phyla/class/order level) of bacteria and fungi acting on soil respiration. The analysis was
196 conducted using the rfPermute package of the R (4.2.2) statistical software. The significant

197 predictors from random forest analysis were further selected for structural equation modeling
198 (SEM) analysis. SEM analysis was applied to determine the direct and indirect contributions of
199 soil properties and the bacterial community to CO_2 emission. SEM analysis was performed using
200 AMOS 22.0 software (SPSS, Chicago, IL, USA). The model fitness was evaluated by χ2 (p >
201 0.05), comparative fit index, and root mean square error of approximation.

202 **Results**

203 Nitrogen and earthworm addition effects on soil respiration

204   Soil respiration varied with Soybean growth period, showing obvious seasonal variation
205 (Fig. 1a). It was the lowest during the early vegetative stage (Jun.23 to Jul.14), reached
206 maximum at the reproductive stage (Jul.21 to Sep.7), and started declining during the maturity
207 period (Sep.7 to Sep.17). Total soil respiration varied during the study from 1.13 to 4.63 mmol
208 m^{-2} s^{-1}, with an average of 2.55 ± 0.12 µmol m^{-2} s^{-1} (Fig. 1b). Compared with C, soil respiration
209 in the NL increased significantly by 25 %. E*NH, E*NL, NH, and E increased by 21%, 21%,
210 12%, and 11%, respectively. Overall, soil respiration was significantly affected by nitrogen
211 addition, but not by earthworm addition or interaction between nitrogen and earthworm addition.
212 (Table 1).

213 Nitrogen and earthworm addition effects on soil properties, plant biomass and agronomic traits

214   Nitrogen addition significantly influenced soil pH, TN, NO$_3^-$-N, and NH$_4^+$-N. Soil moisture
215 was significant in the addition of nitrogen and earthworm, as well as their interaction, while their
216 interaction significantly affected grain yield (Table 1). The E*NL treatment had the highest soil
217 TN, which was also significantly higher than the C and E treatments (Fig. 2a). The soil moisture
218 was significantly lower under NH, NL, and E compared to E*NH (Fig. 2b). In comparison to the
219 control plots (C, E; Fig. 2c), the soil NO$_3^-$-N was considerably greater when nitrogen addition
220 treatments (NH, NL, E*NH, E*NL) were used. Soil NH$_4^+$-N was significantly higher in NH and
221 E*NH than that of other treatments (Fig. 2d). Soil pH of E*NL, NL, NH, E*NH decreased
222 3.38%, 3.03%, 2.17%, 1.64%, and E increased 1.26% compared with C (Fig. 2e). The highest
223 grain yield was observed in E*NH, which was also significantly greater than NH (Fig. 2f).
224 However, neither the addition of nitrogen nor the interaction of earthworms had no significant
225 influence on ST, TC, plant biomass and agronomic traits (except grain yield).

226 Soil microbial community composition for predicting soil respiration

227   Using random forest modeling, we identified the major bacterial and fungal phyla, classes
228 and orders for predicting soil respiration. These taxa include several bacterial and fungal such as
229 Corynebacteriales, Myxococcales, Sordariomycetes, Verrucomicrobia, Thermoleophilia,
230 Agaricomycetes, Gammaproteobacteria, Blastocladiomycetes, and Proteobacteria (Fig. 3).
231   The Spearman's correlation coefficients between the microbial characteristics and the soil
232 respiration as well as soil properties were estimated (Table 2). Soil respiration rate was
233 correlated to bacterial simpson. Particularly, there were significant relationships between the
234 respiration and major bacterial and fungal phyla. The findings demonstrated a strong correlation

235   between soil respiration and the abundances of Blastocladiomycetes and Agaricomycetes in the
236   fungal compositions as well as Gammaproteobacteria, Verrucomicrobia, Thermoleophilia, and
237   Myxococcales in the bacterial compositions. (Fig. 3 and Table 2).

238   Relationships between soil respiration and abiotic and biotic factors

239   Soil respiration showed positively dependent upon Nitrate N ($R^2 = 0.10$, $p < 0.05$, Fig. 4b),
240   aboveground biomass ($R^2 = 0.16$, $p < 0.05$, Fig. 4c), Plant height ($R^2 = 0.15$, $p < 0.05$, Fig. 4e),
241   Grain yield ($R^2 = 0.12$, $p < 0.05$, Fig. 4f), and negatively with soil pH ($R^2 = 0.18$, $p < 0.05$, Fig.
242   4a), but it was not correlated with root biomass (Fig. 4d).
243   Additionally, SR was significantly positively correlated with the relative abundance of
244   Gammaproteobacteria and Corynebacteriales, as well as bacteria Pco1, and negatively correlated
245   with the relative abundance of Myxococcales, Verrucomicrobia and Thermoleophilia (Fig. 5).
246   Notably, the relative abundance of Thermoleophilia, Myxococcales and Verrucomicrobia was
247   low in NL, while the relative abundance of Corynebacteriales and Gammaproteobacteria was
248   high. In general, NL treatment raised the relative abundance of copiotrophs while decreasing the
249   relative abundance of oligotrophs.
250   Structural equation modeling (SEM) further suggested that bacteria richness and microbial
251   composition (Proteobacteria, Myxococcales) had strong direct effects on soil respiration (Fig. 6).
252   However, nitrogen addition mostly had indirect impacts on soil respiration through pH, NO_3^--N,
253   Proteobacteria, Myxococcales, and bacteria richness, while earthworm addition had indirect
254   impacts on soil respiration through NO_3^--N, AGB, GRY and RB. Overall, the most important
255   microbial attributes controlling soil respiration rates were the relative abundances of
256   Proteobacteria and Myxococcales, and bacteria richness.

257   **Discussion**

258   Effects of nitrogen deposition frequency on soil respiration and microbial community

259   In order to increase crop output and soil quality, nitrogen fertilizer is frequently seen as a
260   usual method (He et al., 2022). But fertilization practices can have a big impact on soil CO_2
261   emissions (Wang et al., 2021). In the current investigation, fertilizer treatments considerably
262   improved soil CO_2 emission by 12-25% in comparison to the carbon treatment. This finding was
263   in line with previous study findings that indicated enhanced CO_2 emissions in farmland
264   ecosystems following fertilization (Yan et al., 2020; Lamptey et al., 2019). In addition, NL
265   promoted soil respiration more than NH, indicating that nitrogen inhibited soil respiration with
266   the increase of nitrogen frequency. The addition of nitrogen-promoted CO_2 emission could be
267   attributed to the high level of NO_3^--N and the low level of pH, which increase the bacterial
268   richness and change microbial community composition, thus stimulating soil heterotrophic
269   respiration and CO_2 emission. Similar findings from earlier investigations have been noted, for
270   example, at the 2-N frequency, the addition of N led to a lower, more acidic pH (Ning et al.,
271   2015); When the same total N loading is applied in a single pulse rather than repeatedly, the
272   effects of soil acidification and mineral N toxicity are more likely to be exacerbated (Ning et al.,
273   2015). Plants mainly capture N from the abundant soil N form across N addition gradient 11,

274   therefore NO_3^--N plays an important role as the most abundant available nitrogen in our study.
275   According to previous reports, changes in the soil microbial community might indirectly affect
276   soil respiration by changing the pH of the soil (Whitaker et al., 2014; Liu et al., 2020a; Liu et al.,
277   2020b). NO_3^--N and pH were found to be important environmental factors in explaining changes
278   in bacterial community composition (Chen et al., 2021).
279   In this study, NL treatment obviously increased the bacterial richness compared to C, while
280   NH showed the opposite trend. The result was in accordance with previous researches. For
281   instance, soil respiration rises when nitrogen levels rise, which is compatible with increased
282   bacterial abundance and suggests that this reaction may be driven by an increased metabolic rate
283   (Hagerty et al., 2014). As far as we know, there is almost no research on the impact of nitrogen
284   addition at different frequencies on bacterial richness. Much of the research has been on the
285   effects of adding different levels of nitrogen on microbial richness. For instance, research has
286   shown that adding nitrogen within a specific range might increase the amount of bacteria
287   quantity, but that adding nitrogen in excessive would have an opposite effect (Ma et al., 2007). In
288   our study, low-frequency nitrogen increased bacterial richness relative to high-frequency
289   nitrogen, which is consistent with other results in this study that low-frequency nitrogen played a
290   promoting role relative to high-frequency nitrogen. The increase in NO_3^--N and NH_4^+-N and
291   decrease in pH that nitrogen addition causes are directly correlated with the bacteria richness.
292   Most bacteria prefer a pH that is close to neutral, and they are sensitive to pH variations (Anil et
293   al., 2019). The alkaline soil in the study's location would make an ideal habitat for the soil
294   microbial community to promote soil respiration (Chen et al., 2021). The substrate (such NO_3^--
295   N) that provides nutrients for microorganisms may be related to the rise in soil bacterial
296   community richness (Chen et al., 2021). Nitrification of NH_4^+-N accumulation may result in soil
297   acidification and pH lowering, and the addition of nitrogen enhances this reaction and further
298   lowers pH (Sun et al., 2019). Our research confirms these findings. In the 6th and 7th years
299   following nitrogen application, Cao et al.'s (2020) research shown that HF considerably reduced
300   the detrimental effects of nitrogen addition on plant and soil bacterial diversity. In the present
301   study, NH reduced the negative effect of nitrogen addition on fungal diversity than NL, while the
302   trend of bacterial diversity was the opposite, which may be due to the strong alkaline soil in our
303   experimental site.

304   Effects of earthworm on soil respiration and microbial community

305   Earthworm activity can increase the contents of soil active organic carbon, soil inorganic
306   nitrogen, microbial biomass carbon, and microbial biomass nitrogen (Yu et al., 2007; Li et al.,
307   2002). Additionally, earthworm addition may also increase soil CO_2 emissions (Lubbers et al.,
308   2013). In this study, earthworm addition enhanced soil CO_2 emissions, which is consistent with
309   previous research results (Lubbers et al., 2013; Song et al., 2020; Yang et al., 2019). This could
310   be explained by change in the composition of the microbial community brought on by the
311   addition of earthworms, which is consistent with our findings (Yu et al., 2010). In our study,
312   earthworm addition mainly altered the relative abundance of Myxococcales and
313   Verrucomicrobia, thus affecting soil respiration. Furthermore, our results confirmed that the

314 addition of earthworms promoted seedling respiration rates for a short period of time
315 (Butenschoen et al., 2007; Jennings et al., 2016).
316   In the present study, the addition of earthworms increased bacterial richness and affected
317 the relative abundance of potential keystone taxa as well as the fungal community structure. It
318 has been shown that soluble carbon and other compounds released in the digestive tract of
319 earthworms contribute to bacterial proliferation (Barbosa et al., 2017). Changes in physical and
320 chemical properties such as pH and available nitrogen led to changes in microbial community
321 composition and fungal communities, after the application of earthworm feces (Zhao et al.,
322 2016). Some studies indicated that earthworm addition increased soil pH (Wang et al., 2013),
323 while others showed that earthworm addition decreased soil pH (Xu et al., 2021). This might be
324 because earthworm activity altered the soil's water vapor coordination and acid-base neutral
325 aggregate structure, encouraging soil acid-base balance (Yu et al., 2010).

326 Effects of nitrogen deposition frequency and earthworm on soil respiration

327   In the study, the addition of earthworms and the interaction between earthworms and
328 nitrogen had no significant effect on soil respiration, which may be because the reduction of
329 respiration rate caused by the absorption of sufficient nitrogen by plants and the increase of
330 respiration rate caused by earthworm addition offset each other. Another possible explanation is
331 that earthworm addition is not adapted to the habitat and cannot survive for a long time, so it has
332 only a short-term (1-2 weeks) effect on soil respiration. Nevertheless, the effect of earthworms
333 and nitrogen on soil respiration is additive, which has important practical significance. We offer
334 scientific references for research on how soil respiration in the Yellow River beach area is
335 impacted by soil animals and nitrogen deposition, and we examine whether the short-term (3-4
336 month) effects of earthworms may be ignored.

337 Microbial community and soil properties regulated CO_2 emission

338   Important microbial classification and functional properties have been reported to
339 potentially predict changes in soil respiration. Based on the study of the random forest model, we
340 were able to pinpoint the primary microbial taxa that predict soil respiration. They have been
341 demonstrated to play a significant driving role in the rate of soil respiration. The
342 Corynebacteriaceae upregulation belonging to Actinobacteria was known to be oligotrophic,
343 which is better adapted to conditions with inadequate nutrient and carbon resources. As a result,
344 it plays a part in the degradation of recalcitrant compounds (Fu et al., 2022). The findings of the
345 investigation indicated that a major predictor of soil respiration was the unclassified
346 Myxococcales cluster1-27 (Liu et al., 2019). Proteobacteria have been found to prefer soils with
347 abundant carbon availability, hence this phylum might promote increases in the SOC fractions
348 and respiration. Additionally, SOC fractions and respiration were substantially connected with
349 Gammaproteobacteria, Sordariomycetes, and Agaricomycetes (Wang et al., 2018).
350 Gammaproteobacteria and Corynebacteriales had a beneficial correlation with soil CO_2 emission
351 in the current study, but Myxococcales, Verrucomicrobia, and Thermoleophilia had negative
352 correlations. In the mixed conifer soil, nitrogen addition decreased the absolute abundances of

353   Thermoleophilia and Verrucomicrobia (Liu et al., 2021), which is consistent with our results,
354   suggesting that nitrogen addition may alleviate the cost of nutrient stress and increase microbial
355   growth (Hessen et al., 2004; Li et al., 2019). Copiotrophs and oligotrophs have the ability to use
356   C for respiration, according to widely accepted reports. In general, it has been suggested that
357   oligotrophs have slower respiration rates than copiotrophs (Liu et al., 2108; Chen et al., 2021;
358   Liu et al., 2020). Verrucomicrobia, Thermoleophilia, and Corynebacteriales are categorized as
359   oligotrophs, whilst Myxococcales and Gammaproteobacteria are thought to be potential
360   copiotrophs (Chen et al., 2021). Thermoleophilia and Corynebacteriales, two crucial
361   Actinobacterial groups for forecasting soil respiration, exhibited the lowest sum of relative
362   abundance in the NL treatment compared to other treatments, although Gammaproteobacteria
363   showed an increasing trend. Consequently, the relationship between bacterial community
364   structure and CO_2 emission may be explained by the activity of bacterial communities in
365   response to changes in the oligotroph-to-copiotroph ratio within the community, which may also
366   explain why NL has the greatest CO_2 emissions.
367   The SEM demonstrated a strong association of bacterial community richness and the
368   potential keystone taxa with the respiration rates, indicating that changes in the community
369   composition of soil bacteria could result in predictable variations in soil respiration. Meanwhile,
370   the important taxa may also alter the soil microbial community composition, which may have an
371   impact on the rates of soil carbon respiration. These findings are consistent with recent research
372   emphasizing the crucial function of key taxa and the microbial community composition in soil
373   respiration (Liu et al., 2019; Liu et al., 2018; Wang et al., 2022). Importantly, our SEM revealed
374   that soil properties like pH and NO_3^--N affected soil respiration rates indirectly by changing the
375   soil microbial community rather than directly (Liu et al., 2019; Wang et al., 2022). Our results
376   highlight the importance of core species and community composition as predictors in the
377   development of the soil carbon model (Liu et al., 2019; Wang et al., 2018; Wang et al., 2022;
378   Whitaker et al., 2014). Furthermore, our findings imply that bacteria communities are
379   significantly better than fungus at forecasting changes in soil respiration. This is consistent with
380   the widespread belief that bacterial communities, not fungi, control the majority of ecological
381   activities in agricultural ecosystems (Van Der Heijden et al., 2007), suggesting that future
382   research on major soil groups that regulate carbon emissions should pay more attention to the
383   communities of bacteria. These results can help us anticipate more accurately how the world's
384   carbon emissions will respond to climate change. They may also provide light on how to manage
385   the widespread and highly valuable farming soil carbon storage in the future (Liu et al., 2019).

386   **Conclusions**

387   Our results reveal that nitrogen deposition frequency has a significant impact on CO_2
388   emissions. Changes in the bacterial community composition, which directly affect CO_2
389   emissions, as well as changes in soil pH and NO_3^--N, which have an indirect effect, are both
390   responsible for low-frequency nitrogen's large increase in CO_2 emissions. Earthworm addition
391   briefly increased soil respiration and contributed in an additive way to the reaction of CO_2
392   emissions to the frequency of nitrogen deposition, despite the fact that we did not see a

393 significant response of CO_2 emission to earthworm addition. Furthermore, our study advances
394 our understanding of soil carbon emissions from croplands by highlighting the significance of
395 microbial community composition and keystone species in forecasting soil CO_2 emissions.
396 Overall, this study provides data support and fills the knowledge gap for studying the effects of
397 nitrogen deposition frequency and soil animals on CO_2 emissions in the Huang-Huai-Hai Plain.
398 This study provided further evidence that ecosystem structure and functional traits are influenced
399 by the frequency of nitrogen deposition. More simulations of the nitrogen deposition process will
400 be required in the future in order to better understand the microbiological alterations and
401 mechanisms in ecosystems' carbon cycles.

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Competing Interests

599 The authors declare there are no competing interests.

600

Author contribution

602 • Meiguang Jiang conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the article, and approved the final draft.

605 • Jingyuan Yang performed the experiments, authored or reviewed drafts of the article, and approved the final draft.

607 • Qi Xu performed the experiments, authored or reviewed drafts of the article, and approved the final draft.

609 • Linyu Qi performed the experiments, authored or reviewed drafts of the article, and approved the final draft

611 • Yue Gao performed the experiments, authored or reviewed drafts of the article, and approved the final draft.

613 • Huijie Lu performed the experiments, authored or reviewed drafts of the article, and approved the final draft

615 • Yuan Miao conceived and designed the experiments, authored or reviewed drafts of the article, and approved the final draft

617 • Cancan Zhao conceived and designed the experiments, authored or reviewed drafts of the article, and approved the final draft

619 • Shijie Han conceived and designed the experiments, authored or reviewed drafts of the article, and approved the final draft

621

Data Availability

623 The following information was supplied regarding data availability:

624 The raw data are available in the Supplemental Files.

625 Sequence Data, https://www.ncbi.nlm.nih.gov/sra/PRJNA1008076

Figure 1

Seasonal dynamics and mean values of soil respiration under the six treatments in 2022 (M ± SE, n = 5).

Control (C)、high-frequency nitrogen addition (NH)、low-frequency nitrogen addition (NL)、earthworm addition (E)、earthworm and high-frequency nitrogen addition (E*NH)、earthworm and low-frequency nitrogen addition (E*NL).
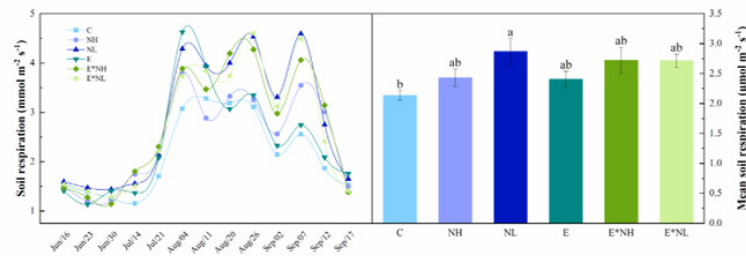
Fig. 1. Seasonal dynamics and mean values of soil respiration under the six treatments in 2022 (M ± SE, n = 5). Control (C)、high-frequency nitrogen addition (NH)、low-frequency nitrogen addition (NL)、earthworm addition (E)、earthworm and high-frequency nitrogen addition (E*NH)、earthworm and low-frequency nitrogen addition (E*NL).

Figure 2

Effects of nitrogen addition (NH, NL) and earthworm addition (E) on Total N (TN), Soil moisture (SM), Nitrate N (NO_3^--N), Ammonium N (NH_4^+-N), Soil pH and Grain yield (GRY) in 2022 (M ± SE, n = 5).

Control (C)、high-frequency nitrogen addition (NH)、low-frequency nitrogen addition (NL)、earthworm addition (E)、earthworm and high-frequency nitrogen addition (E*NH)、earthworm and low-frequency nitrogen addition (E*NL).
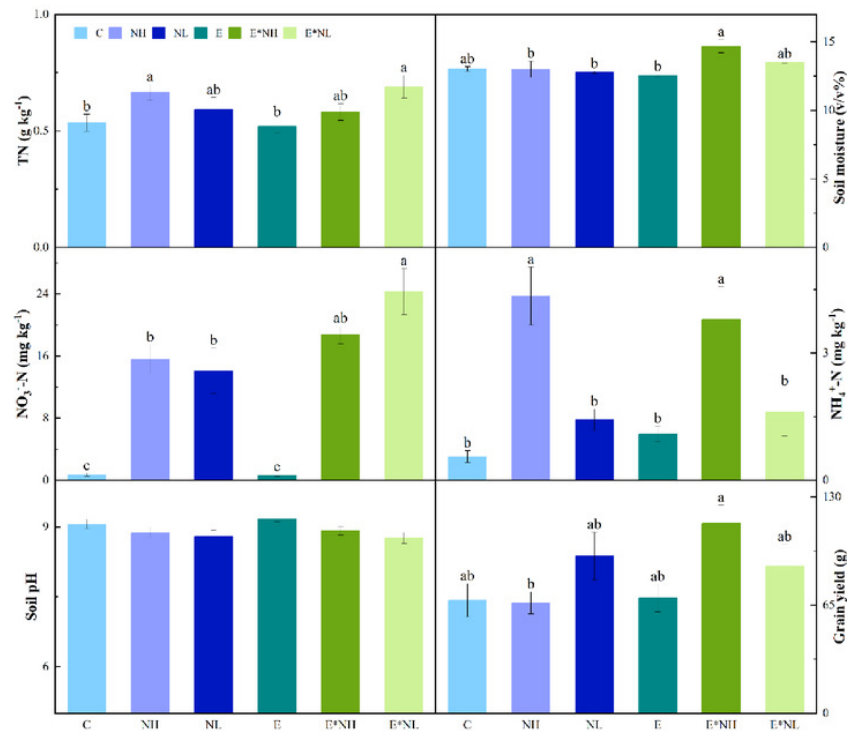
Fig. 2. Effects of nitrogen addition (NH, NL) and earthworm addition (E) on Total N (TN), Soil moisture (SM), Nitrate N (NO_3^--N), Ammonium N (NH_4^+-N), Soil pH and Grain yield (GRY) in 2022 (M ± SE, n = 5).

Figure 3

Predictor importance of major bacterial and fungal phyla/classes as drivers of soil respiration based on random forest model.

Most of them are bacteria, and a few are fungi.
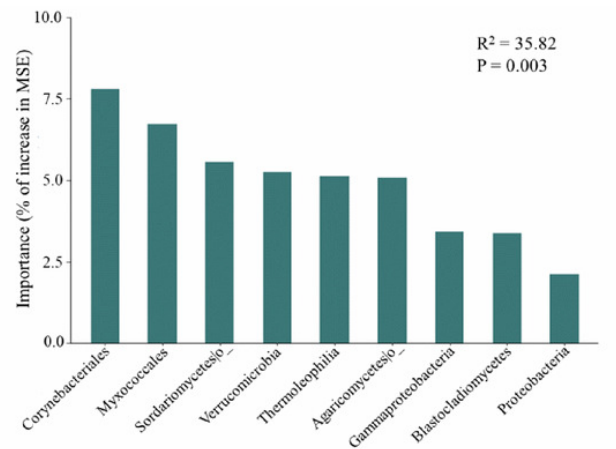
Fig. 3. Predictor importance of major bacterial and fungal phyla/classes as drivers of soil respiration based on random forest model.

Figure 4

Relationships of soil respiration with soil pH (a), Nitrate N (NO_3^--N), Aboveground biomass (AGB), Root biomass (RB), Plant height (PLH), Grain yield (GRY) .

Soil respiration was positively correlated with aboveground biomass, grain yield and plant height, and negatively correlated with NO_3^--N and soil pH.
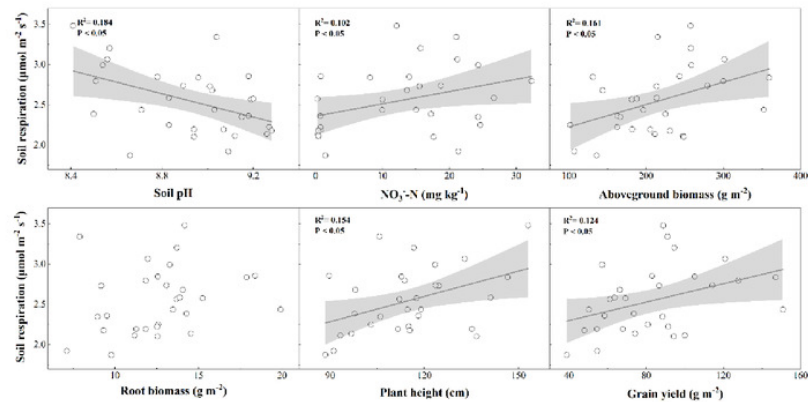
Fig. 4. Relationships of soil respiration with soil pH (a), Nitrate N (NO₃⁻-N), Aboveground biomass (AGB), Root biomass (RB), Plant height (PLH), Grain yield (GRY).

Figure 5

Relationships of soil respiration with Bacterial Pco1(a), Myxococcales (b), Corynebacteriales (c), Gammaproteobacteria (d), Verrucomicrobia (e), Thermoleophilia (f).

SR was significantly positively correlated with the relative abundance of Gammaproteobacteria and Corynebacteriales, as well as bacteria Pco1, and negatively correlated with the relative abundance of Myxococcales, Verrucomicrobia and Thermoleophilia.
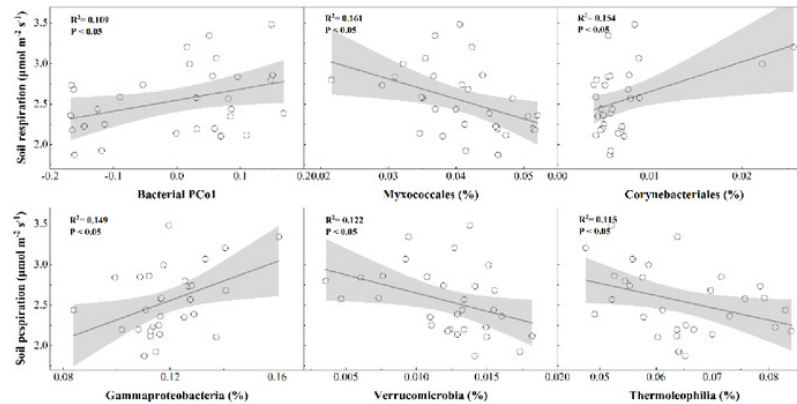
Fig. 5. Relationships of soil respiration with Bacterial Pco1(a), Myxococcales (b), Corynebacteriales (c), Gammaproteobacteria (d), Verrucomicrobia (e), Thermoleophilia (f).

Figure 6

Structural equation modeling (SEM) showing effects of soil abiotic and biotic properties on soil respiration.

Blue and red lines indicate significant positive and negative relationships, respectively. *** p < 0.001; ** p < 0.01; * p < 0.05.
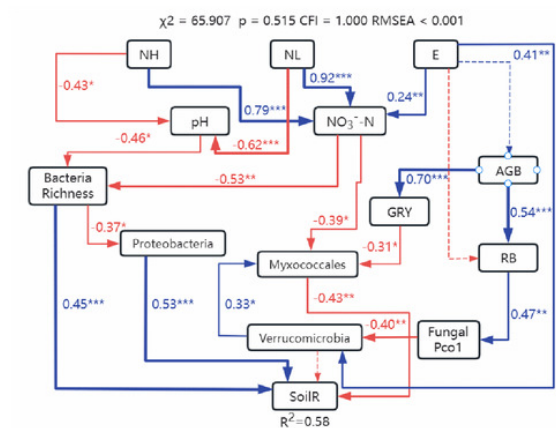
Fig. 6. Structural equation modeling (SEM) showing effects of soil abiotic and biotic properties on soil respiration. Blue and red lines indicate significant positive and negative relationships, respectively. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

Results (F and p values) of two-way ANOVAs on the effects of Nitrogen addition (N), Earthworm addition (E), and their interactions on Soil respiration (SR), Soil temperature (ST), Soil moisture (SM), Soil pH, Total C (TC), Total N (TN), Nitrate N (NO[sub]

Statistical differences are indicated as $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

Variables	N	E	N × E
SR	**5.322***	1.202	1.281
ST	0.210	0.034	0.581
SM	**4.308***	**4.656***	**4.561***
Soil pH	**5.370***	0.186	0.241
TN	**4.566***	0.031	3.157
TC	0.394	0.440	0.529
NO_3^--N	**14.581***	0.417	1.600
NH_4^+-N	**6.207**	1.515	0.915
AGB	0.750	1.967	2.675
RB	0.301	0.121	1.516
PLH	2.137	0.723	1.563
NPP	0.270	0.002	2.326
NGP	0.355	0.002	2.897
GRY	2.838	2.650	**3.651***
W100	0.893	2.929	0.907

2   **Table 1.**   Results (F and p values) of two-way ANOVAs on the effects of Nitrogen addition (N), Earthworm addition (E), and

3   their interactions on Soil respiration (SR), Soil temperature (ST), Soil moisture (SM), Soil pH, Total C (TC), Total N (TN), Nitrate

4   N (NO_3^--N), Ammonium N (NH_4^+-N), Aboveground biomass (AGB), Root biomass (RB), Plant height (PLH), Number of pods

5   per plant (NPP), Number of grains per plant (NGP), Grain yield (GRY), 100-grain weight (W100). Statistical differences are

6   indicated as $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

Spearman's rank correlation coefficients (ρ) between the microbial (bacterial and fungal) characteristics soil properties, and plant biomass as well as soil respiration.

**. Correlation is significant at the 0.01 level; *. Correlation is significant at the 0.05 level.

Richness (number of OTUs), Simpson (alpha diversity), Pco1, Pco2 (beta diversity), Soil respiration (SR), Soil temperature (ST), Soil moisture (SM), Soil pH, Total C (TC), Total N (TN), Nitrate N (NO_3^--N), Ammonium N (NH_4^+-N), Aboveground biomass (AGB), Root biomass (RB).

Microbial community	SR	ST	SM	SWC	Soil pH	TN	TC	NO$_3$-N	NH$_4^+$-N	AGB	RB
Bacterial Richness	-0.022	-0.226	-0.341	-0.192	-0.044	0.153	-0.265	**-0.390***	-0.113	-0.078	-0.154
Bacterial Simpson	**-0.459***	-0.109	-0.023	-0.001	0.096	0.061	0.096	-0.183	-0.254	-0.346	**-0.486****
Bacterial Pco1	0.281	**-0.658****	0.037	0.239	-0.320	0.325	**0.640****	0.080	-0.093	**0.380***	0.285
Bacterial Pco2	0.204	**0.393***	**0.386***	0.051	**-0.411***	0.091	-0.083	**0.706****	0.172	0.167	-0.193
Fungal Richness	-0.146	0.020	-0.155	-0.357	**0.383***	-0.161	-0.324	-0.340	-0.048	-0.087	-0.010
Fungal Simpson	-0.315	-0.104	0.197	0.013	0.024	0.189	0.256	-0.019	-0.010	-0.214	**-0.402***
Fungal Pco1	0.250	-0.232	-0.294	-0.066	-0.081	0.022	-0.096	-0.212	0.061	0.293	**0.503****
Fungal Pco2	0.145	-0.303	0.350	0.063	-0.326	0.206	**0.434***	0.051	-0.003	0.129	-0.064
Proteobacteria	0.276	0.016	0.184	0.304	-0.176	0.123	0.254	**0.373***	0.135	0.142	-0.119
Verrucomicrobia	**-0.439***	0.078	0.004	-0.133	0.134	-0.001	-0.063	-0.196	0.136	-0.217	-0.208
Gammaproteobacteria	**0.380***	0.148	0.315	0.044	-0.221	0.165	0.087	**0.479****	0.199	0.156	-0.076
Thermoleophilia	**-0.399***	**0.530****	-0.050	**-0.562****	**0.500****	**-0.362***	**-0.565****	-0.306	0.016	**-0.402***	-0.194
Myxococcales	**-0.483****	-0.020	-0.285	0.202	**0.366***	-0.184	0.033	**-0.380***	-0.277	**-0.413***	-0.330
Corynebacteriales	0.304	-0.252	-0.087	0.177	-0.044	0.011	0.167	-0.131	0.041	0.077	**0.421***
Blastocladiomycetes	**0.421***	-0.056	0.263	0.165	-0.297	**0.466****	0.067	**0.481****	0.274	0.234	0.013
Sordariomycetes_o_	-0.047	0.075	0.012	-0.237	-0.135	0.089	-0.090	-0.133	-0.007	0.162	-0.120
Agaricomycetes_o_	**-0.427***	-0.017	-0.113	0.272	0.101	0.048	0.009	0.019	0.003	**-0.462***	-0.225

2 **Table 2.** Spearman's rank correlation coefficients (ρ) between the microbial (bacterial and fungal) characteristics soil properties,

3 and plant biomass as well as soil respiration. **. Correlation is significant at the 0.01 level; *. Correlation is significant at the

4 0.05 level. Richness (number of OTUs), Simpson (alpha diversity), Pco1, Pco2 (beta diversity), Soil respiration (SR), Soil

5 temperature (ST), Soil moisture (SM), Soil pH, Total C (TC), Total N (TN), Nitrate N (NO$_3^-$-N), Ammonium N (NH$_4^+$-N),

6 Aboveground biomass (AGB), Root biomass (RB).