The responses of CO_2 emission to nitrogen application and earthworm addition in the soybean cropland

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The impacts of nitrogen application and earthworms on soil respiration and microbial communities in the Huang-Huai-Hai Plain in China have received increasing attention in the literature. However, research on the impact of earthworms and nitrogen application frequency on soil carbon dioxide (CO_2) emission and the microbial community is still limited. We conducted a field study in a farming ecosystem to investigate the effects of earthworm activity and nitrogen application frequency on soil respiration in the Huang-Huai-Hai Plain. Nitrogen application frequency had a significant effect on soil respiration, but neither earthworms nor their interaction with nitrogen application frequency were significant. Low-frequency nitrogen application (NL) significantly increased soil respiration by 25%, while high-frequency nitrogen application (NH), earthworm addition (E), earthworm and high-frequency nitrogen application (E*NH), and earthworm and low-frequency nitrogen application (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. The soil pH and soil NO_3^--N 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 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 frequency nitrogen application.

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Abstract

The impacts of nitrogen application and earthworms on soil respiration and microbial communities in the Huang-Huai-Hai Plain in China have received increasing attention in the literature. However, research on the impact of earthworms and nitrogen application frequency on soil carbon dioxide (CO_2) emission and the microbial community is still limited. We conducted a field study in a farming ecosystem to investigate the effects of earthworm activity and nitrogen application frequency on soil respiration in the Huang-Huai-Hai Plain. Nitrogen application frequency had a significant effect on soil respiration, but neither earthworms nor their interaction with nitrogen application frequency were significant. Low-frequency nitrogen application (NL) significantly increased soil respiration by 25%, while high-frequency nitrogen application (NH), earthworm addition (E), earthworm and high-frequency nitrogen application (E*NH), and earthworm and low-frequency nitrogen application (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. The soil pH and soil NO_3^--N 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 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 frequency nitrogen application.

Introduction

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

raises atmospheric CO_2 concentrations and undermines ecological sustainability (Bond-Lamberty et al., 2010). The carbon budget of terrestrial ecosystems can be seriously affected by even minor changes in soil respiration (Heimann et al., 2008). As one of the most active parts of the terrestrial ecosystem, the agricultural system is crucial to the global carbon cycle (Crippa et al., 2021). Soil carbon emissions from farmland must be understood to predict and manage soil carbon stores (Wu et al., 2019). The main components of soil respiration are microbial and plant root respiration, both of which are regulated by biotic and abiotic factors (Chen et al., 2019; Lei et al., 2021), such as root dynamics, microclimate, substrate availability, nutrition levels, and soil microbial activity (Allison et al., 2010; Talmon et al., 2011; Wagai et al., 2013; Bolat et al., 2016; Wang et al., 2019). These variables have been incorporated into parameterizing models that forecast shifts in the global carbon cycle. However, there remains uncertainty in estimating soil respiration, which limits our capacity to forecast carbon cycling under scenarios of climate change (Liu et al., 2020b). Microbial ecology is one of the most promising fields in the hunt for novel indicators of soil carbon cycling (Liu et al., 2020b). Soil microorganisms play significant roles in predicting CO_2 emission through microbial processes (Liu et al., 2018). Both theoretical and practical evidences point to the possibility of predicting soil carbon fluxes using the functional and taxonomic characteristics of soil microbial communities (Liu et al., 2019; Allison et al., 2010; Liu et al., 2018). For instance, changes in the soil microbial community composition have an impact on soil carbon respiration and fixation (Monteux et al., 2018; Müller et al., 2018). Previous studies have revealed that the compositions of these microbial communities may play a role in regulating CO_2 emissions (Chen et al., 2021; Wang et al., 2019) since copiotrophs have a faster respiration rate than oligotrophs and that proteobacteria and actinobacteria are positively connected with CO_2 emissions (Liu et al., 2018; Chen et al., 2021; Liu et al., 2020). So far, the extent to which the structure and the composition of the microbial community altered CO_2 emission in farming systems has not yet been thoroughly determined.

Fertilization is typically thought to be the primary method for increasing crop yield, it also has a significant impact on the carbon pool and carbon flux in the soil. The structure and function of the world's ecosystems are significantly impacted by changes caused by anthropogenic nitrogen application (Cao et al., 2021). The influence of nitrogen application is still being debated despite many nitrogen addition experiments being carried out to examine how ecosystem carbon exchange mechanisms respond to nitrogen application (Cao et al., 2020; Yang et al., 2020). The frequency of nitrogen amendment is a key factor for simulating nitrogen application. The frequency of nitrogen addition has many effects on the ecosystem. For example, under different nitrogen addition frequencies, nitrogen accumulation, plant nitrogen concentration, plant species loss, and plant biomass are significantly different (Ning et al. 2022). At present, there are few reports on the effects of nitrogen application frequency on soil respiration, particularly when it comes to microbial communities. Hence, nitrogen application frequency experiments included plant, soil and microbial components of ecosystems are needed to fill this gap in understanding (Cao et al., 2020).

In soil formation and function processes, earthworms play a crucial role as keystone detritivores and ecosystem engineers (Fonte et al., 2023; Yang et al., 2019; Fahey et al., 2013).

They can affect soil carbon dynamics since they are ecosystem engineers living in the soil (Jennings et al., 2016). Previous research has demonstrated that soil CO_2 emissions can rise as a results of earthworm invasion (Lubbers et al., 2013). Through their interactions with microbes, macro, and microfauna, earthworms greatly influence the decomposition process and increase heterotrophic activity, which in turn affects soil carbon dioxide emissions (Fisk et al., 2004). Earthworms directly or indirectly affect nitrogen cycle and have the potential to alter ecosystem functions and services in relation to nitrogen cycle (Xue et al., 2022). However, there is still limited research on the effects of nitrogen application frequency and earthworm addition on ecosystem carbon emissions. Therefore, disentangling how nitrogen application frequency and earthworm addition affect CO_2 emission and its relationship with the soil microbial community is of great significance for mediating C cycling in farmland.

Here, we aimed to explore how the microbial community regulates the response of CO_2 emission to different frequencies of nitrogen application and earthworm addition. In this study, field experiment with six treatments was performed in the Huang-Huai-Hai Plain. The specific questions of this study we tried to address are: (1) How does CO_2 emission respond to nitrogen application frequency and earthworms? (2) What were the underlying mechanisms influencing CO_2 emission response to nitrogen application frequency and earthworms?

Materials and methods

Study site

This research was performed at the farm of Jinming Campus of Henan University, Kaifeng City, Henan Province, China (34°49′N, 114°18′E). The crop was soybean and the variety is Kaidou 1104. A permanent 25 × 10 m^2 rainout shelters with steel frames and covered with clear polyethylene roof was built in late summer 2021 to control precipitation inputs each year, to avoid the death of soybean and earthworms caused by extreme rainfall. The appropriate rainfall amounts were selected to simulate natural precipitation in the local area with the long-term rainfall means. The region belongs to temperate continental monsoon climate with an annual mean temperature of 14 ℃ and the annual mean precipitation is of 650 mm (80% occurring between July and August). The soil texture is sandy loam.

Experimental design

The experiment used a randomized block design involving two factors of nitrogen and earthworm, including six treatments: C (control), E (earthworm addition), NH (high-frequency nitrogen application), NL (low-frequency nitrogen application), E*NH (earthworm and high-frequency nitrogen application), and E*NL (earthworm and low-frequency nitrogen application). Each treatment was replicated five times with an area of 1m × 1 m per plot. The total gram of earthworms (*Metaphire guillelmi*) was controlled at 8.0-8.9g/m^2 (about 2-4 earthworms) (Li et al., 2022). Each block was surrounded by glass to prevent earthworms from escaping. Nitrogen (urea) was added by water dissolving and root topdressing. The total amount of nitrogen added was the same as the conventional local field nitrogen application. The experiment included two frequencies of N application (2 times vs. 12 times): High frequency nitrogen was applied once

every seven days from July 15th to September 30th, and low frequency nitrogen was applied once every 30 days from July 15th to September 30th. From seedling stage (VE) to drum stage (R6), high-frequency nitrogen was uniformly added 12 times with 29 N kg·hm^{-2} each time and low-frequency nitrogen was uniformly added twice with 174 N kg·hm^{-2} each time. Earthworms were added in July 15th and August 21st, 2022.

Measurement of soil respiration

Soil respiration and temperature were measured every seven days during the Soybean growing season using a Li-8100 portable soil CO_2 flux system (Li-Cor, Inc. Lincoln, NE, USA) and a thermocouple probe (Li-8100-201, Li-Cor, Inc. Lincoln, NE, USA) connected to the Li-8100 in June 2022 to October 2022. Soil volumetric water content at 0-10cm soil depth was determined adjacent to each collar using a soil detector (TR-6D). All measurements were performed between 9 a.m. and 11:30 a.m. To avoid the respiration of aboveground parts of plants and litter decomposition, all living plants and litter inside the collars were removed by hand two days before soil respiration was measured. If it rains heavily, our measurement would be postponed for two days.

Soil sampling and analysis and plant index measurements

In September 2022. Three soil cores were randomly collected from each plot at a depth of 0-10 cm using a soil corer (inner diameter 5 cm) and mixed into one, sieved through a 2 mm mesh to separate gravel and roots, and divided into three parts. One subsample was stored at 4 °C for the analysis of the chemical properties of soil i.e. the available ammonium and nitrate using a colourimetric method (Smart Chem 200 Discrete Auto Analyser, Systea, Italy). Another subsample was air-dried and ground for analysis of pH、total N (TN) and total carbon (TC). The soil pH was measured with a soil pH meter (TR-6D). The soil TN and TC concentrations were measured by a Vario ELIII Elementar (Elementar Analysensysteme GmbH, Germany) elemental analyzer. The third part was stored at -20 ℃ for the analysis of the microbial community diversity composition spectrum (Wang et al., 2022).

After removing the whole plant from the soil, rinsed it slowly with running water to separate the above and below-ground parts of the plant from the cotyledon nodes. The washed roots were dried in an oven at 65℃ to a constant weight, and the root biomass (RB) was weighed. Aboveground biomass (AGB) was weighed after two weeks of natural air drying. Grain yield (GRY) was measured by removing the mature pods from the plants, placing them in paper bags, and leaving them in a ventilated place for drying to constant weight. The plant height (PLH) was measured by selecting 3 plants from each plot and measuring them with a tape measure. The number of pods per plant (NPP) and number of grains per plant (NGP) were also artificially measured by choosing 3 plants from each plot (Ji et al., 2017). 100 grain weight (W100) was chosen at random from the grain yield of each plot and weighed using a precision scale (Ji et al., 2017).

DNA extraction, PCR amplification, and Illumina sequencing

Data were collected as previously described in Li et al. (2022). Specifically using E.Z.N.A

Soil DNA Kit (Omega Bio-tek, Norcross, Georgia, USA), soil DNA was extracted from each sample in accordance with the manufacturer's protocol. The purity and concentration of the extracted DNA were determined by a NanoDrop-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) (Li et al., 2022). Purified soil DNA was fully pooled together after quantitative determination and then for downstream manipulations (Wang et al., 2022). The V3-V4 of bacterial 16S rRNA genes were amplified with the following universal primer set: upstream primers 338F (5′-ACTCCTACGGGAGGCAGCA-3′) and downstream primers 806R (5′-GGACTA CHVGGGTWTCTAAT3′). For fungi, the primers ITS5 (5′-GGAAGTAAA AGTCGTAACAAGG -3′) and ITS2 (5′-GCTGCG TTC TTCATCGATGC-3′) (Usyk et al., 2017) were used to amplify the ITS_V1 region of the rDNA gene. PCR reactions were performed in 25 μL reaction mixtures containing 5μL 5×reaction buffer, 5μL 5×GC buffer, 2μL 2.5mM dNTPs, 1μL Forwardprimer (10uM), 1μL Reverseprimer (10uM), 2μL DNA Template, 8.75μL ddH_2O, 0.25μL Q5 DNA Polymerase. The reaction conditions were programmed of an initial denaturing step at 98°C for 2 min, denaturation 98°C 15s，annealing 55°C 30s，extension 72°C 30s，final extension 72°C 5min and 10°C hold 25-30cycles. Samples were sequenced in an Illumina MiSeq High-Throughput Sequencing (HTS) platform (Illumina, San Diego, CA, USA) at Personal Biotechnology Co. Ltd Shanghai, China to determine soil microbial community composition.

Statistical analyses

Data were collected as previously described in Wang et al. (2022). Specifically, the sequenced data was performed using QIIME 2 2019.4 with slight modification. Raw sequence data were demultiplexed using the demux plugin followed by primers cutting with cutadapt plugin. Sequences were then merged, filtered and dereplicated using functions of fastq_mergepairs, fastq_filter, and derep_fulllength in Vsearch. All the unique sequences were then clustered at 98% (via cluster_size) followed by chimera removing. At last, the non-chimera sequences were re-clustered at 97% to generate OTU representative sequences and OTU table. Representative sequences were aligned with mafft and used to construct a phylogeny with fasttree. Alpha-diversity metrics (Observed_species, Simpson) were estimated using the diversity plugin with samples were rarefied. Meanwhile, principal coordinates analysis (PCoA) was selected to illustrate the clustering of different samples. In this study, Pco1 and Pco2 were used to represent the β diversity of microbial communities. ASVs were given a taxonomy using the Silva v132 99% OTU reference sequences and the classify-sklearn nave Bayes taxonomy classifier in the feature-classifier plugin (Liu et al., 2020).

Two-way ANOVAs ($p < 0.05$) was used to analyze the significant differences between nitrogen application frequency and earthworms on CO_2 emission and soil properties. One-way ANOVA with Duncan testing ($p < 0.05$) was used to evaluate the significant differences in soil properties and CO_2 emission among the six treatments. Linear regression analysis was used to study the relationship between soil respiration, soil property and soil microbial community under six treatments. Spearman's correlation analyses were performed to assess the relationships between soil properties, respiration, plant biomass and microbial community. Soil chemical

properties data were analyzed with SPSS software (version 26, IBM, Chicago, IL, USA). We conducted a classification random forest analysis to identify the major statistically significant microbial predictors of the composition (relative abundance: number of sequences of major phyla/class/order level) of bacteria and fungi acting on soil respiration. The analysis was conducted using the rfPermute package of the R (4.2.2) statistical software. The significant predictors from random forest analysis were further selected for structural equation modeling (SEM) analysis. SEM analysis was applied to determine the direct and indirect contributions of soil properties and the bacterial community to CO_2 emission. SEM analysis was performed using AMOS 22.0 software (SPSS, Chicago, IL, USA). The model fitness was evaluated by $\chi 2$ ($p > 0.05$), comparative fit index, and root mean square error of approximation.

Results

Nitrogen and earthworm application effects on soil respiration

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

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

Nitrogen application significantly influenced soil pH, TN, NO_3^--N, and NH_4^+-N. No effects of earthworm on soil properties were found. There was interactive effect between N application and earthworm on grain yield was detected (Table 1). The E*NL treatment had the highest soil TN, which was also significantly higher than the C and E treatments (Fig. 2). In comparison to the control treatment (Fig. 2), the soil NO_3^--N was considerably greater in nitrogen application treatments (NH, NL, E*NH, E*NL). Soil NH_4^+-N was significantly higher in NH and E*NH than in other treatments (Fig. 2). Soil pH of E*NL, NL, NH, E*NH decreased by 3.38%, 3.03%, 2.17%, 1.64%, and E increased 1.26% compared with C. The highest grain yield was observed in E*NH, which was also significantly greater than NH (Fig. 2). However, neither the application of nitrogen nor its interaction with earthworms had significant influence on soil temperature, TC, plant biomass and agronomic traits (except grain yield).

Soil microbial community composition for predicting soil respiration

Using random forest modeling, we identified the major bacterial and fungal phyla, classes and orders for predicting soil respiration. These taxa include several bacterial and fungal such as

Corynebacteriales, Myxococcales, Sordariomycetes, Verrucomicrobia, Thermoleophilia, Agaricomycetes, Gammaproteobacteria, Blastocladiomycetes, and Proteobacteria (Fig. 3).

The Spearman's correlation coefficients between the microbial characteristics and the soil respiration as well as soil properties were estimated (Table 2). Soil respiration rate was correlated to bacterial simpson. Particularly, there were significant relationships between the respiration and the relative abundance of major bacterial and fungal phyla. The findings demonstrated a strong correlation between soil respiration and the abundances of Blastocladiomycetes and Agaricomycetes in the fungal compositions as well as Gammaproteobacteria, Verrucomicrobia, Thermoleophilia, and Myxococcales in the bacterial compositions. (Fig. 3 and Table 2).

Relationships between soil respiration and abiotic and biotic factors

Soil respiration showed positive correlation with Nitrate N ($R^2 = 0.10$, $p < 0.05$, Fig. S1), aboveground biomass ($R^2 = 0.16$, $p < 0.05$, Fig. S1), Plant height ($R^2 = 0.15$, $p < 0.05$, Fig. S1), Grain yield ($R^2 = 0.12$, $p < 0.05$, Fig. S1), and negatively with soil pH ($R^2 = 0.18$, $p < 0.05$, Fig. S1), but it was not correlated with root biomass (Fig. S1).

Additionally, soil respiration 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. S2). Notably, the relative abundance of Thermoleophilia, Myxococcales and Verrucomicrobia was low in NL, while the relative abundance of Corynebacteriales and Gammaproteobacteria was high. In general, NL treatment raised the relative abundance of copiotrophs while decreasing the relative abundance of oligotrophs.

Structural equation modeling (SEM) further suggested that bacteria richness and microbial composition (Proteobacteria, Myxococcales) had strong direct effects on soil respiration (Fig. 4). However, nitrogen application mostly had indirect impacts on soil respiration through pH, NO_3^--N, Proteobacteria, Myxococcales, and bacteria richness, while earthworm addition had indirect impacts on soil respiration through NO_3^--N, aboveground biomass, grain yield and root biomass. Overall, the most important microbial attributes controlling soil respiration rates were the relative abundances of Proteobacteria and Myxococcales, and bacteria richness.

Discussion

Effects of nitrogen application frequency on soil respiration and microbial community

Order to increase crop output and soil quality, nitrogen fertilizer is frequently seen as a usual method (He et al., 2022). But fertilization practices can significantly impact on soil CO_2 emissions (Wang et al., 2021). In the study, fertilizer treatments considerably improved soil CO_2 emission by 12-25% in comparison to the control treatment, which is consistence with previous study findings (Yan et al., 2020; Lamptey et al., 2019). In addition, NL promoted soil respiration more than NH, indicating that nitrogen inhibited soil respiration with the increase of nitrogen frequency. Low-frequency nitrogen promotes CO_2 emission, which may be due to the high level of NO_3^--N and low level of pH increase the bacterial richness and change microbial community

composition, thus stimulating soil heterotrophic respiration and CO_2 emission. Similar findings from earlier investigations have been noted, for example, at the 2-N frequency, the application of N led to a lower, more acidic pH (Ning et al., 2015); When the same total N loading is applied in a single pulse rather than repeatedly, soil acidification and mineral N toxicity are more likely to be exacerbated (Ning et al., 2015). Plants mainly capture N from the abundant soil N form (Cao et al., 2021), which can help to explain the NO_3^--N plays a critical role as the most abundant available nitrogen in our study. Previous studies showed both soil microbial community and soil pH value affect soil respiration (Whitaker et al., 2014; Liu et al., 2020a; Liu et al., 2020b). NO_3^--N and pH were found to be important environmental factors in explaining changes in bacterial community composition (Chen et al., 2021).

In this study, NL treatment obviously increased the bacterial richness compared to C, while NH showed the opposite trend. The result was in accordance with previous researches. For instance, soil respiration rises when nitrogen levels rise, which is compatible with increased bacterial abundance and suggests that this reaction may be driven by an increased metabolic rate (Hagerty et al., 2014). As far as we know, there is almost no research on the impact of nitrogen application at different frequencies on bacterial richness. Much of the research has been on the effects of adding different levels of nitrogen on microbial richness. For instance, research has shown that adding nitrogen within a specific range might increase the amount of bacteria quantity, but that adding nitrogen in excessive would have an opposite effect (Ma et al., 2007). In our study, low-frequency nitrogen increased bacterial richness relative to high-frequency nitrogen, which is consistent with other results in this study that low-frequency nitrogen played a promoting role relative to high-frequency nitrogen. The increase in NO_3^--N and NH_4^+-N and decrease in pH after nitrogen application are directly correlated with the bacteria richness. Most bacteria prefer a pH that is close to neutral, and they are sensitive to pH variations (Anil et al., 2019). Nitrification may result in soil acidification, and the application of nitrogen enhances this reaction and further lowers pH (Sun et al., 2019). In our study, acidification of alkaline soil would make an ideal habitat for the soil microbial community to promote soil respiration. The substrate (such NO_3^--N) that provides nutrients for microorganisms may be related to the rise in soil bacterial community richness (Chen et al., 2021), which is consistent with our results. In the 6th and 7th years following nitrogen application, Cao et al. (2020) showed that high frequency considerably reduced the detrimental effects of nitrogen application on plant and soil bacterial diversity. In the present study, NH reduced the negative effect of nitrogen application on fungal diversity than NL, while the trend of bacterial diversity was the opposite, which may be due to the strong alkaline soil in our experimental site.

Effects of earthworm on soil respiration and microbial community

Earthworm activity can increase the contents of soil active organic carbon, soil inorganic nitrogen, microbial biomass carbon, and microbial biomass nitrogen (Yu et al., 2007; Li et al., 2002). Additionally, earthworm addition may also increase soil CO_2 emissions (Lubbers et al., 2013). In this study, earthworm addition enhanced soil CO_2 emissions, which is consistent with previous research results (Lubbers et al., 2013; Song et al., 2020; Yang et al., 2019). This could

be explained by change in the composition of the microbial community after the addition of earthworms (Yu et al., 2010). In our study, earthworm addition had no direct effects on soil respiration but indirectly affected the soil respiration through altering the relative abundance of Myxococcales and Verrucomicrobia. Furthermore, our results demonstrated that the addition of earthworms promoted soil respiration for a short period of time.

In the present study, the addition of earthworms increased bacterial richness and affected the relative abundance of potential keystone taxa as well as the fungal community structure. It has been shown that soluble carbon and other compounds released in the digestive tract of earthworms contribute to bacterial proliferation (Barbosa et al., 2017). Changes in physical and chemical properties such as pH and available nitrogen led to changes in microbial community composition and fungal communities, after the application of earthworm feces (Zhao et al., 2016). Some studies indicated that earthworm addition increased soil pH (Wang et al., 2013), while others showed that earthworm addition decreased soil pH (Xu et al., 2021). This might be because earthworm activity altered the soil's water vapor coordination and acid-base neutral aggregate structure, encouraging soil acid-base balance (Yu et al., 2010).

Effects of nitrogen application frequency and earthworm on soil respiration

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

Microbial community and soil properties regulated CO_2 emission

Important microbial classification and functional properties have been reported to potentially predict changes in soil respiration. Based on the study of the random forest model, we were able to pinpoint the primary microbial taxa that predict soil respiration. They have been demonstrated to be a key factor in determining the rate of soil respiration. The Corynebacteriaceae upregulation belonging to Actinobacteria was known to be oligotrophic, which is better adapted to environments with insufficient nutrient and carbon resources. As a result, it plays a part in the degradation of recalcitrant compounds (Fu et al., 2022). The investigation’s findings indicated that a major predictor of soil respiration was the unclassified Myxococcales cluster1-27 (Liu et al., 2019). Proteobacteria have been found to prefer soils with abundant carbon availability, hence this phylum might encourage increases in the SOC fractions and respiration. Additionally, SOC fractions and respiration were substantially connected with Gammaproteobacteria, Sordariomycetes, and Agaricomycetes (Wang et al., 2018). In our study,

Gammaproteobacteria and Corynebacteriales had a beneficial correlation with soil CO_2 emission, but negatively correlated with Myxococcales, Verrucomicrobia, and Thermoleophilia. In line with our findings, nitrogen application in the mixed conifer soil resulted in a decrease in the absolute abundances of Thermoleophilia and Verrucomicrobia (Liu et al., 2021). This suggests that nitrogen application may alleviate the cost of nutrient stress and increase microbial growth (Hessen et al., 2004; Li et al., 2019). According to generally believed accounts, copiotrophs and oligotrophs have ability to use C for respiration. In general, it has been suggested that oligotrophs have slower respiration rates than copiotrophs (Liu et al., 2108; Chen et al., 2021; Liu et al., 2020). Myxococcales and Gammaproteobacteria are considered potential copiotrophs, but Verrucomicrobia, Thermoleophilia, and Corynebacteriales are classified as oligotrophs (Chen et al., 2021). Thermoleophilia and Corynebacteriales, two crucial Actinobacterial groups for forecasting soil respiration, exhibited the lowest sum of relative abundance in the NL treatment compared to other treatments, although Gammaproteobacteria showed an increasing trend. Consequently, the relationship between bacterial community structure and CO_2 emission may be explained by the activity of bacterial communities in response to changes in the oligotroph-to-copiotroph ratio within the community, which may also explain why NL has the highest CO_2 emissions.

The SEM demonstrated a strong association of bacterial community richness and the potential keystone taxa with the respiration rates, indicating that changes in the community composition of soil bacteria could result in predictable variations in soil respiration. Meanwhile, the important taxa may also alter the soil microbial community composition, which may have an impact on the rates of soil carbon respiration. These findings are consistent with recent research emphasizing the crucial function of key taxa and the microbial community composition in soil respiration (Liu et al., 2019; Liu et al., 2018; Wang et al., 2022). Importantly, our SEM revealed that soil properties like pH and NO_3^--N affected soil respiration rates indirectly by changing the soil microbial community rather than directly (Liu et al., 2019; Wang et al., 2022). Our results highlight the importance of core species and community composition as predictors in the development of the soil carbon model (Liu et al., 2019; Wang et al., 2018; Wang et al., 2022; Whitaker et al., 2014). Moreover, the bacterial communities control the major of ecological activities in agricultural ecosystems (Van Der Heijden et al., 2007), which is consistence with our study. This finding shows that more attention should be paid to the microbial community in regulating carbon emissions. These results can predict how carbon emissions will respond to climate change, providing inspiration for how to manage extensive and valuable agricultural soil carbon storage in the future. (Liu et al., 2019).

Conclusions

Our results reveal that nitrogen application frequency has a significant impact on CO_2 emissions. The direct change of bacterial community composition and the indirect change of soil pH and NO_3^--N are the reasons for the large increase of CO_2 emission in low-frequency nitrogen. Earthworm addition briefly increased soil respiration and contributed in an additive way to the reaction of CO_2 emissions to the frequency of nitrogen application, despite we did not see a

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

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Competing Interests
The authors declare there are no competing interests.

Author contribution
• 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.
• Jingyuan Yang performed the experiments, authored or reviewed drafts of the article, and approved the final draft.
• Qi Xu performed the experiments, authored or reviewed drafts of the article, and approved the final draft.
• Linyu Qi performed the experiments, authored or reviewed drafts of the article, and approved the final draft
• Yue Gao performed the experiments, authored or reviewed drafts of the article, and approved the final draft.
• Huijie Lu performed the experiments, authored or reviewed drafts of the article, and approved the final draft
• Yuan Miao conceived and designed the experiments, authored or reviewed drafts of the article, and approved the final draft
• Cancan Zhao conceived and designed the experiments, authored or reviewed drafts of the article, and approved the final draft
• Shijie Han conceived and designed the experiments, authored or reviewed drafts of the article, and approved the final draft

Data Availability
The following information was supplied regarding data availability:
The raw data are available in the Supplemental Files.

638 Sequence Data, https://www.ncbi.nlm.nih.gov/bioproject/?term=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 application (NH), low-frequency nitrogen application (NL), earthworm addition (E), earthworm and high-frequency nitrogen application (E*NH), earthworm and low-frequency nitrogen application (E*NL).

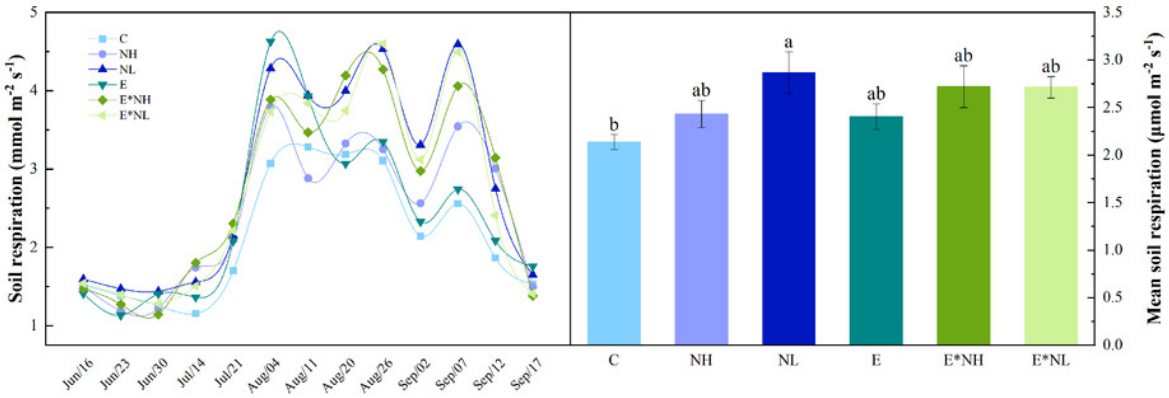

Figure 2

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

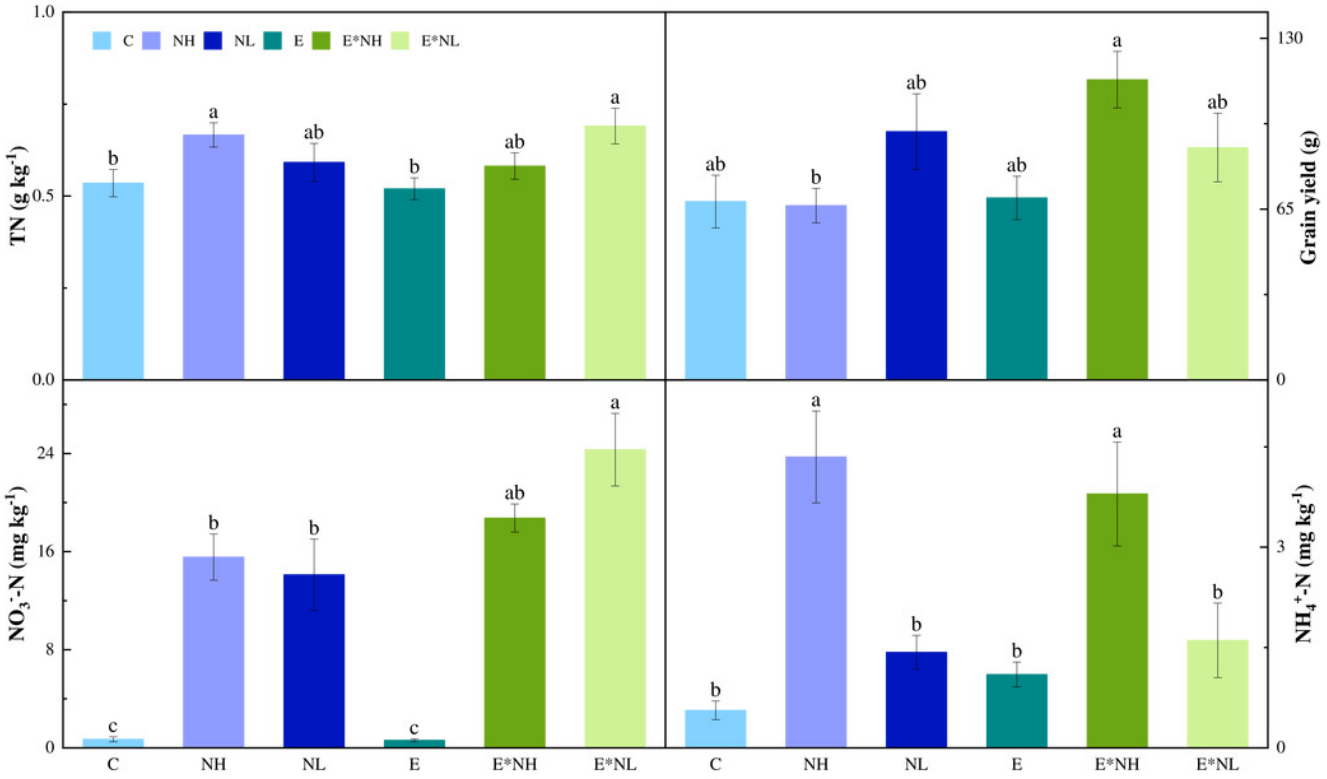

Figure 3

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

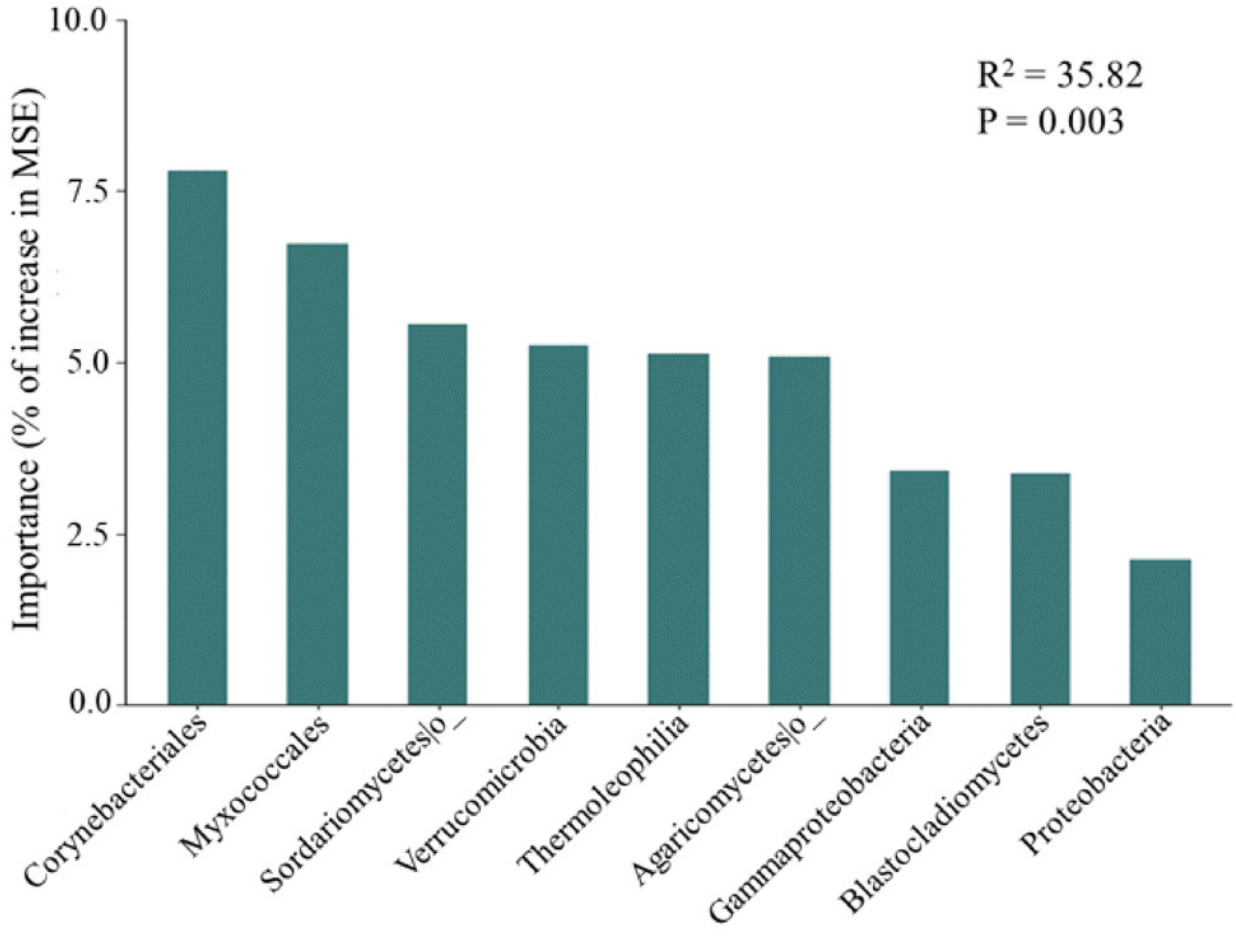

Figure 4

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. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

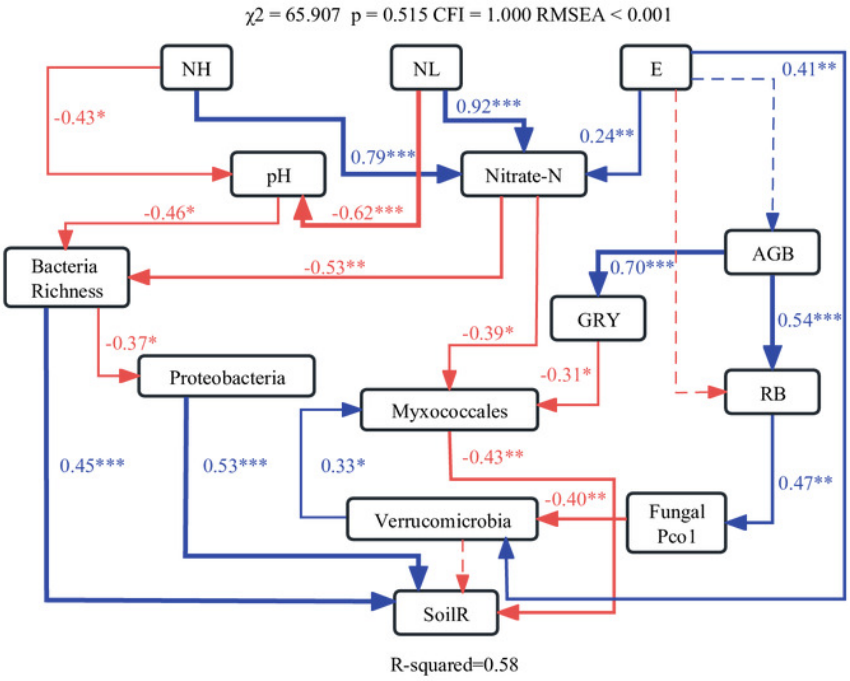
χ2 = 65.907 p = 0.515 CFI = 1.000 RMSEA < 0.001
NH
NL
E
pH
Nitrate-N
AGB
Bacteria Richness
GRY
Proteobacteria
Myxococcales
RB
Verrucomicrobia
Fungal Pco1
SoilR
-0.43*
0.92***
0.41**
0.79***
0.24**
-0.46*
-0.62***
-0.53**
0.70***
0.54***
-0.37*
-0.39*
-0.31*
0.45***
0.53***
0.33*
-0.43**
0.47**
-0.40**
R-squared=0.58

Table 1(on next page)

Results (F and p values) of two-way ANOVAs on the effects of Nitrogen application (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[s

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	2.248	2.202	3.042
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

Table 1. Results (F and p values) of two-way ANOVAs on the effects of Nitrogen application (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_3^--N), Ammonium N (NH_4^+-N), Aboveground biomass (AGB), Root biomass (RB), Plant height (PLH), Number of pods per plant (NPP), Number of grains per plant (NGP), Grain yield (GRY), 100-grain weight (W100). Statistical differences are indicated as *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

Table 2(on next page)

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).

1

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).
7
8

Figure 5

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) .

FS1

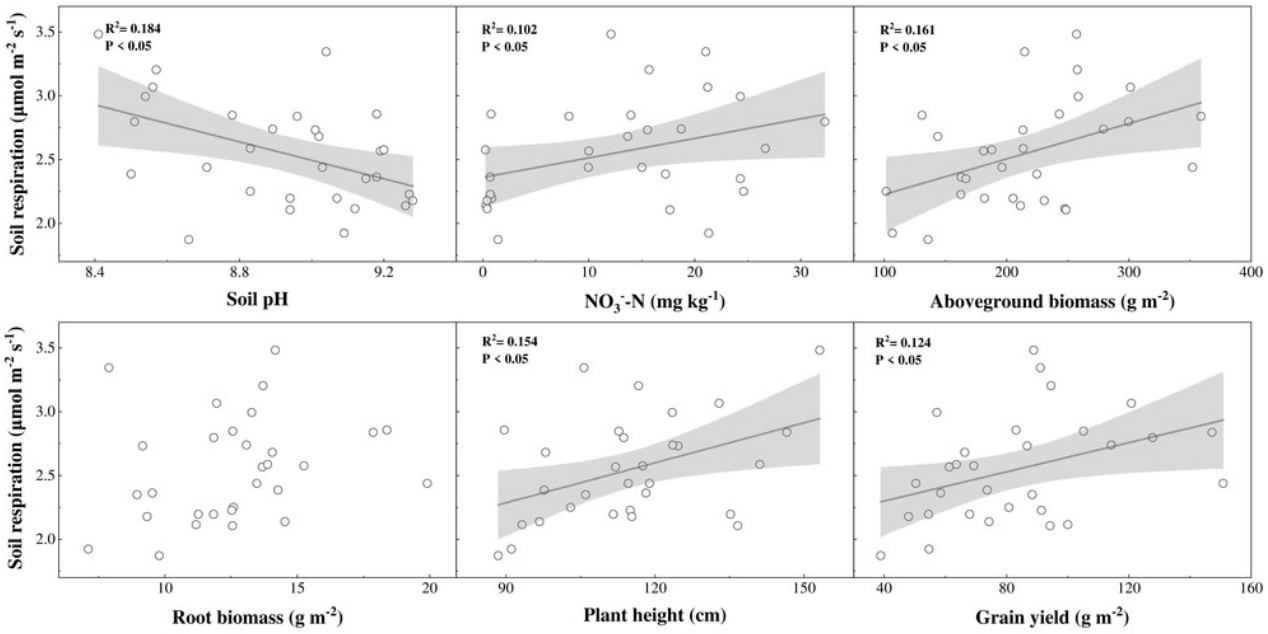

Figure 6

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

FS2