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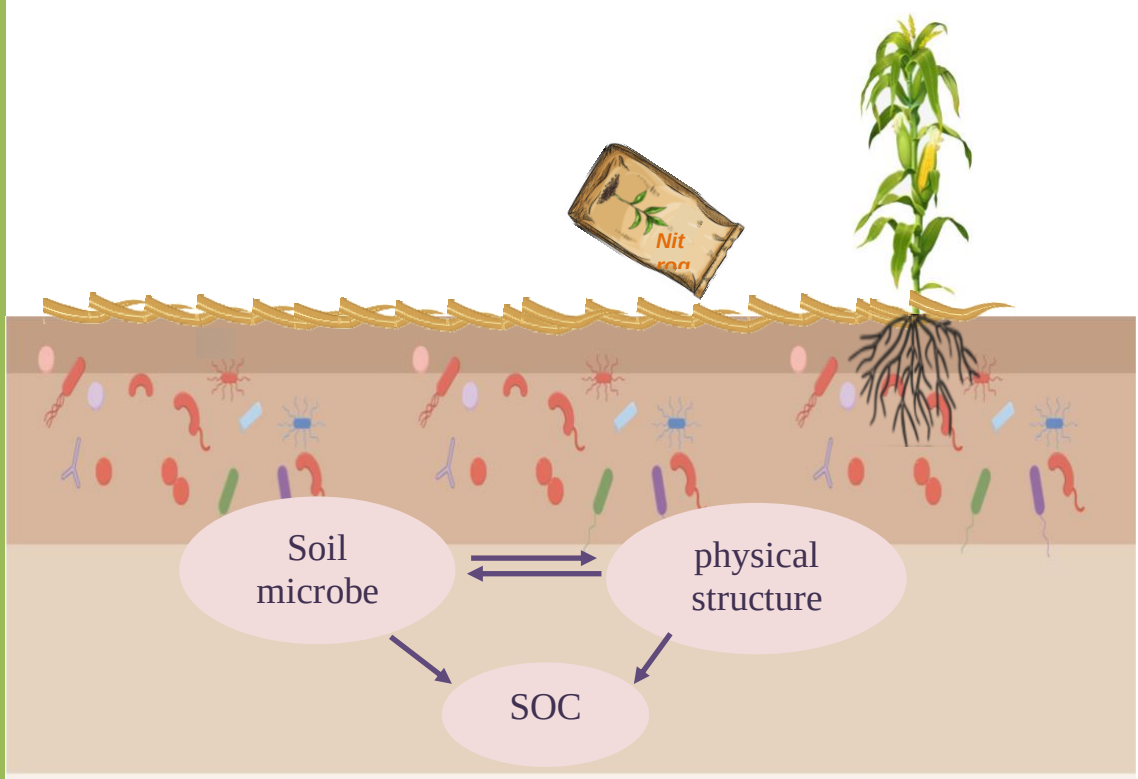
Regulation of soil organic carbon sequestration by microbial community under long-term no-tillage and nitrogen application rates



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Regulation of soil organic carbon sequestration by microbial community under long-term no-tillage and nitrogen application rates

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Abstract

A primary challenge of our era lies in achieving soil organic carbon (SOC) sequestration to provide a diverse array of benefits to the natural environment. Conservation tillage practices have gained significant global recognition to tackle this issue, owing to their impact on the physical-biological characteristics of SOC. However, the soil microbial mechanism of nitrogen (N) fertilizer effect on SOC sequestration in bulk soil under conservation tillage is still unclear. Moreover, the regulation of microorganisms within aggregates on aggregate stability and SOC sequestration remains elusive. The soil aggregate pore properties are also seldom taken into account to regulate carbon sequestration potential by changing soil microbial properties.

In this study, we used a long-term field experiment located at the Dryland Farming Experimental Station in Shouyang, Shanxi Province, in northern China to analyze the soil microbial properties (e.g. microbial community structure, microbial diversity, microbial biomass, microbial interactions, keystone taxa). We also assessed how these soil microbial properties in bulk soil influence SOC sequestration, especially revealing the mechanism of how soil microbial within aggregates affect SOC sequestration by various strategies. To better understand the SOC sequestration potential, we further assessed the effects of pore structure within aggregates on microbial interactions and keystone taxa. We used a 16-year field experiment with no-tillage (NT) and conventional tillage (CT), both of which combined with 105 (N1), 180 (N2), and 210 kg N ha^{-1} (N3) N application. The main results of this thesis are as follows:

(1) Soil microbial properties (e.g. microbial community structure, microbial diversity, microbial biomass, microbial interactions, keystone taxa) were significantly influenced by tillage management, nitrogen application rates and soil depth. The bacterial and fungal diversities of NT were higher than CT and N application decreased their diversities in 0–10 cm bulk soil. NT increased microbial carbon use efficiency (CUE) compared with CT in the 0–10 cm. Microbial CUE increased with increasing N application rate. In addition, under NT, high N application rate reduced the total amount of phospholipid fatty acids (PLFAs) within all aggregates. The bacterial network complexity of NT was higher than CT at lower N application rate but reduced at higher rates. Under NT, increasing N application rate have no impact on properties of soil bacterial co-occurrence networks. Compared to CT, NT increased fungal network complexity under the same N application rates. Increasing N application rate decreased fungal network complexity under two tillage treatments. Compared to CT, NT increased the keystone taxa of Acidobacteria, Planctomycetes, and Bacteroidete under lower N application rate, while reducing the keystone taxa of bacteria and fungi under higher N application rate. Under NT, high N application rate reduced the the keystone taxa of Acidobacteria and Bacteroidetes.

(2) The partial least squares path model showed that bacterial diversity had a positive influence on microbial CUE. Furthermore, particulate organic carbon (POC) and mineral-associated organic matter carbon (MAOC) under NT were

higher than CT and they also increased with increasing N application rate. Increasing microbial CUE induced by N application had the potential to increase POC and MAOC.

(3) Under NT, high N application rate increased SOC by 2.1–3.7 $g \cdot kg^{-1}$ within mega- and macro-aggregates. Actinobacteria were recruited by straw under NT and their biomass increased 1.5–7.8 times within all aggregates compared with CT, where they might participate in aggregate formation via degradation of straw and increasing SOC within mega- and macro-aggregates. Conversely, desulfovibrio biomass within all aggregates was diminished under NT compared with CT, while desulfovibrio possibly directly inhibited soil aggregate formation and decreased SOC within mega- and macro-aggregates under CT. Moreover, under NT, arbuscular mycorrhizal fungi biomass increased by 0.4–1.6 nmol g^{-1} within all aggregates compared with CT in 0–10 cm, potentially indirectly contributing to soil aggregate formation via co-metabolic processes and increasing SOC within mega- and macro-aggregates.

(4) The irregular and elongated pore morphology increased pore connectivity and porosity, and further increased the bacterial network connectivity. The pore connectivity and porosity augments provide favorable conditions for aerobic bacteria survival of Acidobacteria, Planctomycetes and Gemmatimonadetes. Reducing the distribution of (0-10 μm) pores may reduce competition among fungal communities. The decrease in porosity and the distribution of (10-30 μm) pores resulted in a concomitant reduction in the bacterial network connectivity and key bacterial taxa. Furthermore, high N application rates could improve soil carbon(C) sequestration potential (functional gene cbbL) by increasing the C sequestration key microorganisms (Chloroflexi and Proteobacteria) under two tillage practices.

Overall, the thesis revealed the mechanism of how tillage and nitrogen management affect SOC by changing soil microbial properties. We found that SOC sequestration in bulk soil was mainly driven by microbial CUE. N addition can alter the effect of soil microbial diversity on CUE. Furthermore, SOC within soil aggregates was mainly driven by the various survival strategies of Actinobacteria, desulfovibrio biomass and arbuscular mycorrhizal fungi. High N application under long-term NT protects SOC within mega-aggregates by altering aggregate formation through different microbial strategies. In addition, SOC sequestration potential within soil aggregates was mainly driven by microbial keystone taxa. N addition can alter the effect of soil pore structure on microbial co-occurrence patterns and keystone taxa. These results improve our understanding of explaining and predicting the sequestration and potential of SOC within bulk soil and aggregates in tillage systems. From this, we conclude that higher N application rate is the most effective nitrogen management under NT treatments from the perspective of SOC sequestration.

Keywords: No-tillage; Nitrogen; Soil microbial characteristics; Soil physical properties; Soil organic carbon

Résumé

L'un des plus grands défis mondiaux actuels est la séquestration du carbone organique du sol (COS) qui détermine en grande partie les processus clés du sol. Les pratiques de Conservation du sol ont acquis une reconnaissance mondiale significative, en raison de leur impact sur les caractéristiques physico-biologiques du sol et du COS. Cependant, le mécanisme microbien du sol lié à l'effet des engrais azotés (N) sur la séquestration du COS dans le sol en conservation n'est toujours pas clair. De plus, la régulation des microorganismes au sein des agrégats sur la stabilité des agrégats et la séquestration du COS reste insaisissable. Les propriétés de la structure interstitielle du sol sont rarement prises en compte pour réguler les propriétés microbiennes du sol et le potentiel de séquestration du carbone.

Dans cette étude, nous avons utilisé une expérience sur le terrain de long terme, située à la Station expérimentale de culture en zone aride de Shouyang, dans la Province du Shanxi, dans le nord de la Chine pour étudier les propriétés microbiennes du sol (p. ex. structure de la communauté microbienne, diversité microbienne, biomasse microbienne, interactions microbiennes, taxons).

Nous avons également évalué comment ces propriétés microbiennes influencent la séquestration du COS, en particulier en révélant de quelle manière les microbes du sol affectent la séquestration du COS à travers diverses stratégies. Pour mieux comprendre le potentiel de séquestration du carbone dans le sol, nous avons évalué les effets de la structure interstitielle des agrégats sur les interactions microbiennes et les taxons clés. Pour ce faire, nous avons utilisé une expérience de 16 ans sur le terrain avec le travail du sol sans labour (NT) et le travail du sol conventionnel (CT), tous deux combinés à une application de 105 (N1), 180 (N2) et 210 kg de N ha^{-1} (N3). Les principaux résultats de cette thèse sont les suivants:

(1) les propriétés microbiennes du sol (par exemple, la structure de la communauté microbienne, la diversité microbienne, la biomasse microbienne, les interactions microbiennes, les taxons clés) ont été considérablement influencées par la gestion du travail du sol, les taux d'application de l'azote et la profondeur du sol. Les diversités bactériennes et fongiques du NT étaient plus élevées que celles du CT et l'application de N a réduit leurs diversités dans le sol de 0 à 10 cm. NT augmente l'efficacité d'utilisation du carbone microbien (EUC) par rapport au CT dans les 0-10 cm. Le CUE microbien augmentait avec l'augmentation du taux d'application de l'azote. De plus, sous NT, un taux d'application élevé de N a réduit la quantité totale d'acides gras phospholipides (agp) dans tous les agrégats. La complexité du réseau bactérien du NT était plus élevée que celle du CT à des taux d'application de N plus faibles, mais la réduisait à des taux plus élevés. Sous NT, l'augmentation du taux d'application de N n'a aucun impact sur les propriétés des réseaux de co-occurrence bactérienne du sol. Comparativement au CT, le NT a augmenté la complexité du réseau fongique avec les mêmes taux d'application de N. L'augmentation du taux d'application de N a réduit la complexité du réseau fongique sous deux traitements du sol. Comparativement à la CT, la NT a augmenté les taxons keystone d'acidobactéries, de planctomycètes et de bactérioidete avec un taux d'application de N plus faible, tandis qu'il a réduit les taxons keystone de

bactéries et de champignons avec un taux d'application de N plus élevé. Sous NT, un taux d'application élevé de N a réduit les taxons clés des acidobactéries et des bactérioidétes.

(2) Le modèle de chemin des moindres carrés partiels a montré que la diversité bactérienne avait une influence positive sur les EUC. De plus, le carbone organique particulaire (COP) et le carbone organique associé aux minéraux (COAM) sous NT étaient plus élevés que le CT et ils augmentaient également avec l'augmentation du taux d'application de N. Que l'augmentation des marqueurs microbiens induits par l'application de l'azote avait le potentiel d'augmenter le COP et le COAM.

(3) sous NT, le taux d'application élevé de N a augmenté le COS de 2,1-3,7 $g \cdot kg^{-1}$ dans les méga - et les macro-agrégats. Les actinobactéries ont été recrutées par paille sous NT et leur biomasse a augmenté de 1,5 à 7,8 fois dans tous les agrégats par rapport au CT, où elles pourraient participer à la formation d'agrégats par la dégradation de la paille et l'augmentation du COS dans les méga- et les macro-agrégats. Inversement, la biomasse de désulfovibrio dans tous les agrégats était diminuée sous NT par rapport au CT, tandis que le désulfovibrio inhibait peut-être directement la formation d'agrégats de sol et diminuait le COS dans les méga - et les macro-agrégats sous CT. De plus, sous NT, la biomasse des champignons mycorhiziens à arbuscules a augmenté de 0,4-1,6 nmol g^{-1} dans tous les agrégats comparativement au CT dans les 0-10 cm, ce qui pourrait contribuer indirectement à la formation d'agrégats de sol par le biais de processus co-métaboliques et à l'augmentation du COS dans les méga - et les macro-agrégats.

(4) la morphologie irrégulière et allongée des pores améliore la connectivité des pores et la porosité, augmente encore la connectivité du réseau des bactéries du sol. L'augmentation de la connectivité interstitielle et de la porosité offre des conditions favorables à la survie des bactéries aérobies des acidobactéries, des planctomycètes et des gemmatimonadètes. La réduction de la distribution des pores (0-10 μm) peut réduire la concurrence entre les communautés fongiques. La diminution de la porosité et de la distribution des pores (de 10 à 30 μm) a entraîné une réduction concomitante de la connectivité du réseau bactérien et des principaux taxons bactériens. De plus, des taux d'application élevés de N pourraient améliorer le potentiel de séquestration du C dans le sol (gène fonctionnel cbbL) en augmentant la séquestration des microorganismes clés (Chloroflexi et protéobactéries) sous deux méthodes de travail du sol.

Dans l'ensemble, la thèse révèle la façon dont le travail du sol et la gestion de l'azote influent sur la du COS en modifiant les propriétés microbiennes du sol. Nous avons constaté que la séquestration du COS dans le sol était principalement provoquée par des marqueurs microbiens. L'addition d'azote peut modifier l'effet de la diversité microbienne du sol sur les repères. De plus, la du COS dans les agrégats du sol était principalement motivée par les diverses stratégies de survie des actinobactéries, de la biomasse de désulfovibrio et des champignons mycorhiziens à arbusculaires. L'application d'N élevé sous NT à long terme protège le COS dans les méga-agrégats en modifiant la formation d'agrégats à travers les communautés microbiens. De plus, le potentiel de séquestration du COS dans les agrégats du sol a

été principalement déterminé par les taxons microbiens keystone. L'addition d'azote peut modifier l'effet de la structure interstitielle du sol sur les profils de co-occurrence microbienne et les taxons keystone. Ces résultats améliorent notre compréhension pour expliquer et prévoir la séquestration et le potentiel du COS dans le sol en vrac et les agrégats dans les systèmes de travail du sol. À partir de cela, nous concluons que le taux d'application d'N plus élevé est la gestion de l'azote la plus efficace dans le cadre du NT du point de vue de la séquestration du COS.
Mots clés: travail du sol; azote; Caractéristiques microbiennes du sol; Les propriétés physiques du sol; Carbone organique du sol

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Mengni Zhang
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Chapter 1

General introduction

1. Background

One of the critical challenges of humans is to attain high soil organic carbon sequestration for mitigating climate change with reduced investment and maintaining ecosystem sustainability (Lal et al., 2015; Moinet et al., 2023; Rodrigues et al., 2023). Conventional tillage (CT) necessitates two or more annual intensive tillage operations at a depth of between 20 and 30 cm, leading to soil fragmentation, collapse, compaction, and soil carbon loss in China. However, conservation agriculture (CA) and nitrogen addition as two common management strategies have received widespread attention to solving this issue because of their effects on carbon (C) sequestration in soil ecosystems. CA is based on the principles of minimum tillage, soil mulching by crop straw at least 30%, and implementation of a crop rotation system (Kassam et al., 2019). Applying solely the three defining principles of CA is frequently insufficient, necessitating the incorporation of supplementary techniques such as effective nitrogen management to enhance productivity and optimize the functionality of CA systems (Thierfelder et al., 2018; Zhang et al., 2014).

Fig. 1-1 Conservation agriculture is composed of four principles (Lal, 2018).

The area of CA has experienced a significant worldwide increase since the 1970s, resulting in a growth of its area to 180.44 M ha by 2015 (Kassam et al., 2019). In the past few years, 12.5% of the global cropland area is CA (Kassam et al., 2019). However, the regional distribution of tillage practices varies. Table 1-1 presents the percentage of land area implementing CA across different continents. CA accounts for approximately 73.7% of the total global area in America, while Asia's share is merely 7.70% (Kassam et al., 2019).

The inception of CA research in China can be traced back to the 1970s (Zheng et al., 2014). However, China encounters specific challenges in the adoption and implementation of CA, primarily due to its heterogeneous cropping systems and the comparatively larger quantities of residues and stubbles left in the fields. Nonetheless, the demand for CA in China has been steadily rising, driven by its economic benefits, limited natural resources, and diminishing agricultural labor

force. Consequently, CA has emerged as a pivotal agricultural technology within the Chinese context, the area of CA has increased by more than four times in the past 15 years, and the area has increased to 9.0 M ha (Kassam et al., 2019).

Table 1-1 Cropland area under CA (M ha) by region in 2015/16; CA area as % of global total cropland (Kassam et al., 2019).

Region	CA Cropland area	Percent of global CA cropland area
South America	69.9	38.7
North America	63.18	35.0
Australia & NZ	22.67	12.6
Asia	13.93	7.7
Russia & Ukraine	5.7	3.2
Europe	3.56	2.0
Africa	1.51	0.8
Global total	180.44	100

Since 2008/09, the increased interest of farmers in the CA farming system approach to sustainable production and agricultural land management. The CA area in China witnessed a significant increase from 1.3 to 9.0 Mha from 2008 to 2016 (Kassam et al., 2019). However, the CA area in China is increasing, but it is still much lower compared to the USA (43.2 Mha), Brazil (32 Mha), Canada (19.9 Mha), and Australia&New Zealand (22.6 Mha) in 2016 (Kassam et al., 2019). The main reason is the challenge of achieving SOC sequestration and sustaining high crop yield with CA and the knowledge about how CA influences SOC sequestration via microbial mechanism and soil physical properties is lacking.

Changes in SOC sequestration influences some soil quality, such as production, soil erosion control, improvement in water and air quality, nutrient cycling, soil carbon dynamics, and biodiversity. Due to this factor, extensive research has been conducted on the impact of CA on SOC sequestration; however, conflicting outcomes have emerged (Niu et al., 2019; Stewart et al., 2017).

1.1. Local agricultural context

In the Loess Plateau region of China, the topography is undulating, characterized by numerous mountains and hills, resulting in fragmented cultivated land and limited mechanization (Qin et al., 2024). Furthermore, due to its relatively low level of economic development, farmer use traditional agricultural production methods (manual labor and basic farming tools for cultivation and crop growth). Farmers primarily engage in manual farming with small-scale cultivation areas that entail high labor intensity.

The Loess Plateau is predominantly characterized by sandy loam cinnamon soil, which exhibits poor water and soil retention properties. This type of soil is less fertile due to lower organic matter content and nutrient availability, thereby imposing limitations on crop growth (An et al., 2010). Furthermore, the region faces water scarcity issues with precipitation being the primary source of irrigation for rain-fed agriculture (Sun et al., 2019).

The agricultural production in the Loess Plateau is still dominated by traditional agriculture. Many farmers choose to go out for work or engage in non-agricultural

industries, resulting in the reduction of the agricultural labor force. CA does not need to loosen the soil, which saves the labor and time cost of tilling the soil compared to traditional farming methods (Lal, 2018). In addition, due to the relatively low level of development in the region, farmers are generally in a relatively difficult economic situation and a relatively low level of education, and they usually focus on crop yield and economic benefits. Therefore, when choosing the type and amount of nitrogen fertilizer, they tend to use the chemical fertilizer urea which can improve the yield and quality, and try to control the cost to ensure the convenience and economic efficiency of agricultural production. According to (Fao, 1999), the total use of chemical fertilizer in China ranks first in the world and the average N addition rate is 235 kg N ha^{-1}. Farmers in this area have experienced that fertilization with 210 kg N ha^{-1} can obtain high yields of maize. However, the previous fertilization study in this study region showed that N uptake of maize plants, fertilizer N availability, and yield reached the maximum when N application rate was 105 kg N ha^{-1} (Wang et al., 2001). The excessive utilization of nitrogen fertilizer not only poses a threat to environmental conservation but also exerts adverse effects on soil and agricultural product quality. The over-application of nitrogen fertilizer can lead to its accumulation in the soil, thereby elevating the risk of nitrogen loss and leakage, consequently polluting groundwater and water bodies. Therefore, these farmers must receive pertinent agricultural technical training and support that enable them to comprehend and proficiently employ appropriate types and quantities of nitrogen fertilizers, thus fostering sustainable agricultural development.

1.2. Process of soil aggregation

Aggregates are the basic units of soil structure and are composed of pores and solid material produced by the rearrangement of particles, flocculation, and cementation. Aggregate formation involves numerous factors: vegetation, soil fauna, microorganisms, cations, and interactions between clay particles and organic matter (Kumar et al., 2019).

The hierarchical model for classifying soil aggregates suggests that larger aggregates are composed of smaller units, which are formed from even smaller aggregates (Tisdall and Oades, 1982). At present, there are two main views on the formation process of micro-aggregates. One perspective posits that the micro-aggregates' nucleus is mineral. Microaggregates are created through the interaction between mineral surfaces and polyvalent metal cations as well as organic ligands ((Totsche et al., 2018). The stabilizing agents seem to be OM compounds that act as gluing agents and cementing agents, such as oxides, hydroxides, and calcium carbonates, sulfates (Totsche et al., 2018). The other viewpoint posits that the core is organic, and in some cases even biological. Microaggregates are believed to arise from the encapsulation of organic debris by fine mineral particles (Totsche et al., 2018). Micro-aggregates are bonded to each other by transient binders such as polysaccharides and polycyclins to form macro-aggregates.

1.3. The effect of tillage management and nitrogen fertilizer on soil microbial community

Soil microbial community is one of the important factors controlling SOC sequestration (Chen et al., 2015). There are different methods to determine the soil microbial community. The initial studies primarily employed the plate counting technique, wherein microorganisms were isolated and cultured to analyze the microbiological community structure based on specific phenotypes (Li et al., 2008). However, this method has limitations in assessing microbial community diversity comprehensively due to its inability to capture more than 1% of soil microorganisms that can be cultivated. Furthermore, only the microbes that grow rapidly in the soil can be measured through the plate counting technique, which would significantly underestimate the true state of the microbes. Consequently, it only provides a limited representation of microbial information.

Phospholipid Fatty Acids (PLFAs) are utilized to identify microbial biomass in soil without the need for pre-culturing of microorganisms, as they remove phospholipid components that remain relatively constant (Zelles, 1999). Microorganisms typically produce specific PLFAs through various biochemical pathways, and these phospholipids degrade slowly only after cell death, making them a reliable indicator of the soil's microbial population. Despite the numerous benefits it offers, this approach has a couple of limitations: it is limited to identifying species at the generic level and alterations in labeled phospholipid fatty acids can result in variations in estimations of microbial communities.

At the end of the last century, the majority of scholars gradually applied molecular biology techniques to in-depth analysis of soil microbial community composition, diversity and function, and usually applied high-throughput sequencing or amplicon technology to determine nucleic acid polymerase chain reaction amplification products (J. Liu et al., 2014). The approach successfully detected a significant proportion (90-99%) of soil microbial populations that cannot be cultured. The advantages and disadvantages of these methods are shown in Table 1-2 below:

Some previous studies have used PLFA and high-throughput sequencing to explain microbial community properties (Chen et al., 2022, 2015). Currently, extensive research is being conducted on the microbial characteristics associated with tillage practices and nitrogen application. However, knowledge about the relationship between microbial properties and SOC sequestration is still limited under tillage management and nitrogen application.

Six et al., (2004) found that the stabilization of SOC relies on the microbial processes that lead to the formation of durable micro-aggregates and non-hydrolyzable compounds, which effectively occlude SOC loss. The opposite findings also indicated that the processes of microbial community assembly play a crucial role in determining the rate and efficiency of microbial growth, while finally limiting the formation of SOC (Anthony et al., 2020). Therefore, the relationship between microbial properties (microbial community, soil microbial activity, microbial diversity, and microbial function genes) and SOC still needs further study under tillage management and nitrogen application.

Table 1-2 Pros and cons of traditional and biochemical approaches for investigating microbial community (Fakruddin and Mannan, 2013; Nkongolo and Narendrula-Kotha, 2020).

Method	Advantages	Disadvantages
Plate counts	Fast	Unculturable microorganisms not detected
	Cost-effective	Bias towards fast growing individuals
		Bias towards fungal species that produce large quantities of spores
Phospholipid fatty acid (PLFA) analysis/Fatty acid methyl ester analysis (FAME)	Culturing of microorganisms is not required	If fungal spores are used, more material is needed
	Direct extraction from soil	Can be influenced by external factors
	Follow specific organisms or communities	results can easily be confounded by the presence of other microorganisms.
sequencing technologies	Simple and widely used; large throughput; High yield; short run time;	high instrument cost

1.4. The effect of tillage management and nitrogen fertilizer on soil porosity

The arrangement of soil pores plays a crucial role in regulating important functions within soil, such as water holding capacity, carbon, and related rhizosphere functions (Caplan et al., 2017). Agricultural management practices are a key factor affecting soil porosity. The long-term adoption of no-tillage (NT) practice as one of CA, which resulted in an increase in the proportion of coarse and fine mesopores (Sekaran et al., 2021), while a noticeable decline of 3% in overall porosity was observed within the top 20 cm of soil under NT compared to long-term conventional tillage (CT) (Mondal and Chakraborty, 2022). This decline was accompanied by a substantial decrease (20-32%) in macroporosity and a moderate rise (4-7%) in microporosity. In addition, tillage management could also influence the vertical distribution of soil pores (Fig. 1-2).

Currently, numerous studies have been conducted on the impact of NT on soil porosity (Mondal and Chakraborty, 2022; Sekaran et al., 2021); however, limited research has been carried out regarding the influence of nitrogen application on soil porosity structure. Chen et al., (2019) found nitrogen addition can have a negative effect on soil aggregation by decreasing the content of glomalin-related soil protein (GRSPs) associated with fine root growth. However, one study found that the effects of enhanced root growth are believed to be responsible for a significant portion of the alterations observed in the intra-aggregate pore structure following nitrogen

fertilization (Caplan et al., 2017). Therefore, it is imperative to investigate the impact of nitrogen application rates on soil pore structure under conservation tillage.

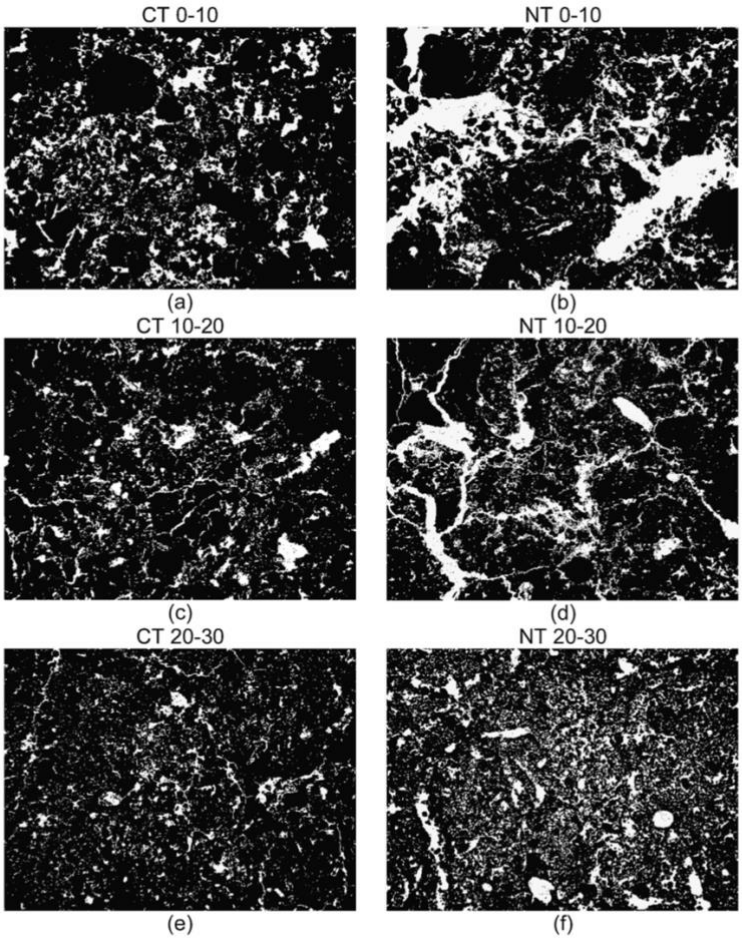

Fig. 1-2 2D micromorphologic images for the conventional tillage (CT) and no-tillage (NT) systems, in the layers 0–10 (a, b), 10–20 (c, d) and 20–30 cm (e, f). The pores are presented in white (Pires et al., 2017).

1.5. The effect of tillage management and nitrogen fertilizer on soil organic carbon fraction

The formation of soil organic matter is a multifaceted process that involves various physical, chemical, and biological mechanisms. Depending on the method used for soil fractionation, the SOC can be categorized into different fractions such as fast-turnover, particulate organic carbon (POC) and slow-turnover, mineral-bound organic matter (MAOC) (Chen et al., 2021). These intricate carbon compounds of organic origin exhibit diverse impacts on the sequestration of soil carbon.

Frequent disruption of soil structure caused by CT practices can often lead to accelerated mineralization and decomposition of SOC. Nevertheless, under the NT

system, a significant increase in total organic carbon content (Prasad et al., 2016) and greater formation of POC & MAOC have been observed as a result of substantial C input from abundant crop residues, surpassing those seen in the CT system (Sithole et al., 2019; Six et al., 2000; Pinto et al., 2021). Previous research has indicated that the impact of conservation tillage on enhancing SOC varies across different soil layers (Rodrigues et al., 2023; Valkama et al., 2020). However, there is a dearth of studies examining the enhancement of POC and MAOC in distinct soil layers under conservation tillage.

Furthermore, the impact of nitrogen application on the dynamics of these two carbon pools may vary significantly. The addition of nitrogen can impede microbial decomposition, resulting in an accumulation of POC alongside increased plant carbon input. Although elevated POC levels are frequently observed following nitrogen additions, this is not always the prevailing outcome (Cusack et al., 2011b; Wang et al., 2014). Numerous previous studies have examined the response of MAOC to nitrogen addition across diverse ecosystems, consistently observing an increase in MAOC with increasing N addition (Chen et al., 2018a; Cusack et al., 2011a). However, a study conducted in a subtropical forest revealed that the addition of N had a significant impact on the reduction of MAOC (J. Chen et al., 2020b). This can be attributed to losses of MAOC caused by leaching of dissolved organic carbon (DOC), destabilization of MAOC due to soil acidification, or decreased microbial growth rate and efficiency resulting in reduced formation of microbially-derived MAOC. At present, there are some research on two carbon pools under conservation tillage, but the effects of nitrogen application on two carbon pools under long-term no-till tillage are lacking.

As the basic functional unit of soil structure, aggregate is the core area that provides physical protection for soil carbon pool. The bulk soil can be categorized into different fractions: three aggregate size classes [mega-aggregates (>2000 μm), macro-aggregates (250–2000 μm), and micro-aggregates (<250 μm)] according to the differences in the forms of cementing agents that polymerize with soil particles (Tisdall and Oades, 1982). Micro-aggregates are composed of mineral, organic, and biotic elements that are bound together by cementing and gluing agents (Totsche et al., 2018). These micro-aggregates are then interconnected into larger mega- and macro-aggregates through temporary binding agents (roots, hyphae, particularly arbuscular mycorrhizal hyphae, and some fungi) (Amézketa, 1999; Tisdall and Oades, 1982). Compared to micro-aggregates, the mega-aggregates exhibit higher vulnerability to soil disturbance (Six et al., 2000). The disruption of these aggregates leads to a decline in aggregate stability as well as the loss of both particulate and dissolved organic C (Chaplot and Cooper, 2015).

According to a recent study conducted by (Piazza et al., 2020), it was found that the adoption of conservation agriculture along with a high nitrogen application rate resulted in the accumulation of SOC within occluded micro-aggregates. Conversely, Sithole et al. (2019) demonstrated that nitrogen application rates had minimal impact on both soil aggregate stability and SOC levels within aggregates under NT practices. The inconsistent effects observed regarding varying nitrogen application rates on soil aggregate stability and SOC in NT systems may be attributed to the lack of clarity surrounding the microbial mechanisms involved in these processes.

1.6. The effect of tillage management and nitrogen fertilizer on soil microbial carbon use efficiency

Microbial carbon use efficiency (CUE) that is the proportion of carbon assimilated by microorganisms and incorporated into biomass rather than being released through respiration, which affect the carbon stock (Agumas et al., 2021; Spohn et al., 2016a). Microbial CUE may be subject to variation due to the presence of microbial communities with varying rates of organic matter decomposition and absorption (M.P. Waldrop and Firestone, 2004). Most investigations solely concentrated on the impact of microbial community and biomass on microbial CUE (Keiblinger, et al., 2010; Waldrop and Firestone, 2004) while disregarding the crucial contribution of microbial diversity to microbial CUE (Domeignoz-Horta et al., 2020). Therefore, investigating the influence of nitrogen application on microbial CUE by examining its impact on microbial diversity and community structure may offer a holistic approach to uncovering the effects of nitrogen application on carbon cycling.

NT practice and nitrogen addition are two common agricultural practices that could change soil microbial CUE by changing soil properties (Kallenbach et al., 2019; Widdig et al., 2020). Some studies have suggested that the microbial CUE may be higher in NT systems compared with CT (Kallenbach et al., 2019; Sauvadet et al., 2018) while contrasting findings were reported by others (van Groenigen et al., 2013). One possible explanation for the varying outcomes could be attributed to the potential impact of nitrogen application on microbial CUE (Kallenbach et al., 2019; van Groenigen et al., 2013), with variations in application rates observed across these studies. Additionally, it is worth noting that nitrogen application has the potential to influence microbial growth and respiration by altering soil nutrient availability, particularly in terms of nitrogen, as microbial cells require a balanced composition of carbon and nitrogen (Manzoni et al., 2012). Furthermore, the restriction on nitrogen leads to an increase in respiration or excretion of C rather than microbial growth, resulting in a decrease in microbial CUE (Qiao et al., 2019). Previous research has demonstrated that retaining straw under NT practices can reduce soil nitrogen availability (Gentile et al., 2011; Thierfelder et al., 2018). These findings suggest that the application of nitrogen is a useful approach to increase the enhancement of microbial CUE within NT systems.

1.7. The effect of tillage management and nitrogen fertilizer on microbial mechanism of soil organic carbon sequestration

Soil microorganisms actively participate in the degradation of plant residues and play a predominant role, accounting for 85-90%, in the formation, stabilization, and transformation of SOC (Trivedi et al., 2019). The fixation effect of soil microorganisms on SOC can be summarized as follows: (1) incorporation of their residues and metabolites into SOC; (2) provision of physical and chemical protection to SOC through their composition and secretions, thereby facilitating soil carbon sequestration. In recent years, the pivotal role played by soil microorganisms in promoting SOC accumulation has received increasing attention from scientific researchers.

The presence of different types of microorganisms plays a crucial role in fixing soil organic carbon SOC within agricultural ecosystems (Yang et al., 2022; Zheng et al., 2023). Specific groups of microbes are responsible for both forming and

maintaining SOC levels (Jeewani et al., 2021). Additionally, the research conducted by Sul et al., (2013) revealed a robust positive correlation between microbial diversity and SOC content. This relationship arises from the fact that reduced levels of microbial diversity impede the breakdown process of straw lignin, thereby hindering the conversion and accumulation of external carbon within the soil environment.

In addition to microbial communities and diversity, microbes harbor a diverse array of functional genes, including those involved in carbon sequestration which significantly impact the sequestration of SOC. For example, the 1, 5-diphosphate carboxylase/oxygenase and cbbM encoded by the cbbL gene of carbon-sequestration bacteria are key enzymes in the Calvin cycle (Selesi et al., 2007; Zhou et al., 2023). At present, there are no uniform results on the study of microbial functional genes related to the effects of conservation tillage and soil carbon conversion (Devine et al., 2014; Hao et al., 2019; Lu et al., 2019). The application of nitrogen also exerts an influence on the genes associated with carbon sequestration, yet no consistent conclusions have been reached (Qin et al., 2021; Zhou et al., 2019).

The physiological characteristics of microorganisms also play a pivotal role in the process of organic carbon fixation. For instance, filamentous microorganisms like arbuscular mycorrhizal fungi and actinomycetes actively intertwine with soil particles, thereby providing physical protection to SOC (Bhatti et al., 2017; Jeewani et al., 2021). The abundance of arbuscular mycorrhizal fungi and actinomycetes is generally enhanced under no-tillage practices (Barbosa et al., 2019; Zaitlin et al., 2004), while it is reduced by nitrogen addition (Barbosa et al., 2019; Zaitlin et al., 2004). Therefore, further investigation is required to understand the impact of nitrogen addition on the physical protection of organic carbon through the regulation of filamentous microorganisms in no-tillage systems.

2. Objective

In this study, long-term experiment fields (northern China) were valorized to study the effect of tillage management and nitrogen application rates on soil microbial properties, physical properties, and organic carbon sequestration. The research technology roadmap of our study is shown in Fig. 1-3. Under NT management, nitrogen application rates can change soil carbon sequestration by soil pore structure and microbial properties in our study. The first part is to explain how the microbial community and diversity affect SOC fraction. Then, the second part is to analyze microbial regulation of aggregate stability and carbon sequestration. The last part is to reveal the effect of microbial interaction on carbon sequestration potential. Finally, based on the above research, this thesis aims to reveal the microbial mechanism induced by tillage management and nitrogen application rates on carbon sequestration through changing soil physical properties and microbial properties.

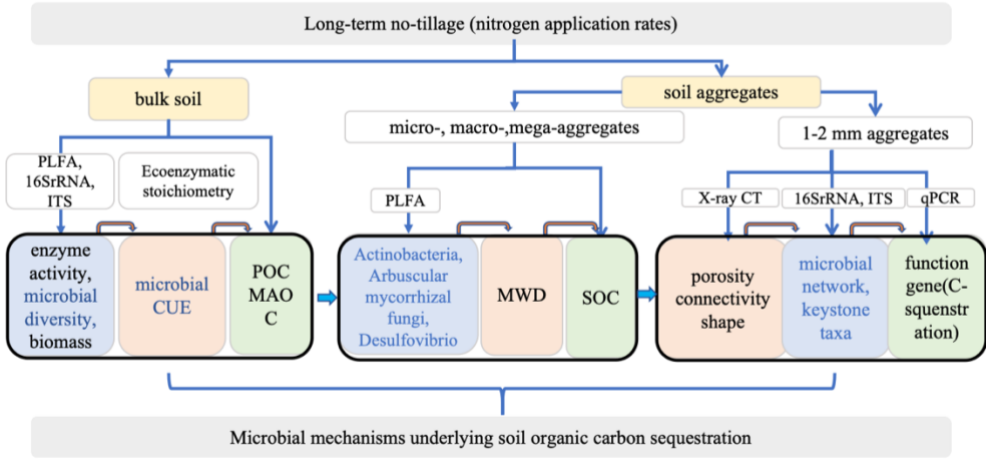

Fig. 1-3 The technology roadmap of this thesis

To expound the above main objective, the following specific aims of this thesis are as follows:

(1) Does microbial diversity and microbial use efficiency affect soil organic carbon sequestration in bulk soil under long-term no-tillage system?

(2) To understand what kind of microbes affect soil organic carbon sequestration in soil aggregates under long-term no-tillage system.

(3) To investigate how soil aggregate pores affect soil organic carbon sequestration by changing microbial interactions under long-term no-tillage system.

3. Outline

This dissertation is structured into the following 5 chapters.

Chapter I General introduction.

In this chapter, the global description of the thesis is shown. The main contents were the general background development of CA, the local agriculture context, the process of soil aggregation, and further the effect of CA on soil microbial characteristics, and soil porosity, soil organic carbon sequestration. The problems and knowledge gaps were described in the part.

Chapter II Nitrogen addition mediates the effect of soil microbial diversity on microbial carbon use efficiency under long-term tillage practices

The objective of this chapter was to explain the effect of CA on organic carbon sequestration in bulk soil was discussed from the perspective of microorganisms. A long-term field experiment was established in 2003 in northern China. Soil microbial properties (e.g. microbial biomass, microbial diversity, microbial community structure, microbial CUE) were determined under conventional tillage with residue removal (CT), and no-tillage with residue mulch (NT), both of which combined with 105 (N1), 180 (N2), and 210 kg N ha^{-1} (N3) N application. The results showed bacterial and fungal diversities were more responsible for soil microbial CUE than their biomass. Furthermore, soil microbial CUE increased soil POC and MAOC contents.

Chapter III Microbial regulation of aggregate stability and carbon sequestration under long-term conservation tillage and nitrogen application

In this chapter, determine microbial communities within soil aggregates by PLFA and reveal how microbial properties within aggregates influences SOC within aggregates. All bulk soil samples were collected from two tillage practice (CT and NT) and three nitrogen application rates and then were divid into three aggregate size classes. The results showed high nitrogen application under long-term no-tillage protects SOC within mega-aggregates by altering aggregate formation through multiple microbial strategies.

Chapter IV Soil pore structure regulates microbial co-occurrence patterns and keystone taxa under long-term conservation tillage

In this chapter, X-ray computed tomography was used to calculate the shape, porosity, and connectivity of the pore network in soil aggregate scale and to reveal how soil pore structure influences microbial co-occurrence patterns and SOC sequestration potential. All soil aggregate samples were collected from two tillage practices (CT and NT) and two nitrogen application rates. We found that pore shape and connectivity regulate key bacteria and microbial networks. High N application rates could improve soil C sequestration potential (functional gene cbbL) by increasing the C sequestration key microorganisms (Chloroflexi and Proteobacteria) under two tillage practices.

Chapter V General discussion and conclusions

In this chapter, the meaning, importance, and relevance of general results were delved into. We stated the answers to the main research question, made recommendations for future research on the topic, and showed what new knowledge we have contributed.

Chapter 2

Nitrogen addition mediates the effect of soil microbial diversity on microbial carbon use efficiency under long-term tillage practices

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Abstract

Tillage practices can influence soil microbial carbon use efficiency (CUE), which is critical for carbon cycling in terrestrial ecosystems. The effect of tillage practices could also be regulated by nitrogen (N) addition. However, the soil microbial mechanism about N fertilizer effect on microbial CUE under no-tillage is still unclear. We investigated how N fertilizer regulates the effect of tillage management on microbial CUE through changing microbial properties and further assessed the impact of microbial CUE on particulate (POC) and mineral-associated organic matter carbon (MAOC) using a 16-yr field experiment with no-tillage (NT) and conventional tillage (CT), both of which combined with 105 (N1), 180 (N2), and 210 kg N ha^{-1} (N3) N application. We found that microbial CUE increased with increasing N application rate. NT increased microbial CUE compared with CT in the 0-10 cm. The bacterial and fungal diversities of NT were higher than CT and N application decreased their diversities in 0-10 cm. The partial least squares path model showed that bacterial and fungal diversity had a significant influence on microbial CUE. Furthermore, POC and MAOC under NT were higher than CT and they also increased with increasing N application rate. It suggested that increasing microbial CUE induced by N application had the potential to increase POC and MAOC. Overall, this study highlights that N addition can alter the effect of soil microbial diversity on CUE, which further improves our understanding of explaining and predict the fractions of SOC (i.e., POC and MAOC) in tillage systems.

Keywords: Microbial community; Microbial carbon use efficiency; Nitrogen; No-tillage; Soil organic carbon

1. Introduction

Soil biodiversity loss induced by agricultural practices threatened soil organic carbon storage (De valença et al., 2017; Huang et al., 2019), which is crucial to the determination of carbon (C) cycling in ecological systems (Chen et al., 2017; Novara et al., 2017). The C stock is also susceptible to microbial carbon use efficiency (CUE) that is the fraction of C taken up by microbial cells and retained in biomass as opposed to being respired (Li et al., 2014; J. Li et al., 2019; Zhou et al., 2020). Conservation tillage and nitrogen (N) addition are two common agricultural practices that could change soil microbial CUE by changing soil properties (e.g., temperature, moisture, and N availability) (Domeignoz-Horta et al., 2020; Kallenbach et al., 2019; Manzoni et al., 2012; Widdig et al., 2020). Conservation tillage could induce N limitation because straw applied has a wide C/N ratio (Thierfelder et al., 2018) and needs more N to relieve N deficiency. However, conservation tillage and N addition have opposite effects on the diversity and structure of microbial community; for instance, no-tillage can increase the ratio of fungi to bacteria (F:B) and soil microbial diversity compared with conventional tillage, while N addition could decrease them (Dai et al., 2018; Liu et al., 2018; Zhang et al., 2012). When studying the combined effect of N addition and tillage practice, Li et al. (2020) found that N addition in no-tillage system had higher soil microbial diversity than conventional tillage. These results indicate that tillage

practice and N addition had interaction effect on microbial community. The microbial community could also influence microbial CUE (Nunes et al., 2020; Sinsabaugh et al., 2016; Wang et al., 2020). However, the combined impact of N addition and tillage practices on microbial CUE from the perspective of microbial community is lacking. Hence, it is essential to explore the soil microbial mechanism responsible for the effect of N application on microbial CUE to better understand carbon sequestration under tillage management.

No-tillage is one of the typical conservation tillage practices and numerous studies have investigated its effect on microbial CUE (Kallenbach et al., 2019; Mo et al., 2021; H. Yang et al., 2020). Some studies have indicated that no-tillage increased microbial CUE compared with conventional tillage (Kallenbach et al., 2019; Mo et al., 2021; Sauvadet et al., 2018), but no effect was also found (Van Groenigen et al., 2013). A possible reason for the different effects is that N application could influence microbial CUE (Kallenbach et al., 2019; Mo et al., 2021; Van Groenigen et al., 2013) and its application rate is different among these studies. N application can also affect microbial growth and respiration by changing soil nutrient availability, particularly for N, because microbial cells need to balance C and N compositions (Manzoni et al., 2012). Moreover, the limitation of N increases overflow respiration or C excretion rather than microbial growth, which further decreases microbial CUE (Qiao et al., 2019). Previous studies showed that no-tillage with straw retention could decrease soil N availability (Gentile et al., 2011; Thierfelder et al., 2018). These findings indicate that N application is a promising way to induce no-tillage systems to increase microbial CUE.

Microbial CUE can be influenced by microbial populations that have different rates of organic matter decomposition and absorption (Mark P. Waldrop and Firestone, 2004). Adu and Oades. (1978)) found that fungi played a more important role than bacteria on microbial CUE. The main reason is that the C:N variation range of fungi is generally wider than that of bacteria and fungi have a higher demand for C element than bacteria (Keiblinger et al., 2010). However, other studies showed insignificant differences in the effect of microbial CUE induced by fungi and bacteria (Six et al., 2006; Thiet et al., 2006). One reason for these conflicting results is that N application could also influence microbial CUE by stimulating microbial activity and decreasing microbial respiration metabolism (Lee and Schmidt, 2014; Liu et al., 2018; Thiet et al., 2006) and the difference N application rates under these studies could contribute to the discrepancy. Another reason is that these studies only focused on the influence of microbial populations and biomass on microbial CUE (Keiblinger et al., 2010; Mark P. Waldrop and Firestone, 2004) and ignored the key role of microbial diversity on microbial CUE (Domeignoz-Horta et al., 2020). Hence, studying the impact of N application on microbial CUE based on its effects on microbial diversity and community structure could provide a comprehensive perspective to reveal the influence of N application on C cycling.

Furthermore, the increase of microbial CUE is an effective means of increasing SOC sequestration (Bradford et al., 2013; Haddix et al., 2016). SOC fractions, especially for particulate (POC) and mineral-associated organic matter carbon

(MAOC), are more sensitive to microbial CUE than total SOC (Averill and Waring, 2018; Chen et al., 2018; Ye et al., 2018). Averill and Waring. (2018) found that substrate use efficiency can also directly affect C cycling through changing POC and MAOC. In addition, N addition significantly influenced soil POC and MAOC (J. Chen et al., 2020b; Y. Chen et al., 2021; Ye et al., 2018). However, it remains unclear how N application regulates the effect of soil microbial CUE on POC and MAOC under tillage management. Therefore, studying the effects of N application is essential to understanding the role of soil microbial CUE on carbon sequestration potential.

Here we investigated the influence of N application on microbial CUE under tillage practices from a microbiological perspective. We hypothesized that: (a) the increase of microbial CUE induced by N application under no-till was higher than under conventional tillage, and (b) microbial diversity plays a more important role than microbial biomass in microbial CUE. The main objectives of this study were to: (a) evaluate the influence of tillage management and N application on soil microbial diversity, community compositions, and soil microbial CUE, (b) reveal how N application influences soil microbial CUE by regulating microbial diversity, community structure, and biomass, and (c) assess the influence of microbial CUE on soil POC and MAOC under tillage management with different N application rates.

2. Materials and methods

2.1. Study site

We conducted a continuous field experiment from 2003 to 2019 at Shouyang Experimental Station (113.11°E, 37.97°N), Jinzhong City, Shanxi Province, Northern China. The climate of the station is continental monsoon and its average annual potential precipitation and evaporation is 484 mm and 1750 mm, respectively (B. Wang et al., 2019). There were average 131 days about annual frost-free season. The soil type in the experimental site was sandy loam cinnamon soil developed from Calcaric-Fluvic Cambisols (S. Li et al., 2020b). Soil physical and chemical properties were initially presented in Table 2-1.

Table 2-1 Soil physical and chemical properties in 0-25 cm layer in 2003.

Soil layer (cm)	Available soil nutrient (mg kg^{-1})			SOC (g kg^{-1})	Bulk density (g cm^{-3})	pH	C/N
	N	P	K				
0-10	58	8.3	96	22.7	1.06	8.3	19
10-25	52	6.9	93	19.8	1.2	8.4	19

2.2. Experimental design

The long-term experiment was conducted in 2003 using a randomized block design with three replicates. There were 18 plots, and each plot was 5 m × 5 m in size. The continuous cultivated crop was spring maize that was planted in March and harvested in November.

Three N fertilizer rates were applied under two tillage treatments in this study. The two tillage practices were NT (no-tillage with the maize straw mulching after harvesting, seeded with a no-till planter, N fertilizer was applied in small holes with 10 cm depth between two maize in each row, about 5 cm from the maize seed/maize plant in April) and CT (conventional tillage with maize straw removed, the plots were plowed twice to 0.25 m depth after harvesting and before seeding, respectively, and fertilized before plow in April) (S. Li et al., 2020b). The three N fertilizer rates were 105 kg N ha^{-1} (N1), 180 kg N ha^{-1} (N2), and 210 (N3) kg N ha^{-1} with urea. According to (Fao, 1999), the total use of chemical fertilizer in China ranks first in the world and the average N addition rate is 235 kg N ha^{-1}. However, the previous fertilization study in this study region showed that N uptake of maize plants, fertilizer N availability, and yield reached the maximum when N application rate was 105 kg N ha^{-1} (Wang et al., 2001). Hence, it is essential to explore the effect of high N addition under tillage practices due to the problem of high N addition in China. In addition, the row spacing was 0.6 m and plant spacing was 0.3 m.

2.3. soil sampling

The soil samples were collected from depths of 0–10 cm and 10–25 cm using a 10 cm diameter soil auger on 1 August 2019. The sampling date corresponded to the tasseling stage. During transport to the laboratory, all soil samples were stored in 4°C incubator. Plant tissues and rocks in soil samples were removed. The fresh soil was divided into several samples and put in a refrigerator at 4°C and soil subsamples for microbiological analysis were put in another refrigerator at − 80 °C before further analyses.

2.4. Soil analysis

2.4.1 Enzyme activities and microbial biomass

We determined the soil microbial biomass nitrogen (MBN) and carbon (MBC) by the chloroform fumigation-extraction method with an extraction efficiency of 0.45 (Cleveland and Liptzin, 2007; Jenkinson et al., 2004). The activities of β-1, 4-N-acetyl-glucosaminidase (NAG), β-1, 4-glucosidase (BG), and leucine aminopeptidase (LAP) in the soil samples were assayed with microplate-scale fluorometric procedures (Sinsabaugh et al., 1997). The BG, NAG, and LAP can produce assimilable nutrients from the major organic sources of C (e.g., β-linked glucans) and N (e.g., protein and amino polysaccharides) (Sinsabaugh et al., 2013). One gram of fresh soil sample was homogenized in 125 mL 50 mM Tris buffer. Buffer, soil sample solution, and substrate were dispensed into a 96-well microplate. Then, the microplates were cultured in a dark incubator for 4 h at 25°C. Finally, we added 1 μl of 1 M NaOH to each well to stop the reaction. The microplates were determined using an automated fluorometer (BioTek Synergy H1 microplate reader, Winooski, VT, USA) with excitation at 365 nm and emission at 450 nm (Saiya-Cork et al., 2002).

2.4.2 Ecoenzymatic stoichiometry and CUE estimation

We used ecoenzyme activity, labile organic matter, and the C:N ratio of microbial biomass to calculate the CUE according to the stoichiometric modeling (Geyer et al., 2019; Sinsabaugh et al., 2016; Sinsabaugh and Shah, 2012). Labile organic matter was determined as the contents of DOC and N extracted from non-fumigated samples (Geyer et al., 2019). The CUE calculated from stoichiometric models was similar to it according to direct measurements of bacterial and fungal growth and respiration (Geyer et al., 2019; Sinsabaugh et al., 2016).

The microbial CUE was calculated according to the following equation:

$$CUE = CUEMAX[SC:N/(SC:N+kN)] \quad (1)$$

where SC:N = (1/EEAC:N)(BC:N/LC:N), SC:N is a scalar ratio that reflects the capability of the microbes to modify the disparity between the composition of microbial biomass and the basic composition of the available resources by the allocation of enzymatic activities. The value of half-saturation constant KN is 0.5. According to the thermodynamic constraints, CUEmax is set at 0.6. EEAC:N represents the ratio of C-acquiring activity to N-acquiring activity, EEAC:N = BG/(NAG + LAP). LC:N represents the molar C:N ratio of labile substrate. BC:N represents C:N of microbial biomass.

The threshold element ratios (TER) were calculated as follows:

$$TERC:N = LC:N \times EEAC:N \quad (2)$$

2.4.3 PLFA analysis

We used phospholipid fatty acid (PLFA) analysis to assess microbial biomass and community structure. The modified Bligh and Dyer method was applied to extract PLFAs (Börjesson et al., 1998). We placed 5 g freeze-dried soil in a chloroform-methanol-citrate buffer mixture overnight and then extracted lipids from it. The lipids were poured into the SPE Tubes (DSC-Si, Discovery®, Sigma-Aldrich) and separated into neutral lipids, glycolipid, and phospholipid. In addition, we added PLFA 19:0 (Larodan Malmö, Sweden) to the phospholipid fraction as an internal standard. PLFAs were transesterified to fatty acid methyl esters by 1 ml 0.2 M methanolic-KOH (Chowdhury and Dick, 2012). We analyzed the extracts using an Agilent 6890 gas chromatograph furnished with a flame-ionization detector (Agilent Technologies, Palo Alto, CA, United States). Fungal biomass was the sum of PLFAs 18:1ω9c and 18:2ω6c (Frostegård and Bååth, 1996; White et al., 1996). PLFAs (a15:0, a17:0, i14:0, i16:0, i15:0, i17:0) were used as markers for Gram-positive bacteria, whereas PLFAs (16:1ω11c, 16:1ω9c, 18:1ω7c, 18:1ω5c, cy19:0, and cy17:0) were used to markers Gram-negative bacteria (Brockett et al., 2012; Frostegård and Bååth, 1996). Actinomycetes biomass was the sum of 10Me16:0 and 10Me18:0 biomass (Willers et al., 2015). The sum of Actinomycetes, G^-, and G^+ biomass was total bacterial biomass.

2.4.4 DNA extraction

The GMO food DNA Extraction Kit (Illumina MiSeq 250 PE, Auwigene Company, Beijing, China) was used to extract the microbial DNA of soil samples

following the manufacturer's instructions. The total DNA concentration and quality were checked using a spectrophotometer (NanoDrop, ND2000, ThermoScientific, United States) and agarose gel electrophoresis and the DNA samples were placed at −40 °C for further analysis.

2.4.5 16S rRNA gene amplicon sequencing and ITS amplicon sequencing

The hypervariable bacterial V3-V4 region of 16S rRNA gene was amplified with the following forward/reverse primer 338F/806R (5′-ACTCCTACGGGAGGCAGCAG-3′)/(5′-GGACTACVVGGGTATCTAATC-3′) (Lee et al., 1993). The ITS2 region of fungi was amplified with the following forward/reverse primer set: ITS1F/ITS2R (CTTGGTCATTTAG AGGAAGTAA/GCTG-CGTTCTTCATCGATGC) (Luan et al., 2015). The thermal-cycling conditions were as follows: 95 °C (3 min), followed by 30 cycles of 98 °C (20 s), 58 °C (15 s), 72 °C (20 s) and final elongation at 72 °C (5 min). The PCR products were detected using 1% agarose gel electrophoresis, then purified with an AxyPrep DNA gel Extraction Kit (Axygen Biosciences, Union City, CA, United States). Amplicon libraries were quantified using a Fluorometer (Applied Biosystems 7500, Thermo Fisher Scientific, United States), after which amplicons were sequenced (Illumina MiSeq PE250, Allwegene Technologies, China).

2.4.6 Soil fractions separation

We used the soil wet-sieving method to separate different soil fractions (Curtin et al., 2019; Fang et al., 2019). To separate soil organic matter into labile C fraction and stable C fraction, we performed a combined density and particle size fractionation (Herath et al., 2014; Six et al., 1998). The two soil C fractions are as follows: light fraction, defined as f-POM, and the heavy fraction that contained aggregate protected organic matter (o-POM, > 53 μm fraction) and mineral protected organic matter (MAOM< 53 μm fraction) (Fang et al., 2019). The soil was isolated light fraction and heavy fraction by density fractionation using sodium polytungstate (SPT, IMBROS, Australia) (Herath et al., 2014; Six et al., 1998).

All soil fractions were dried at 60 °C. Soil fractions were acidified with 1.0 M HCl for decomposing the carbonate. Then, soil samples were dried for 8 hours at 60℃ and sieved with a 0.149 mm sieve after drying. The SOC of soil sample was determined by using an elemental analyzer (Vario Macro C/N, Elementar, Germany).

2.5. Statistics

The data were analyzed by two-way ANOVA to compare the effects of tillage management, N addition rates, and their interaction on enzyme activities, microbial CUE, PLFAs, microbial diversity and bacteria and fungi relative abundance in each soil depth. The student’s t-test was also applied to evaluate the significance of differences within two tillage treatments or two soil depths. The significance of differences within three N application rates was assessed by one-way ANOVA with the least significant difference (LSD) tests in each soil depth under the same tillage treatment. We used Shapiro-Wilk and Levene's test to detect the normality

distribution and homoscedasticity of data before conducting ANOVA. Statistical analyses were conducted using SPSS software version 20.0 (SPSS Inc., Chicago, United States). We used Quantitative Insights Into Microbial Ecology (QIIME) version 1.9.1 to process the sequences (Caporaso, 2010). Operational taxonomic units clustering at 97% of identity were collected using UCLUST in QIIME software. We used principal coordination analysis (PCoA) based on the Bray-Curtis distance in R (v. 3.4.1) to evaluate changes in bacterial and fungal community structure. The statistical significance of differences was evaluated by permutational multivariate analysis of variance based on the Bray-Curtis distance metrics (with a significance level of $p < 0.05$). The relationships among agricultural practices, soil microbial diversity and community structure, microbial biomass, microbial CUE, and soil POC were explored using partial least squares path modeling (PLS-PM). In our path model, path coefficients and coefficients of determination (R2) were confirmed by R (v.3.4.1) with the 'plspm' package (Ai et al., 2018). The Goodness of Fit ($0.40 < GoF < 1.00$) was carried out to evaluate the model's fit (W. Wang et al., 2021). The value of Goodness of Fit was 0.69 in our study.

3. Results

3.1. Changes in enzyme activities and microbial CUE

The interaction effect of tillage and N management on soil enzyme activities was significant ($p < 0.05$) (Table 2-S1). NT significantly increased BG and NAG activities under each N application rate relative to CT in 0-10 cm ($p < 0.05$) (Fig. 2-1). However, an insignificant difference was observed for BG between the two tillage treatments in the 10-25 cm soil layer (Table 2-S1). There was an insignificant difference in the activities of BG and LAP under CT between different soil layers, while the two enzyme activities under NT in the 0–10 cm soil layer were significantly higher than that in the 10–25 cm soil layer ($p < 0.05$) (Table 2-S2). Moreover, the activities of BG and NAG of N2 were higher than of N1 and N3 under the two tillage treatments in the 0-10 cm and 10-25 cm soil layers (Fig. 2-1). Moreover, the average value of LAP activity under CT treatment was higher than that of NT ($p < 0.05$) in 0-25 cm and it was higher under N2 than under N1 and N3 for CT treatment in the 0-10 cm layers (Fig. 2-1c-d).

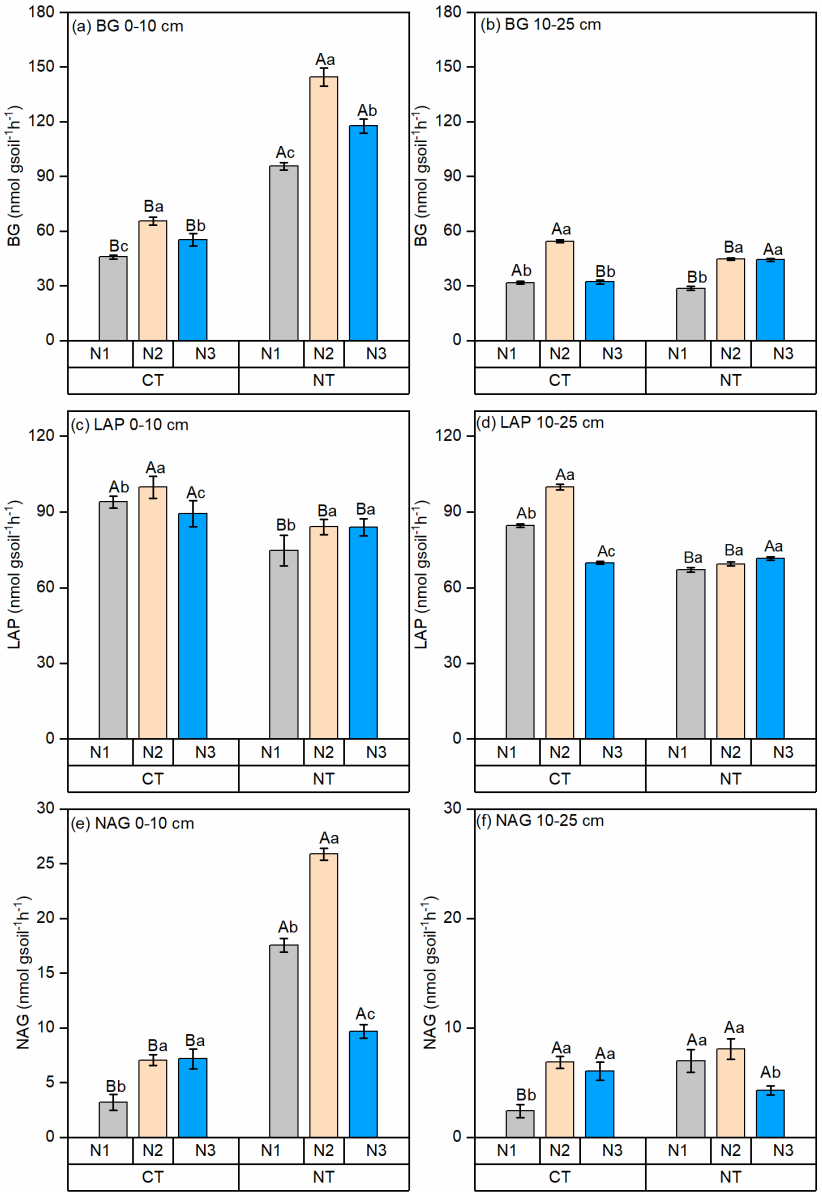

Fig. 2-1 The effects of tillage (T) and nitrogen (N) on enzyme activity. Vertical bars indicate the standard error of means (n = 3). Different capital letters indicate significant differences ($p < 0.05$) between two tillage treatments under the same nitrogen addition rate; Different lowercase letters indicate significant differences ($p < 0.05$) among nitrogen addition rates under the same tillage treatment. BG, β-glucosidase; NAG, N-acetyl-β-glucosaminidase; LAP, Leucyl aminopeptidase; N1, nitrogen addition at 105 kg N ha^{-1}; N2, nitrogen addition at 180 kg N ha^{-1}; N3, nitrogen addition at 210 kg N ha^{-1}; CT, conventional tillage; NT, no tillage.

The microbial CUE was significantly affected by tillage practice and N management, but their interaction effect was not significant (Table 2-S3). The microbial CUE of NT under each N application rate was higher than CT in the 0-10 cm layers (Fig. 2-2). The microbial CUE increased with increasing N application under two tillage treatments in 0-25 cm soil layers. These results showed that increasing N application rates under NT could enhance microbial CUE.

3.2. Soil microbial community

The PLFA contents of the total and grouped soil microorganisms under tillage and N application treatments are shown in Fig. 2-3. Total PLFAs, bacteria, fungi, and actinomycetes PLFAs in the 0–10 cm layer were greater than in the 10–25 cm layer under two tillage treatments (Table 2-S6). The average values of total PLFAs, bacteria, fungi, and actinomycetes PLFAs were higher under NT than CT in the 0–10 cm soil layer ($p < 0.05$) (Fig. 2-3). Moreover, only fungi and the F:B ratio were significantly affected by N application rates in the 0-25 cm layer and the interaction of tillage and N management also had a significant influence in 0-10 cm layer ($p < 0.05$) (Table 2-S5). Overall, the total PLFAs were increased by 19.2% under NT compared with CT in the 0-10 cm soil layer and not significantly affected by N level under NT in the 0-25 cm layer (Fig. 2-3). For each grouped soil microorganism, the average values of bacterial PLFAs and actinomycetes PLFAs under NT were increased by 21.2% and 24.4% in the 0-10 cm layer, respectively, compared with CT, but insignificantly affected by N level under each tillage treatment at both depths (Fig. 2-3). The fungal PLFAs of N2 were the highest than N1 and N3 under NT, while there was no effect of N application rate under CT in the 0–10 cm layer (Fig. 2-3). Moreover, the G+:G- ratio was insignificantly affected by soil depth (Table 2-S6), tillage treatments, and N application rates (Table 2-S5). The G+:G- ratio increased with increasing N application rates under NT and there was no effect of N application rate under CT at 0–10 cm (Fig. 2-3). In addition, the F:B ratio was insignificantly affected by tillage management (Table 2-S5). N2 produced a higher F:B ratio than N1and N3 under NT, whereas N application rate did not affect F:B ratio under CT in both depths (Fig. 2-3).

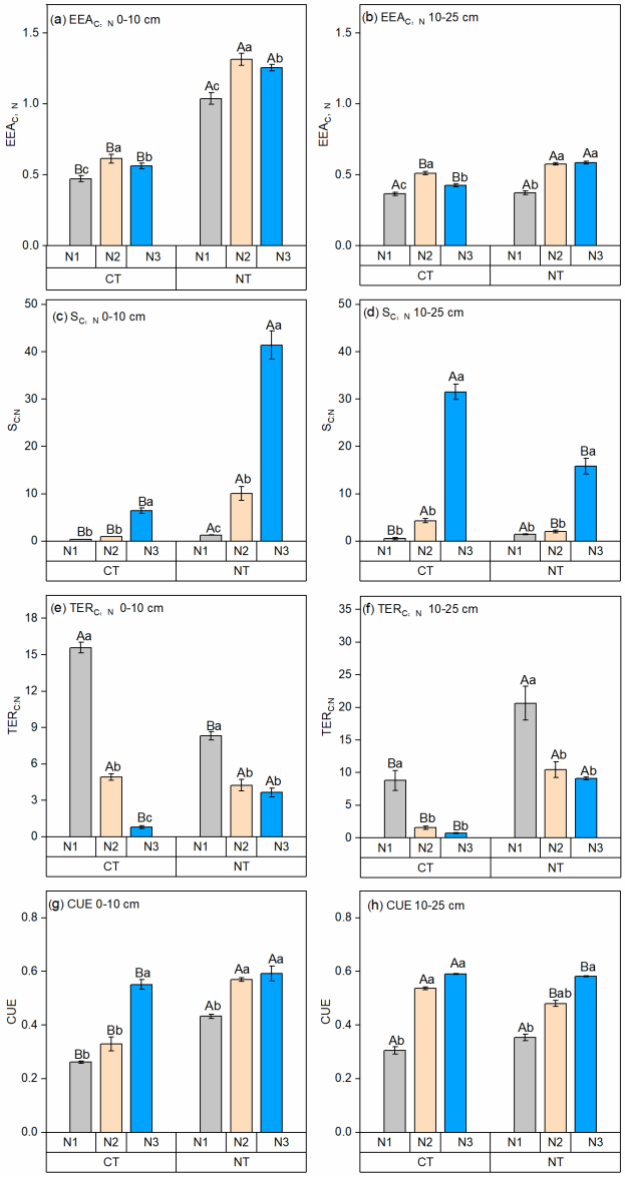

Fig. 2-2 The effects of tillage (T) and nitrogen (N) on carbon use efficiency (CUE), element-requiring enzymatic activity ratio (EEAC:N), threshold element ratio (TERC:N), and scalar index (SC:N). Vertical bars indicate the standard error of means (n = 3). Different capital letters indicate significant differences ($p < 0.05$) between two tillage treatments under the same nitrogen addition rate; Different lowercase letters indicate significant differences ($p < 0.05$) among nitrogen addition rates under the same tillage treatment. N1, nitrogen addition at 105 kg N ha^{-1}; N2, nitrogen addition at 180 kg N ha^{-1}; N3, nitrogen addition at 210 kg N ha^{-1}; CT, conventional tillage; NT, no-tillage.

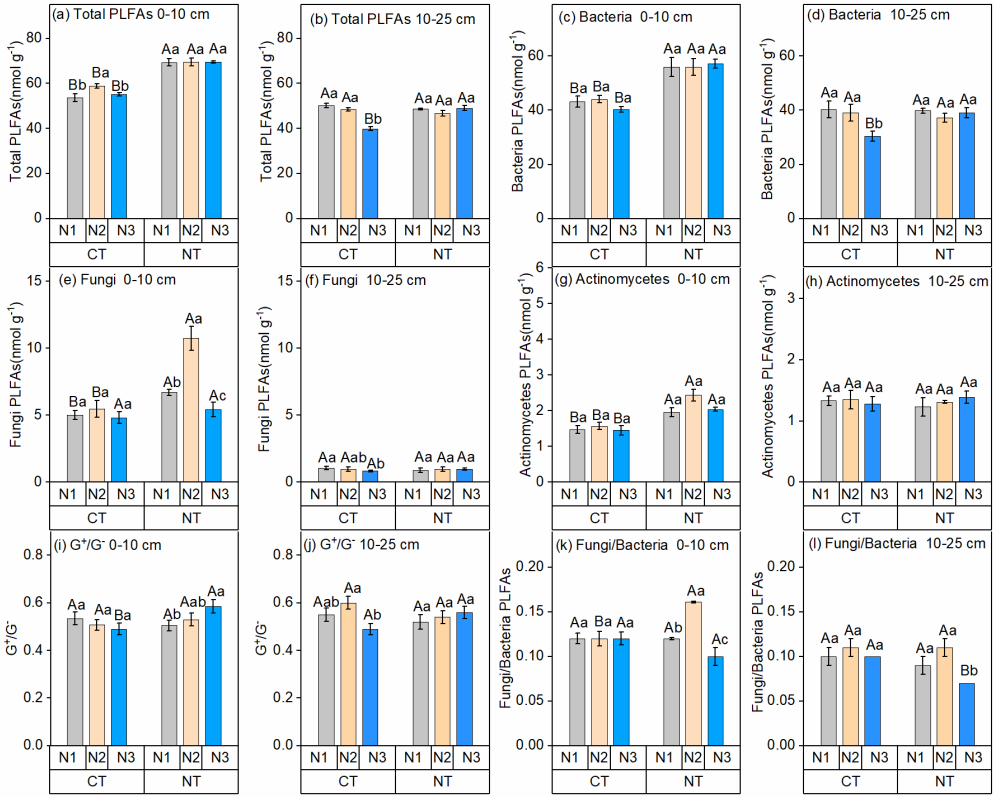

Fig. 2-3 The effects of tillage (T) and nitrogen (N) on PLFAs. Vertical bars indicate the standard error of means (n = 3). Different capital letters indicate significant differences ($p < 0.05$) between two tillage treatments under the same nitrogen addition rate; Different lowercase letters indicate significant differences ($p < 0.05$) among nitrogen addition rates under the same tillage treatment. N1, nitrogen addition at 105 kg N ha^{-1}; N2, nitrogen addition at 180 kg N ha^{-1}; N3, nitrogen addition at 210 kg N ha^{-1}; CT, conventional tillage; NT, no-tillage.

3.3. Soil bacteria community compositions

According to 16S rRNA gene sequences, each sample ranged from 31458 to 172704 sequences at a 97% sequence identity threshold. Overall, a total of 8232 OTUs were identified. Actinobacteria (14.5%–32.6% relative abundance), Proteobacteria (16.5%–28.7% relative abundance), Acidobacteria (15.5%–37.1% relative abundance), Chloroflexi (10.5%–21.6% relative abundance), and Gemmatimonadetes (4.0%–6.9% relative abundance) were considered the dominant phyla associated with residue decomposition (Fig. 2-4). These five phyla accounted for 96.4% of all sequence reads.

N application, tillage, and N × tillage interaction significantly influenced the bacterial (16S) community compositions (Table 2-S7). For the dominant phyla, the relative abundances of Acidobacteria increased with soil depth, while the relative abundances of Proteobacteria, Bacteroidetes, and Actinobacteria declined with soil

depth (Table 2-S8). Compared with CT, NT increased the relative abundances of Bacteroidetes and Proteobacteria in the 0–25 cm layer (Fig. 2-S1). The relative abundances of Bacteroidetes increased with an increase in N application under two tillage treatments in the 0–10 cm soil layer. Furthermore, the relative abundances of Chloroflexi of N1 were higher than N2 and N3 under NT at both depths.

3.4. Soil fungi community compositions

The histogram of fungal community structure revealed structural and abundance differences among N application rates and tillage treatments (Fig. 2-4). Soil fungal communities included 2074 OTUs and there were five phyla of eumycota with an abundance > 0.01%. Ascomycota and Mortierellomycota were the two most dominant, accounting for > 60% of all phyla.

In more detail, the abundance of Basidiomycota and Glomeromycota was higher under NT than under CT in 0-10 cm layers (Fig. 2-S2). The relative abundances of Glomeromycota decreased with an increase in N application under NT in the 0-10 cm layers.

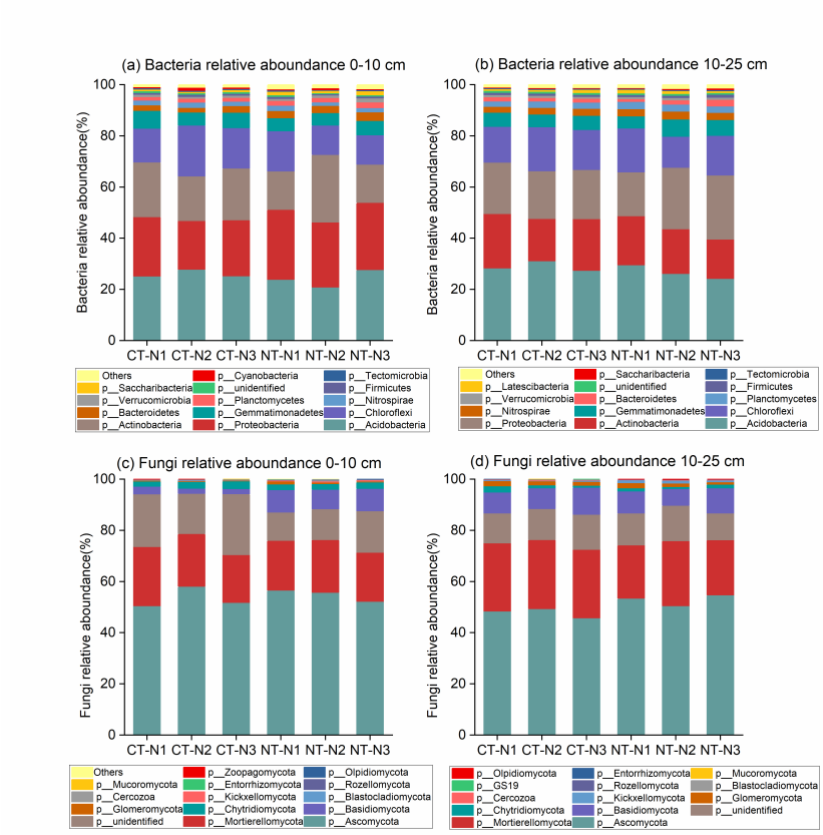

Fig. 2-4 The effects of tillage (T) and nitrogen (N) on the relative abundance of bacteria and fungi. Relative abundance of bacterial and fungus for the taxonomic levels of the phylum. Values are means (n = 3).

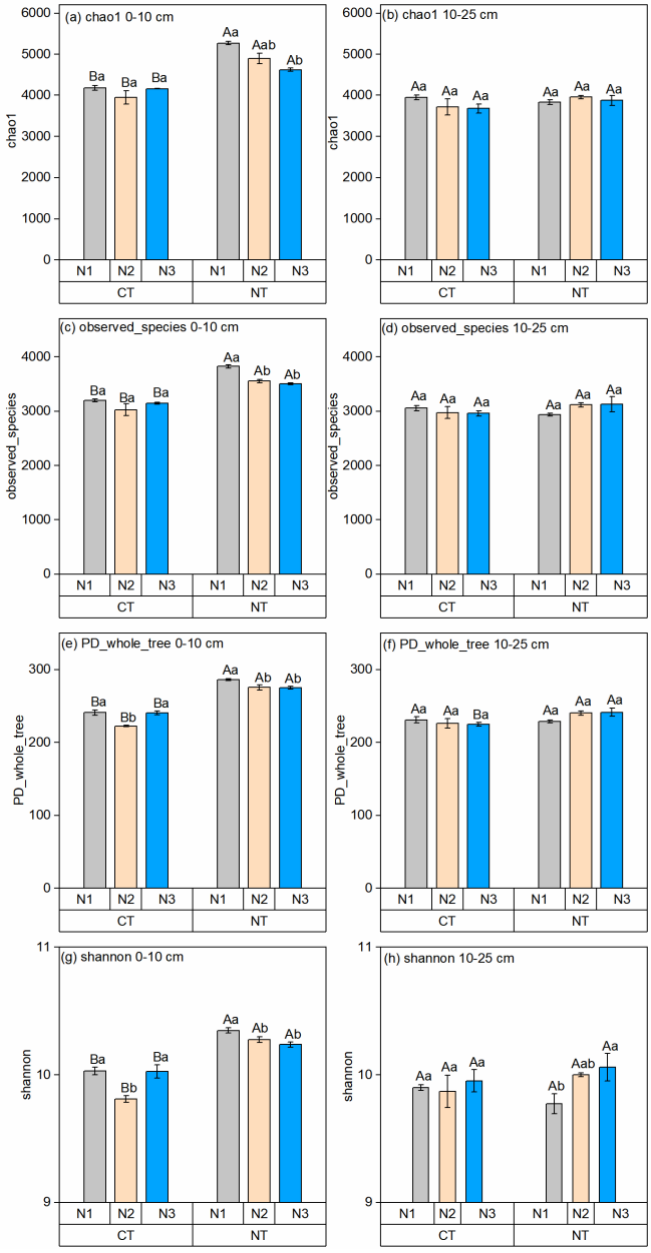

Fig. 2-5 The effects of tillage (T) and nitrogen (N) on bacterial diversity. Vertical bars indicate the standard error of means (n = 3). Different capital letters indicate significant differences ($p < 0.05$) between two tillage treatments under the same nitrogen addition rate; Different lowercase letters indicate significant differences ($p < 0.05$) among nitrogen addition rates under the same tillage treatment. N1, nitrogen addition at 105 kg N ha^{-1}; N2, nitrogen addition at 180 kg N ha^{-1}; N3, nitrogen addition at 210 kg N ha^{-1}; CT, conventional tillage; NT, no-tillage.

3.5. Diversity of soil bacteria and fungi

The diversity of soil bacteria and fungi was significantly affected by tillage practice and N management in 0-10 cm layer. However, soil fungal diversity was only affected by their interactions in 0-10 cm layer ($p < 0.05$). NT significantly increased soil bacterial diversity compared with CT in 0-10 cm under each N addition rate ($p < 0.05$) (Fig. 2-5). Soil bacterial diversity decreased with soil depth under NT ($p < 0.05$) (Table 2-S12). N application also significantly affected bacterial diversity under NT, whereas N application had no effect under CT in the 0–10 cm layer (Fig. 2-5). Bacterial diversity decreased with an increase in N application rates under NT in the 0–10 cm layer, while N application had no influence on bacterial diversity under two tillage treatments in the 10–25 cm layer. Similarly, NT significantly enhanced the average value of soil fungi diversity compared with CT in 0-10 cm layer (Fig. 2-6). Soil fungal diversity decreased as the soil depth under NT and decreased with an increase in N application rates under NT in 0-10 cm layer (Table 2-S14 and Fig. 2-6). However, the fungal diversity of CT was not influenced by soil depth. N application also had no influence on fungal diversity under two tillage treatments in 10–25 cm soil layer.

Principal component analysis of bacterial composition at the phylum level showed that two principal components accounted for 47.7% and 42.4% of the overall variances among these treatments in the 0–10 cm and 10-25 cm layers, respectively (Fig. 2-7). We also found that PCoA of the fungal composition showed that two principal components accounted for 46.5% and 39.2%, respectively. We revealed that the two fractions (CT and NT) formed their clusters separated by PC1 in both soil layers. For fungi, the samples under the three N application rates of CT clustered closely, while samples within the NT differed more distinctly in both soil layers.

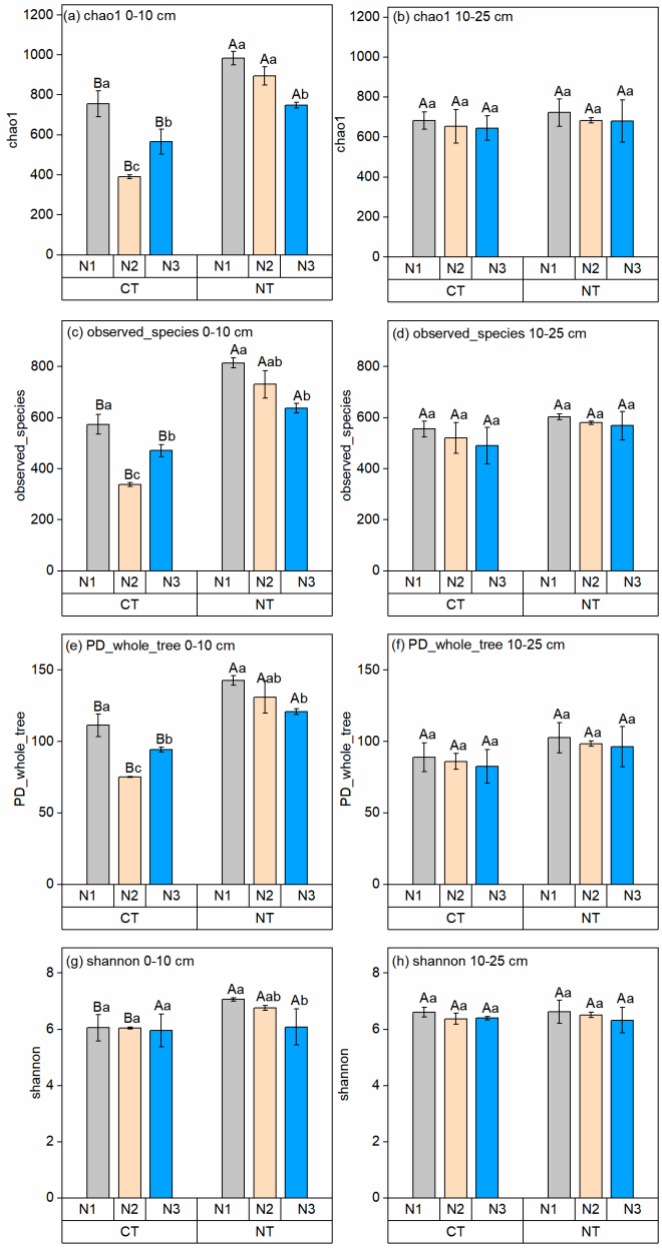

Fig. 2-6 The effects of tillage (T) and nitrogen (N) on fungal diversity. Vertical bars indicate the standard error of means (n = 3). Different capital letters indicate significant differences ($p < 0.05$) between two tillage treatments under the same nitrogen addition rate; Different lowercase letters indicate significant differences ($p < 0.05$) among nitrogen addition rates under the same tillage treatment. N1, nitrogen addition at 105 kg N ha^{-1}; N2, nitrogen addition at 180 kg N ha^{-1}; N3, nitrogen addition at 210 kg N ha^{-1}; CT, conventional tillage; NT, no-tillage.

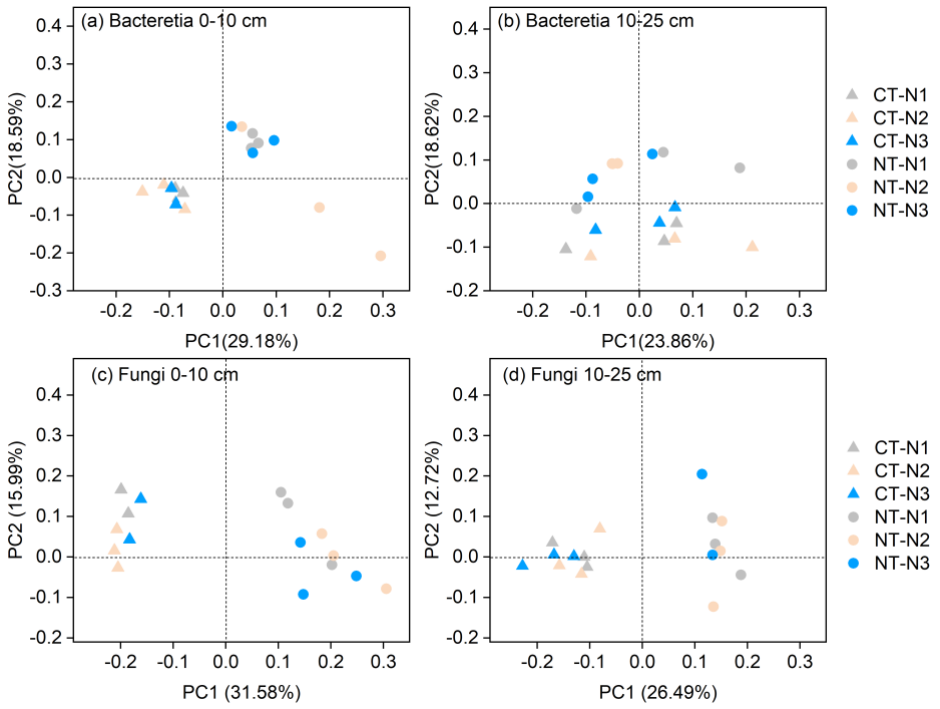

Fig. 2-7 Principal coordinate analysis (PCoA) of the bacterial and fungal compositions among tillage (T), nitrogen (N), and soil depth (D). N1, nitrogen addition at 105 kg N ha-1; N2, nitrogen addition at 180 kg N ha-1; N3, nitrogen addition at 210 kg N ha-1; CT, conventional tillage; NT, no-tillage.

3.6. Soil fractions

Tillage practice, N management, and their interaction had a significant influence on soil POC and MAOC contents in 0-25 cm layer ($p < 0.05$) (Table 2-S15). The POC and MAOC contents decreased with depth (Table 2-S16). NT increased the POC and MAOC contents by 12.1% and 10.1% compared with CT in the 0–10 cm layer, respectively (Fig. 2-8). The POC and MAOC contents increased with increasing N addition and the rate of increase under NT was higher than under CT in the 0–10 cm layer. However, tillage and N treatment had no influence on MAOC in the 10–25 cm layer.

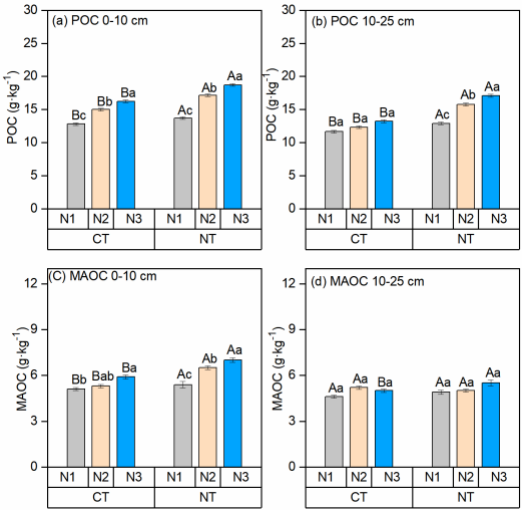

Fig. 2-8 The effects of tillage (T) and nitrogen (N) on soil POC and MAOC. Vertical bars indicate the standard error of means (n= 3). Different capital letters indicate significant differences ($p < 0.05$) between two tillage treatments under the same nitrogen addition rate; Different lowercase letters indicate significant differences ($p < 0.05$) among nitrogen addition rates under the same tillage treatment. N1, nitrogen addition at 105 kg N ha^{-1}; N2, nitrogen addition at 180 kg N ha^{-1}; N3, nitrogen addition at 210 kg N ha^{-1}; CT, conventional tillage; NT, no-tillage.

3.7. PLS-PM analysis

We established a partial least squares path model to better integrate the interrelationships among N application, tillage practices, microbial communities, soil enzyme activities, soil microbial CUE, POC, and MAOC (Fig. 2-9). The indirect effect of tillage treatments (0.38) on soil microbial CUE was larger than that of N application (0.13). We further found that tillage management and N application affected microbial CUE through changing soil bacterial diversity, fungal community structure, and fungus diversity more than bacterial and fungal biomass. The responses of microbial CUE to bacterial and fungal diversity were also different. Moreover, the results showed that microbial CUE and soil enzyme activities had a direct effect on soil POC.

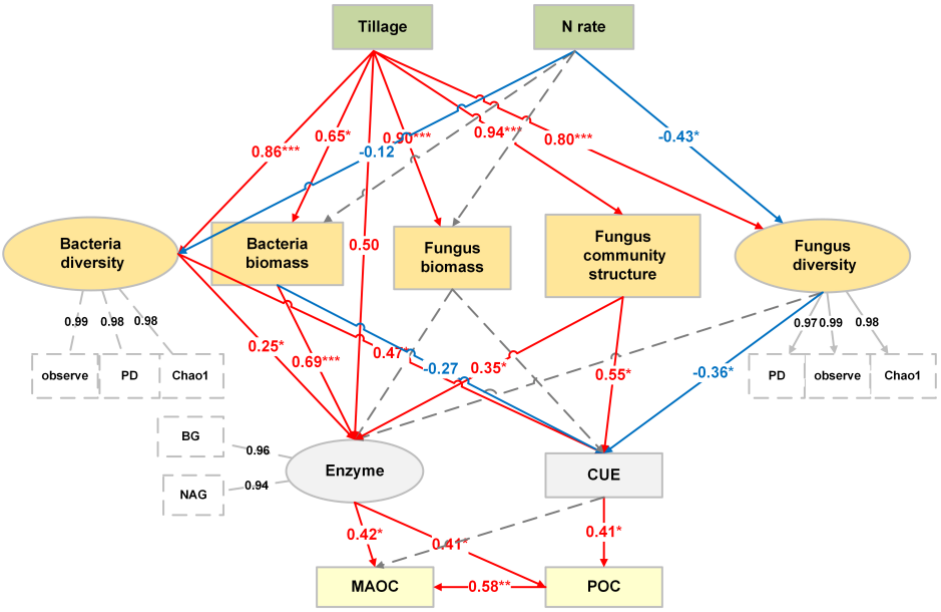

Fig. 2-9 Directed graph of the partial least squares path model (PLS-PM). Each box represents an observed (i.e., Bacteria biomass) or each oval represents a latent variable (e.g., Bacteria diversity). The loading of Bacteria diversity, Fungus diversity, and enzyme activities that create the latent variables are shown in the dashed rectangle. Path coefficients are reflected by the widths of the arrows and the numbers next to the arrows. Red and blue arrows indicate positive and negative effects, respectively. Dashed arrows indicate that coefficients do not differ significantly ($p > 0.05$).

4. Discussion

4.1. Effect of tillage and N addition on soil microbial diversity and community structure

Soil microbial communities are essential to maintaining soil ecosystem function and can be affected by tillage and N application (Bärlocher and Boddy, 2016; Keszthelyi et al., 2008). We found that NT treatment increased bacterial and fungal diversity in 0-10 cm layer compared to CT treatment (Fig. 2-5). The difference between CT and NT could be due to the decrease of soil physical disturbance and protection from fungal hyphae and their mycelial network under the no-tillage system (Ceja-Navarro et al., 2010; Verbruggen and Toby Kiers, 2010; Y. Wang et al., 2017). Another reason is that no-tillage with straw mulching could increase soil microbial diversity by increasing soil organic matter and carbon source inputs in surface soil (Y. Li et al., 2020). However, straw mulching less affected soil microorganisms in 10-25 cm than in 0-10 cm depth under no-tillage (Sun et al., 2016), which could lead to an insignificant difference in the bacterial and fungal diversity in 10-25 cm layer between NT and CT treatments. This is the main reason

why the effect of the two tillage practices on microbial diversity is inconsistent in 0-10 cm and 10-25 cm soil layers.

Furthermore, soil fungal and bacterial diversity decreased with increasing N application rates in the 0–10 cm layer and was higher under NT treatment than under CT (Figs. 2-5 and 2-9). One possible reason is that the straw in no-tillage has a wide C/N ratio (Thierfelder et al., 2018), which leads to an N limitation under this tillage system because microbe needs more N under this condition. A previous meta-analysis showed that appropriate N addition (<100 kg N $ha^{-1}year^{-1}$) is essential to stimulate microbial growth in no-tillage systems because it regulates soil C/N (Thierfelder et al., 2018; Zhou et al., 2017). However, excessive N fertilization suppresses the diversity of soil microbes because of the toxic effect of urea (Omar and Ismail, 1999; Chao Wang et al., 2018). In this study, the highest N application rate (210 kg N ha^{-1}) could induce toxicity, resulting in lesser microbial diversity. In addition, CT had lower soil SOC (Li et al., 2010) and C/N ratio compared with NT (Fiorini et al., 2020), which leads to carbon limiting for microorganisms. Hence, the effect of N application had a smaller effect on microbial diversity under CT than NT. The previous study also showed that the N application level (100–200 kg N ha^{-1}) decreased soil microbial diversity under no-tillage (Y. Li et al., 2020a), which confirms our conclusion under the N application level (105–210 kg N ha^{-1}) (Figs. 2-5 and 2-6). In addition, increasing N application rates under no-tillage practice had a negative effect on some dominant flora such as Chloroflexi (Fig. 2-S1) that plays a vital role in the decomposition of refractory C compounds (Yue Li et al., 2019; Piazza et al., 2019). These results further indicate that N application needs to be considered when studying the effect of tillage management on SOC from the perspective of microbial properties.

Tillage management could also influence the vertical distribution of soil microbial communities (Nunes et al., 2020). We found no difference in enzyme activities, total PLFAs, and bacterial and fungal diversity among soil layers under CT treatment (Figs. 2-1, 2-3, 2-5, and 2-6). The main reason was that soil microbial communities in different soil layers would be similar to each other after homogenization induced by plowing under CT (Sun et al., 2018). However, fungal and bacterial diversity decreased as soil depth increased under NT (Tables. 2-S12 and 2-S14), which was supported by the previous study (Jumpponen et al., 2010). This was likely because no-tillage creates heterogeneous soil (Sun et al., 2018). Our previous study in this experiment site showed that soil depth had a significant influence on soil moisture, bulk density, porosity, and aggregate stability in 0-20 cm soil depth (S. Li et al., 2020a), which could lead that microbial properties are different with changing in topsoil depth. In addition, some previous studies also showed the difference in enzyme activities and microbial properties under different topsoil depths (Fierer et al., 2003; Mathew et al., 2012). Hence, altered soil physicochemical properties under conservation tillage created significantly different habitats for microbe and resulted in the change of soil microbial community structure and diversity (Mathew et al., 2012).

Moreover, the decrease rate of fungal and bacterial diversity with increasing soil depth was higher under N1 than N2 and N3 for NT treatment (Figs. 2-5 and 2-6), indicating that a low N rate can enhance topsoil bacterial and fungal diversity under

NT. Hence, it is not sufficient to only consider the surface layer when investigating bacterial and fungal diversity response to N application rates in no-tillage systems.

4.2. Relationship of soil microbial characteristic and microbial CUE

Soil microbial CUE can affect soil C cycling (Spohn et al., 2016b). We found that NT increased the soil microbial CUE compared with CT in the 0-10 cm layer (Fig. 2-2). One possible reason was that NT could decrease soil temperature by surface mulching and further increase microbial CUE (Apple et al., 2006; Wetterstedt and Agren, 2011). We also found that NT decreased average soil temperature in the two soil layers (Table 2-S17). The second reason could be that NT continuously supplied labile organic substrates for microbial biomass by residue application, resulting in higher CUE of NT than CT (Álvaro-Fuentes et al., 2013). POC is a labile organic substrate and NT increased POC compared with CT treatment (Fig. 2-8). In addition, bacteria diversity had positive relationships with microbial CUE (Figs. 2-9). NT could also increase microbial CUE by increasing bacteria diversity. Microbial CUE increased with increasing N application under both tillage treatments in 0-25 cm (Fig. 2-2). The reason is that N addition can reduce microbial respiration metabolism (Liu et al., 2018b; Spohn et al., 2016b; Thiet et al., 2006) and increase microbial biomass (Jha et al., 2020), resulting in higher microbial CUE. Moreover, although N fertilizer was only applied at 10 cm depth under no-tillage in this study, N addition had a significant influence on the microbial CUE in deeper soil layer (10-25 cm). The main reason is that nitrate nitrogen can transport with soil water movement, resulting in nitrogen leached into deeper soil depth (S. Li et al., 2021b). In addition, our previous study showed that NT increased the soil porosity of > 55 μm diameter pores compared to the CT treatment, which indicated that NT could increase soil water infiltration and nitrogen leaching (S. Li et al., 2020a).

Furthermore, although a recent study showed that microbial diversity drives CUE in artificial soil (Domeignoz-Horta et al., 2020), to the best of our knowledge, few experimental studies have directly demonstrated the interaction effect of tillage management and N application on microbial CUE in a field experiment. In this study, the PLS-PM showed that bacteria diversity, fungal diversity, and fungal community structure could play more critical roles than their biomass in increasing microbial CUE (Figs. 2-9, 2-S3 and 2-S4). We also found that the bacterial and fungal diversity had different influences on microbial CUE under two tillage and these relationships were regulated by N application under no-tillage (Figs. 2-9 and 2-S4). Bacterial diversity positively influenced microbial CUE, whereas fungal diversity had an adverse impact on microbial CUE (Fig. 2-9). The difference points to the importance of studying the diversity of fungal and bacterial communities separately for predicting soil C cycling. In addition, microbial network complexity drives carbon cycling with direct feedback effects on multiple ecosystem functions (Morriën et al., 2017; Wagg et al., 2019; Zhou et al., 2010), which could also influence microbial CUE. Further research should be undertaken to explore the effect of bacterial and fungal networks on microbial CUE.

4.3. The influence of microbial CUE on soil POC and MAOC fractions

POC is a functional soil component for stable soil organic carbon (Witzgall et al., 2021). In contrast to POC, MAOC is more protected (physically or chemically), making it not easy to mineralize (Abramoff et al., 2018). We found that high N application (210 kg N ha^{-1}) increased POC and MAOC content under two tillage practices (Fig. 2-8), which is similar to the previous study (Ye et al., 2018). The possible reason was that plant biomass (Stewart et al., 2016; Thomas et al., 2010; Chao Wang et al., 2018) and microbial residues (J. Chen et al., 2020a) increased with increasing N application. However, some discrepant findings showed that N addition decreased (Ye et al., 2018) or had no significant influence on MAOC (Yuan et al., 2020). The main reason for the inconsistent results could be that microbial residues controlled the variation of soil MAOC pool and the microbial residues were different due to different N application rates (Averill and Waring, 2018; J. Chen et al., 2020a; Su et al., 2020; Y. Yang et al., 2020).

Microbial CUE increased with increasing POC and MAOC due to the increment of N application rates (Fig. 2-S5) and N addition also increased microbial CUE, POC, and MAOC content in some previous studies (Ye et al., 2018). These findings suggested that increasing N application rates is an efficient measure to increase POC and MAOC by enhancing microbial CUE under CT and NT practices. However, the increasing rate of POC or MAOC with microbial CUE under NT was higher than under CT because of the regulation of N addition (Fig. 2-S5). A possible reason was that there was N limitation under NT (Zhang et al., 2015) because the straw applied in this tillage system has a wide C/N ratio and microbe needs more N (Thierfelder et al., 2018). Therefore, this study further highlights the critical role of N addition in regulating the effect of microbial CUE on soil organic carbon fractions under tillage practices.

5. Conclusions

N application could alter the effects of tillage practices on soil microbial diversity, community composition, biomass, and CUE. Bacterial and fungal diversities were more responsible for soil microbial CUE than their biomass. Although microbial CUE was more susceptible to tillage management than N application, it increased with an increase in N application rate under the two tillage practices. Furthermore, soil microbial CUE increased soil POC and MAOC contents and N application also increased the two SOC fractions. This research underscores the importance of N application to reveal the effect of tillage management on POC and MAOC from the perspective of soil microbial properties, which contributes to understanding the potential C sequestration benefits of increasing N addition under no-tillage.

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Chapter 3

Microbial regulation of aggregate stability and carbon sequestration under long-term conservation tillage and nitrogen application

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Abstract

The stability of aggregates plays a significant role in soil organic carbon (SOC) sequestration in conservation agriculture soils. However, the regulation of microorganisms within aggregates on aggregate stability and SOC sequestration remains elusive. By dividing the soil into three aggregate size classes [mega-aggregates (>2000 μm), macro-aggregates (250-2000 μm), and micro-aggregates (<250 μm)], we evaluated the response of aggregate stability, SOC and microbial communities within aggregates to long-term conservation tillage, which consisted of two tillage methods (conventional tillage and no-tillage) and three nitrogen application rates (105, 180, and 210 kg N ha^{-1}). Under no-tillage treatment, high nitrogen application rate increased SOC by 2.1-3.7 $g \cdot kg^{-1}$ within mega- and macro-aggregates but reduced the total amount of phospholipid fatty acids (PLFAs) within all aggregates. Under conventional tillage, high N application rate increased mean weight diameter (MWD) and reduced total PLFAs within all aggregates only in 0-10 cm. With the same nitrogen application rate, no-tillage increased MWD by 8.7%-42.7%, SOC content within mega-aggregates by 7.3%-27.8% and within macro-aggregates by 13.2%-28.3% when compared with conventional tillage. Actinobacteria were recruited by straw under no-tillage and their biomass increased 1.5-7.8 times in all aggregates compared with conventional tillage, where they might participate in aggregate formation via degradation of straw and increasing SOC within mega- and macro-aggregates. Conversely, desulfovibrio biomass within all aggregates was diminished under no-tillage compared with conventional tillage, while desulfovibrio possibly directly inhibited soil aggregate formation and decreased SOC within mega- and macro-aggregates under conventional tillage. Moreover, under no-tillage, arbuscular mycorrhizal fungi biomass increased by 0.4-1.6 nmol g^{-1} within all aggregates compared with conventional tillage in 0-10 cm, potentially indirectly contributing to soil aggregate formation via co-metabolic processes and increasing SOC within mega- and macro-aggregates. Overall, high nitrogen application under long-term no-tillage protects SOC within mega-aggregates by altering aggregate formation through the microbial communities, providing information that may be useful in developing management strategies to enhance carbon sequestration in agricultural soils.

Keywords: No-tillage, Nitrogen, Aggregate-associated organic carbon, Actinobacteria, Arbuscular mycorrhizal fungi, Desulfovibrio

1. Introduction

There has been a dramatic increase in recent years in investigations into soil organic carbon (C) sequestration, as its storage contributes to soil fertility and climate change mitigation (Chowaniak et al., 2020; Lu et al., 2009). The essential function of soil aggregates in protecting the sequestration of soil organic carbon (SOC) has been widely appreciated. Nevertheless, the structural disruption of soil aggregates caused by tillage practices has resulted in a yearly depletion of SOC from cropland at a rate of 0.3-1.0 Pg (Chappell et al., 2016). Long-term conservation agriculture through the use of straw return, no-tillage (FAO, 2012), and appropriate

nitrogen application (Lu et al., 2009; McConkey et al., 2002) has been practiced to improve SOC concentrations, aggregate stability, and microbial community biomass (Hati et al., 2021; Piazza et al., 2020). Notably, microbial decomposition of SOC weakens the stability of aggregates, but microbial ectomycorrhizal hyphae and secretions may also facilitate aggregate formation (Ji et al., 2019). Hence, it is essential to comprehend the interplay between microorganisms within aggregates, aggregate formation and SOC accumulation is essential for exploring the mechanisms of SOC sequestration in long-term conservation tillage.

The sequestration of SOC was considered to be determined by the chemical molecular structure of organic materials for a long time (Sollins et al., 1996). Nevertheless, recent studies indicated that the intrinsic properties of SOC have a minor role while physicochemical and microbiological effects play a significant role in protecting SOC (Kan et al., 2021; Six et al., 2000; Six and Paustian, 2014). To demonstrate this recognition, soil aggregates were divided into three size classes [mega-aggregates (>2000 μm), macro-aggregates (250-2000 μm), and micro-aggregates (<250 μm)](F. Li et al., 2019). Micro-aggregates consist of mineral, organic, and biotic components that are held together by cementing and gluing agents (Totsche et al., 2018), These micro-aggregates are further bound into mega- and macro-aggregates by transient binding agents (mainly polysaccharides) and by temporary agents (i.e., fine roots, fungi hyphae, dead bacteria, and glomalin) (Amézketa, 1999; Tisdall and Oades, 1982). The mega-aggregates were more vulnerable to damage from soil disturbance than micro-aggregates, owing to larger pore space, greater infiltration rate, and higher quality (Six et al., 2000). The disruption of the aggregates resulted in a reduction in the stability of aggregates and the losses of particulate and dissolved organic C (Chaplot and Cooper, 2015).

By covering with straw and mitigating soil disturbance, long-term no-tillage practice increased C input and alleviated aggregate disruption compared with long-term conventional tillage (Jha et al., 2020; Song et al., 2022). Additionally, nitrogen addition enhanced C input by improving crop production (Liu and Greaver, 2010), but high nitrogen availability also has a potentially negative impact on C cycling by decreasing bacterial abundance, phenol oxidase activity or modifying the fungal communities composition (Paungfoo-Lonhienne et al., 2015; Wang et al., 2019). Therefore, the objective of this work was to investigate the effects of long-term conservation tillage on the stability of aggregate, SOC and microbial communities within aggregates, as well as to reveal the regulation of microbial communities on aggregate stability and SOC accumulation. Soil samples were collected from a long-term conservation tillage experiment station. The conservation tillage experiment consisted of two treatments (conventional tillage and no-tillage) and three nitrogen (N) application rates (105, 180, and 210 kg N ha^{-1}). Our hypotheses included: i) The implementation of long-term no-tillage and high nitrogen application rate possibly influence the stability of aggregates and SOC within aggregates; ii) Microorganisms potentially regulate aggregate stability and SOC within aggregates via multiple pathways.

2. Literature review

2.1 The effect of tillage and nitrogen application on the distribution of soil aggregates

The modulation of soil aggregate size distribution significantly influences ecological interactions and C cycling within the soil (Nie et al., 2014; Tisdall and Oades, 1982), which is influenced by agricultural treatments, particularly tillage practices (Hati et al., 2021; Liu et al., 2021). Numerous studies and meta-analyses consistently indicated that tillage intensity affected aggregate size distribution. In particular, conservation tillage had a greater potential to form macroaggregates and increase means weight diameter (MWD) (Kumar et al., 2019; Li et al., 2023; Liu et al., 2021). Furthermore, nitrogen fertilizer was indispensable components in contemporary agriculture. However, the influence of high nitrogen fertilization on the stability of soil aggregates can vary, either diminishing, augmenting, or exhibiting no discernible effect, even in instances of heightened C residue input (Z. Chen et al., 2019; Piazza et al., 2020; Zhang et al., 2021). Recent studies by Zhang et al., 2021 and Sithole et al., 2019 have indicated that varying nitrogen application rates do not appear to significantly influence soil aggregate stability under no-tillage. The underlying mechanisms behind these effects remain inadequately comprehended.

2.2 The effect of tillage and nitrogen application on the soil aggregates organic carbon

Long-term no-tillage practice increased C input by covering with straw and mitigating soil disturbance compared with long-term conventional tillage (Jha et al., 2020; Song et al., 2022). The impact of nitrogen addition on C input remains inconsistent (Liu and Greaver, 2010; Paungfoo-Lonhienne et al., 2015; Wang et al., 2019). It was found that the combination of straw return and appropriate nitrogen application increased SOC accumulation (Alvarez and Alvarez, 2005; Lin, 2018; Poirier et al., 2009). Conversely, high nitrogen availability in soils after nitrogen addition led to a decline in SOC accumulation because SOC mineralization exceeded residue C incorporation in some studies (Khan et al., 2007; Poirier et al., 2009). Thus, it is imperative to explore the effects of long-term no-tillage and nitrogen application on SOC sequestration at the aggregate scale.

A study suggested that conservation agriculture with a high nitrogen application rate promoted SOC accumulation in occluded micro-aggregates (Piazza et al., 2020). Alternatively, Sithole et al. (2019) showed that nitrogen application rates had little effect on soil aggregate stability and SOC within aggregates under no-tillage. The inconsistent impact of varying nitrogen application rates on soil aggregate stability and SOC under no-tillage systems may be attributed to the lack of clarity in the underlying microbial mechanisms involved.

2.3 The effect of tillage and nitrogen application on the soil microbial community

Long-term no-tillage practices increased the biomass of bacteria, and fungi (Wang et al., 2017), specifically arbuscular mycorrhizal fungi (Dai et al., 2015) and actinomycetes (B. Zhang et al., 2014), resulting in the recycling of organic matter and improving soil structure (Bhatti et al., 2017; Jeewani et al., 2021).

Actinomycetes release proteases and cellulases to decompose organic matter (Bhatti et al., 2017). Additionally, these microorganisms produce hyphae and mycelia that promote the aggregation of soil particles, thereby enhancing soil structure (Forster, 1990). The arbuscular mycorrhizal fungi act as biofertilizers and extend plant root systems, improving the uptake of soil water and nutrients (Diagne et al., 2020). Meanwhile, arbuscular mycorrhizal fungi promoted non-nutritional effects on plants, such as metabolic process alterations, soil structure improvements, and the stimulation of plant defense mechanisms against biotic and abiotic stresses (Vilela and Damásio, 2021). However, increasing nitrogen application rates decreased the biomass of bacteria, and fungi (T. Zhang et al., 2018), especially for arbuscular mycorrhizal fungi and actinomycetes biomarkers (Dai et al., 2015; Wang et al., 2017). The influence of long-term tillage practices and nitrogen fertilization on microbial communities across various soil aggregate size classes remains limited.

2.4 The relationship among aggregates, microorganisms, and soil organic carbon accumulation

Soil aggregates can be considered substantial microbial incubators (Bach et al., 2018). Different aggregate sizes have different physical and chemical conditions, so a number of studies have examined microorganism distribution according to aggregate size (Trivedi et al., 2015; Xue et al., 2021; Zheng et al., 2018). Bacteria have commonly been linked to micro-aggregates (Bach et al., 2018; Helgason et al., 2010). Micro-aggregates, which are characterized by processed organic matter exhibiting a low ratio of carbon and nitrogen, along with reduced predation pressure and increased water availability, potentially provide a favorable ecological niche for bacterial communities within soil (Davinic et al., 2012; Totsche et al., 2018). However, Yang et al., 2019 indicated a higher presence of bacteria in macro-aggregates. These varying findings could potentially be attributed to the utilization of different sieving methods and the application of distinct techniques for quantifying microorganisms, such as biochemical or molecular approaches (Helgason et al., 2010; Trivedi et al., 2015; Yang et al., 2019). In relation to fungi, numerous studies have associated increased fungal abundance with macro-aggregates (Bach et al., 2018; Baumert et al., 2018; Helgason et al., 2010), supporting the concept of macroaggregate-microaggregate formation theory (Six et al., 2000). On the contrary, the microorganisms exert both direct and indirect influences on the ongoing processes of soil aggregate formation (Barbosa et al., 2019; Guhra et al., 2019). Bacteria play a crucial role in the formation of both macro-aggregates and micro-aggregates, whereas fungi primarily contribute to the formation of macro-aggregates (Totsche et al., 2018). Fungi, particularly arbuscular mycorrhizal fungi, have garnered considerable attention in studies concerning soil aggregation (Philippot et al., 2023). This is primarily due to the filamentous nature of these fungi, which facilitates effective entanglement and interweaving of soil particles. Furthermore, fungi play a crucial role in the production of binding agents (Leifheit et al., 2014). Therefore, it is necessary to understand the role of various microorganisms on soil aggregates across different scale gradients under long-term no-tillage system.

Microbial communities are essential in transforming and storing organic C in soils (Schaeffer et al., 2015; Six et al., 2006). Fungi play a more significant role than

bacteria in the accumulation of necromass, thereby contributing to the increase in SOC under different agricultural practices (Li et al., 2015a; Yang et al., 2022). In addition, the presence of bacteria rather than fungi exhibited a positive correlation with the concentrations of C within aggregates (Navas et al., 2021). Hence, the intricate relationship among microorganisms within aggregates, aggregate formation, and SOC accumulation remains unclear. Such understanding of this intricate relationship under long-term no-tillage with varying nitrogen application rates is currently lacking.

3. Materials and methods

3.1 Experimental field site

The experiment was conducted in the field from 2003 to 2019 at Shouyang Experiment Station in Shanxi Province, the north of China (113.11°E, 37.97°N). The station has a continental monsoon climate. The mean annual rainfall is 483 mm and the evaporation is 1751 mm at the station (S. Li et al., 2021a). Approximately 129 days per year are frost-free. The warmest month (July) experiences an average daily temperature of 28.2°C, whereas the coldest month (January) witnesses a temperature as low as -11.4°C (Wang et al., 2019), and the mean annual temperature is recorded at 7.4 °C (S. Li et al., 2021a). The soil type is sandy loam Cinnamon, classified as Calcaric-Fluvic Cambisols (IUSS Working Group WRB, 2014). The physical and chemical characteristics of soil initially were displayed in Table 2-S1.

3.2 Experimental design

Since 2003, a block-randomized design was used with 3 replications in the long-term experiment. The experiment consisted of 18 plots, each measuring 25 m2 (5 m by 5 m). Moreover, the plant-to-plant spacing and row-to-row spacing were 0.3 and 0.6 m, respectively. During the months of March and November, maize was continuously planted.

Under long-term no-tillage and conventional tillage treatments, nitrogen fertilizer was employed at different rates. The no-tillage treatment consisted of no-till sowing with a no-till planter and mulching with harvested maize stalks. In April, nitrogen fertilizer was applied in tiny holes with a depth of 10 cm between two rows of maize (S. Li et al., 2021a). The conventional tillage treatment involved two plowings to a depth of 25 cm in pre-sowing and post-harvest, with the application of fertilizer prior to plowing and the removal of maize residue. All three rates of nitrogen application used urea, including 105, 180, and 210 kg N ha−1 for N1, N2, and N3, respectively. The average application rate of chemical fertilizer in China is 235 kg N ha-1, ranking it first worldwide as reported by Fao. (1999). In the northern region of China, an application rate of 180 kg N ha-1 is recommended to achieve a balanced nitrogen level and minimize leaching in a high-yielding maize system (Guo et al., 2017; Jiang et al., 2020; Jin et al., 2012). However, the maximum fertilizer nitrogen availability and yield in Northern China's Shouyang Experiment Station were observed at an nitrogen application rate of 105 kg N ha-1 according to a previous study (Wang et al., 2001). Therefore, 210 kg N ha-1 was defined as a high nitrogen application rate in this study. China's excessive nitrogen addition issue makes it imperative to examine the impact of high nitrogen application rates under tillage.

3.3 Soil sampling

Soil samplings were collected in 18 plots at depths of 0-10 cm and 10-25 cm. In each replicate plot, four undisturbed soil blocks near the center point were mixed together to form a homogeneous sample. In total, 36 soil subsamples were taken. The sampling was conducted on 1 August 2019, during the tasselling stage, a critical period for maize growth and has the highest relative abundance of soil microbes (Ayiti et al., 2022; Jia et al., 2021).

The soil samples were packed in ice boxes and taken to the lab immediately. Then we carefully broke soil samples along the breaking point. Stones, crop residues, and roots were removed from the soil samples using tweezers.

3.3.1 Aggregate screening determination

Soil samples were sieved by using field humidity dry screening (Nie et al., 2014) because we analyzed the weight water content of all samples (between 8.3% and 11.2%), which could minimize the interference to microbial community structure (Bach, 2014). Soil samples of 300 g (approximately 10% moisture) were placed on screens (2 mm and 0.25 mm) and sieved up and down 3 cm 60 times in 3 min. Thus, three aggregate sizes were obtained, including mega-aggregates (>2000 μm), macro-aggregates (250-2000 μm), and micro-aggregates (<250 μm). The aggregates were then weighed and the MWD was calculated (Li et al., 2015b).

After separation, we splited aggregates samples into two groups. One portion was immediately freeze-dried and preserved in a refrigerator (-20°C) for phospholipid fatty acid analysis (PLFA) within one week. Another part was used to determine SOC content.

3.3.2 Soil phospholipid fatty acid analysis

We utilized PLFA analysis to determine the microbial community biomass. The modified Bligh and Dyer method was used (Börjesson et al., 1998). A chloroform-methanol-citrate buffer was used to extract PLFA from 5 g freeze-dried soil samples. Separating neutral lipids, glycolipids, and phospholipids from extracted lipids was done with solid-phase extraction tubes. The phospholipid fraction was internally standardized with PLFA 19:0 (Larodan Malmö, Sweden). Next, transesterification of PLFAs to fatty acid methyl esters. Then the extracts were analyzed by gas chromatography (Agilent Technologies, USA) (Chowdhury and Dick, 2012).

Fungal biomass was calculated as the total of PLFAs 18:2ω6c and 18:1ω9c (Frostegård and Bååth, 1996). Gram-negative bacteria were identified using PLFAs (16:1ω11c, 16:1ω9c, 18:1ω7c, 18:1ω5c, cy19:0, and cy17:0), whereas gram-positive bacteria were identified using PLFAs (a17:0, i17:0, i16:0, a15:0, i15:0, and i14:0) (Moore-Kucera and Dick, 2008; Zhang et al., 2022). PLFA markers assigned for Actinomycetes are 10Me19:0, 10Me18:0, 10Me17:0, and 10Me16:0 (Zelles, 1999). The FAME marker 16:1ω5c was assigned to arbuscular mycorrhizal fungi (Frostegård et al., 2011), whereas the i17:1ω5c was as a biomarker for desulfovibrio (Bossio et al., 2006). The actinomycetes, gram-positive bacteria, and gram-negative bacteria biomass were summed as total bacterial biomass. In addition, actinomycetes, desulfovibrio, and arbuscular mycorrhizal fungi were three representative microorganisms that manifested significant differences across the treatments.

3.4 Statistics

The impact of tillage, nitrogen application rates, and their interaction on PLFAs, SOC, microbe biomass carcon (MBC), and MWD within all soil aggregates were determined by two-way analysis of variance (ANOVA) analysis. Additionally, using student's t-test to test whether tillage or soil depths differed significantly. Employing a one-way ANOVA with Duncan's test at 0.05 significance level, differences in application rates of three nitrogen were examined. Before conducting ANOVA, we utilized the Shapiro-Wilk and Levene tests to determine the normal distribution and homoscedasticity of data. Moreover, we used the linear model to evaluate the correlation between microbial community biomass and MWD or SOC. Data were analyzed by SPSS software 20.0 (SPSS Inc).

4 Results

4.1 Distribution of soil aggregates

The macro-aggregates size class was the main category across all treatments, constituting over 40.7% of all soil aggregates. The distribution of soil aggregates did not differ significantly between soil layers (Table 3-S2). The tillage, nitrogen application rates, and their interaction significantly influenced the proportion of mega- and micro-aggregates, while only tillage had an effect on the percentage of macro-aggregates (Table 3-S3). Under the same nitrogen application rate, no-tillage boosted its mega- and macro-aggregate proportions by 6.4-58.1% and 9.4-25.0%, respectively, while decreasing the proportion of micro-aggregates by 29.3-51.0% compared with conventional tillage. Increasing nitrogen application rates under conventional tillage resulted in an increase in the percentages of mega-aggregates, while the proportion of micro-aggregates decreased (Fig. 3-1). By contrast, nitrogen application rates under no-tillage did not influence the aggregate distribution.

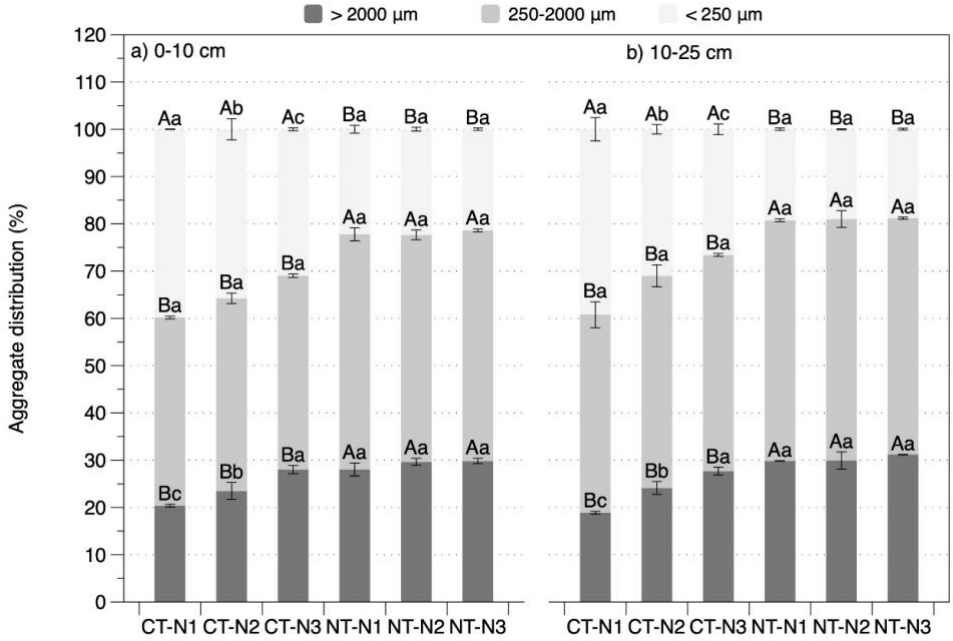

Fig. 3-1 Soil aggregate distribution under long-term tillage and nitrogen application. Vertical bars indicate the standard error of means (n = 3). Different capital letters indicate significant differences ($p < 0.05$) between two tillage treatments under the same nitrogen application rate; different lower-case letters indicate significant differences ($p < 0.05$) among nitrogen application rates under the same tillage treatment. N1, nitrogen application rate at 105 kg N ha-1; N2, nitrogen application rate at 180 kg N ha-1; N3, nitrogen application rate at 210 kg N ha-1; CT, conventional tillage; NT, no-tillage.

In 0-25 cm, tillage, nitrogen application rates, and their interaction significantly influenced soil MWD (Table 3-S3). No-tillage increased MWD by 8.7%-42.7% under the same nitrogen application rate compared with conventional tillage (Fig. 3-2). Moreover, as nitrogen application rates increased, the MWD under conventional tillage increased, while the MWD in no-tillage was unaffected.

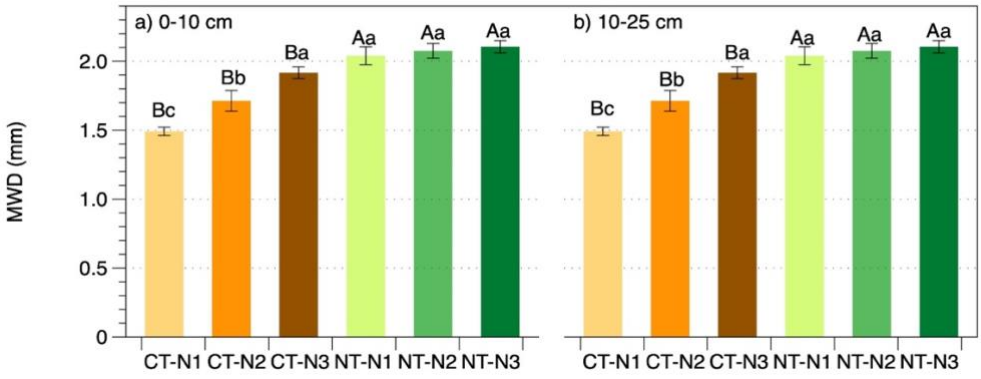

Fig. 3-2 The mean weight diameter under long-term tillage and nitrogen application. Vertical bars indicate the standard error of means (n = 3). Different capital letters indicate significant differences ($p < 0.05$) between two tillage treatments under the same nitrogen application rate; different lower-case letters indicate significant differences ($p < 0.05$) among nitrogen application rates under the same tillage treatment. MWD, mean weight diameter; N1, nitrogen application rate at 105 kg N ha^{-1}; N2, nitrogen application rate at 180 kg N ha^{-1}; N3, nitrogen application rate at 210 kg N ha^{-1}; CT, conventional tillage; NT, no-tillage.

4.2 Soil organic carbon within soil aggregates

The SOC content within aggregates was not affected by the soil layer but was notably influenced by nitrogen application and tillage (Table 3-S4 and 3-S5). In 0-10 cm, under the same nitrogen application rate, no-tillage enhanced SOC content within mega-aggregates by 17.7%-27.8% and macro-aggregates by 15.9%-22.5%, while reducing SOC content within micro-aggregates by 18.0%-28.6%, compared with conventional tillage (Fig. 3-3). With increasing nitrogen application, the SOC content within all aggregates increased significantly by 2.1 to 3.7 g·kg^{-1} under no-tillage, but under conventional tillage, the SOC content increased only within micro-aggregates.

In 10-25 cm, tillage and nitrogen application rates had similar but weaker effects on SOC within aggregates than in 0-10 cm (Fig. 3-3). With increasing nitrogen application, only the SOC within mega- and macro-aggregates under no-tillage was significantly increased.

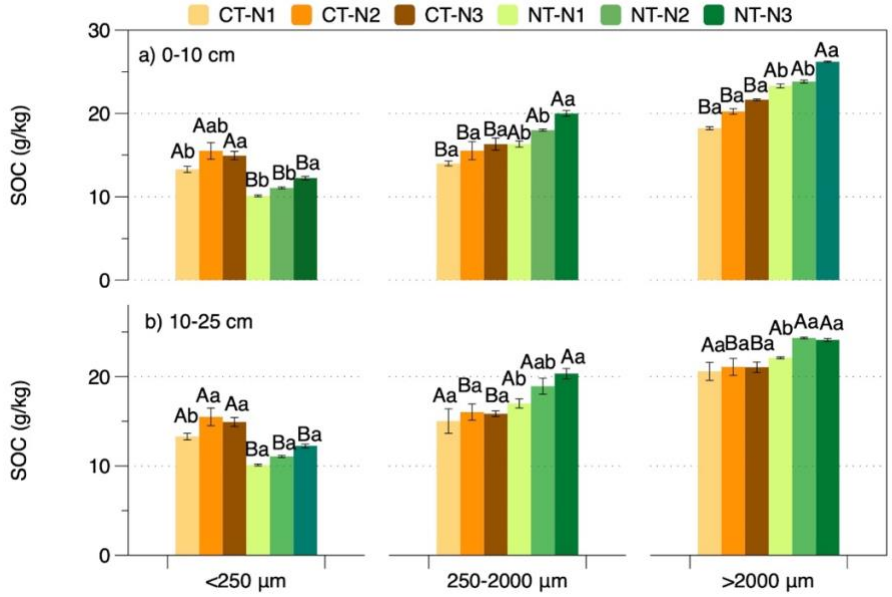

Fig. 3-3 The soil organic carbon content within soil aggregates under long-term tillage and nitrogen application. Vertical bars indicate the standard error of means (n = 3). Different capital letters indicate significant differences ($p < 0.05$) between two tillage treatments under the same nitrogen application rate; different lower-case letters indicate significant differences ($p < 0.05$) among nitrogen application rates under the same tillage treatment. SOC, soil

organic carbon; N1, nitrogen application rate at 105 kg N ha^{-1}; N2, nitrogen application rate at 180 kg N ha^{-1}; N3, nitrogen application rate at 210 kg N $ha^{-1;}$ CT, conventional tillage; NT, no-tillage.

4.3 Soil microbial community

The mean concentrations of bacterial, fungal, and total PLFAs biomass within aggregates in 0-10 cm were significantly higher by 29.7%, 31.1%, and 44.1%, respectively, for various treatments as compared with those in 10-25 cm (Table 3-S6).

For the 0-10 cm layer, bacterial, fungal, and total PLFAs were significantly impacted by tillage, nitrogen application rates, and their interaction (Table 3-S7). Compared with conventional tillage, no-tillage enhanced considerably total PLFAs within micro- (N2 and N3), mega-, and macro-aggregates under the same nitrogen application rate and also significantly increased fungal biomass within all aggregates (Table 3-S8). For bacterial biomass, no-tillage significantly reduced them within micro-aggregate under N1, but significantly increased them within macro- and micro-aggregates under N2 and micro-aggregates under N3, compared with conventional tillage. Specifically, Fig. 3-S4 depicts the PLFA profiles in each soil aggregate size class of different treatments. Compared with conventional tillage, no-tillage decreased desulfovibrio PLFAs (i17:1ω5c) within all aggregates by 8.6-15.1 nmol g^{-1} (Fig. 3-4). However, no-tillage increased actinomycetes within all aggregates by 2.1-7.8 times and arbuscular mycorrhizal fungi within all aggregates by 0.4-1.6 nmol g^{-1}, as well as increased gram-negative bacteria within mega- and macro-aggregates and gram-positive bacteria within mega- and macro-aggregates compared with conventional tillage (Table 3-S8).

Moreover, increasing nitrogen application rates decreased bacterial, fungal, and total PLFAs within all aggregates of conventional tillage by 13.0%-46.0%, 10.9%-46.7%, and 6.6%-35.0%, respectively (Table 3-S8). Under no-tillage, increasing nitrogen application rates decreased total PLFAs, bacterial, and fungal biomass within mega-aggregates by 17.2%-21.1%, 21.9%-26.5%, and 16.6%-19.6%, respectively. Specifically, under conventional tillage, increasing nitrogen application rates decreased the biomass of arbuscular mycorrhizal fungi, actinomycetes, desulfovibrio, gram-negative, and gram-positive bacteria within all aggregates. Increasing nitrogen application rates under no-tillage decreased the biomass of actinomycetes, gram-negative bacteria, and arbuscular mycorrhizal fungi within mega-aggregates by 14.9%-20.2%, 19.1%-21.8%, and 22.1%-32.8%, respectively.

For 10-25 cm, tillage and nitrogen application rates significantly affected total PLFAs, bacterial, and fungal biomass (Table 3-S9). Under the same nitrogen application rate, no-tillage significantly decreased the total PLFAs by 7.3%-32.8% and bacterial biomass by 7.8%-46.1% within all aggregates compared with conventional tillage (Table 3-S10). Specifically, compared with conventional tillage, no-tillage increased actinomycetes biomass within all aggregates, while decreasing the biomass of arbuscular mycorrhizal fungi (under N2 and N3), desulfovibrio, gram-positive, and gram-negative bacteria within all aggregates.

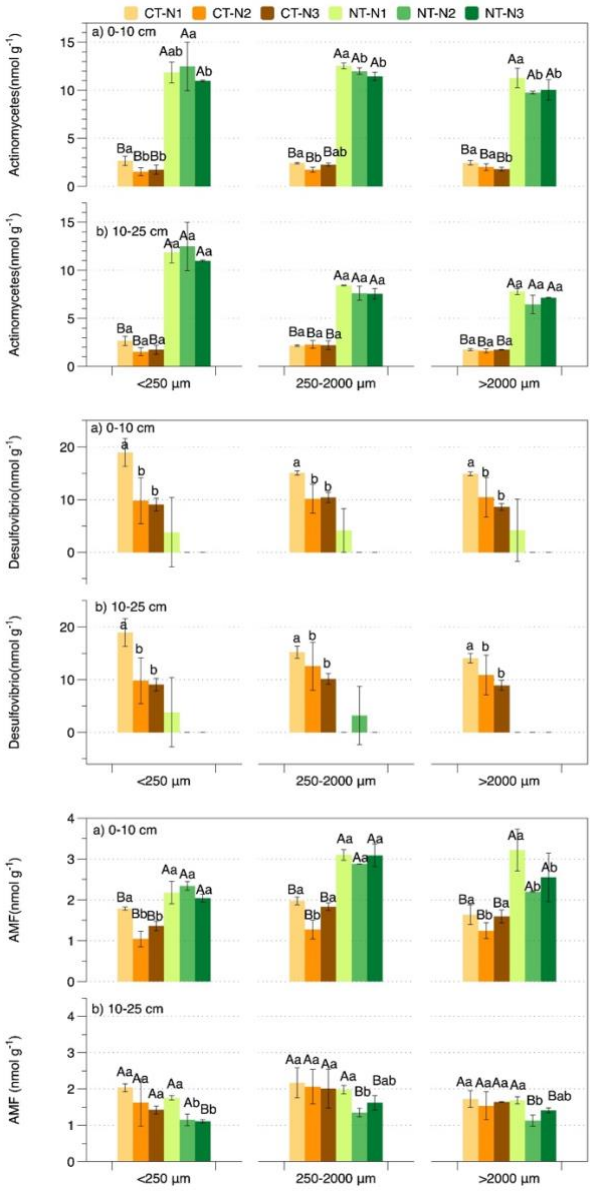

Fig. 3-4 Actinomycetes, Desulfovibrio, and arbuscular mycorrhizal fungi within soil aggregates under long-term tillage and nitrogen application. Vertical bars indicate the standard error of means (n = 3). Different capital letters indicate significant differences ($p < 0.05$) between two tillage treatments under the same nitrogen application rate; different lower-case letters indicate significant differences ($p < 0.05$) among nitrogen application rates under the same tillage treatment. AMF, Arbuscular mycorrhizal fungi; N1, nitrogen

application rate at 105 kg N ha^{-1}; N2, nitrogen application rate at 180 kg N ha^{-1}; N3, nitrogen application rate at 210 kg N ha^{-1}; CT, conventional tillage; NT, no-tillage.

Moreover, under conventional tillage, increasing nitrogen application rates had little influence on the fungal, bacterial, and total PLFAs within all aggregates (Table 3-S10). However, under no-tillage, increasing nitrogen application rates reduced total PLFAs by 10.3%-30.8% and fungal biomass by 15.4%-31.9% within all aggregate, as well as bacterial biomass by 13.8%-26.4% in mega-aggregates. Specifically, under no-tillage, increasing nitrogen application rates decreased arbuscular mycorrhizal fungi and gram-negative bacteria biomass within all aggregates.

5 Discussion

5.1 The effect of long-term no-tillage on mean weight diameter and soil organic carbon content within all aggregates

Soil aggregation is a crucial parameter for understanding the functional and structural quality of soils (Gupta and Germida, 2015). Compared with long-term conventional tillage, long-term no-tillage enhanced the MWD and the percentages of mega- and macro-aggregates, while decreasing the proportion of micro-aggregates under the same nitrogen application rate (Figs. 3-1 and 3-2), which supports the hypothesis that long-term no-tillage enhances soil aggregation. Two mechanisms could explain these results. Firstly, long-term no-tillage reduced soil disturbances, which improved the formation of macro-aggregates (Fig. 3-1). Secondly, compared with conventional tillage, more crop straw was invested under no-tillage (Fig. 3-S1), which first decomposed into small pieces of particle organic C as a nucleation center for large aggregation. Moreover, crop straw provides energy for microbes to generate binding agents (polysaccharides and glutamate) (Hati et al., 2021), thereby facilitating the formation of large aggregates.

An interesting observation was that the impact of nitrogen application on aggregate fractions differed between long-term conventional tillage and no-tillage systems (Fig. 3-1). Under long-term conventional tillage, an increase in nitrogen application rates, even without straw input, resulted in an elevation of root biomass (Fig. 3-S1) and root secretion, thereby augmenting the exudation of organic matter and fostering the formation of mega-aggregates (Figs. 3-1 and 3-2). Similarly, nitrogen addition stimulated soil aggregation in terrestrial ecosystems in a meta-analysis (Lu et al., 2021). However, under long-term no-tillage, the aggregate distributions were not influenced by nitrogen application rates, likely because straw returns to the field which contributes more to aggregate formation than nitrogen fertilizer application.

SOC within aggregates as characteristics and monitoring instruments for terrestrial ecosystems (Six and Paustian, 2014). Compared with long-term conventional tillage, long-term no-tillage decreased SOC content within micro-aggregates and raised SOC content within mega- and macro-aggregates (Fig. 3-3), and comparable study results were achieved by Du et al. (2013). One explanation for the higher SOC content within mega- and macro-aggregates under no-tillage versus

conventional tillage is that mega- and macro-aggregates created by the cementation of organic materials are the principal SOC reservoirs (Six et al., 2000). Long-term no-tillage enhanced the percentages of mega- and macro-aggregates to defend SOC from microbial assault (Fig. 3-1). The negative impact of long-term conventional tillage on SOC sequestration was attributed to the decrease of mega- and macro-aggregates (Fig. 3-1). Moreover, it is plausible that the observed lower SOC content within micro-aggregates under no-tillage versus conventional tillage. Under long-term conventional tillage, where, mega- and macro-aggregates undergo rapid turnover, micro-aggregates are deemed safer and preferred enrichment areas for microorganisms, as confirmed by MBC data (Fig. 3-S3). A significantly higher concentration of MBC was found in micro-aggregates under conventional tillage versus no-tillage (Fig. 3-S3). Additionally, microbial-derived C tended to dominate in micro-aggregates (Totsche et al., 2018). Hence, SOC content within micro-aggregates was higher under long-term conventional tillage versus long-term no-tillage. Higher nitrogen application rates increased SOC content within micro-aggregates under conventional tillage and all aggregates under no-tillage (Fig. 3-3). This implies that increasing nitrogen application rates alone has little impact on C sequestration in mega- and macro-aggregates, but that a combination of C and nitrogen inputs is necessary to increase exogenous C sequestration within all soil aggregates. Previous studies also have shown that nitrogen fertilizer applications promote similar accumulation patterns of organic C (+4-8%) around the world due to chemical reactions and plant growth (Aguilera et al., 2013; Piazza et al., 2020). Furthermore, we found this effect diminishes as soil layers deepen (Fig. 3-3), implying that deeper soil layers may mitigate the impact of nitrogen application rates on the microbial biomass that relies on SOC as a survival resource.

5.2 The effect of long-term no-tillage on microbial communities within all aggregates

Compared with long-term conventional tillage systems, total PLFAs in mega- and macro-aggregates were higher under long-term no-tillage in 0-10 cm (Table 3-S8). The result indicates that no-tillage is more favorable for total microbial biomass within mega- and macro-aggregates. The findings may be explained by the fact that long-term no-tillage promotes the formation of mega- and macro-aggregates, which are nutrient-rich and suited for microorganisms. Thus, long-term no-tillage practice significantly enhanced the total PLFAs within mega- and macro-aggregates compared with long-term conventional tillage. Specifically, compared with conventional tillage, no-tillage induced a general positive effect on fungi (arbuscular mycorrhizal fungi) and bacteria (actinomycetes) within all aggregates in 0-10 cm layer (Table 3-S8), and several investigations confirmed this finding (Guo et al., 2015; Li et al., 2020). Our result may be interpreted with two possible reasons: i) compared with conventional tillage, no-tillage generally protects the fungal mycorrhizae from damage (Zhang et al., 2012). Fungal hyphae can grow in soil pores (>10 μm) (Effmert et al., 2012) and extend into all soil aggregate. ii) compared with conventional tillage, no-tillage has higher soil moisture content and

milder soil temperatures, making it more conducive to fungal and bacterial colonization (Zhang et al., 2022).

In contrast, for the deeper soil layer, long-term conventional tillage treatment increased total PLFAs and bacteria biomass within all aggregates compared with long-term no-tillage (Table 3-S10). This may be due to conventional tillage creating more favorable conditions for microbial colonization in deeper soils by loosening the soil and enhancing soil aeration, thereby improving nutrient transport and microbial growth (Mello Ivo and Mielniczuk, 1999). Additionally, tillage can influence the distribution of microorganisms in different soil layers. Specifically, conventional tillage promotes microbial growth in deeper soil layers due to the deeper root system and higher organic matter concentrations (Ji et al., 2013; Moraes et al., 2020). Consequently, while long-term no-tillage may be conducive to microbial biomass in the surface layer, long-term conventional tillage may be more conducive to microbial biomass in deeper soil layers.

To our knowledge, microbial responses to nitrogen application in diverse ecosystems exhibited significant variability (Cui et al., 2020; Stewart et al., 2018; T. Zhang et al., 2018). Nitrogen application rates regulate the impact of long-term no-tillage on microbial biomass within micro- and macro-aggregates (Table 3-S8). This might be because increasing nitrogen application under no-tillage significantly reduced the ratio of carbon and nitrogen within micro- and macro-aggregates (Fig. 3-S2) and alleviated microbial nitrogen limitation (Thierfelder et al., 2018). Moreover, higher nitrogen application rates reduced fungal (arbuscular mycorrhizal fungi), bacterial (gram-negative, gram-positive, actinomyces, desulfovibrio), and total microbial biomass within mega-aggregates under both tillage practices in 0-10 cm (Table 3-S8). As the toxicity of urea, excessive nitrogen application has suppressed the soil microbial activity (Lian et al., 2018; Wang et al., 2018). In soils with continuous input of exogenous litter, long-term nitrogen addition significantly reduced gram-negative bacteria biomass by increasing the stress index of bacteria (Wang et al., 2018). Furthermore, nutrient enrichment may inhibit a subset of generalist microbial taxa, such as the mycorrhizal microbial groups that assist crop roots in obtaining nitrogen from the soil (Lu et al., 2022). Driven by the soil nutrient status, the altered microbial biomass and community composition affected soil microstructure and aggregate stability (Zhang et al., 2023).

5.3 Microbial strategies that regulate partly the characteristics of soil aggregates

To investigate the microbial strategies that regulate the characteristics of soil aggregates, we have selected three scenarios from microbial communities significantly impacted by long-term tillage and nitrogen application for a thorough discussion.

5.3.1 Actinomycetes readily enhancing the stability and soil organic carbon content of soil aggregates

Long-term no-tillage significantly increased the biomass of actinomycetes within all aggregates compared with long-term conventional tillage (Fig. 3-4), and a prior

study also supported our finding (B. Zhang et al., 2014). As a heterotrophic gram-positive bacterium, actinomycetes obtain the required C source by secreting cellulolytic, ligninolytic, phenoloxidase, and peroxidase (Bhatti et al., 2017). The returned maize straw in the no-tillage system provided sufficient cellulose and lignin for actinomycetes growth (Samson et al., 2020). Fu et al. (2022) also demonstrated that actinomycetes were the overwhelmingly dominant bacterial group for maize straw-carbon degradation. Moreover, actinomycetes had a significant positive correlation with MWD (Fig. 3-5). This may owe to the positive feedback effect of actinomycetes degrading straw on the formation of soil aggregates. Actinomycetes have been found to degrade straw-derived C into microbial-derived C or convert it to low-molecular-weight C (Mitra et al., 2022; Su et al., 2020b). These re-formed C components could be wrapped more easily by soil particles and adsorbed by minerals to form soil aggregates (Bhatti et al., 2017). Additionally, actinomycetes, as filamentous heterotrophic bacteria, entwined soil particles with mycelia and mucilages (Ren et al., 2022), thereby contributing to the formation and stabilization of soil aggregates. Furthermore, straw degradation by actinomyces significantly increased SOC content within mega- and macro-aggregates (Fig. 3-S5), the predominant fraction where straw residues were located (Six et al., 2000). Therefore, in the presence of a large amount of straw, these actinomycetes that prefer to degrade straw were recruited, thereby may contribute to an increase in the stability and SOC content of aggregates (Fig. 3-6).

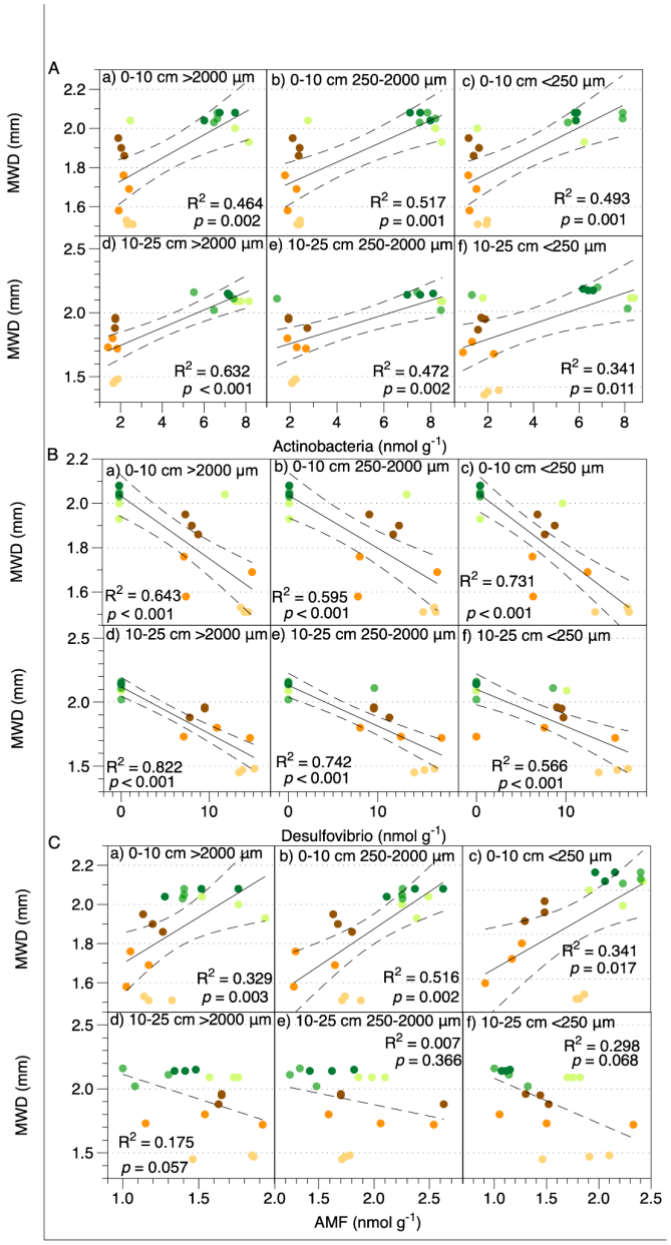

Fig. 3-5 Relationships between Mean weight diameter and Actinomycetes (A), Desulfovibrio (B), and Arbuscular mycorrhizal fungi (C) at different aggregate sizes and soil layers. Linear regression is shown as a black solid line. The black dashed line represents no significance. MWD, mean weight diameter; AMF, Arbuscular mycorrhizal fungi; N1, nitrogen application rate at 105 kg N ha^{-1}; N2, nitrogen application rate at 180 kg N ha^{-1}; N3, nitrogen application rate at 210 kg N ha^{-1}; CT, conventional tillage; NT, no-tillage.

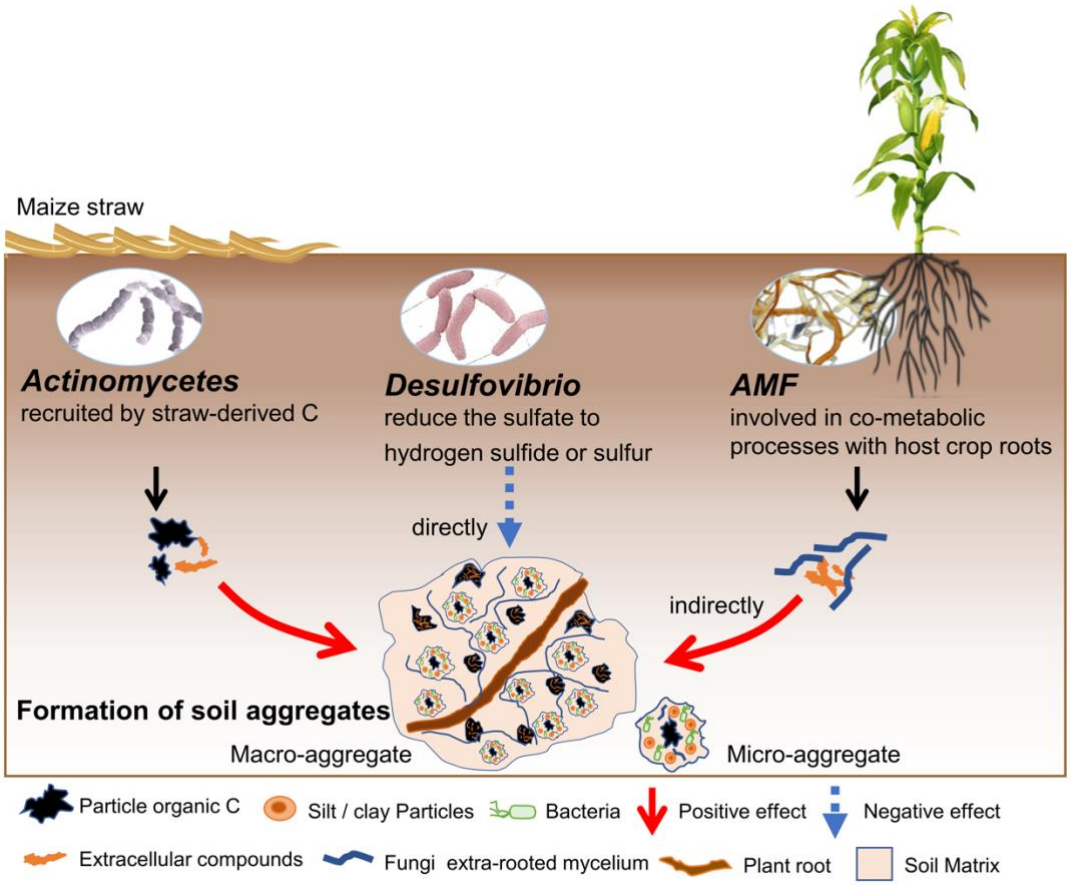

Fig. 3-6 Conceptual diagram illustrating the microbial regulation of soil aggregate formation. Actinomycetes were readily recruited by straw-derived carbon, thereby contributing to an increase in the stability and soil organic carbon content of soil aggregates. Desulfovibrio possibly limits the formation process of soil aggregates directly by reducing the inorganic colloidal substance sulfate to hydrogen sulfide or sulfur. Since Arbuscular mycorrhizal fungi (AMF) are unable to survive independently of the host roots, they are mainly involved in co-metabolic processes with the crop roots, which might indirectly contribute to the formation of soil aggregates.

5.3.2 Desulfovibrio possibly limited the process of soil aggregate formation directly

Long-term conventional tillage significantly increased desulfovibrio biomass within all aggregates compared with long-term no-tillage (Fig. 3-4). As anaerobic gram-negative bacteria, desulfovibrio will convert the sulfate in soil aggregates to hydrogen sulfide or sulfur to obtain energy for growth and reproduction (Voordouw, 1995). Sulfate was an inorganic cementing substance in soil (Totsche et al., 2018), so its reduction directly inhibited the formation of aggregates under conventional

tillage, leading to a decrease in soil aggregate stability (Fig. 3-5). However, increasing nitrogen application rates significantly reduced desulfovibrio biomass within all soil aggregate under conventional tillage (Fig. 3-4). There might be a toxic effect on soil bacteria caused by an increase in nitrogen application (Ramirez et al., 2010; Yue et al., 2016). Decreased soil pH and increased nitrate and nitrite inhibited significantly desulfovibrio growth (Greene et al., 2003). Curiously, the desulfovibrio biomass was only detected in the low nitrogen application rate under no-tillage (Fig. 3-4). The reason for this could be related to the environment surrounding the experimental site or to the competitive relationship between desulfovibrio and other microorganism in the soil (Ayiti et al., 2022). Further analysis of detailed microbial community interactions is required. Moreover, Desulfovibrio had significant negative correlations with SOC and MWD (Fig. 3-S5 and 3-5), suggesting that the reduction of desulfovibrio biomass improved the soil aggregates stability and increased SOC within mega-and macro-aggregates. Thus, the increase in desulfovibrio biomass possibly inhibited the formation of soil aggregates directly and limited SOC sequestration (Fig. 3-6).

5.3.3 Arbuscular mycorrhizal fungi might indirectly affect soil aggregate stability through ecological interactions

Compared with long-term conventional tillage, long-term no-tillage increased arbuscular mycorrhizal fungi biomass within all aggregates in 0-10 cm while decreasing them in 10-25 cm (Fig. 3-4). As an obligate symbiotic fungus, the prerequisite for arbuscular mycorrhizal fungi to grow and complete its life cycle is establishing a close and mutually beneficial relationship with crop root system (Rodrigues and Rodrigues, 2019). The arbuscular mycorrhizal fungi would use the photosynthetic compounds transported by the roots of the crops for growth and reproduction, which will, in turn, provide nutrients through the fungal hyphae to crops (Kokkoris et al., 2020; Vilela, 2021). Compared with conventional tillage, no-tillage resulted in higher soil compactness (Leghari et al., 2016) and nutrient accumulation (Tshuma et al., 2021) in surface soil, which allowed for greater maize root distribution in this layer (Ji et al., 2013; Moraes et al., 2020). This explained why no-tillage contained a higher arbuscular mycorrhizal fungi biomass within soil aggregates than conventional tillage in 0-10 cm but lower arbuscular mycorrhizal fungi biomass in 10-25 cm. Increased nitrogen application rates under no-tillage practices caused a decrease in arbuscular mycorrhizal fungi (Fig. 3-4), which was attributed to nutrient enrichment reducing the dependence of the crop root system on arbuscular mycorrhizal fungi (Lu et al., 2022, 2020).

In addition to nutritional effects, alterations in arbuscular mycorrhizal fungi communities also have non-nutritional effects on crops, such as modifying the soil microstructure of the crop root zone (Vilela and Damásio, 2021). However, the relationship between arbuscular mycorrhizal fungi and soil aggregates remains controversial. For example, Ji et al. (2019) revealed that arbuscular mycorrhizal fungi improved soil aggregate stability via its glomalin amounts and mycelial biomass. In contrast, a comparative study showed that single arbuscular mycorrhizal

fungi species (Gl. intraradices) did not affect soil aggregation (del Mar Alguacil et al., 2004).

Only at 0-10 cm did we observe a significant positive correlation between arbuscular mycorrhizal fungi and MWD (Fig. 3-5), which could be attributed to the different vertical distributions of arbuscular mycorrhizal fungi community composition across soil layers (Moll et al., 2016). Family and operational taxonomic unit-level arbuscular mycorrhizal fungi communities differed depending on soil layers, while they were more complex and rich on surface soil (Sosa-Hernández et al., 2018). There are some explanations for the positive association between arbuscular mycorrhizal fungi and MWD. One reason is that the secreted products (e.g., mucilage, polysaccharides, and other extracellular compounds) produced by arbuscular mycorrhizal fungi during its interaction with other soil microbial communities may contribute indirectly to the formation of soil aggregates (Barbosa et al., 2019; Anika Lehmann et al., 2017). Another reason is that the extra-rooted mycelium produced by arbuscular mycorrhizal fungi acts as a temporary binder for the formation of soil aggregates as it acquires soil nutrients for plant growth (Parihar et al., 2020). Since arbuscular mycorrhizal fungi cannot survive independently of the host roots (A. Lehmann et al., 2017), they primarily participate in co-metabolic processes with the crop roots, which might indirectly contribute to the formation of soil aggregates (Fig. 3-6).

5.4 Implications

Exploring the relationship between soil aggregate microstructure and microbial communities provided a deep recognition of mechanisms controlling SOC sequestration under long-term no-tillage and nitrogen application. Protecting SOC by aggregates has been considered the primary factor limiting the microorganisms' access to SOC (Zhang et al., 2023). We quantified the associations between microbial communities' characteristics within aggregates and soil aggregates stability, demonstrating that changes in the biomass of microorganisms within the aggregates might directly or indirectly affect aggregate stability, thereby regulating the sequestration of SOC within the soil aggregates (Fig. 3-6). Especially in mega- and macro-aggregates containing more abundant primary photosynthetic products (e.g. straw residues), microbial processes had a closer relationship with the stability of macro-aggregates than in micro-aggregates (Wilpiszeski et al., 2019). This mechanism of microbial regulation of soil aggregate formation processes is more common in soils with long-term agricultural management because of the legacy effect of soil microorganisms (Sauvadet et al., 2018). The knowledge of how microorganisms regulate soil aggregate formation spatially and temporally still awaits future research. To gain a better understanding of this process, a more in-depth analysis of the interactions between different microbial communities (Lin et al., 2019; Liu et al., 2021) and the application of high-resolution methods (Elisa Korenblum et al., 2022; Weng et al., 2022) will be required. Overall, this research offers new perspectives on quantifying the involvement of microorganisms in

forming soil aggregates and exploring the mechanisms by which this process regulates SOC sequestration.

6. Conclusions

Based on our long-term experiment and hypothesis, the development of no-tillage with high nitrogen application rate in agricultural ecosystems provides a more sustainable strategy to improve soil quality compared to conventional tillage. In contrast to the general assumption that aggregates provide a protective function for soil organic carbon against microbial degradation, this study quantifies the relationship between microbial properties within aggregates and aggregate stability based on a rare long-term no-tillage experiment with varying nitrogen application rates.

The initial significant discovery indicated that nitrogen application rates did not affect the aggregate distributions under long-term no-tillage. This suggests that the incorporation of straw into the field may play a more substantial role in aggregate formation than the application of nitrogen fertilizers. The second finding also clearly indicated that higher nitrogen application rates led to a significant increase in soil organic carbon content exclusively within micro-aggregates under long-term conventional tillage, while all aggregates exhibited this increase under long-term no-tillage practice. The current data highlight the importance of nitrogen application rates in understanding the carbon sequestration in mega- and macro-aggregates under long-term no-tillage systems.

In addition, this study has identified the combination of long-term no-tillage practice and lower nitrogen application rates has a positive impact on the surface soil microbial community, specifically arbuscular mycorrhizal fungi and actinomycetes. Most importantly, under long-term tillage and nitrogen application, we identified microbial strategies that regulate the characteristics of soil aggregates via multiple pathways. The actinomycetes biomass possibly participated in aggregate formation via straw degradation and increased soil organic carbon content within mega- and macro-aggregates. Conversely, the desulfovibrio biomass might inhibit the formation of soil aggregates and decrease soil organic carbon content within mega- and macro-aggregates. Arbuscular mycorrhizal fungi biomass potentially indirectly contributes to soil aggregate formation through co-metabolic processes with crop roots and increases soil organic carbon content within mega- and macro-aggregates.

Among the farmland ecosystems, the combination of no-tillage and high nitrogen application rate agricultural ecosystems improved soil aggregate stability by altering aggregate formation through the microbial communities, and enhanced soil organic carbon stock within aggregates. This work shed light on microbial regulation of aggregate stability, allowing for a more precise forecast of soil organic carbon dynamics within aggregates under long-term conservation tillage.

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Chapter 4

Soil pore structure regulates microbial co-occurrence patterns and keystone taxa under long-term conservation tillage

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Abstract

The pore structure of soil plays a significant role in carbon (C) and nitrogen (N) cycling in conservation agriculture soils. However, the regulation of pore structure of soil on microbial co-occurrence patterns and the cycling of carbon and nitrogen remains elusive. We investigated the effects on the microstructure, the bacterial and fungal community's co-occurrence patterns, and N and C-cycling functional genes following addition of N application rates (105 and 210 kg N ha-1) under two long-term conventional tillage (CT) and no-tillage (NT). Compared to CT, NT improved bacterial network complexity at lower N application rates but reduced it at higher rates. Under NT, increasing N application rate have no impact on properties of soil bacterial co-occurrence networks. Compared to CT, NT increased fungal network complexity under the same N application rates. Under same tillage treatments, increasing N application rate decreased fungal network complexity. Compared to CT, NT increased the keystone taxa of Acidobacteria, Planctomycetes, and Bacteroidete under lower N application rate, while reduced the keystone taxa of bacteria and fungi under higher N application rate. Under NT, high N application rate reduced the the keystone taxa of Acidobacteria and Bacteroidetes. The irregular and elongated pore morphology enhances pore connectivity and porosity, further augments the network connectivity of soil bacteria. The pore connectivity and porosity augments provide favorable conditions for aerobic bacteria survival of Acidobacteria, Planctomycetes and Gemmatimonadetes. Reducing the distribution of (0-10 μm) pores may reduce competition among fungal communities. The decrease in porosity and the distribution of (10-30 μm) pores resulted in a concomitant reduction in the bacterial network connectivity and key bacterial taxa. Furthermore, high N application rates could improve soil C sequestration potential (functional gene cbbL) by increasing the C sequestration key microorganisms (Chloroflexi and Proteobacteria) under two tillage practice. Some key bacterial taxa can directly or indirectly modulate nitrogen-fixing genes under tillage systems. Overall, this study highlights that N addition can alter the effect of soil pore structure on microbial co-occurrence patterns and keystone taxa, which further improves our understanding to explain and predict the cycling of carbon and nitrogen in tillage systems.

Keywords: No-tillage, Nitrogen, soil pore structure, microbial co-occurrence patterns, N and C-cycling functional genes

1. Introduction

The pore structure of soil plays a vital role in soil biology, including microbial activity and root growth, as well as important processes such as carbon (C) and nutrient cycling, which ultimately determine the ecosystem services provided by soil (Powlson et al., 2011; Bünemann et al., 2018). The characteristics of soil pores are susceptible to tillage management and are essential for nutrient cycling, as well as the storage and transformation of soil organic carbon (SOC) (Zhao et al., 2018). However, the specific biophysical mechanism by which soil aggregates pore impact SOC sequenstration remains unclear (Kan et al., 2020; Vogel et al., 2022).

Compared with conventional tillage (CT), no-tillage (NT) is frequently linked to reduced soil loss, increased SOC, improved soil structure, and enhanced flow of soil moisture and gases, especially when combined with straw mulching, although

primarily in the surface 0-10 cm layer (Mondal and Chakraborty, 2022). Previous studies have demonstrated that residue retention-based NT practices enhance total porosity by primarily increasing macroporosity and mesoporosity (He et al., 2009; Gao et al., 2019). This has a positive impact on the ongoing formation of pores, including biopores resulting from residue decomposition and faunal activity, which significantly affect soil transport functions (Hartmann et al., 2012). However, it should be noted that NT practices may also lead to compacted surface soil, potentially restricting root growth and impeding the movement of air and water (Nunes et al., 2015). Conversely, CT disrupts pore continuity, loosens the surface soil, and promotes the formation of compacted layers beneath the surface (Blanco-Canqui and Ruis, 2018; Jayaraman et al., 2021). NT coupled with straw mulching and nitrogen (N) fertilizer application enhanced soil aggregate stability by providing both aggregate forming and stabilizing agents (Hati et al., 2021). The application of N fertilization resulted in an augmentation of intra-aggregate porosity and a concomitant shift towards increased pore space accumulation in larger aggregates, likely attributed to the substantial stimulation of root growth (Caplan et al., 2017). Nevertheless, the specific alterations in soil pore structure under NT conditions due to varying rates of N application, and their potential implications for carbon storage and nitrogen cycling, remain uncertain.

The recent advancements in X-ray μ-CT technology have facilitated the capture of detailed 3D images of soil structures, enabling a more precise assessment of soil heterogeneity at micro-scales and facilitating the identification and characterization of soil pores (Bouckaert et al., 2013; Müller et al., 2018). Pores are of most importance in the regulation of air, water, and nutrient movement within the soil, consequently influencing the micro-environmental conditions essential for microbial activity (Strong et al., 2004; Or et al., 2007). The presence of macropores enhances water infiltration and aeration, thereby fostering microbial activity and the decomposition of organic matter. This decomposition process results in the release of vital nutrients, such as nitrogen, phosphorus, and sulfur, into the soil (Josa et al., 2013; Sun et al., 2020). Xia et al. (2022) have found that the distribution of soil pore sizes significantly affects both the compositions and networks of the soil microbial community. Besides porosity and pore size distribution, one of the most important parameters for soil functions is pore connectivity (Rabot et al., 2018). It affects both air permeability (Marcos Paradelo et al., n.d.) and saturated hydraulic conductivity (Sandin et al., 2017; Zhang et al., 2019). Furthermore, the connectivity of pore is also essential for soil as a habitat for a myriad of organisms and for the accessibility of SOC for these organisms and their aeration status (Kravchenko et al., 2015; Negassa et al., 2015; Rabbi et al., 2016). However, there is a significant research gap when it comes to studying the impact of soil pore connectivity on the relationships among soil microbial keystone taxa, microbial function and interaction attributes under NT conditions.

In recent years, the microbial network has been used to analyze co-occurrences and interactions between members of microbial communities (Banerjee et al., 2016; Ishimoto et al., 2021). The analysis of microbial network architecture using topological indices helps identify key microbial species, including hubs, connectors, and keystones, which exert significant influence on community structure and

function, irrespective of their abundance (Barberán et al., 2012; Banerjee et al., 2018). Agricultural management practices have a significant impact on the complexity and stability of soil microbial communities by influencing the abundance of keystone taxa (Zheng et al., 2022). These key-stone phylotypes were also positively associated with plant growth and multiple functional genes related to nutrient cycling (Fan et al., 2021). For instance, Zheng et al. (2022) found the increased soil porosity and organic matter content under NT can provide habitats and substrates for a greater diversity of microbial species, leading to more complex microbial interactions and co-occurrence patterns. Straw reduced negative correlations between bacteria and fungi in soil, suggesting competition between microbes dominated in nutrient-limited regions (Banerjee et al., 2016). Furthermore, the addition of nitrogen also provides essential nutrients that mitigate microbial competition. In brief, the impact of tillage practice on the coexistence dynamics of microbial communities has been extensively explored, yet the impact of N fertilizer has not been adequately investigated (Li et al., 2021).

It is now recognized that microbial compositional information does not always inform function (Jansson and Hofmockel, 2020). A number of processes and key microbes are involved in the C and N cycling of soil. C-fixing microorganisms play a crucial role in this process by selectively utilizing C sources, thereby influencing the renewal and cycling of organic matter (Yousuf et al., 2012). For example, the crucial enzyme of the Calvin–Benson–Bassham (CBB) cycle is ribulose-1, 5-bisphosphate carboxylase/oxygenase (RuBisCO) encoded by cbbL and cbbM (C fixation functional key genes). The composition of C-fixing bacterial communities is typically influenced by factors such as land use practices and climatic conditions (Selesi et al., 2007; Yuan et al., 2012). Currently, there is no consensus on the impact of conservation tillage on microbial functional genes associated with soil C transformation (De Vries et al., 2015; Hao et al., 2019; Lu et al., 2019).

For soil N cycling process, the nifH gene is commonly employed to investigate N-fixing microorganisms (J. Hu et al., 2021) and the quantification of amoA-AOA and amoA-AOB genes serves as a means to characterize the nitrification process (Hayden et al., 2010; Keshri et al., 2015). Compared CT, NT increased the abundance of the nifH gene in the soil, which due to NT with crop residue provides C substrate for soil nitrogenase growth (Tang et al., 2021). Many studies indicate that NT can increase the abundance of both AOA amoA (Souza et al., 2013; Munroe et al., 2016), but a study showed that AOA amoA abundance was unaffected by tillage (Segal et al., 2017). Additionally, N fertilization significantly affects the community structure and abundance of N-fixing bacteria and nitrifying bacteria (Jorquera et al., 2014). The nifH, AOA-amoA gene abundances increased due to improved available N at low N rates but were suppressed by salt toxicity and acidification at high N rates (Ning et al., 2015). However, the specific impact of NT on the abundance of C and N functional genes under varying N additions, is still not well understood. Conceptually, the importance of pores as contributors to the cycling of soil C and N is well recognized, increased porosity was found to be associated with enhanced soil functional gene abundance (Du et al., 2023). However, there is still a deficiency in our current comprehension of the biophysical mechanisms underlying C and N cycling.

Therefore, the objective of this work was to investigate the effects of long-term conservation tillage on the soil pore structure, microbial co-occurrence patterns, as well as to reveal the regulation of the soil pore structure on microbial co-occurrence patterns and C and N cycling. It can also help identify microbial taxa that are important for maintaining healthy soil and inform soil management practices to optimize soil health and productivity. Soil samples from a long-term conservation tillage station were collected. The conservation tillage experiment consisted of two tillage treatments (CT and NT) and two N application rates (105 and 210 kg N ha–1). We hypothesized that i) Under long-term NT practice, N addition possibly influence on soil pore properties, affect the characteristics of community networks and decrease negative interactions between microbes. ii) High N addition under NT can decrease the pore size range of <30 μm, enhance copiotrophic actinomycetes, firmicutes, proteobacteria community. iii) pore morphology and distribution play important roles in maintaining network complexity and the keystone taxa.

2. Material and methods

2.1 Study site

We conducted a continuous field experiment from 2003 to 2019 at Shouyang Experimental Station (113.11°E, 37.97°N), Jinzhong City, Shanxi Province, Northern China.

2.2 Experimental design

The long-term experiment was conducted in 2003 using a randomized block design with three replicates. There were 12 plots, and each plot size was 25 m^2 (5 m by 5 m).

Two nitrogen fertilizer rates were applied under two tillage treatments in this study and the two tillage practices were NT (no-tillage with the maize straw mulching after harvesting, seeded with a no-till planter, N fertilizer was applied in small holes with 10 cm depth between two maize in each row, about 5 cm from the maize seed/maize plant in April) and CT (conventional tillage with maize straw removed, plowed twice to 0.25 m depth after harvesting and before seeding, respectively, and fertilized before plow in April) (Li et al., 2020). The two N fertilizer rates were 105 kg N ha^{-1} (N1) and 210 (N3) kg N ha^{-1} with urea.

2.3 Soil sampling

From depths of 0–10 cm and 10–25 cm on 1 August 2019, undisturbed soil samples were crushed by hand along the natural planes of weakness and collected. 1–2 mm soil samples (5 replicates per treatment) were obtained by gently crushing the soil columns manually, ensuring breakage along natural planes of weakness. The microorganisms, including bacteria and fungi, are believed to inhabit pores with a size ranging from 0 to 100 μm (Effmert et al.,2012). The pore sizes from 10 to 50 μm were described using the 1–2 mm samples as small sample volumes were less dominated by continuous and tube-like macropores, that is, root biopores. This size of aggregate (1–2 mm samples) was a compromise considering both the microbial community of the aggregate and the potential resolution in X-ray computed tomography scanning (Ananyeva et al., 2013). The sampling date corresponded to the tasselling stage. The fresh soil was divided into several samples. During

transport to the laboratory, soil subsamples for microbiological analysis were put in a refrigerator at -80 °C before further analyses. Other soil samples were stored in a 4°C incubator. Afterward, the aggregates were air-dried and X-rayed within one month after sampling.

2.4. Soil structure analysis by 3D-imaging

2.4.1. X-ray tomography

Aggregate samples were scanned using skyscan-1172 high-resolution desktop micro-computed tomography (Belgium) within the Chemical Engineering research unit at the University of Liege. The scanner was set at 100 kV/100 μA. An aluminum/copper (0.5 mm/0.04 mm) filter was positioned between the aggregate and the detector to minimize beam hardening artifacts. 2D image slices were reconstructed after a 180º rotation at 0.2ºangular incremental steps and combined into a 3D image consisting of 1440 slices, the pixel size being 4.91 μm. Five 1-2 mm aggregates were selected from each soil for the micro-computed tomography study. The image slices were pre-processed to remove the ring artifact and compensate for misalignment, and then constructed using NRecon software (Skyscan, Belgium). After reconstruction, the greyscale value ranged from 0 to 255, corresponding to the attenuation coefficient.

2.4.2. Image processing

We processed the image data using the Fiji distribution of the open-access software ImageJ (Schneider et al., 2012). Unless otherwise stated, default parameter settings were used for all processing steps.

Image preprocessing, segmentation, and quantification have previously been detailed in (Zhou et al., 2016) and are only briefly described here. For the aggregate-scale samples, a region of interest of 200 × 200 × 200 voxel3. A 3-D median filter was used to reduce noises before segmentation. Images were segmented by a method ostus (Vogel and Kretzschmar, 1996).

2.4.3 Aggregate Pore Architecture Analysis

Pore morphology parameters (three-dimensional fractal dimension, roundness, and connectivity) were determined using the "BongJ plugin" (Peng et al., 2023). The pore shape factor (F value) was calculated following Wadell (1932) and classified into three categories: regular ($F \geq 0.5$), irregular ($0.2 < F < 0.5$) and elongated ($F \leq 0.2$)(Zhou et al., 2012). The porosities of elongated, irregular and regular pores were denoted by Pelongated, Pirregular and Pregular, respectively. The 3D fractal dimension (FD) reflects the tortuosity or complexity of a geometrical shape. A larger FD indicates a more complex pore structure. The degree of anisotropy (DA) can be used to represent the porosity roundness, and when its value approaches 0, the pore shape is closer to a circle. Connectivity was calculated according to the connectivity density (CD) in the plugin, with a higher value representing a higher soil pore connectivity. The methodological details for image analysis of FD, DA, and CD have been described in a previous study (Doube et al., 2010).

Peng et al., (2023) The porosity and pore number were calculated with the '3d object counter' plugin in the ImageJ. Pores in this study were classified into three classes based on the equivalent diameter: ultramicropores (0-10 μm), micropores

(10-30 μm), mesopores (30-75 μm)(Liang et al., 2019), belong to storage pores, and storage pores are pores mostly help retain soil water and they do not drain water under gravity which then makes water available for plant roots and soil organisms (Sekaran et al., 2021).

2.4.4 Network construction and analyses

The co-occurrence network was obtained from 16S rRNA gene amplicon sequencing and ITS amplicon sequencing (same as Chapter 1 2.4.5). The co-occurrence network was constructed using the Molecular Ecological Network Analyses Pipeline (MENA, http://ieg2.ou.edu/ MENA/main.cgi) (Deng et al., 2012; Zheng et al., 2022). The network of fungi and bacteria was built separately for each of the treatments. Because the difference in microbial community composition between soil depths is small relative to the difference between treatments (Table S1), our network analysis combined the data from both soil depths in each treatment. This approach resulted in six replicates per treatment, i.e. the appropriate sample requirement for network analysis (Deng et al., 2012; Zheng et al., 2022). To improve network reliability, only OTUs that had average relative abundances > 0.01% (Zhang et al., 2022) were retained. Network topological properties such as average degree, geodesic distance, modularity and clustering coefficient were obtained; the use of these network topological properties can be found in (Deng et al., 2012). The average degree describes the average number of neighbors per node; geodesic distance is the shortest path length to connect any two nodes; modularity denotes the extent that a network is divided into modules; the clustering coefficient represents the extent to which neighbors of a node in a network tend to cluster together (Banerjee et al., 2016; Yuan et al., 2021). The topological role of each node was determined using the among-module connectivity (Pi) and the within-module connectivity (Zi) (Zhou et al., 2011). The threshold values of Pi and Zi for assorting network nodes are 0.62 and 2.5, respectively. Module hubs and connectors have been suggested to be as putative keystone taxa (Deng et al., 2012). Gephi (version 0.9.2) was used to visualize the co-occurrence network.

2.4.5 Quantification of cbbL, cbbM, nifH and amoA genes

Soil genomic DNA was extracted from 0.2 5 g of each homogenized frozen soil sample using the Extraction Kit (Tiangen Biochemical Technology Company, Beijing, China) following the manufacturer's instructions. For all samples, successful isolation of DNA was verified by 1.0% (w/v) agarose gel electrophoresis, and the purity and concentration of the extracted DNA was concurrently determined by the NanoDrop-2000 spectrophotometer (Thermo Scientific, USA). All DNA samples were stored in an ultra-low temperature freezer (-80°C) before molecular analysis.

The abundances of functional genes were determined using an ABI7500 Fluorescence Quantitative PCR system (Applied Biosystems, USA). Each sample is diluted 10-fold, and then the dilution DNA 2ul is taken as the reaction amount. The primer sets and thermal procedures used for real-time quantitative PCR are shown in Table 4-S2. Dilute the plasmid standard product by ten-fold the gradient from 101-105 and take 2ul for each gradient as a template to establish a standard curve. The fluorescent data were collected at the elongation step. To confirm the specificity of

amplification, a melting curve analysis was conducted at the end of each qPCR program. The amplification efficiencies of the target genes ranged from 82.0% to 93.0% with R2 values of 0.998 ~ 1.000.

3 Analysis

3.1 soil pore structure

We found that tillage and nitrogen treatments both affect soil pore properties (Table 4-1 and Fig. 4-1). Under low nitrogen level condition (N1), at 0-10 cm, the average pore values of Euler, Tb and Regular pore measured under CT were found to be higher than those under NT (Table 4-1). Conversely, the connectivity, porosity, irregular porosity, and elongated pores were higher under NT compared to CT. The impact of tillage on soil pore properties under N3 was found to be insignificant, with the exception of a decrease in elongated pores observed under NT compared to CT. In addition, at a depth of 10-25 cm, the tillage practices had minimal impact on these aforementioned indicators, with the exception of a decrease in Connectivity and Porosity observed under NT compared to CT.

Under CT, the average Euler, Tb and Regular pore values of N1 at 0-10 cm were found to be higher than those of N3 (Table 4-1). Conversely, the mean Connectivity, Irregular pore, and Elongated pore values of N1 were lower than those of N3. These findings suggest that increased nitrogen application in the topsoil layer leads to a reduction in pore diameter and regular pores under CT, while simultaneously promoting pore complexity and biological pores. Under NT, the average Porosity and Regular pore values of N1 at a depth of 0-10 cm were significantly greater than those of N3. These findings suggest that enhanced nitrogen application results in diminishing regular porosity and porosity under NT. Similar trends were observed at a depth of 10-25 cm.

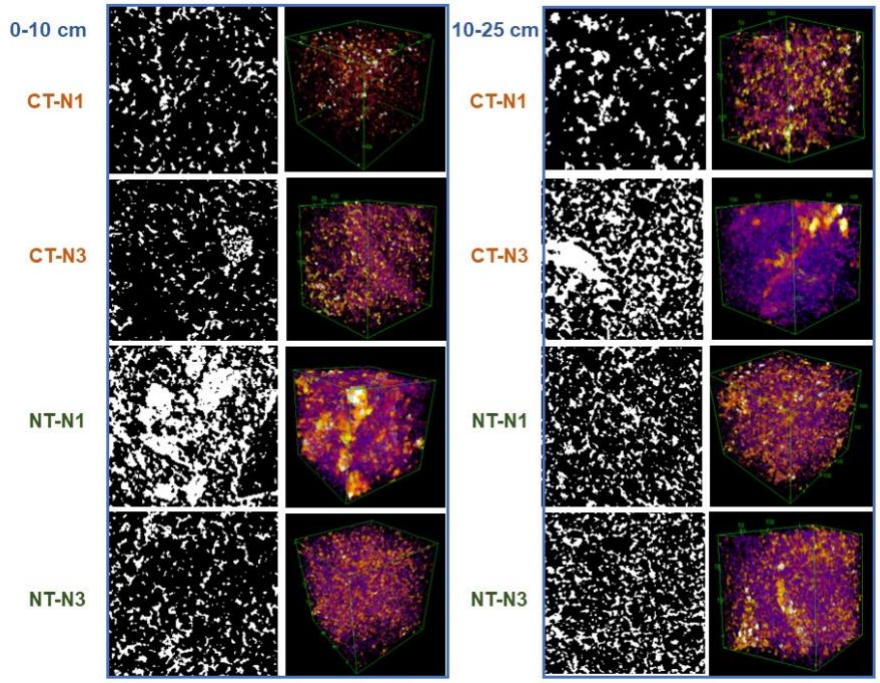

Fig. 4-1 Representative 2D visualization and 3D structures of soil aggregates from study soils. The white part of the rectangular body is the pore, and the black part is the soil matrix under image resolution (2D). The purple and yellow part of the rectangular body is the pore under image resolution (3D). N1, nitrogen addition at 105 kg N ha^{-1}; N3, nitrogen addition at 210 kg N ha^{-1}; CT, conventional tillage; NT, no tillage.

Table 4-1. Soil structure properties under long-term tillage and N application treatment. Capital letters indicate differences between two tillage treatments within the same nitrogen treatment; Different lowercase letters indicate significant differences ($p < 0.05$) among nitrogen treatments within the same tillage treatment. DA, degree of anisotropy; FD, fractal dimension; Tb.th, thickness. N1, nitrogen addition at 105 kg N ha^{-1}; N3, nitrogen addition at 210 kg N ha^{-1}; CT, conventional tillage; NT, no-tillage.

Treatment	Soil depth	Euler ch.	Connectivity	DA	FD	Porosity (%)
CT-N1	0-10 cm	-27187+5225 Aa	26531+5076 Bb	0.19+0.01 Aa	2.82+0.04 Aa	13.5+0.07 Ba
CT-N3	0-10 cm	-50308+2258 Ab	49856+2253Aa	0.17+0.01 Aa	2.81+0.01 Aa	14.1+0.02 Aa
NT-N1	0-10 cm	-63880+2434 Ba	64627+2391 Aa	0.19+0.02 Aa	2.86+0.03 Aa	35.1+0.07 Aa
NT-N3	0-10 cm	-57761+3169 Aa	57595+3193 Aa	0.18+0.01 Aa	2.86+0.04 Aa	12.6+0.04 Ab
CT-N1	10-25 cm	-34799+9946 Aa	34320+1003 Aa	0.2+0.01 Aa	2.82+0.01 Aa	7.69+0.02 Bb
CT-N3	10-25 cm	-48109+1197 Aa	48459+1215 Aa	0.22+0.02 Aa	2.84+0.03 Aa	37.9+0.04 Aa
NT-N1	10-25 cm	-38544+9392 Aa	38806+8954 Aa	0.2+0.01 Aa	2.84+0.07 Aa	28.1+0.01 Aa
NT-N3	10-25 cm	-38395+2510 Aa	38594+2458 Ba	0.17+0.01 Aa	2.82+0.03 Aa	20.5+0.03 Bb

Continue the table 4-1.

Treatment	Soil depth	Regular pore	Irregular pore	Elongated pore
CT-N1	0-10 cm	31.2+ 3.1Aa	26.4+ 1.8Aa	42.4+ 2.9Ab
CT-N3	0-10 cm	21.3+ 1.0Ab	21.8+ 2.1Ba	56.9+ 2.6Aa
NT-N1	0-10 cm	23.4+ 1.3Ba	29.8+ 3.2Aa	46.8+ 3.2Ab
NT-N3	0-10 cm	22.0+ 2.1Aa	28.3+ 2.3Aa	49.7+ 2.6Ba
CT-N1	10-25 cm	31.6+ 2.5Aa	17.0+ 1.2Aa	51.3+ 4.3Bb
CT-N3	10-25 cm	14.6+ 1.5Bb	18.6+ 1.1Aa	66.8+ 3.2Aa
NT-N1	10-25 cm	15.4+ 1.2Bb	15.8+ 2.1Aa	68.8+ 4.9Aa
NT-N3	10-25 cm	23.6+ 1.9Aa	20.2+ 1.9Aa	56.2+ 3.1Bb

3.2 Bacterial and fungal co-occurrence network and keystone taxa

The Bacterial and fungal networks for tillage and nitrogen application were constructed, and their topological characteristics were calculated to describe the structure of each network (Tables 4-2 and 4-3). More nodes, more links, and a higher average K mean a more complex network (Deng et al., 2012).

With these thresholds, the bacterial network sizes under no-tillage and nitrogen application were similar to the control group, but total links were significantly distinct (from 2780 to 12177 links) among the six networks (Table 4-2). Compared to CT, NT improved bacterial total links, Negative links, Positive links, Average degree, and Avg clustering coefficient (avgCC), and Density reduced bacterial Av g path length (GD) and Harmonic geodesic distance (HD), Modularity (no. of modules) under N1. Compared to CT, NT reduced bacterial links, Negative links, Positive links, Average degree, and avgCC and increased bacterial GD, HD, and Modularity (no. of modules) in N3 conditions (Fig. 4-2). Under CT, bacterial links, Negative links, Positive links, Average degree, avgCC, and Density increased with increasing N application rates while bacterial GD and HD, Modularity (no. of modules) decreased with increasing N application rates. Under NT, bacterial network properties were not affected by nitrogen level.

With these thresholds, the fungal network sizes under no-tillage and nitrogen application were similar to the control group but total links were significantly distinct (from 657 to 1333 links) among the six networks (Table 4-3). Compared to CT, NT increased fungal total nodes, links, Negative links, fungal Average degree, fungal avgCC, and decreased fungal GD under the same N application rates (Fig. 4-2). Compared to CT, NT increased fungal Positive links in N1 conditions and decreased fungal Positive links in N3 conditions. Fungal total nodes and fungal links, Positive links decreased with increasing N application rates under CT and NT treatment. Fungal negative links decreased with increasing N application rates under CT, fungal negative links increased with increasing N application rates under NT.

For bacterial networks, from the Zi-Pi plot (Fig. 4-3), we found 1 and 6 bacterial nodes sinking into “module hubs” and 0 and 13 nodes sinking into “connectors” under CT-N1 and CT-N3, respectively (Table 4-4). Members from Actinobacteria, Acidobacteria, Planctomycetes, Chloroflexi, Gemmatimonadetes, and Proteobacteria were identified as keystone bacterial taxa. We found 2 and 1 bacterial nodes sinking into “module hubs” and 3 and 1 nodes sinking into “connectors” under NT-N1 and NT-N3, respectively. Members from Bacteroidetes, Acidobacteria, Proteobacteria, Actinobacteria, and Planctomycetes were identified as keystone bacterial taxa.

For fungal networks, we classified 5 and 2 fungal nodes as “module hubs” and 2 and 4 nodes as “connectors” under CT-N1 and CT-N3, respectively (Fig. 4-3 and Table 4-5). Members from Ascomycota, Basidiomycota, Cercozoa, and Blastocladiomycota, were identified as keystone fungal taxa. We classified 0 and 0 fungal nodes as “module hubs” and 2 and 2 nodes as “connectors” under NT-N1 and NT-N3. Members from Glomeromycota, Ascomycota, Chytridiomycota, and Basidiomycota were identified as keystone fungal taxa. Tables 4 and 5 further summarized more details of these hubs and connectors.

Table 4-2 Topological properties of soil bacterial co-occurrence networks under long-term tillage and N application treatment. N1, nitrogen addition at 105 kg N ha^{-1}; N2, nitrogen addition at 180 kg N ha^{-1}; N3, nitrogen addition at 210 kg N ha^{-1}; CT, conventional tillage; NT, no tillage.

Communities	CT-N1	CT-N3	NT-N1	NT-N3
Similarity threshold (st)	0.98	0.96	0.98	0.98
Total nodes	909	934	897	896
Total links	2780	6736	3906	4070
R^2 of power-law[b]	0.502	0.643	0.527	0.509
Average degree/connectivity (avgK)	6.117	14.424	8.709	9.085
Avg clustering coefficient (avgCC)	0.57	0.652	0.613	0.597
Av g path length (GD)	13.583	7.978	12.585	11.925
Geodesic efficiency (E)	0.119	0.185	0.122	0.127
Harmonic geodesic distance (HD)	8.414	5.392	8.174	7.904
Centralization of degree (CD)	0.014	0.065	0.027	0.028
Centralization of betweenness (CB)	0.102	0.089	0.239	0.12
Centralization of stress centrality (CS)	1.2E+11	9419.7	1.9E+14	9.2E+11
Centralization of eigenvector centrality (CE)	0.248	0.15	0.183	0.162
Density (D)	0.007	0.015	0.01	0.01
Reciprocity	1	1	1	1
Transitivity (Trans)	0.665	0.671	0.699	0.693
Connectedness (Con)	0.432	0.754	0.707	0.675
Efficiency	0.987	0.981	0.988	0.987
Hierarchy	0	0	0	0
Lubness	1	1	1	1
Modularity (no. of modules)	0.84 (86)	0.68 (45)	0.80 (59)	0.78 (67)
Negative links	1349	3342	1862	1830
Positive links	1431	3395	2045	2241

Table 4-3 Topological properties of soil fungal co-occurrence networks under long-term tillage and N application treatment. N1, nitrogen addition at 105 kg N ha^{-1}; N2, nitrogen addition at 180 kg N ha^{-1}; N3, nitrogen addition at 210 kg N ha^{-1}; CT, conventional tillage; NT, no tillage.

Communities	CT-N1	CT-N3	NT-N1	NT-N3
Similarity threshold (st)	0.98	0.98	0.99	0.99
Total nodes	505	376	527	446
Total links	1004	657	1333	1191
R^2 of power-law[b]	0.804	0.882	0.842	0.783
Average degree/connectivity (avgK)	3.976	3.495	5.059	5.341
Avg clustering coefficient (avgCC)	0.447	0.422	0.509	0.54
Av g path length (GD)	7.234	7.194	5.813	6.491
Geodesic efficiency (E)	0.179	0.192	0.226	0.215
Harmonic geodesic distance (HD)	5.582	5.214	4.416	4.646
Centralization of degree (CD)	0.03	0.025	0.03	0.038
Centralization of betweenness (CB)	0.118	0.1	0.034	0.065
Centralization of stress centrality (CS)	18.522	17.33	13.003	162.732
Centralization of eigenvector centrality (CE)	0.273	0.307	0.268	0.253
Density (D)	0.008	0.009	0.01	0.012
Reciprocity	1	1	1	1
Transitivity (Trans)	0.555	0.514	0.609	0.629
Connectedness (Con)	0.414	0.317	0.26	0.315
Efficiency	0.985	0.978	0.969	0.968
Hierarchy	0	0	0	0
Lubness	1	1	1	1
Modularity (no. of modules)	0.84 (67)	0.86 (56)	0.85 (75)	0.84 (64)
Negative links	532	376	803	952
Positive links	473	283	530	240

Table 4-4 The keystone taxa identified as module hubs and connectors in the soil bacterial networks. N1, nitrogen addition at 105 kg N ha^{-1}; N2, nitrogen addition at 180 kg N ha^{-1}; N3, nitrogen addition at 210 kg N ha^{-1}; CT, conventional tillage; NT, no-tillage.

Treatment	OTU	Role	Phylum	Order	Degree	Pi	Zi	Betweeness centrality	Clustering coefficient
CT-N1	OTU_1601	module nubs	Proteobacteria	Xanthomonadales	8	0.000	2.794	5285.0	0.393
CT-N3	OTU_318	module nubs	Proteobacteria	Xanthomonadales	9	0.000	2.776	5565.0	0.556
CT-N3	OTU_5635	module nubs	Chloroflexi	unidentified	71	0.000	3.925	12342.1	0.344
CT-N3	OTU_351	module nubs	Acidobacteria	unidentified	9	0.000	2.776	5565.0	0.556
CT-N3	OTU_6100	module nubs	Chloroflexi	Chloroflexales	8	0.000	2.577	3308.0	0.464
CT-N3	OTU_415	module nubs	Acidobacteria	unidentified	71	0.000	3.925	12342.1	0.344
CT-N3	OTU_5829	module nubs	Chloroflexi	unidentified	20	0.000	3.447	3678.1	0.368
NT-N1	OTU_7989	module nubs	Acidobacteria	Blastocatellales	15	0.000	2.539	13706.4	0.543
NT-N1	OTU_3587	module nubs	Planctomycetes	unidentified	15	0.000	2.539	13706.4	0.543
NT-N3	OTU_779	module nubs	Proteobacteria	Xanthomonadales	14	0.000	2.770	8676.0	0.473
CT-N3	OTU_347	connectors	Proteobacteria	Xanthomonadales	49	0.635	0.499	7477.1	0.565
CT-N3	OTU_400	connectors	Actinobacteria	Solirubrobacterales	21	0.621	-0.910	4649.4	0.490
CT-N3	OTU_6030	connectors	Chloroflexi	unidentified	75	0.644	1.194	6835.9	0.375
CT-N3	OTU_207	connectors	Acidobacteria	unidentified	60	0.626	0.673	5922.0	0.541
CT-N3	OTU_5411	connectors	Chloroflexi	Anaerolineales	60	0.626	0.673	5922.0	0.541
CT-N3	OTU_440	connectors	Acidobacteria	unidentified	60	0.626	0.673	5922.0	0.541
CT-N3	OTU_41	connectors	Gemmatimonadetes	Gemmatimonadales	32	0.646	-0.573	8740.9	0.474
CT-N3	OTU_984	connectors	Planctomycetes	unidentified	60	0.626	0.673	5922.0	0.541
CT-N3	OTU_5112	connectors	Chloroflexi	AKYG1722	49	0.635	0.499	7477.1	0.565
CT-N3	OTU_5302	connectors	Chloroflexi	unidentified	49	0.635	0.499	7477.1	0.565
CT-N3	OTU_5486	connectors	Actinobacteria	Acidimicrobiales	75	0.644	1.194	6835.9	0.375

Continue the table 4-4.

Treatment	OTU	Role	Phylum	Order	Degree	Pi	Zi	Betweeness centrality	Clustering coefficient
CT-N3	OTU_5766	connectors	Chloroflexi	Caldilineales	11	0.645	-0.523	26760.6	0.345
CT-N3	OTU_5315	connectors	Chloroflexi	JG30-KF-CM45	68	0.645	0.857	16327.4	0.360
NT-N1	OTU_840	connectors	Acidobacteria	Blastocatellales	8	0.656	-1.249	11089.5	0.321
NT-N1	OTU_4293	connectors	Bacteroidetes	Cytophagales	4	0.625	-1.342	4765.5	0.167
NT-N1	OTU_1341	connectors	Acidobacteria	unidentified	8	0.656	-1.249	11089.5	0.321
NT-N3	OTU_5250	connectors	Actinobacteria	Acidimicrobiales	4	0.625	-1.723	2799.8	0.167

Table 4-5 The keystone taxa identified as module hubs and connectors in the soil fungal networks. N1, nitrogen addition at 105 kg N $ha^{-1;}$ N2, nitrogen addition at 180 kg N ha^{-1}; N3, nitrogen addition at 210 kg N ha^{-1}; CT, conventional tillage; NT, no tillage.

Treatment	OTU	Role	Phylum	Order	Degree	Pi	Zi	Betweeness centrality	Clustering coefficient
CT-N1	OTU_1759	module nubs	Cercozoa	unidentified	6	0.000	2.660	1597.00	0.133
CT-N1	OTU_1947	module nubs	Basidiomycota	Filobasidiales	8	0.000	2.571	1750.14	0.321
CT-N1	OTU_20	module nubs	Blastocladiomycota	Blastocladiales	8	0.000	2.571	1750.14	0.321
CT-N1	OTU_2216	module nubs	unidentified	unidentified	5	0.000	2.773	1638.08	0.100
CT-N1	OTU_300	module nubs	Ascomycota	Onygenales	8	0.219	2.635	4854.52	0.071
CT-N3	OTU_1017	module nubs	#N/A	#N/A	6	0.000	3.032	1005.98	0.133
CT-N3	OTU_2121	module nubs	Ascomycota	Capnodiales	6	0.000	2.556	2175.21	0.200
CT-N1	OTU_1639	connectors	unidentified	unidentified	17	0.630	0.255	12645.76	0.221
CT-N1	OTU_1663	connectors	Mortierellomycota	Mortierellales	15	0.658	-0.232	15556.20	0.324
CT-N3	OTU_1021	connectors	unidentified	unidentified	5	0.640	-0.553	1900.11	0.200
CT-N3	OTU_1059	connectors	Ascomycota	Pleosporales	9	0.642	-0.336	7361.86	0.278

Continue the table 4-5.

Treatment	OTU	Role	Phylum	Order	Degree	Pi	Zi	Betweeness centrality	Clustering coefficient
CT-N3	OTU_1947	connectors	Basidiomycota	Filobasidiales	3	0.667	-1.095	1201.20	0.000
CT-N3	OTU_2034	connectors	Ascomycota	Hypocreales	10	0.620	-0.723	3455.98	0.267
NT-N1	OTU_2533	connectors	Basidiomycota	Tremellales	5	0.640	-1.209	3202.09	0.100
NT-N1	OTU_965	connectors	Glomeromycota	Glomerales	5	0.640	-0.744	2540.40	0.200
NT-N3	OTU_1146	connectors	Ascomycota	Xylariales	5	0.640	-1.184	2120.95	0.100
NT-N3	OTU_284	connectors	Basidiomycota	Agaricales	13	0.639	-0.321	3318.86	0.308

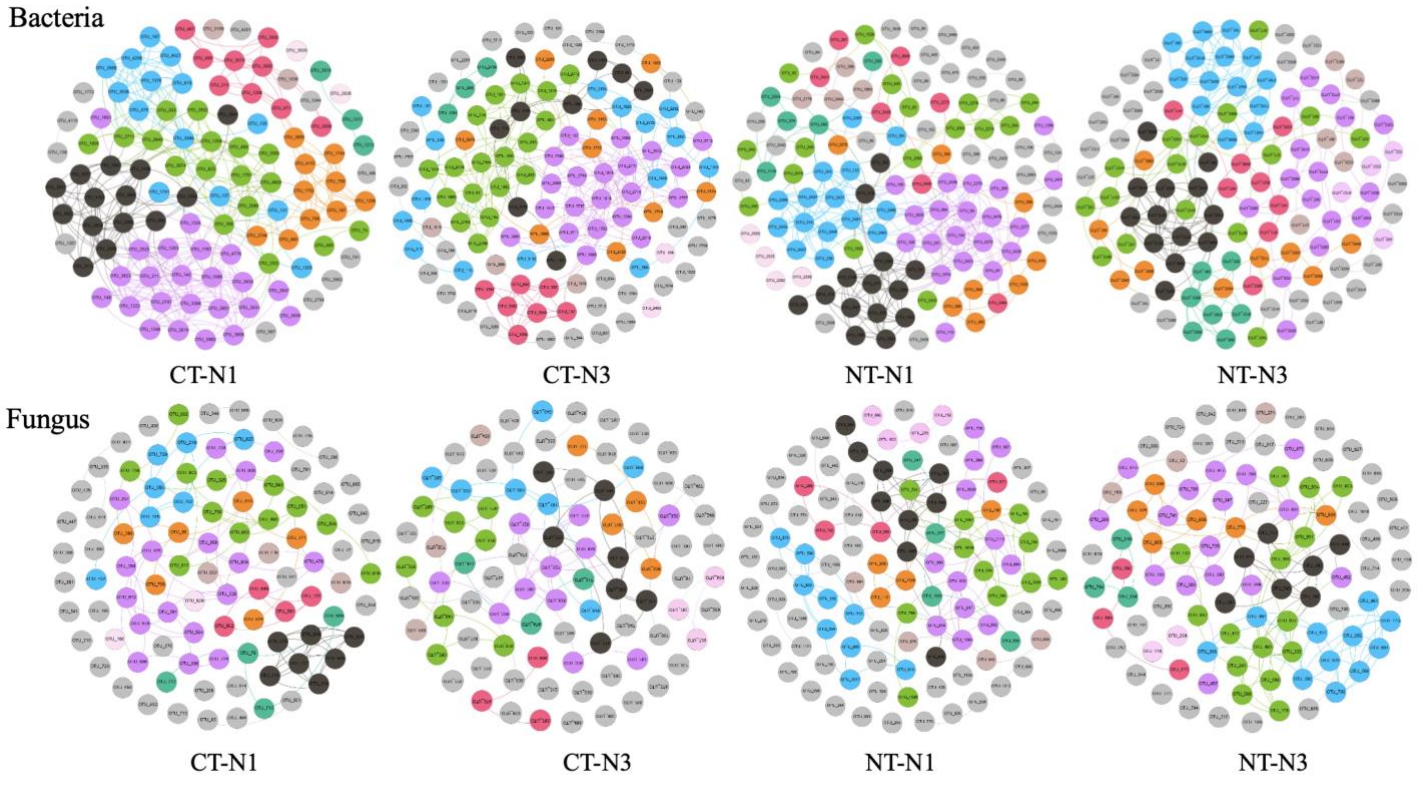

Fig. 4-2 Bacterial and fungal community co-occurrence patterns. N1, N application rates at 105 kg N ha^{-1}; N3, N application rates at 210 kg N ha^{-1}; CT, conventional tillage; NT, no-tillage.

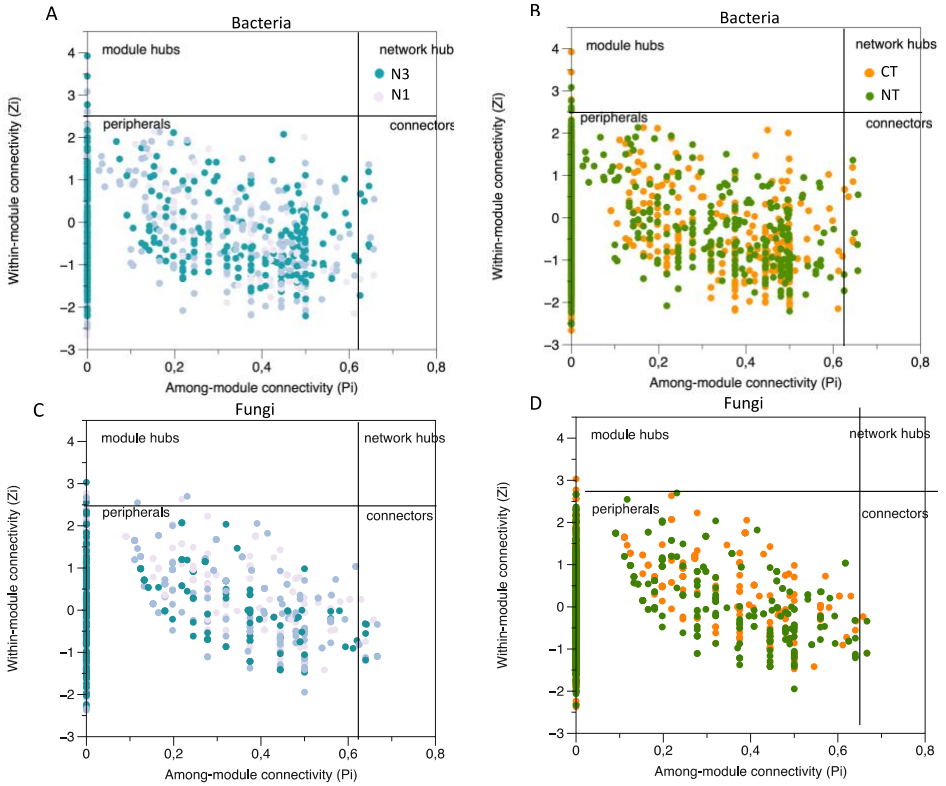

Fig. 4-3 Zi-Pi plots showing the distribution of OTUs based on their topological roles in bacterial (A) and fungal (B) networks. The threshold values of Zi and Pi for categorizing OTUs were 2.5 and 0.62, respectively. Nodes are defined as peripherals (Pi ≤ 0.62, Zi ≤ 2.5), module hubs (Pi ≤0.62, Zi > 2.5), connectors (Pi > 0.62, Zi ≤ 2.5) and network hubs (Pi > 0.62, Zi > 2.5). N1, N application rates at 105 kg N ha^{-1}; N3, N application rates at 210 kg N ha^{-1}; CT, conventional tillage; NT, no-tillage.

3.3 Abundance of nifH, AOAamoA, cbbL, and cbbM genes

The abundance of the nifH, AOAamoA, cbbL, and cbbM genes differed across tillage practices and N application rates, but was not impacted by soil depth (Fig. 4-4). In 0-10 cm, compared with CT, the copy numbers of nifH (10.4-86.4%), AOAamoA (21.2-157.0%), and cbbM (43.4-76.4%) genes were significantly higher in NT. Under the same tillage treatment, compared to N1, N3 decreased the nifH (78.5-87.3%), AOAamoA (49.7-76.3%), and cbbM (38.3-49.8%) gene abundance. However, the copy numbers of cbbL (50.1-90.9%) genes were significantly lower in NT than those in CT. Under the same tillage treatment, compared to N1, N3 increased the cbbL (12589-73316%) gene abundance (Fig. 4-4). Similar trends were observed at a depth of 10-25 cm.

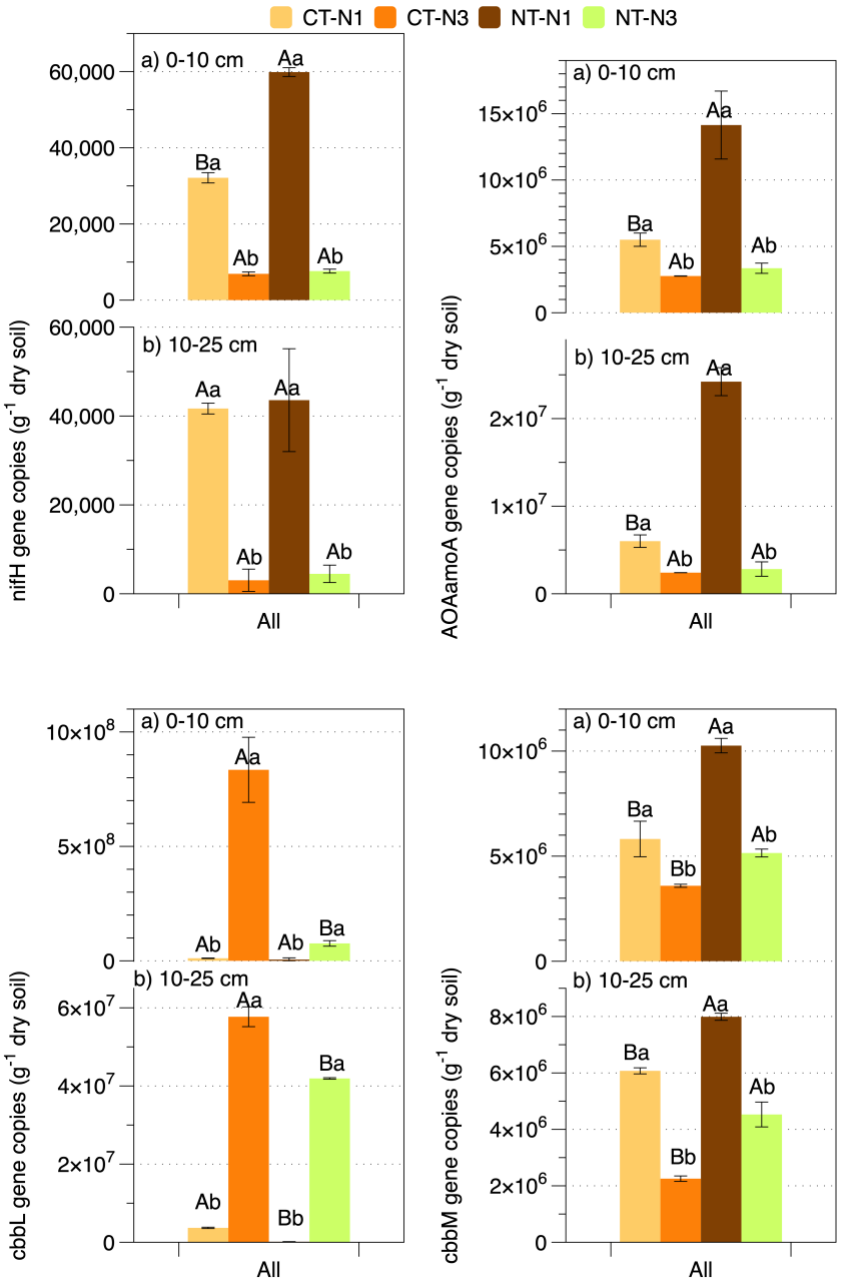

Fig. 4-4 Abundance of nifH, AOAamoA, cbbL, and cbbM genes under long-term tillage and N application treatment. Capital letters indicate differences between two tillage treatments within the same nitrogen treatment. N1, nitrogen addition at 105 kg N ha^{-1}; N3, nitrogen addition at 210 kg N ha^{-1}; CT, conventional tillage; NT, no tillage.

4 Discussion

4.1 The effect of long-term no-tillage and lower nitrogen application improved the microstructure of macroaggregates

We found that tillage and nitrogen both change soil pore properties. Under low nitrogen condition, we observed that pore connectivity was higher under NT than CT (Fig. 4-1 and Table 4-1), which indicated that NT can increase soil pore connectivity and porosity, and some studies have the same result (Schlüter et al., 2020; Li et al., 2021). This can be attributed primarily to the protective effect of NT on maize roots against disturbance caused by conventional tillage practices, which facilitates the formation of elongated and irregularly shaped pores (Table 4-1). Consequently, this improves both pore connectivity and overall porosity (Pagliai et al., 1984; Pires et al., 2022). An indirect reason is the accumulation of soil organic carbon in the topsoil under NT (Blanco-Canqui and Ruis, 2018), which leads to a higher soil structure stability and increase soil pore connectivity. In addition, under low nitrogen application rate, the total porosity and the percentage of 0-10 μm and 10-30 μm were higher under NT than CT (Table 4-1 and Fig. 4-S1), which indicated that under low nitrogen application rate, NT can increase soil total porosity and storage pore volume compared CT. The reason main may be long-term no-tillage may lead to compaction in the upper soil layers, resulting in a conversion of some macropores into micropores (Sasal et al., 2006). Some studies also reported that NT has a higher storage pore volume than CT at a soil depth of 10 cm (Burgos Hernández et al., 2019). Meta-analysis also showed small but significant reduction in total porosity, large decrease (19.5–32.3%) in macroporosity and moderate increase (4.4–7.3%) in microporosity were recorded under NT in comparison to the CT up to 20 cm depth (Mondal and Chakraborty, 2022).

We found that tillage had no significant effect on topsoil pore properties under N3. In our study, the soil C/N ratio was more than 10, N addition may somewhat mitigate the N limitation in soil microbes and increase microbial biomass. In the case of high nitrogen addition, the presence of increased organic matter in CT enhances soil structure stability (Zhang et al., 2022). Thus, nitrogen addition could counteract the adverse effects of CT on soil pore structure. Furthermore, nitrogen supplementation can augment nutrient availability for plant growth, stimulate plant growth and root development (Zhang et al., 2022), enhance mechanical forces within the soil matrix, and potentially mitigate the impacts of tillage measures. However, in 10-25 cm, under the condition of N3, it is observed that the porosity, percentage of 10-30 μm (Fig. 4-S1), and connectivity of NT are comparatively lower than those of CT. This is because the addition of high nitrogen stimulates the root length under CT than NT in 10-25 cm (Leghari et al., 2016). Moreover, in NT, N fertilizer is applied at a depth of 10 cm between two maize seeds (Zhang et al., 2022), potentially affecting the effectiveness of N fertilizer due to variations in fertilization depth. Consequently, the impact on root growth and development of plants within the 10-25 cm is considerably limited. Therefore, CT results in increased porosity within the 10-25 cm layer, exhibiting higher porosity and connectivity compared to long-term NT.

Soil pore connectivity, total porosity and the porosity of (0-10 μm and 10-30 μm) pores were higher for higher N application rates than lower N application rates under

CT, while increasing N application rates had opposite effect on soil pore properties under NT (Table 4-1). This phenomenon is mainly attributed to the different nitrogen requirements between NT and CT systems. Under long-term CT without straw return, soil N limitation may result in N competition between crops and microorganisms. Therefore, the promotion of maize root development under CT system is facilitated by higher nitrogen availability. As a result, long and irregular pores (Table 4-1) are more likely to form within root channels, thereby enhancing pore connectivity and overall porosity (Rasa et al., 2012).

4.2 The effect of long-term no-tillage and nitrogen application on bacterial and fungal co-occurrence network and key microbial communities

The cooccurrence patterns of microbial communities change under changing environmental conditions, we found that NT improved bacterial total links, Negative links, Positive links, Average degree, and avgCC, and Density under N1 (Table 4-2). This implies that under low N application rates, no-tillage with straw return can decrease the distance between nodes, increase the complexity of the bacterial and promote the stability of bacterial network (Hu et al., 2021). Similarly, NT increased fungal total nodes, links, Negative links, Average degree, and avgCC under the same N application rates compared to CT (Table 4-3). These results may be explained by the fact that i) following straw input, bacteria and fungi aggressively participate in the decomposition of organic material of crop straw (Delgado-Baquerizo et al., 2016; Zhao et al., 2016) and microorganisms use resources through interactions (Dang et al., 2015; Hu et al., 2021). ii) high soil moisture in no-tillage indirectly releases soil nutrient diffusion limitations for plant and microbial growth, altering substrate availability for bacterial and fungal communities (Hawkes et al., 2011; Sorensen et al., 2013; Zhang et al., 2018) and subsequently affecting soil bacterial and fungal community networks. However, NT reduced bacterial links, Negative links, Positive links, Average degree, and avgCC under N3 compared to CT. This implies that under high N application rates, no-tillage with straw mulching weakened the interactions between soil microorganisms. This may be owing to the decrease in competition and inhibition where both carbon and nitrogen are sufficient, i.e. eutrophic conditions (Bronstein, 1994; Cao et al., 2018). Moreover, the fungal negative links were higher under NT than CT, indicating a higher level of fungal competition under no-tillage. This result could be due to the high amount of straw returned to the field, which increased the C/N ratio and caused microbial competition for nitrogen (Cline et al., 2018).

Under CT, high N application rate raised bacterial links, Negative links, Positive links, Average degree, avgCC, and Density (Table 4-2), which is consistent with previous studies (Li et al., 2021). There are two reasons that can explain: i) high N application rates may have alleviated competition and favored many trophic levels. ii) High N application rates alleviate N limitation, improve crop growth, increase the abundance of labile and refractory organic compounds exuded by roots (Lin, 2018) and facilitate cooperation between microorganisms. Under NT, increasing N application rate have no impact on properties of soil bacterial co-occurrence networks. However, fungal total nodes and fungal links, and Positive links dropped with increasing N application rates under CT and NT treatment. This may be due to

fungi having lower nitrogen requirements than bacteria (Zechmeister-Boltenstern et al., 2015) and high N application rates inhibit fungal growth, which reduces association within fungal taxa. Moreover, fungal negative links increased with increasing N application rates under NT. Negative links can promote network stability (Coyte et al., 2015; Zhou et al., 2020). Our results illustrate that high nitrogen application rates promotes the establishment of stable fungal networks under NT system.

Keystone taxa confer greater biotic connectivity to the community and thus can be indicators of community shifts and compositional turnover (Berry and Widder, 2014). We found there are Actinobacteria, Acidobacteria, Planctomycetes, and Proteobacteria were identified as keystone bacterial taxa under CT and NT (Table 4-4). Only Bacteroidetes were identified as keystone bacterial taxa under NT. The reason is that Bacteroidetes are known to be involved in the degradation of complex organic matter, such as plant residues, into simpler compounds that can be utilized by other microorganisms in the soil (Larsbrink and McKee, 2020). NT typically has higher levels of surface crop residues, which can provide a source of carbon and nutrients for microorganisms, including Bacteroidetes. In contrast, CT involves the disruption of soil structure and no straw mulching, which can lead to a decrease in soil organic matter and a lower abundance of Bacteroidetes. Additionally, CT may select microorganisms such as Chloroflexi and Gemmatimonadetes that are better adapted to the disturbance and nutrient fluctuations associated with this management practice. Therefore, the presence or absence of Bacteroidetes may reflect differences in soil organic matter inputs and soil management practices between NT and CT systems. Only Chloroflexi and Gemmatimonadetes have been identified as keystone bacterial taxa under CT, this is because they have nitrogen-fixing function (West-Roberts et al., 2021), they can supplement the phenomenon of nitrogen deficiency in CT.

We also found that there are different keystone fungal taxa between CT and NT systems, only Ascomycota and Basidiomycota were all in CT and NT systems (Table 4-5). Cercozoa was only found in CT. Cercozoa, a category of soil protists, regulate bacterial decomposition, the flow of nutrients in soil and they dominated arid soils (Wu et al., 2022), so we found that Cercozoa were only found in CT, due to CT has less soil moisture than NT because it is not covered with straw. Only Glomeromycota and Chytridiomycota were identified in NT. Glomeromycota forms an arbuscular mycorrhizal symbiosis with the roots of most plants (Stürmer et al., 2018), enhancing nutrient absorption, particularly phosphorus while contributing to the carbon cycle by transferring carbon from the plant to the soil. They also create soil aggregates, improving soil structure under NT (Ji et al., 2019). The presence of Chytridiomycota showed a strong positive association with an average pore size within the range of <20 μm (Hanrahan-Tan et al., 2023). These organisms play important functional roles in decomposing organic matter, regulating biological populations, and potentially contributing to the resilience of soil communities at an ecosystem level (Hanrahan-Tan et al., 2023). The results indicated that Chytridiomycota was the dominant bacterial group in NT system.

Under low nitrogen levels, compared to CT, NT with straw return resulted in increased abundance of Acidobacteria, Planctomycetes, and Bacteroidetes (Table 4-

4). Acidobacteria and Bacteroidetes in agricultural soils have been associated with enhanced degradation of complex organic compounds (Ai et al., 2015; Wang et al., 2019; Larsbrink and McKee, 2020). Therefore, the adoption of NT with straw return provides a greater carbon source and promotes the growth of Acidobacteria and Bacteroidetes. Acidobacteria produces abundant extracellular polymeric substances (EPS) with a distinct sugar composition, which serve as a nutritional source for other microorganisms and support the growth and development of Planctomycetes (Costa et al., 2020), which aligns with our findings highlighting the key role of Acidobacteria and Planctomycetes in the microbial community. Despite the unknown function of Planctomycetes, they exhibit a diverse range of hydrolytic capabilities (Ivanova and Dedysh, 2012; Faria et al., 2018). Furthermore, (Ivanova et al., 2018) revealed distinct responses of various Planctomycetes groups to the induction of cellulose, xylan, pectin, and chitin, as evidenced by transcriptome analysis. Under conditions of high nitrogen, compared to CT, NT resulted in a reduction in the abundance of key soil bacterial and fungal groups, potentially attributed to the inhibitory effects of excessive nitrogen addition on the proliferation of dominant bacteria and fungi under no-tillage system (Zhang et al., 2022).

Under CT, with the increase of nitrogen application, nitrogen could be enough to supply microorganisms and crops, and the key bacterial groups Chloroflexi, Acidobacteria, Proteobacteria, Gemmatimonadetes, Planctomycetes were increased. Among them, Gemmatimonadetes prefers neutral pH to acidic pH (Mujakić et al., 2022). Consequently, under CT, the abundance of Gemmatimonadetes increased as nitrogen application rates were elevated. Under no-tillage conditions, the application of nitrogen resulted in a reduction of the key bacterial groups Acidobacteria and Bacteroidetes. This observation can be attributed to the oligotrophic nature of Acidobacteria (Guo et al., 2018), which is supported by other research findings. Furthermore, Acidobacteria has been found to exhibit a negative correlation with NO_3^-−N concentration (Luchibia et al., 2020). Our results align with recent studies that have highlighted the inhibitory effect of nitrogen application on Acidobacteria (Dai et al., 2018). Furthermore, although no-tillage straw can provide a substantial amount of carbon sources, it exerts a beneficial effect on the growth of Bacteroidetes, which possess cellulose-degrading capabilities (Cheng et al., 2023). However, in intensive agriculture settings, Bacteroidetes generally exhibit a pronounced negative response to excessive nitrogen fertilizer input (Isobe et al., 2019), aligning with our findings that the key bacterial group exhibited a decline concomitant with increasing nitrogen application rates under no-till conditions (Table 4-4).

4.3 Interactions among pore structure, soil key microbes, microbial community networks and function genes

4.3.1 The effect of pore structure on microbial community networks and soil key microbes

Our findings demonstrate that the irregular and elongated pore morphology increase pore connectivity and porosity (Fig. 4-S2 and Fig. 4-5), thereby promoting soil connectivity and permeability (Zhao et al., 2020). Moreover, this feature further increases the network connectivity of soil bacteria (Fig. 4-S3). The pore connectivity and porosity augment provide favorable physical conditions for aerobic bacteria

survival of Acidobacteria, Planctomycetes and Gemmatimonadetes (Table 4-4). Notably, our study aligns with previous evidence suggesting that a decrease in apertural network connections leads to an overall reduction in bacterial abundance due to heightened sensitivity toward carbon flux decline resulting from dispersed and zigzag diffusion pathways (Borer et al., 2018).

Moreover, the mycelia of fungi can extend into pores ranging from 0-10 μm in order to absorb nutrients (Wu et al., 2024). High nitrogen application led to an increase in the distribution of pores within the range of 0-10 μm (Fig. 4-S1), thereby weakening negative interactions between them under CT (Table 4-3). The results suggest that high nitrogen application can enhance the distribution of pores within the 0-10 μm range, thereby reducing competition among fungal communities and creating favorable conditions for the survival of fungal communities.

The decrease in porosity within the range of 10-30 μm resulted in a concomitant reduction in the network connectivity among soil bacteria and key groups of bacteria (Fig. 4-S5), such as the aerobic Acidobacteria group (Table 4-4). One plausible explanation for this observation is that diminished soil porosity leads to increased physical isolation between microorganisms, thereby limiting direct contact between them (Xia et al., 2022). Furthermore, a decrease in porosity within the specified range also impedes water flow, gas diffusion, and nutrient exchange (Neira et al., 2015), which may further constrain microbial associations.

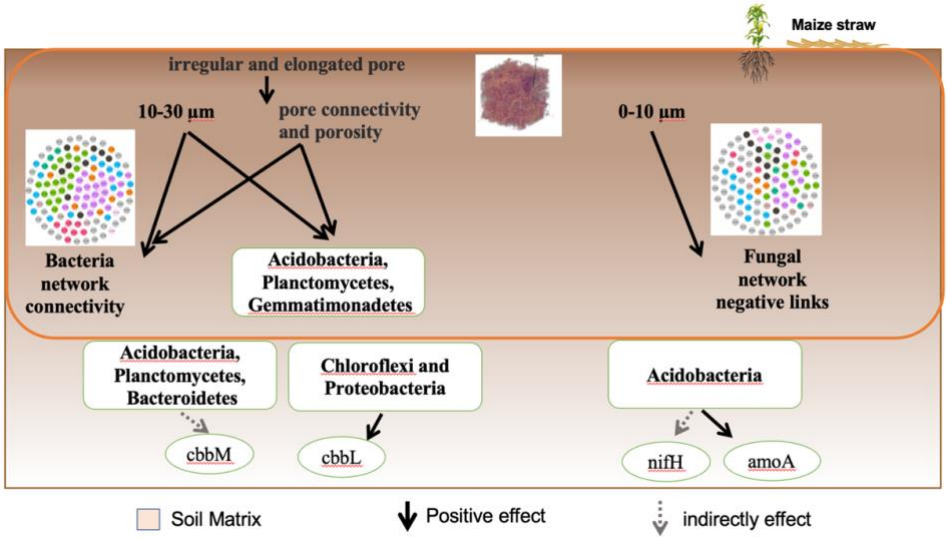

Fig. 4-5 Conceptual diagram illustrating the soil pore structure regulation of soil microbial co-occurrence patterns and the cycling of carbon and nitrogen. The augments of pore connectivity and porosity provides favorable conditions for aerobic bacteria such as Acidobacteria, Planctomycetes and Gemmatimonadetes. Reducing the distribution of (0-10 μm) pores reduced competition among fungal communities. The decrease in porosity and the the distribution of (10-30 μm) pores contributing to reduction in the key bacterial taxa and bacteria network connectivity. Key microorganisms like Chloroflexi and Proteobacteria directly enhance soil carbon sequestration potential through functional gene cbbL, while Acidobacteria indirectly or directly modulate nitrogen-fixing genes.

4.3.2 The effect of soil key microbes on function genes

4.3.2.1 Key microorganisms directly or indirectly influence carbon sequestration genes

Chloroflexi employ the Calvin cycle for CO2 fixation (Hanada, 2003), and Proteobacteria have emerged as the predominant phylum in carbon-fixing bacterial communities (Wang et al., 2021). Studies revealed that the majority of cbbL genes may be associated with unidentified microbial populations; however, these predominant cbbL gene sequences are more likely to be attributed to Proteobacteria (Li et al., 2019; Liu, 2016). According to our research findings, the increasing application of nitrogen under two tillage practice resulted in a significant augmentation of the key bacterial groups Chloroflexi and Proteobacteria (Table 4-4), while exhibiting a notable upward trend in the functional gene cbbL (Fig. 4-4). This indicated that high N application rates could improve soil C sequestration potential by increasing the C sequestration key microorganisms.

In addition, under conditions of high nitrogen availability, NT practices exhibit a significant reduction in the abundance of Proteobacteria bacteria and a decrease in cbbl content when compared to CT (Table 4-4 and Fig. 4-4). The reason why no-tillage reduces cbbL may be that conventional tillage results in a soil gas diffusion rate about four times that of no tillage, suggesting that cbbL-carrying bacteria are able to capture more carbon dioxide under conventional tillage system (Lu, 2019). Under low nitrogen conditions, the functional gene cbbL exhibited a higher expression level in NT compared to CT practices. The effect of no-tillage on soil C sequestration potential was regulated by N application rate. In addition, we found compared with CT, NT increased the cbbM genes although no-tillage have been shown to increase the abundance of key bacterial groups such as Acidobacteria, Planctomycetes, and Bacteroidetes (Ai et al., 2015; Faria et al., 2018; Larsbrink and McKee, 2020), it remains unclear whether these groups harbor cbbM genes based on experimental studies. However, by modulating microbial communities, these cbbM genes may potentially represent novel CO2-fixing groups that have not yet been successfully cultured (Li, 2020).

4.3.2.2 Nutrient abundance reduces nitrogen fixing genes

A nutrient-rich soil environment may induce microorganisms carrying the nifH gene to shift from photoautotrophy to mutualism with crops (Fu et al., 2022). Therefore, we observed a decrease in the functional gene nifH under no-tillage conditions with increasing nitrogen application. This may be because the abundance of key bacterial groups Acidobacteria and Bacteroidetes decreased with increasing nitrogen application rates under no-tillage (Table 4-4), leading to microbial structural adjustments that may indirectly impact the functional gene nifH.

Currently, there is limited empirical evidence supporting the nitrogen-fixing ability of Acidobacteria, and the presence of nifH gene within this phylum is infrequent (Kielak et al., 2016). However, our findings demonstrate that under no-tillage, an increase in nitrogen application leads to a reduction in Acidobacteria, a pivotal bacterial group, as well as the functional gene nifH. This decline may be attributed to the presence of the dinitase reductase-encoding gene (nifH) within the genome of a specific acid bacterium (Ward et al., 2009). In addition, Acidobacteria

is a bacterium involved in nitrification (Cheng et al., 2023). Our results also found that under no-tillage conditions, with the increase of nitrogen application rate, the key bacterial group Acidobacteria decreased, and the amoA functional gene, which represents the strength of nitrification ability, also decreased. Cheng et al., (2023) also showed that amoA abundance was positively correlated with the nitrification ability of soil microorganisms, which supported our view. Under CT, an increase in nitrogen application can sufficiently supply microorganisms (Chloroflexi and Proteobacteria) with nitrogen. However, despite the nitrogen-fixing capacity of Chloroflexi and Proteobacteria, which are key microorganisms (West-Roberts et al., 2021; Zhao et al., 2023), they did not enhance the expression of the nitrogen-fixing gene nifH.

Furthermore, under low nitrogen conditions, no-tillage resulted in increased abundance of the functional gene nifH compared to conventional tillage (Fig. 4-4). On one hand, the incorporation of no-till straw into the field may have stimulated soil nitrogen-fixing microorganisms' activity and subsequently enhanced soil nitrogenase growth due to increased availability of carbon substrate from crop residue (Tang et al., 2021). On the other hand, in the context of low nitrogen levels, incorporating straw into the soil results in an elevated soil carbon to nitrogen ratio, thereby diminishing nitrogen availability. Consequently, microorganisms compete with crops for nitrogen sources (Thierfelder et al., 2018) and thus enhancing the expression of genes responsible for atmospheric nitrogen reduction to bioavailable ammonium can potentially alleviate this limitation. Overall, our investigation revealed that key bacterial taxa the ability to directly or indirectly modulate nitrogen-fixing genes.

5. Conclusion

N application could alter the effects of tillage practices on soil pore properties microbial co-occurrence network and key microbial communities, and function gene. The irregular and elongated pore morphology enhances pore connectivity and porosity, further augments the network connectivity of soil bacteria. The pore connectivity and porosity augments provide favorable conditions for aerobic bacteria survival of Acidobacteria, Planctomycetes and Gemmatimonadetes. Reducing the distribution of pores within the range of 0-10 μm may reduce competition among fungal communities and creat favorable conditions for the survival of fungal communities. The decrease in porosity within the range of 10-30 μm resulted in a concomitant reduction in the network connectivity among soil bacteria and key bacterial taxa, such as the aerobic Acidobacteria group. Furthermore, High N application rates could improve soil C sequestration potential (functional gene cbbL) by increasing the C sequestration key microorganisms (Chloroflexi and Proteobacteria) under two tillage practice. Some key bacterial taxa can directly or indirectly modulate nitrogen-fixing genes under tillage systems. This research underscores the importance of N application to reveal the effect of tillage management on microbial co-occurrence network and key microbial communities, from the perspective of soil physical structure properties, which contributes to understanding the potential C and N sequestration benefits of increasing N addition under no-tillage.

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Chapter 5

General discussion and conclusion

1. General discussion

Globally, there is a rapid advancement in the adoption of conservation farming methods, which significantly contribute to preserving both plant productivity and environmental integrity (Chimsah et al., 2020; Farmaha et al., 2022; Sairam, 2023). However, the effects of conservation tillage on organic carbon sequestration are still debated (Li et al., 2021; Luo et al., 2010; Niu et al., 2019). Li et al., (2021) conducted a comprehensive analysis using data from 95 global studies and found that, within the depth range of 0 to 40 cm, NT exhibited significantly higher levels in each soil organic carbon fraction compared to CT. Conversely, the implementation of no-tillage practices led to an a decrease by 3.30±1.61 t ha^{-1} within the surface soil layer ranging from 20 to 40 cm deep. Niu et al., (2019) reported that the implementation of a 3-year no-tillage regime with residue removal did not result in a significant increase in carbon storage within the 0–30 cm soil profile. Overall, there was inconformity observed enhancement in total carbon storage below a depth of 40 cm as a result of adopting no-tillage. Nitrogen application and the duration were one of the main factors affecting the response ratio of soil organic carbon components (Li et al., 2021). Therefore, further investigation is warranted to explore the impact of nitrogen application on soil organic carbon and its components. This research is crucial for gaining a comprehensive understanding of the implications for long-term tillage management in terms of soil carbon sequestration.

1.1 The effect of no-tillage and nitrogen application on microbial properties

Soil C sequestration is affected by a variety of factors (Kravchenko and Guber, 2017; Six et al., 2000; Wu et al., 2024). Soil microbial communities play a pivotal role in the carbon cycle and are influenced by tillage practices and nitrogen application (Li et al., 2021; Yan et al., 2016). Our findings indicate that no-tillage significantly elevated the content of total phospholipid fatty acids (PLFA) and bacteria in the topsoil layer compared to conventional tillage, while the impact of nitrogen application rate on total PLFA and bacteria was not significant (Fig. 2-3). It is worth noting that there is a dearth of research examining the effects of tillage practices and N application rates on microbial communities within soil aggregates.

Our research revealed that tillage practices have a significant impact on the distribution of microorganisms within aggregates across various soil layers. Specifically, no-till practices were found to promote higher content of total microbial biomass in surface macroaggregates, as well as increased content of fungi (AMF) and bacteria (actinomycetes) within all aggregates (Table 3-S8). Conversely, conventional tillage practices were found to favor higher content of PLFAs and bacteria in all aggregates located in the deeper layers of the soil (Table 3-S10). Furthermore, higher nitrogen application rates resulted in reduced content of fungi, bacteria, and overall microbial biomass within all aggregates in the 0-10 cm soil layer under both two tillage systems (Table 3-S8).

Prior research has primarily examined the impacts of tillage and nitrogen application on microbial communities, but lacks in-depth investigation of the intricate interactions and diversity of bacteria and fungi. Our study revealed that NT treatment resulted in an increase in bacterial and fungal diversity within the 0-10 cm

layer when compared to CT treatment (Tables 2-S6 and 2-S7). Furthermore, the diversity of soil fungi and bacteria decreased with increasing N application rates at the 0-10 cm layer, which was higher in NT treatment than in CT treatment (Figs. 2-5 and 2-9). The application of a high rate of nitrogen in our study, specifically 210 kg N ha^{-1}, led to a decrease in microbial diversity. Furthermore, the levels of soil organic carbon (Li et al., 2010) and the C/N ratio (Fiorini et al., 2020) in CT soils were lower compared to NT soils, resulting in limitations on microbial carbon availability. Consequently, the impact of CT on microbial diversity was found to be less pronounced than that of NT. Our findings indicate the importance of considering nitrogen application in the study of how tillage management impacts microbial properties. Additionally, tillage practice influences the vertical distribution of soil microbial communities (Nunes et al., 2020). Specifically, we observed no variation in bacterial and fungal diversity between soil layers under CT (Figs. 2-5 and 2-6). However, fungal and bacterial diversity decreased as soil depth increased under NT. Furthermore, the rate of decline in fungal and bacterial diversity with soil depth was greater under the N1 treatment compared to the N2 and N3 treatments. Hence, it is imperative to include deep soil analysis in the investigation of bacterial and fungal diversity response to nitrogen application in no-tillage systems.

Soil microorganisms typically live together in complex systems that influence soil function (Banerjee et al., 2016). Changes in environmental conditions can alter the co-occurrence pattern of microbial communities, impacting the stability of microbial networks and C cycling processes (de Vries et al., 2018; Zheng et al., 2018). Our findings indicate NT increased fungal network complexity compared to conventional tillage under the same nitrogen application (Table 4-3), by improving soil moisture, nutrient diffusion, and substrate availability through the use of straw (Hawkes et al., 2011; Y. Zhang et al., 2018). In addition, the practice of no-tillage with straw returning increase bacterial network complexity and promote microorganism competition for nutrients under low N application (Table 3-2), but under high N application, no-tillage straw mulching reduces bacterial community interaction compared to conventional tillage due to reduced competition in eutrophic conditions (Bronstein, 1994; Cao et al., 2018). Furthermore, variations in the impact of nitrogen application on the resilience of bacterial networks were observed across two tillage practice. Conversely, the intricacy of fungal networks exhibited a decline with escalating nitrogen application levels under two tillage practice. This phenomenon could be attributed to the comparatively lower nitrogen demand of fungi in comparison to bacteria (Zechmeister-Boltenstern et al., 2015) and the suppression of fungal growth by elevated nitrogen application rates.

Furthermore, in addition to microbial community interactions, Keystone taxa are known to exert significant influence on the composition and function of the microbiome (Berry and Widder, 2014). Our study revealed distinct key fungal taxa present in two tillage systems. Specifically, Cercozoa and Blastocladiomycota were exclusively detected in the CT system(Fig. 4-5), while Glomeromycota and Chytridiomycota were only identified in the NT system. These differences can be attributed to variations in the survival strategies and environmental tolerances of the microorganisms. Furthermore, the impact of no-tillage on key bacterial taxa exhibited divergent patterns depending on the nitrogen application rates. Specifically,

under lower nitrogen application rates, NT led to a heightened presence of Acidobacteria, Planctomycetes, and Bacteroidetes. Acidobacteria and Bacteroidetes in comparison to CT (Fig. 4-4). Conversely, under higher nitrogen application rates, the implementation of NT resulted in a reduction in the prevalence of key bacterial and fungal taxa within the soil (Figs. 4-4 and 4-5), potentially attributable to the excessive nitrogen input hindering the proliferation and activity of microbial populations (Zhang et al., 2022). As nitrogen application rates increased, there was an increase in key bacteria taxa under CT (Fig. 4-4). However, excessive nitrogen led to a decrease in key taxa under NT with straw returning, as the practice of returning straw provided sufficient carbon and nitrogen nutrients. Our study highlights that under no-tillage conditions, a high application rate of nitrogen has a detrimental impact on key bacterial taxa. Our study revealed a decrease in the expression of the functional gene nifH with increasing nitrogen application rates under two tillage practices. This phenomenon may be attributed to shifts in key bacterial groups, resulting in alterations to the microbial structure that indirectly impact the expression of the functional gene nifH. These findings provide valuable insights for further research into the roles of unidentified microorganisms.

1.2 Interactions between microorganisms and soil structure

Agricultural practices have the potential to influence microbial properties through the disruption of soil structure, thereby impacting microbial properties directly and contributing to a feedback effect on SOC sequestration (Kravchenko and Guber, 2017; Six et al., 2000; Wu et al., 2024). Soil structure, defined as the spatial organization of solids and pores at various scales, is considered within the context of chemical heterogeneity in the solid phase. Our study offers a comprehensive examination of soil structure by considering both the solid phase and pore space dimensions, addressing limitations of prior single-focused investigations. Based on the soil aggregate formation mechanism (Six et al., 2000), aggregates are considered to represent three hierarchical organization stages of the soil solid phase, with each stage involving binders (Gupta and Germida, 2015). Our research indicates that the implementation of the long-term NT system resulted in an increase in the proportion of macro-aggregates and mean weight diameter while decreasing the percentage of micro-aggregates under the same nitrogen application rate (Fig. 3-1 and 3-2). Different N application rates had varying effects on aggregate composition in CT and NT systems. In CT conditions, increased N application led to higher root biomass and exudates, promoting organic matter exudation and macro-aggregate formation (Fig. 3-1). However, there was no significant impact of N application rate on aggregate distribution in long-term no-tillage. The significance of straw returning to field in aggregate formation outweighs the impact of nitrogen application rates, which may account for the observed phenomenon.

However, from the perspective of pores, soil structure is defined not by the shape, size, and spatial arrangement of original soil particles and aggregates, but by the amalgamation of different types of pores (Vogel et al., 2022). While previous research primarily concentrated on the impact of tillage practices on pore structure, this study delves deeper into the influence of nitrogen application rates on pore structure, specifically porosity and pore morphology, across two tillage practices. Our findings indicate that under conditions of low nitrogen application rates, no-

tillage practices enhance pore connectivity and porosity in comparison to conventional tillage (Table 4-1), attributed to the protective role of no-tillage on maize roots. Moreover, increasing nitrogen application under CT conditions can improve the porosity and connectivity of pores within aggregates (Table 4-1) by promoting root development in maize, leading to the formation of long and irregular root channels that enhance pore connectivity and overall porosity (Pagliai et al., 1984; Rasa et al., 2012). Variations in nitrogen application rates influence the impact of tillage practices on pore distribution in this study. Specifically, under low nitrogen application rates, no-tillage practices were found to significantly enhance pore distribution within the 10-30 μm range compared to conventional tillage (Fig. 4-S1), while a decrease was observed within the 0-10 μm range. Conversely, at high nitrogen application rates, no-tillage led to a decrease in pore distribution within the 10-30 μm range and an increase within the 0-10 μm range. These phenomena may be mainly attributed to the differential effects of different tillage methods (Liu et al., 2014; Mondal and Chakraborty, 2022) on straw input, root growth and development, and different nitrogen application rates (Averill and Waring, 2018; Q. Chen et al., 2020; Witzgall et al., 2021).

To the best of our knowledge, limited research has been conducted to directly assess the impact of soil pore structure on microbial properties through the measurement of pore characteristics. In recent times, X-ray computed tomography (μCT) has proven effective in capturing intricate 3D features of soil porosity without causing damage (Yu et al., 2018; Zhao et al., 2020). In this investigation, the direct measurement approach was employed to quantify soil aggregate pore structure. The findings of our study indicate that increased pore connectivity and porosity contribute to the improved network connectivity of soil bacteria and fungi. This is attributed to a reduction in physical spatial isolation and the creation of optimal physical environments for key microorganisms, specifically aerobic bacteria like Acidobacteria, Planctomycetes and Gemmatimonadetes (Borer et al., 2018). Furthermore, our research indicates that pore distribution plays a significant role in shaping co-occurrence patterns and key taxa of bacteria and fungi. As noted by (Wu et al., 2024), fungi hyphae have the capability to penetrate pores ranging from 0-10 μm to acquire nutrients. Our findings align with this point that a decrease in pore distribution between 0-10 μm can reduce key fungal taxa by limiting nutrient availability (Fig. 4-S1). Under the two tillage practices, high nitrogen application rates could enhance the pore distribution in the range of 0-10 μm, thus creating favorable conditions for the community stability of fungal communities (Fig. 4-S1). We also found that reducing pore distribution in the range of 10-30 μm resulted in a reduction of key soil bacterial taxa, specifically aerobic Acidobacteria, and a decrease in fungal network connectivity. This phenomenon can be attributed to the impaired water flow, gas diffusion, and nutrient exchange caused by decreased porosity within this range (Neira et al., 2015). Consequently, the diminished porosity within this range restricts the physical interaction among microorganisms, thereby limiting direct contact between them (Xia et al., 2022).

We investigated the influence of soil pore structure on microbial characteristics and reciprocally examined the impact of microorganisms on soil structure. Our research quantifies the relationship between microbial characteristics within

aggregates and aggregate stability under long-term no-tillage experiments with varying nitrogen application rates. Specially, our research revealed a significant increase in actinomycetes biomass within all aggregates under NT compared to CT practice (Fig. 3-4). Furthermore, a positive correlation was observed between actinomyces and mean weight diameter (Fig. 3-5). Therefore, the presence of abundant straw may attract straw-degrading actinomycetes, potentially enhancing aggregate stability. Furthermore, the extended exposure to CT treatment resulted in a notable augmentation of Desulfovibrio biomass within all aggregates when compared to NT (Fig. 3-4). Desulfovibrio play a crucial role in the conversion of sulfate present in soil aggregates into either hydrogen sulfide or sulfur (Voordouw, 1995). Notably, sulfate functions as an inorganic binding agent within soil (Totsche et al., 2018), and its reduction directly hampers aggregate formation under CT practice, consequently leading to a decline in soil aggregate stability (Fig. 3-5). For AMF, only at 0-10 cm did we observe a significant positive correlation between AMF and mean weight diameter (Fig. 3-5). Since AMFs cannot survive independently of host roots(Lehmann et al., 2017), they are primarily involved in co-metabolic processes with crop roots, which may indirectly contribute to the formation of soil aggregates (Fig. 3-6). Due to the legacy effects of soil microorganisms, this mechanism of microbial regulation of soil aggregate formation processes is frequently observed in soils under long-term agricultural management. Our research offers novel perspectives on quantifying the role of microorganisms in the formation of soil aggregates.

1.3 Microorganisms regulate soil organic carbon sequestration

Investigating the correlation between soil aggregate microstructure and microbial community is crucial for comprehending the mechanisms governing soil organic carbon sequestration under long-term no-tillage practices and nitrogen application. The Protection of SOC within aggregates is recognized as a primary factor limiting microbial access to SOC (Zhang et al., 2023). Our study revealed that the actinomycetes in 0-25 cm and AMF in 0-10 cm notably increase SOC content within both macro- and mega-croaggregates (Fig. 3-S5). Conversely, the presence of Desulfovibrio led to a significant reduction in SOC content within macro- and mega-croaggregates. In summary, our study shows that changes in microbial biomass in soil aggregates influence the sequestration of SOC by impacting aggregate stability (Fig. 3-6). This study offers a novel approach to understanding the role of microorganisms in the formation of soil aggregates and the regulation of SOC sequestration.

These key microorganisms of carbon sequestration bacteria such as Chloroflexi (Hanada, 2003) and Proteobacteria are conducive to soil carbon fixation (Wang et al., 2021). Previous research has indicated that the carbon-fixing cbbL gene sequences are predominantly associated with the Proteobacteria phylum (Yang Li et al., 2019; Liu, 2016), a notion that is also supported by our findings. We have discovered that the increasing application of nitrogen under CT significantly enhances the key bacterial taxa, namely Chloroflexi and Proteobacteria and increases the expression of functional gene cbbL (Fig. 4-4), which indicating that nitrogen application may effectively enhance carbon sequestration potential. In addition, under high N nitrogen application rate, the abundance of Proteobacteria and the cbbl content were

significantly lower in soils under NT compared to CT (Fig. 4-4). Under low nitrogen application rate, NT treatment showed higher expression of the functional gene cbbM. Overall, NT practices had varying effects on carbon sequestration potential depending on nitrogen application rates. Furthermore, while current research has demonstrated that NT can enhance the presence of important bacterial taxa such as Acidobacteria, Planctomycetes, and Bacteroidetes (Ai et al., 2015; Faria et al., 2018; Larsbrink and McKee, 2020), the presence of the cbbM gene within these populations has yet to be determined. Experimental studies have demonstrated that the cbbM gene signify a novel carbon dioxide fixation gene that has yet to be effectively cultured (Li et al., 2020). Our study highlights the impact of key microorganisms and their carbon sequestration genes on SOC sequestration under different tillage and nitrogen application conditions from a microbial perspective.

In addition to the contribution of various soil microbial communities to carbon sequestration, enhancing soil microbial carbon use efficiency (CUE) is also a viable strategy for promoting carbon sequestration (Bradford et al., 2013; Haddix et al., 2016). Previous research that solely examined the impact of microbial population and biomass on microbial CUE (Waldrop and Firestone, 2004) failed to fully acknowledge the significant role of microbial diversity in microbial CUE (Domeignoz-Horta et al., 2020). Based on current research, there is a notable absence of field experiments that directly illustrate the relationship between tillage management and nitrogen application on microbial CUE. Our analysis utilizing Partial Least Squares Path Modeling indicated that bacterial diversity, fungal diversity, and fungal community structure had a greater impact on enhancing microbial CUE compared to their biomass (Fig. 2-9, Figs. 2-S3 and 2-S4). In addition, we found differences in the effects of bacterial and fungal diversity on microbial CUE under the two tillage practices and that these relationships were modulated by N application (Figs. 2-9 and 2-S4), which suggests the importance of considering fungal and bacterial community diversity independently in predicting soil C cycling.

Particulate (POC) and mineral-associated organic matter carbon (MAOC) in SOC components exhibit higher sensitivity to microbial CUE than total SOC (Averill & Waring, 2018; Chen et al., 2018; Ye et al., 2018). POC plays an important role in stabilizing organic carbon in soil (Witzgall et al., 2021). Compared with POC, MAOC is less prone to mineralization due to more (physical or chemical) protection (Abramoff et al., 2018). Our findings indicate that increasing the application rate of nitrogen proved to be an effective strategy for enhancing POC and MAOC content through the stimulation of microbial CUE in both tillage systems. However, it was observed that microbial CUE led to a more pronounced increase in POC and MAOC levels under NT compared to CT due to the modulation of nitrogen addition (Fig. 2-S5). This discrepancy may be attributed to nitrogen deficiency in the NT system, as evidenced by the high carbon-to-nitrogen ratio of the straw utilized in this tillage practice, which in turn heightens microbial nitrogen demand (Thierfelder et al., 2018). Therefore, it can be inferred that a high N application rate is the most effective under NT for enhancing carbon sequestration.

1.4 Implications

We found that adequate nitrogen addition significantly enhanced is the most effective under NT for enhancing carbon sequestration. The increase of organic carbon improves soil fertility and affects crop productivity. Two meta-analyses found that adequate N addition significantly increased NT yield compared with CT (Li et al., 2024). Therefore, advocating suitable nitrogen application in NT system may be a favorable strategy to improve soil quality and crop productivity.

Farmers are willing to adopt no-tillage practices due to their potential for improving crop yield and soil fertility, as well as reducing labor requirements through the incorporation of straw in situ. However, they acknowledge certain drawbacks associated with this technology: (1) Long-term no-tillage may lead to compaction of surface soil particles and subsequent deterioration of its physical properties due to the absence of tillage and soil stirring. This issue can be mitigated by implementing regular deep plowing techniques; (2) Prolonged use of no-tillage practices can facilitate the occurrence and spread of pests and diseases, thereby negatively impacting crop yield and management costs. To address this concern, farmers can disrupt the transmission chain of pests and diseases by adopting crop rotation or intercropping strategies involving corn, soybean, or peanut cultivation.

1.5 Limitation

The microbiological CUE data is derived from Ecoenzymatic stoichiometry and not directly measured. Enzymatic measurement of CUE using O^{18} labels is necessary to further validate our conclusions.

To gain a deeper understanding of the microbial mechanisms involved in soil aggregate formation, there is a lack of data on stabilizing agents (oxides, hydroxides, calcium carbonates and sulfates) and transient binders (including polysaccharides and polycyclins).

Soil animals, as predators of soil microorganisms, also have a significant impact on soil structure; however, our study did not consider their influence, and future research should quantify the extent of their impact on both soil animals and microorganisms.

This experiment only utilized one test site's soil without conducting multi-point networking experiments; therefore, the universality of these results requires verification in other soil types and climate conditions. Additionally, conservation tillage's effect on deep soil organic carbon content remains controversial; we only studied up to 25 cm depth in this paper, so further investigation into deeper soils is necessary.

2. General conclusion and perspectives

Our results demonstrated significant impacts of nitrogen application rate under tillage management on soil microbial characteristics, including microbial biomass, microbial diversity, microbial community interactions, as well as key bacteria and fungi. Additionally, X-ray computed tomography was employed to investigate the influence of soil pore structure on the interaction of soil microbial communities. To address the existing knowledge gap regarding the response of microbial characteristics to N application rate in carbon sequestration under conservation

tillage practices, we characterized microbial CUE and functional genes related to carbon sequestration while evaluating the underlying mechanisms.

Our findings suggest that the responses of soil microbial characteristics to different rates of nitrogen application under conservation tillage can provide valuable insights into their impact on soil organic carbon dynamics. Bacterial and fungal diversity exerted a greater influence on soil microbial CUE than their biomass. Furthermore, an increase in soil microbial CUE was found to be associated with elevated content of POC and MAOC, which were also enhanced by nitrogen application. Subsequent analysis revealed that high rates of nitrogen application could enhance the potential for soil carbon sequestration by promoting key microorganisms involved in carbon fixation, such as Chloroflexida and Proteobacteria (functional gene cbbL). These results indicate that soil microbial biomass may not serve as an effective indicator for explaining the effects of different tillage practices on soil carbon sequestration under varying rates of nitrogen application. The biomass of actinomycetes and arbuscular mycorrhizal fungi directly or indirectly facilitated the accumulation of SOC within macro-aggregates within both CT and NT treatments. Conversely, Desulfovibrio reduced the content of SOC within macro-aggregate. Microbial processes exhibited stronger associations with large aggregates compared to microaggregates, suggesting that different-sized soil aggregates can significantly influence the capacity for storing organic carbon. To gain a comprehensive understanding regarding the relationship between microbes and carbon sequestration, as well as explore this relationship from a structural perspective, it is crucial to accurately determine the soil aggregates distribution and pore characteristics.

Soil aggregate distribution and soil porosity characteristics are important aspects for a comprehensive analysis of soil structure. Therefore, it is crucial to study the effects of N addition on them under tillage management. Compared with long-term conventional tillage, long-term no-tillage could increase the proportion of macro-aggregates and improve aggregate stability. Under no-tillage conditions, N application rate did not significantly affect aggregate distribution. In addition, no-tillage can improve pore connectivity and porosity compared to conventional tillage. Under no-tillage, increasing N application rate decreased porosity, pore connectivity and porosity of 0-10 μm and 10-30 μm. Therefore, in order to more accurately reveal the effect of N application rate on soil structure in conservation agriculture, both aggregate and pore distribution must be considered. We made it possible to study the effect of pore structure on microbial characteristics using X-ray computed tomography. The irregular elongated pore morphology enhanced pore connectivity and volume, and further promoted soil bacterial network connectivity. This enhancement is conducive to the support of environmental conditions for the survival and development of aerobic bacterial taxa. Reducing pores in the range of 0-10 μm could reduce the competition among fungal taxa and create favorable environmental conditions for the survival of fungal taxa. However, porosity reduction in the range of 10 to 30μm can lead to a decrease in the stability of soil bacterial network. Therefore, under no-tillage conditions, appropriate N application rate could reduce the competition among fungal taxa and increase the ecological stability of soil microbial communities. In addition to soil structure influencing microbial characteristics, we examined how microbial characteristics in turn affect

soil structure. Microbes modulate soil aggregate characteristics through diverse mechanisms in response to long-term agricultural management practices. The biomass of actinomycetes and arbuscular mycorrhizal fungi might participate in the formation of macro-aggregate through straw degradation. On the contrary, the biomass of Desulfovibrio might inhibit the formation of large particle assemblages. The combination of no-tillage and high nitrogen application improved the stability of soil aggregates and the storage of SOC within aggregates by altering aggregate formation by microbial communities.

Although increasing nitrogen application rate reduced pore connectivity, porosity, (0-10 μm) and (10-30 μm) porosity, bacterial interaction and microbial diversity under long-term conservation tillage. With the increases of N application rate, the contents of CUE, POC, MAOC and macro-aggregate organic carbon were increased, and carbon-fixing key microorganisms were increased to improve soil C sequestration potential under long-term conservation tillage. From this, we conclude that high N application rate is the most effective N application rate under conservation tillage in terms of soil C sequestration. Based on the results of this study, the following are suggested to better understand the potential benefits of conservation tillage:

(1) Evaluate the changes of soil microbial characteristics with increasing N application rate under conservation tillage.

(2) The responses of soil structure to N application rate under conservation tillage were analyzed from two aspects of soil structure.

(3) Reveal the effects of soil pore structure on soil microbial community interactions and key microorganisms. Investigate the effects of soil microorganisms on soil structure.

(4) Consider the effects of soil microbial characteristics on microbial CUE of soil microorganisms. The soil microbial CUE was affected by the changes of microbial community. The efficiency of different microorganisms on straw decomposition and fixation was not consistent. Therefore, when studying the effects of soil microbial characteristics on soil microbial CUE, it is not reasonable to only consider the changes in microbial biomass, and it is necessary to determine microbial diversity at the same time.

(5) Establish the relationship between soil microbial characteristics and soil carbon sequestration genes.

(6) Reveal the effects of microbial characteristics on C fixation combined with soil properties (soil pore structure, aggregate stability, and aggregate distribution). Our results showed that soil microbial characteristics decreased with increasing nitrogen application rates. Therefore, it is necessary to strengthen the research on the effects of N application rate management on microbial characteristics under conservation tillage because high N application rate could cause soil and groundwater pollution. Nitrogen application rate is another important factor affecting the adaptability of tillage methods. Therefore, understanding the effect of N application rate on soil C fixation can improve agricultural production and avoid some of the disadvantages caused by tillage and fertilization management.

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Supplementary information for Chapter II

Table 2-S1 *F*-values and *p*-values (in parentheses) for significance tests of the effects of tillage (T) and nitrogen (N) treatments on soil extracellular enzyme activities in farmland in North China. BG, β-glucosidase; NAG, N-acetyl-β-glucosaminidase; LAP, Leucyl aminopeptidase. **p* < 0.05; ***p* < 0.01; ****p* < 0.001.

			sampling	
Variable	Soil depth	Factor	F	P value
BG	0-10 cm	T	26276.7	(<0.001)***
		N	2570.6	(<0.001)***
		T×N	467.0	(<0.001)***
	10-25 cm	T	0.7	0.406
		N	522.7	(<0.001)***
		T×N	174.3	(<0.001)***
NAG	0-10 cm	T	809.2	(<0.001)***
		N	135.8	(<0.001)***
		T×N	209.0	(<0.001)***
	10-25 cm	T	22.4	(<0.001)***
		N	6.3	(<0.001)***
		T×N	26.1	(<0.001)***
LAP	0-10 cm	T	770.8	(<0.001)***
		N	91.9	(<0.001)***
		T×N	67.9	(<0.001)***
	10-25 cm	T	1051.7	(<0.001)***
		N	304.3	(<0.01)**
		T×N	393.7	(<0.001)***

Table 2-S2 *T*-values and *p*-values (in parentheses) for significance tests of the effects of soil depths on soil extracellular enzyme activities in farmland in North China. BG, β-glucosidase; NAG, N-acetyl-β-glucosaminidase; LAP, Leucyl aminopeptidase. **p* < 0.05; ***p* < 0.01; ****p* < 0.001.

			sampling	
Variable	Treatment	Factor	T	P value
BG	CT	Depth	3.3	(0.004)**
	NT	Depth	10.5	(<0.001)***
NAG	CT	Depth	1.3	0.204
	NT	Depth	4.1	(0.001)**
LAP	CT	Depth	2.1	0.051
	NT	Depth	6.7	(<0.001)***

Table 2-S3 *F*-values and *p*-values (in parentheses) for significance tests of the effects of tillage (T) and nitrogen (N) treatments on soil microbial CUE in farmland in North China. CUE: The variations of carbon use efficiency; $EEA_{C:N}$: element-requiring enzymatic activity ratio, $TER_{C:N}$: threshold element ratio, and $S_{C:N}$: scalar index. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

			sampling	
Variable	Soil depth	Factor	F	P value
$EEA_{C:N}$	0-10 cm	T	14221.5	(<0.001)***
		N	522.3	(<0.001)***
		T×N	70.2	(<0.001)***
	10-25 cm	T	732.3	(<0.001)***
		N	1475.4	(<0.001)**
		T×N	235.1	(<0.001)***
$S_{C:N}$	0-10 cm	T	12.0	(0.002)**
		N	10.6	(0.005)**
		T×N	5.6	(0.019)*
	10-25 cm	T	14.2	(0.003)**
		N	91.4	(<0.001)***
		T×N	10.8	(0.002)**
$TER_{C:N}$	0-10 cm	T	11.7	(0.005)**
		N	172.5	(<0.001)***
		T×N	41.1	(<0.001)***
	10-25 cm	T	8.6	(0.021)**
		N	3.9	(0.049)**
		T×N	0.1	0.918
CUE	0-10 cm	T	25.5	(<0.001)***
		N	18.9	(<0.001)***
		T×N	3.8	0.052
	10-25 cm	T	0.4	0.495
		N	18.9	(<0.001)***
		T×N	0.3	0.745

Table 2-S4 *T*-values and *p*-values (in parentheses) for significance tests of the effects of soil depth on soil microbial CUE in farmland in North China. CUE: The variations of carbon use efficiency; $EEA_{C:N}$: element-requiring enzymatic activity ratio, $TER_{C:N}$: threshold element ratio, and $S_{C:N}$: scalar index. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

			sampling	
Variable	Treatment	Factor	T	P value
$EEA_{C:N}$	CT	Depth	3.7	(0.002)**
	NT	Depth	12.6	(<0.001)***
$S_{C:N}$	CT	Depth	-1.8	0.08
	NT	Depth	1.4	0.158
$TER_{C:N}$	CT	Depth	1.1	0.253
	NT	Depth	-2.3	(0.033)***
CUE	CT	Depth	-1.8	0.088
	NT	Depth	1.2	0.239

Table 2-S5 *F*-values and *p*-values (in parentheses) for significance tests of the effects of tillage (T) and nitrogen (N) treatments on soil PLFAs. **p* < 0.05; ***p* < 0.01; ****p* < 0.001.

			sampling	
Variable	Soil depth	Factor	F	P value
Total PLFAs	0-10 cm	T	85.0	(<0.001)***
		N	2.3	0.138
		T×N	2.3	0.139
	10-25 cm	T	5.1	(0.042)*
		N	0.5	0.584
		T×N	11.2	(0.002)**
Bacteria	0-10 cm	T	68.5	(<0.001)***
		N	0.1	0.901
		T×N	1.6	0.242
	10-25 cm	T	12.2	(0.004)**
		N	1.0	0.374
		T×N	28.9	(<0.001)***
Fungus	0-10 cm	T	53.1	(<0.001)***
		N	20.7	(<0.001)***
		T×N	8.9	(0.004)**
	10-25 cm	T	1.9	(0.192
		N	6.6	(0.012)**
		T×N	0.3	0.713
Actinomycetes	0-10 cm	T	68.4	(<0.001)***
		N	0.5	0.578
		T×N	0.5	0.604
	10-25 cm	T	5.9	(0.032)*
		N	3.9	(0.047)*
		T×N	13.9	(0.001)***
F:B	0-10 cm	T	1.5	0.237
		N	24.6	(<0.001)***
		T×N	18.1	(<0.001)***
	10-25 cm	T	0.1	0.872
		N	7.0	(0.01)**
		T×N	14.4	(0.001)***
G^+:G^-	0-10 cm	T	4.4	0.056
		N	0.9	0.399
		T×N	6.7	(0.011)*
	10-25 cm	T	0.1	0.691
		N	3.5	0.060
		T×N	1.9	0.181

Table 2-S6 *T*-values and *p*-values (in parentheses) for significance tests of the effects of soil depth on soil PLFAs in farmland in North China. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

			sampling	
Variable	Treatment	Factor	T	P value
Total PLFAs	CT	Depth	3.3	(0.004)*
	NT	Depth	9.7	(<0.001)***
Bacteria	CT	Depth	2.7	(0.015)*
	NT	Depth	8.6	(<0.001)***
Fungus	CT	Depth	5.8	(<0.001)***
	NT	Depth	5.3	(<0.001)***
Actinomycetes	CT	Depth	4.0	(0.001)***
	NT	Depth	9.1	(<0.001)***
F:B	CT	Depth	5.5	(<0.001)***
	NT	Depth	2.0	0.055
G^+:G^-	CT	Depth	-1.6	0.115
	NT	Depth	0.01	0.998

Table 2-S7 *F*-values and *p*-values (in parentheses) for significance tests of the effects of tillage (T) and nitrogen (N) treatments on soil five phyla of bacteria. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

			sampling	
Variable	Soil depth	Factor	F	P value
Acidobacteria	0-10 cm	T	69.5	(<0.001)***
		N	20.7	(<0.001)***
		T×N	4.9	(0.028)*
	10-25 cm	T	85.3	(<0.001)***
		N	82.9	(<0.001)***
		T×N	101.7	(<0.001)***
Proteobacteria	0-10 cm	T	0.3	0.556
		N	9.1	(0.004)**
		T×N	10.5	(0.002)**
	10-25 cm	T	14.3	(0.003)**
		N	2.9	0.092
		T×N	38.7	(<0.001)***
Actinobacteria	0-10 cm	T	0.4	0.521
		N	8.6	(0.005)**
		T×N	6.9	(0.01)**
	10-25 cm	T	85.5	(<0.001)***
		N	108.1	(<0.001)***
		T×N	69.1	(<0.001)***
Chloroflexi	0-10 cm	T	48.7	(<0.001)***
		N	75.0	(<0.001)***
		T×N	65.4	(<0.001)***
	10-25 cm	T	23.8	(<0.001)***
		N	45.7	(<0.001)***
		T×N	2.3	0.139
Gemmatimonadetes	0-10 cm	T	1.4	0.249
		N	2.3	0.135
		T×N	4.2	(0.04)*
	10-25 cm	T	0.1	0.729
		N	25.6	(<0.001)***
		T×N	10.8	(0.002)**
Bacteroidetes	0-10 cm	T	238.7	(<0.001)***
		N	33.1	(<0.001)***
		T×N	88.4	(<0.001)***
	10-25 cm	T	612.1	(<0.001)***
		N	77.6	(<0.001)***
		T×N	10.0	(<0.001)***

Table 2-S8 *T*-values and *p*-values (in parentheses) for significance tests of the effects of soil depth on soil five phyla of bacteria in farmland in North China. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

			sampling	
Variable	Treatment	Factor	T	P value
Acidobacteria	CT	Depth	2.2	(0.041)*
	NT	Depth	3.5	(0.003)***
Proteobacteria	CT	Depth	-3.9	(0.001)***
	NT	Depth	-2.7	(0.015)*
Actinobacteria	CT	Depth	-0.8	0.39
	NT	Depth	2.9	(0.01)**
Chloroflexi	CT	Depth	3.5	(0.002)**
	NT	Depth	2.5	(0.02)*
Gemmatimonadetes	CT	Depth	1.4	0.17
	NT	Depth	0.1	0.894
Bacteroidetes	CT	Depth	-3.6	(0.002)**
	NT	Depth	-0.5	0.562

Table 2-S9 *F*-values and *p*-values (in parentheses) for significance tests of the effects of tillage (T) and nitrogen (N) treatments on soil five phyla of eumycota. *$p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

			sampling	
Variable	Soil depth	Factor	F	P value
Ascomycota	0-10 cm	T	0.2	0.627
		N	1.1	0.359
		T×N	0.8	0.462
	10-25 cm	T	4.4	0.056
		N	0.1	0.941
		T×N	0.9	0.427
Mortierellomycota	0-10 cm	T	0.1	0.742
		N	0.2	0.812
		T×N	0.1	0.844
	10-25 cm	T	2.0	0.174
		N	0.2	0.766
		T×N	0.2	0.811
Basidiomycota	0-10 cm	T	11.2	(0.006)**
		N	0.1	0.885
		T×N	0.03	0.962
	10-25 cm	T	0.2	0.608
		N	1.9	0.189
		T×N	0.2	0.787
Chytridiomycota	0-10 cm	T	0.05	0.816
		N	0.2	0.820
		T×N	0.05	0.948
	10-25 cm	T	0.8	0.362
		N	2.3	0.142
		T×N	2.6	0.115
Glomeromycota	0-10 cm	T	9.0	(0.011)*
		N	5.1	(0.024)*
		T×N	3.1	0.080
	10-25 cm	T	0.5	0.49
		N	2.3	0.13
		T×N	0.2	0.75

Table 2-S10 *T*-values and *p*-values (in parentheses) for significance tests of the effects of soil depth on soil five phyla of eumycota in farmland in North China. **p* < 0.05; ***p* < 0.01; ****p* < 0.001.

			sampling	
Variable	Treatment	Factor	T	P value
Ascomycota	CT	Depth	2.4	(0.026)*
	NT	Depth	0.7	0.472
Mortierellomycota	CT	Depth	-2.2	(0.035)*
	NT	Depth	-1.0	0.327
Basidiomycota	CT	Depth	-6.9	(<0.001)***
	NT	Depth	0.01	0.986
Chytridiomycota	CT	Depth	1.3	0.184
	NT	Depth	2.4	(0.024)*
Glomeromycota	CT	Depth	-5.9	(<0.001)***
	NT	Depth	-2.8	(0.011)*

Table 2-S11 *F*-values and *p*-values (in parentheses) for significance tests of the effects of tillage (T) and nitrogen (N) treatments on soil bacterial diversity. **p* < 0.05; ***p* < 0.01; ****p* < 0.001.

			sampling	
Variable	Soil depth	Factor	F	P value
Chao1	0-10 cm	T	45.4	(<0.001)***
		N	3.1	0.083
		T×N	0.5	0.571
	10-25 cm	T	2.9	0.112
		N	1.0	0.382
		T×N	3.0	0.084
Observed sequences	0-10 cm	T	54.7	(<0.001)***
		N	3.7	0.054
		T×N	0.2	0.765
	10-25 cm	T	1.8	0.195
		N	0.4	0.626
		T×N	4.1	(0.044)*
PD-whole-tree	0-10 cm	T	54.2	(<0.001)***
		N	2.8	0.096
		T×N	0.1	0.909
	10-25 cm	T	14.6	(0.002)**
		N	0.7	0.487
		T×N	5.4	(0.021)**
Shannon	0-10 cm	T	23.4	(<0.001)***
		N	3.7	0.055
		T×N	0.3	0.727
	10-25 cm	T	0.6	0.461
		N	4.2	(0.041)*
		T×N	2.9	0.094

Table 2-S12 *T*-values and *p*-values (in parentheses) for significance tests of the effects of soil depth on soil bacterial diversity in farmland in North China. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Variable	Treatment	Factor	sampling	
			T	P value
Chao1	CT	Depth	2.0	0.061
	NT	Depth	10.4	(<0.001)***
Observed sequences	CT	Depth	1.7	0.099
	NT	Depth	8.1	(<0.001)***
PD-whole-tree	CT	Depth	2.7	(0.014)*
	NT	Depth	7.2	(<0.001)***
Shannon	CT	Depth	1.4	0.155
	NT	Depth	4.1	(0.001)***

Table 2-S13 *F*-values and *p*-values (in parentheses) for significance tests of the effects of tillage (T) and nitrogen (N) treatments on soil fungal diversity. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Variable	Soil depth	Factor	sampling	
			F	P value
Chao1	0-10 cm	T	146.0	(<0.001)***
		N	33.6	(<0.001)***
		T×N	15.6	(<0.001)***
	10-25 cm	T	0.5	0.813
		N	5.1	(0.024)*
		T×N	0.1	0.948
Observed sequences	0-10 cm	T	223.9	(<0.001)***
		N	31.8	(<0.001)***
		T×N	14.0	(0.001)***
	10-25 cm	T	0.2	0.616
		N	2.0	0.172
		T×N	0.243	0.788
PD-whole-tree	0-10 cm	T	125.8	(<0.001)***
		N	18.7	(<0.001)***
		T×N	7.1	(0.009)**
	10-25 cm	T	0.1	0.785
		N	1.4	0.283
		T×N	0.7	0.487
Shannon	0-10 cm	T	4.8	(0.048)**
		N	6.0	(0.015)*
		T×N	0.1	0.855
	10-25 cm	T	0.02	0.875
		N	0.8	0.438
		T×N	0.1	0.865

Table 2-S14 *T*-values and *p*-values (in parentheses) for significance tests of the effects of soil depth on soil fungal diversity in farmland in North China. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

			sampling	
Variable	Treatment	Factor	T	P value
Chao1	CT	Depth	-0.8	0.392
	NT	Depth	4.3	(<0.001)***
Observed sequences	CT	Depth	-1.1	0.273
	NT	Depth	4.1	(0.001)***
PD-whole-tree	CT	Depth	-0.3	0.762
	NT	Depth	5.5	(<0.001)***
Shannon	CT	Depth	-1.5	0.133
	NT	Depth	0.6	0.536

Table 2-S15 *F*-values and *p*-values (in parentheses) for significance tests of the effects of tillage (T) and nitrogen (N) treatments on soil POC and MAOC *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

			sampling	
Variable	Soil depth	Factor	F	P value
POC	0-10 cm	T	2933.5	(<0.001)***
		N	1415.1	(<0.001)***
		T×N	133.4	(<0.001)***
	10-25 cm	T	3612.5	(<0.001)***
		N	1276.6	(<0.001)***
		T×N	289.6	(<0.001)***
MAOC	0-10 cm	T	335.4	(<0.001)***
		N	218.3	(<0.001)***
		T×N	37.3	(<0.001)***
	10-25 cm	T	28.1	(<0.001)***
		N	50.0	(<0.001)***
		T×N	6.0	(<0.001)***

Table 2-S16 *T*-values and *p*-values (in parentheses) for significance tests of the effects of soil depth on soil POC and MAOC in farmland in North China. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

			sampling	
Variable	Treatment	Factor	T	P value
POC	CT	Depth	4.1	(0.001)***
	NT	Depth	1.2	0.235
MAOC	CT	Depth	3.2	(0.005)**
	NT	Depth	4.3	(0.001)***

Table 2-S17 Differences in soil temperature among tillage (T) and nitrogen (N). Capital letters indicate differences between two tillage treatments within the same nitrogen treatment. N1, nitrogen addition at 105 kg N ha^{-1}; N2, nitrogen addition at 180 kg N ha^{-1}; N3, nitrogen addition at 210 kg N ha^{-1}; CT, conventional tillage; NT, no tillage.

Soil depth	treatment	Soil temperature
0-10 cm	CT-N1	21.2±0.2A
	CT-N2	21.3±0.1A
	CT-N3	21.0±0.2A
	NT-N1	19.9±0.3B
	NT-N2	20.5±0.2B
	NT-N3	20.6±0.4B
10-25 cm	CT-N1	21.9±0.1A
	CT-N2	21.9±0.2A
	CT-N3	21.6±0.2A
	NT-N1	19.9±0.3B
	NT-N2	20.9±0.2B
	NT-N3	20.9±0.4B

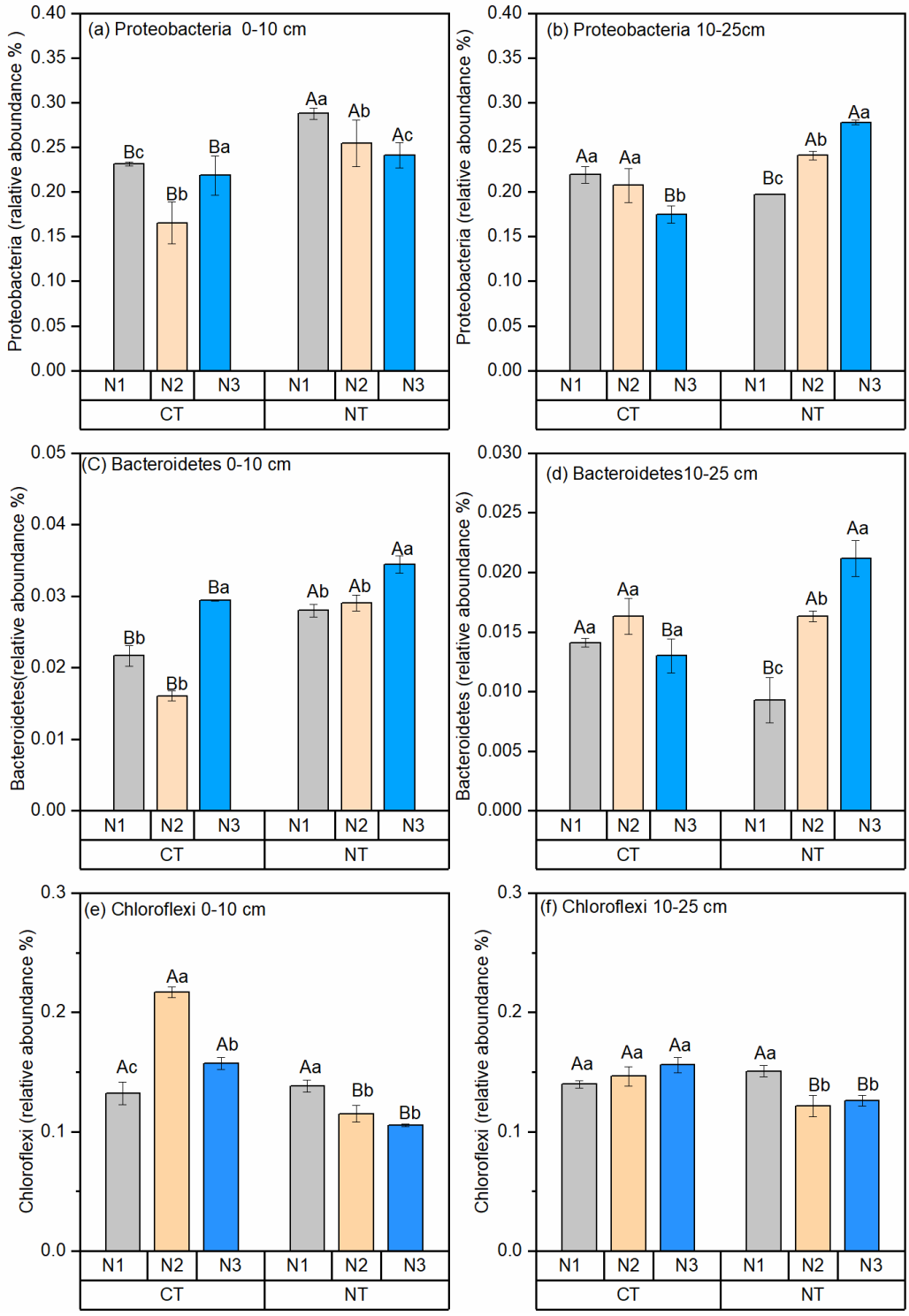

Fig. 2-S1 Differences in Proteobacteria and Bacteroidetes among tillage (T) and nitrogen (N). Vertical bars indicate standard error of means (n = 3). Capital letters indicate differences between two tillage treatments within the same nitrogen treatment; Different lowercase letters indicate significant differences ($p < 0.05$) among nitrogen treatments within the same tillage treatment. N1, nitrogen addition at 105 kg N ha^{-1}; N2, nitrogen addition at 180 kg N ha^{-1}; N3, nitrogen addition at 210 kg N ha^{-1}; CT, conventional tillage; NT, no tillage.

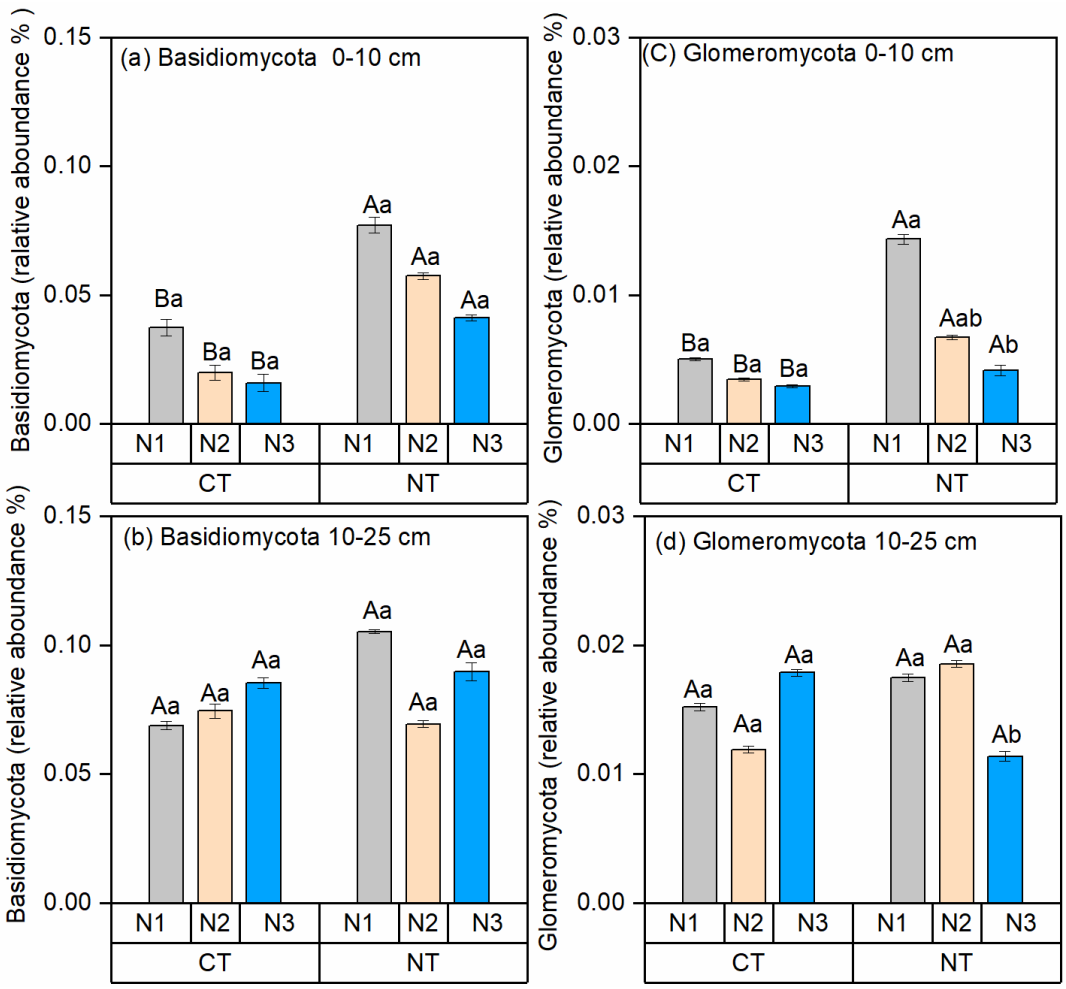

Fig. 2-S2 Differences in Basidiomycota and Glomeromycota among tillage (T) and nitrogen (N). Vertical bars indicate standard error of means (n = 3). Capital letters indicate differences between two tillage treatments within the same nitrogen treatment; Different lowercase letters indicate significant differences ($p < 0.05$) among nitrogen treatments within the same tillage treatment. N1, nitrogen addition at 105 kg N ha^{-1}; N2, nitrogen addition at 180 kg N ha^{-1}; N3, nitrogen addition at 210 kg N ha^{-1}; CT, conventional tillage; NT, no tillage.

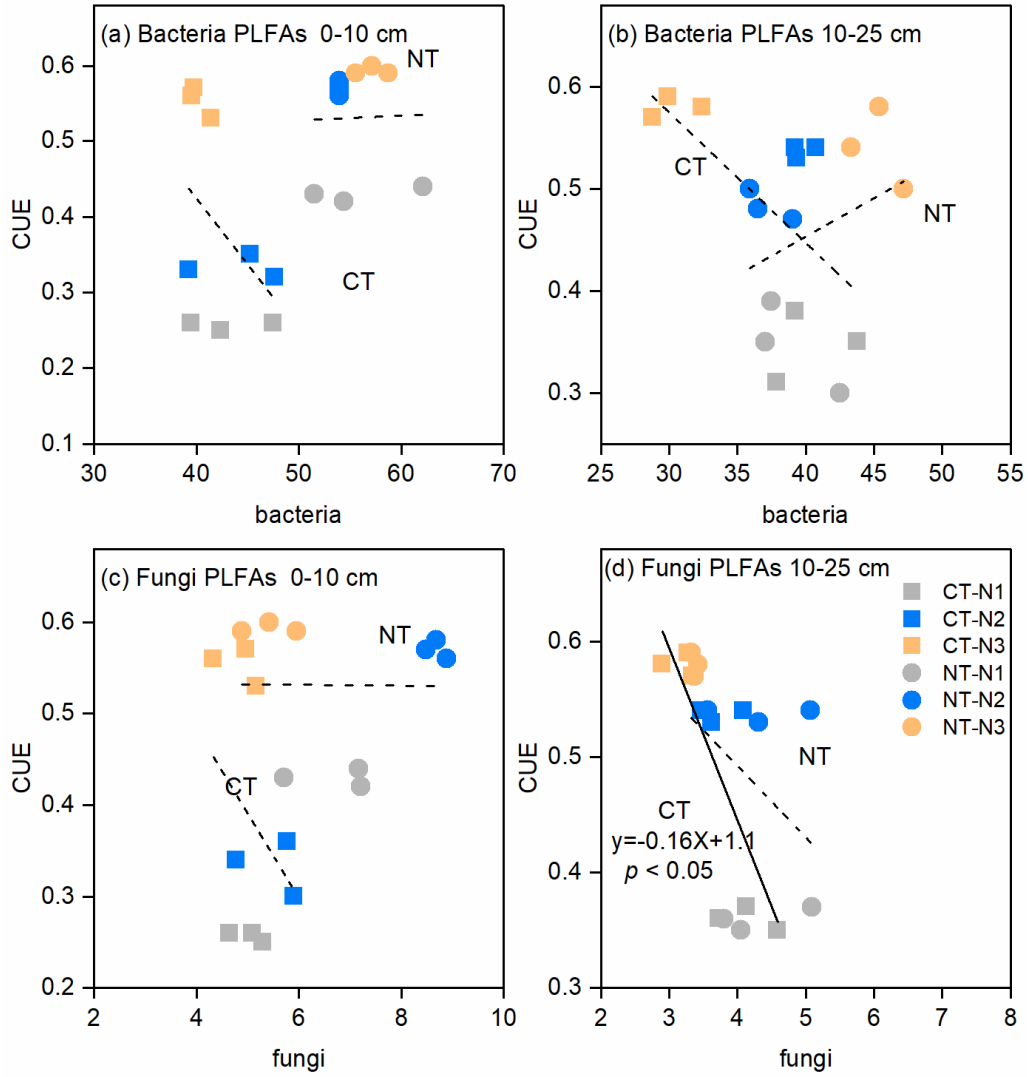

Fig. 2-S3 Soil bacteria and fungi PLFAs as a function of soil microbial CUE among treatments of tillage (T) and nitrogen (N) in farmland in North China. Linear regression is shown as a black solid line. N1, nitrogen addition at 105 kg N ha^{-1}; N2, nitrogen addition at 180 kg N ha^{-1}; N3, nitrogen addition at 210 kg N ha^{-1}; CT, conventional tillage; NT, no tillage.

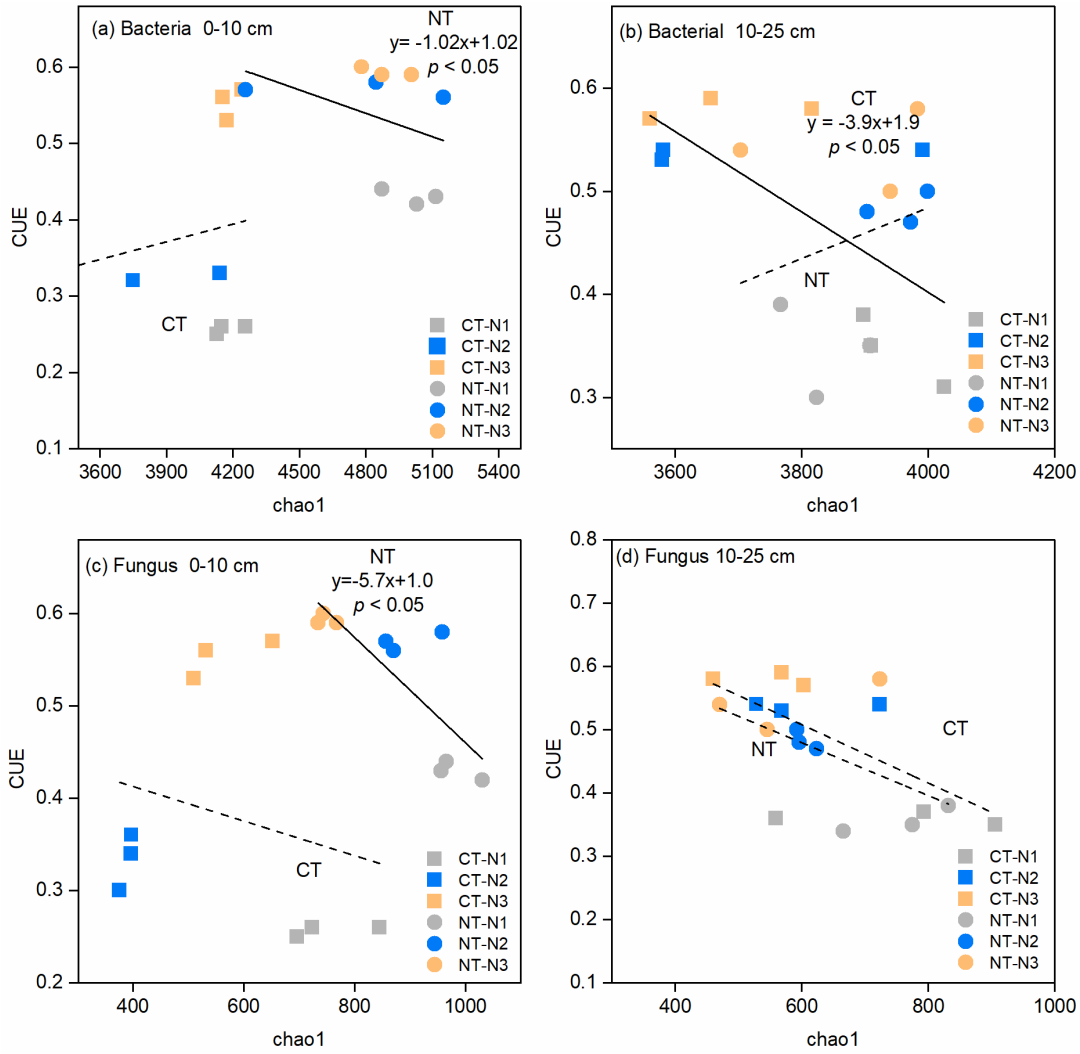

Fig. 2-S4 CUE as a function of soil bacterial and fungal diversity among treatments of tillage (T) and nitrogen (N) in farmland in North China. Linear regression is shown as a black solid line. The black dashed line represents no significance. N1, nitrogen addition at 105 kg N ha^{-1}; N2, nitrogen addition at 180 kg N ha^{-1}; N3, nitrogen addition at 210 kg N ha^{-1}; CT, conventional tillage; NT, no tillage.

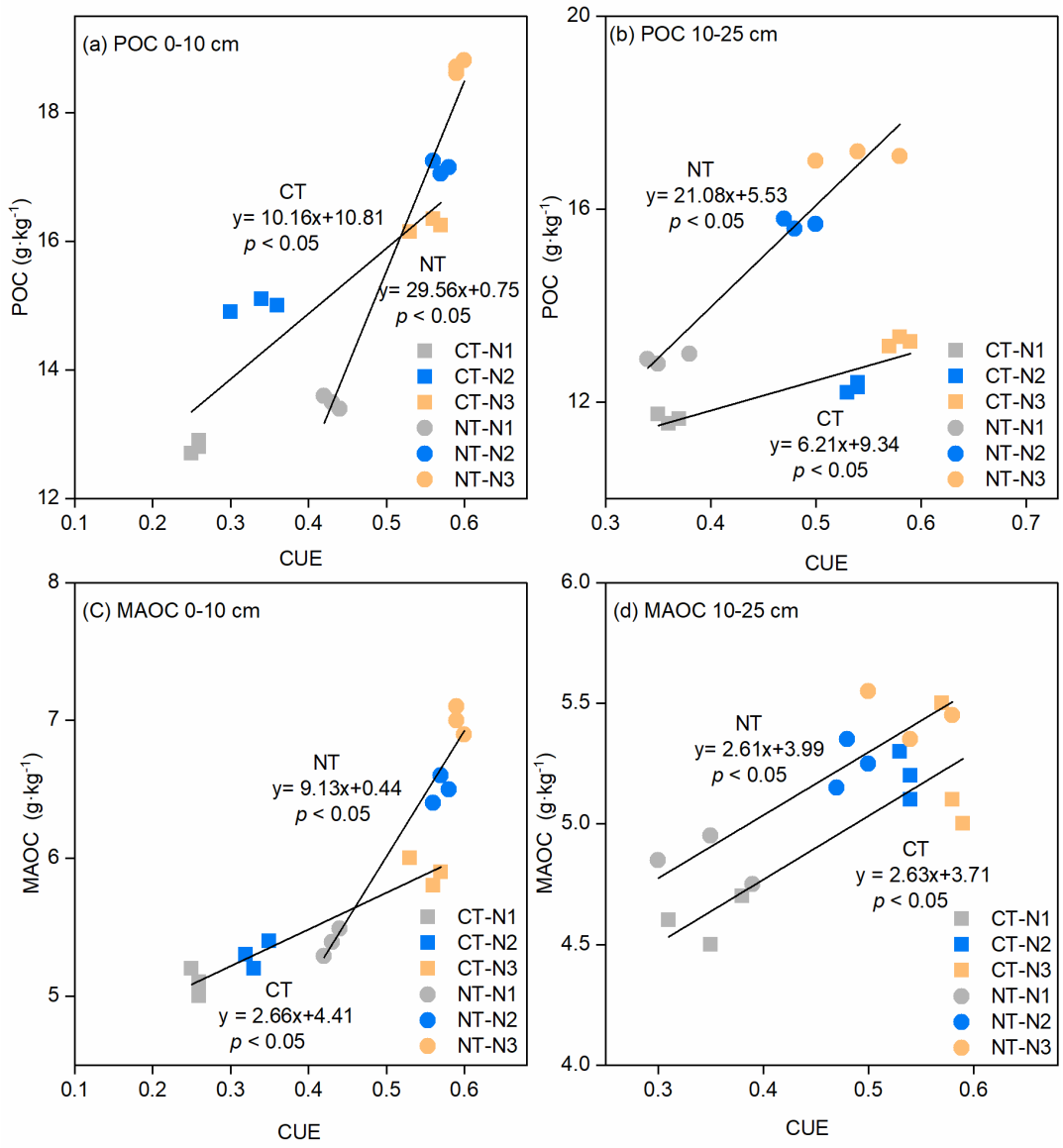

Fig. 2-S5 POC and MAOC as a function of soil microbial CUE among treatments of tillage (T) and nitrogen (N) in farmland in North China. Linear regression is shown as a black solid line. N1, nitrogen addition at 105 kg N ha^{-1}; N2, nitrogen addition at 180 kg N ha^{-1}; N3, nitrogen addition at 210 kg N ha^{-1}; CT, conventional tillage; NT, no tillage.

Supplementary information for Chapter III

Table 3-S1. Soil physical and chemical properties in 0-25 cm layer in 2003.

Table 3-S2. T-values and p-values for significance tests of the effects of soil depths on soil aggregate distribution.

Table 3-S3. F-values and p-values for significance tests of the effects of tillage and nitrogen application on soil aggregate distribution.

Table 3-S4. T-values and p-values for significance tests of the effects of soil depths on soil organic carbon within soil aggregates.

Table 3-S5. F-values and p-values for significance tests of the effects of tillage and nitrogen application on soil organic carbon within soil aggregates.

Table 3-S6. T-values and p-values for significance tests of the effects of soil depths on the amount of phospholipid fatty acid analysis within soil aggregates.

Table 3-S7. F-values and p-values for significance tests of the effects of tillage and nitrogen application on the amount of phospholipid fatty acid analysis within soil aggregates in 0-10 cm layer.

Table 3-S8. The amount of total phospholipid fatty acid analysis within soil aggregates under long-term tillage and nitrogen application in 0-10 cm soil layer.

Table 3-S9 F-values and p-values for significance tests of the effects of tillage and nitrogen application on the amount of phospholipid fatty acid analysis within soil aggregates in 10-25 cm layer.

Table 3-S10 The amount of total phospholipid fatty acid analysis within soil aggregates under long-term tillage and nitrogen application in 10-25 cm soil layer.

Fig. 3-S1 The straw input and roots input under long-term tillage and nitrogen application.

Fig. 3-S2 The ratio of carbon and nitrogen within soil aggregates under long-term tillage and nitrogen application.

Fig. 3-S3 The microbial biomass carbon within soil aggregates under long-term tillage and nitrogen application.

Fig. 3-S4 Phospholipid fatty acid analysis profiles within soil aggregates under long-term tillage and nitrogen application.

Fig. 3-S5 Relationships between soil organic carbon and Actinomycetes, Desulfovibrio, and Arbuscular mycorrhizal fungi at different aggregate sizes and soil layers

Table 3-S1 Soil physical and chemical properties in 0-25 cm layer in 2003.

Soil layer (cm)	Soil particle size distribution (%)			Available soil nutrient (mg kg^{-1})			SOC (g kg^{-1})	Bulk density (g cm^{-3})
	>0.020 mm	0.002-0.020 mm	<0.002 mm	N	P	K		
0-10	58.5	35.7	5.8	58	8.3	96	22.7	1.06
10-25	59.6	34.6	5.8	52	6.9	93	19.8	1.2

Table 3-S2 *T*-values and *p*-values for significance tests of the effects of soil depths on soil aggregate distribution. Mega-aggregates (>2000 μm); macro-aggregates (250-2000 μm); micro-aggregates (<250 μm); MWD, mean weight diameter. *p < 0.05; **p < 0.01; ***p < 0.001.

		sampling	
Variable	Factor	*T*-value	*p*-value
mega-aggregate	Depth	1.217	0.232
macro-aggregate	Depth	-1.995	0.054
micro-aggregate	Depth	1.217	0.232
MWD	Depth	-0.582	0.564

Table 3-S3 *F*-values and *p*-values for significance tests of the effects of tillage and nitrogen application on soil aggregate distribution. Mega-aggregates (>2000 μm); macro-aggregates (250-2000 μm); micro-aggregates (<250 μm); MWD, mean weight diameter; T, the effect of tillage; N, the effect of nitrogen application; T×N, interactive effects of tillage and nitrogen application. *p < 0.05; **p < 0.01; ***p < 0.001.

		sampling	
Variable	Factor	*F*-value	*p*-value
mega-aggregates	T	158.741	0.000***
	N	38.934	0.000***
	T×N	12.868	0.000***
macro-aggregates	T	104.888	0.000***
	N	0.829	0.446
	T×N	2.694	0.084
micro-aggregates	T	371.110	0.000***
	N	24.727	0.000***
	T×N	15.796	0.000***
MWD	T	320.190	0.000***
	N	52.113	0.000***
	T×N	20.789	0.000***

Table 3-S4 *T*-values and *p*-values for significance tests of the effects of soil depths on soil organic carbon within aggregates. SOC, soil organic carbon. *p < 0.05; **p < 0.01; ***p < 0.001.

		sampling	
Variable	Factor	*T*-value	*p*-value
SOC	Depth	0.928	0.355

Table 3-S5 *F*-values and *p*-values for significance tests of the effects of tillage and nitrogen application on soil organic carbon within soil aggregates. Mega-aggregates (>2000 μm); macro-aggregates (250-2000 μm); micro-aggregates (<250 μm); SOC, soil organic carbon; T, the effect of tillage; N, the effect of nitrogen application; T×N, interactive effects of tillage and nitrogen application. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

				sampling	
Variable	Soil depth	Soil aggregate class	Factor	*F*-value	*p*-value
SOC	0-10 cm	mega-aggregates	T	87.472	0.000***
			N	248.879	0.000***
			T×N	14.112	0.001***
		macro-aggregates	T	34.775	0.001***
			N	13.009	0.001***
			T×N	0.796	0.473
		micro-aggregates	T	222.019	0.000***
			N	25.849	0.000***
			T×N	5.080	0.025*
SOC	10-25 cm	mega-aggregates	T	78.102	0.001***
			N	8.769	0.004**
			T×N	3.997	0.050*
		macro-aggregates	T	62.038	0.000***
			N	9.710	0.003**
			T×N	3.425	0.067
		micro-aggregates	T	51.524	0.000***
			N	25.167	0.000***
			T×N	25.543	0.000***

Table 3-S6 *T*-values and *p*-values for significance tests of the effects of soil depths on the amount of phospholipid fatty acid analysis within soil aggregates. PLFAs , the total amount of phospholipid fatty acids; AMF, arbuscular mycorrhizal fungi; GramPos, Gram-positive bacteria; GramNeg, Gram-negative bacteria. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

		sampling	
Variable	Factor	*T*-value	*p*-value
Total PLFAs	Depth	6.481	0.000***
Fungus	Depth	7.790	0.000***
Bacteria	Depth	5.410	0.000***
AMF	Depth	4.440	0.000***
GramPos	Depth	4.851	0.000***
GramNeg	Depth	7.102	0.000***
Actinomycetes	Depth	3.064	0.003**
Desulfovibrio	Depth	0.271	0.787

Table 3-S7 *F*-values and *p*-values for significance tests of the effects of tillage and nitrogen application on the amount of phospholipid fatty acid analysis within soil aggregates in 0-10 cm layer. PLFAs, the total amount of phospholipid fatty acids; AMF, arbuscular mycorrhizal fungi; GramPos, Gram-positive bacteria; GramNeg, Gram-negative bacteria; T, the effect of tillage; N, the effect of nitrogen application; T×N, interactive effects of tillage and nitrogen application. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

		sampling	
Variable	Factor	*F*-value	*p*-value
Total PLFAs	T	14.948	0.000***
	N	11.516	0.000***
	T×N	6.632	0.003**
Fungus	T	12.621	0.001***
	N	5.629	0.006**
	T×N	6.605	0.003**
Bacteria	T	1.123	0.295
	N	14.321	0.000***
	T×N	4.382	0.018*
AMF	T	121.915	0.000***
	N	7.927	0.001***
	T×N	0.908	0.410
GramPos	T	50.814	0.000***
	N	4.135	0.022*
	T×N	3.170	0.051
GramNeg	T	85.299	0.000***
	N	10.062	0.000***
	T×N	6.064	0.004**
Actinomycetes	T	793.086	0.000***
	N	0.815	0.449
	T×N	1.495	0.237
Desulfovibrio	T	185.330	0.000***
	N	20.707	0.000***
	T×N	1.230	0.301

Table 3-S8 The amount of phospholipid fatty acids within soil aggregates for different tillage and nitrogen application in 0-10 cm soil layer. Vertical bars indicate one standard error of means (n= 3). Different lowercase letters mean significant differences at the $\alpha = 0.05$ level. GramPos, Gram-positive bacteria; GramNeg, Gram-negative bacteria; N1, nitrogen application rate at 105 kg N ha^{-1}; N2, nitrogen application rate at 180 kg N ha^{-1}; N3, nitrogen application rate at 210 kg N ha^{-1}; CT, conventional tillage; NT, no-tillage.

Soil aggregate size classes	Treatments	GramNeg (nmol g^{-1})	GramPos (nmol g^{-1})	Fungi (nmol g^{-1})	Bacteria (nmol g^{-1})	Total PLFAs (nmol g^{-1})
>2000 μm	CT-N1	19.91 ± 2.60 Ba	10.57 ± 1.19 Ba	8.32 ± 0.08 Ba	61.37 ± 5.01 Aa	72.54 ± 5.76 Ba
	CT-N2	14.17 ± 0.27 Bb	7.52 ± 0.17 Bb	6.36 ± 0.43 Bb	45.24 ± 5.49 Ab	50.62 ± 5.99 Bb
	CT-N3	14.22 ± 2.37 Bb	8.72 ± 0.88 Bb	6.18 ± 0.61Bb	43.38 ± 5.45 Ab	55.83 ± 6.71 Bb
	NT-N1	25.75 ± 2.82 Aa	13.67 ± 0.22 Aa	9.02 ± 0.49 Aa	59.55 ± 4.40 Aa	82.88 ± 5.26 Aa
	NT-N2	20.54 ± 0.57 Ab	11.62 ± 0.36 Ab	7.25 ± 0.38 Ab	43.74 ± 1.02 Aab	65.41 ± 1.33 Ab
	NT-N3	21.91 ± 3.03 Aab	12.10 ± 1.40 Aab	7.52 ± 0.94 Ab	46.53 ± 5.76 Ab	68.59 ± 6.27 Ab
250-2000 μm	CT-N1	20.82 ± 0.89 Ba	11.52 ± 1.30 Ba	8.36 ± 0.06 Ba	60.86 ± 5.92 Aa	80.87 ± 1.67 Ba
	CT-N2	14.67 ± 0.43 Bb	7.09 ± 0.32 Bb	6.02 ± 0.68 Bb	44.53 ± 3.90 Bb	58.99 ± 3.98 Bb
	CT-N3	17.76 ± 2.61 Bab	11.09 ± 1.75 Ba	7.81 ± 0.47 Ba	54.22 ± 4.42 Ab	70.37 ± 8.61 Bab
	NT-N1	27.27 ± 1.32 Aab	14.36 ± 0.91 Aa	8.52 ± 0.04 Aa	63.09 ± 5.46 Aa	86.93 ± 3.84 Aa
	NT-N2	28.41 ± 1.01 Ab	14.21 ± 0.90 Aa	10.18 ± 0.96 Aa	59.20 ± 1.97 Aa	86.55 ± 2.82 Aa
	NT-N3	25.23 ± 1.98 Ab	14.89 ± 1.31 Aa	8.48 ± 0.72 Aa	54.70 ± 5.72 Aa	81.50 ± 7.68 Aa
<250 μm	CT-N1	23.85 ± 3.08 Aa	13.95 ± 2.01 Aa	7.01 ± 0.38 Ba	74.69 ± 10.44 Aa	93.36 ± 13.47 Aa
	CT-N2	11.82 ± 1.58 Bb	7.92 ± 0.66 Bb	4.56 ± 0.28 Bb	39.79 ± 2.87 Bb	50.46 ± 4.84 Bb
	CT-N3	13.43 ± 2.60 Bb	8.33 ± 0.92 Bb	4.78 ± 0.26 Bb	42.74 ± 0.19 Bb	56.73 ± 0.41Bb
	NT-N1	23.36 ± 2.52 Aa	12.38 ± 1.35 Aa	7.92 ± 0.22 Aa	53.31 ± 4.52 Ba	74.75 ± 1.66 Aa
	NT-N2	25.17 ± 0.47 Aa	13.84 ± 0.88 Aa	8.18 ± 0.28 Aa	56.36 ± 3.75 Aa	85.39 ± 8.20 Aa
	NT-N3	25.26 ± 0.10 Aa	13.22 ± 0.12 Aa	8.14 ± 0.11 Aa	53.09 ± 0.21 Aa	77.25 ± 0.20 Aa

Table 3-S9 *F*-values and *p*-values for significance tests of the effects of tillage and nitrogen application on the amount of phospholipid fatty acid analysis within soil aggregates in 10-25 cm layer. PLFAs, the total amount of phospholipid fatty acids; AMF, arbuscular mycorrhizal fungi; GramPos, Gram-positive bacteria; GramNeg, Gram-negative bacteria; T, the effect of tillage; N, the effect of nitrogen application; T×N, interactive effects of tillage and nitrogen application. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

		sampling	
Variable	Factor	*F*-value	*p*-value
Total PLFAs	T	38.480	0.000***
	N	6.091	0.004**
	T×N	0.179	0.837
Fungus	T	26.581	0.000***
	N	6.738	0.003**
	T×N	0.714	0.495
Bacteria	T	52.300	0.000***
	N	5.999	0.005**
	T×N	0.344	0.710
AMF	T	8.211	0.006**
	N	6.180	0.004**
	T×N	4.455	0.017*
GramPos	T	1.478	0.230
	N	4.118	0.022*
	T×N	0.284	0.754
GramNeg	T	7.243	0.010**
	N	6.083	0.004**
	T×N	0.362	0.698
Actinomycetes	T	151.617	0.000***
	N	1.791	0.178
	T×N	1.097	0.342
Desulfovibrio	T	127.368	0.000***
	N	8.792	0.001**
	T×N	6.710	0.003**

Table 3-S10 The amount of phospholipid fatty acids within soil aggregates for different tillage and nitrogen application in 10-25 cm soil layer. Vertical bars indicate one standard error of means (n= 3). Different lowercase letters mean significant differences at the $\alpha = 0.05$ level. GramPos, Gram-positive bacteria; GramNeg, Gram-negative bacteria; N1, nitrogen application rate at 105 kg N ha^{-1}; N2, nitrogen application rate at 180 kg N ha^{-1}; N3, nitrogen application rate at 210 kg N ha^{-1}; CT, conventional tillage; NT, no-tillage.

Soil aggregate size classes	Treatments	GramNeg (nmol g^{-1})	GramPos (nmol g^{-1})	Fungi (nmol g^{-1})	Bacteria (nmol g^{-1})	Total PLFA (nmol g^{-1})
>2000 μm	CT-N1	14.82 ± 0.18 Aa	9.15 ± 0.27 Aa	6.06 ± 0.12 Aa	50.65 ± 3.20 Aa	63.67 ± 2.92 Aa
	CT-N2	13.71 ± 2.56 Aa	8.71 ± 0.74 Aa	5.53 ± 0.29 Aa	45.04 ± 0.35 Aa	57.07 ± 5.07 Aa
	CT-N3	13.90 ± 0.79 Aa	9.38 ± 0.61 Aa	6.00 ± 0.71 Aa	45.12 ± 4.29 Aa	59.48 ± 5.12 Aa
	NT-N1	14.01 ± 0.16 Ba	8.59 ± 0.15 Ba	5.21 ± 0.65 Ba	33.00 ± 2.30 Ba	50.05 ± 3.57 Ba
	NT-N2	10.39 ± 1.27 Bb	6.92 ± 0.78 Ba	3.55 ± 0.30 Bb	24.29 ± 2.54 Bb	38.47 ± 4.96 Bb
	NT-N3	12.96 ± 0.52 Bab	8.01 ± 0.13 Ba	4.41 ± 0.46 Bab	28.44 ± 0.69 Bab	43.41 ± 0.88 Bb
250-2000 μm	CT-N1	17.75 ± 0.52 Aa	11.04 ± 1.40 Aa	6.76 ± 0.03 Aa	58.16 ± 6.01 Aa	72.79 ± 6.14 Aa
	CT-N2	18.51 ± 0.13 Aa	11.51 ± 1.09 Aa	7.54 ± 0.60 Aa	56.53 ± 4.67 Aa	71.61 ± 6.11 Aa
	CT-N3	16.87 ± 2.33 Aa	6.69 ± 0.50 Ab	4.97 ± 0.54 Aa	48.08 ± 0.26 Aa	68.35 ± 5.35 Aa
	NT-N1	16.92 ± 0.21 Ba	10.01 ± 0.42 Ba	5.57 ± 0.11 Ba	36.83 ± 1.88 Ba	56.41 ± 3.13 Ba
	NT-N2	13.69 ± 1.75 Bb	9.08 ± 0.31 Bab	4.29 ± 0.25 Bb	35.73 ± 0.13 Ba	50.60 ± 3.27 Bab
	NT-N3	13.75 ± 1.61 Bb	8.56 ± 0.69 Bb	4.30 ± 0.38 Bab	30.20 ± 3.14 Ba	45.91 ± 4.32 Bb
<250 μm	CT-N1	18.58 ± 2.71 Aa	11.51 ± 0.15 Aa	6.66 ± 0.78 Aa	59.57 ± 4.30 Aa	74.16 ± 6.24 Aa
	CT-N2	13.83 ± 0.10 Ab	9.01 ± 0.47 Aab	4.69 ± 0.35 Aa	37.07 ± 1.09 Aa	52.35 ± 1.01 Aa
	CT-N3	12.94 ± 1.24 Ab	8.45 ± 0.99 Ab	4.97±0.41 Aa	42.81 ± 2.47 Aa	54.92 ± 3.55 Aa
	NT-N1	16.05 ± 0.20 Ba	9.70 ± 0.44 Ba	5.13±0.32 Ba	39.49 ± 0.30 Ba	55.57 ± 4.53 Ba
	NT-N2	13.07 ± 0.99 Ab	8.87 ± 0.80 Aa	4.18±0.46 Aab	34.17 ± 0.35 Ba	48.54 ± 0.55 Bb
	NT-N3	10.81 ± 0.20 Bc	7.57 ± 0.50 Ba	3.57±0.11 Bb	24.79 ± 0.87 Ba	38.44 ± 0.96 Bb

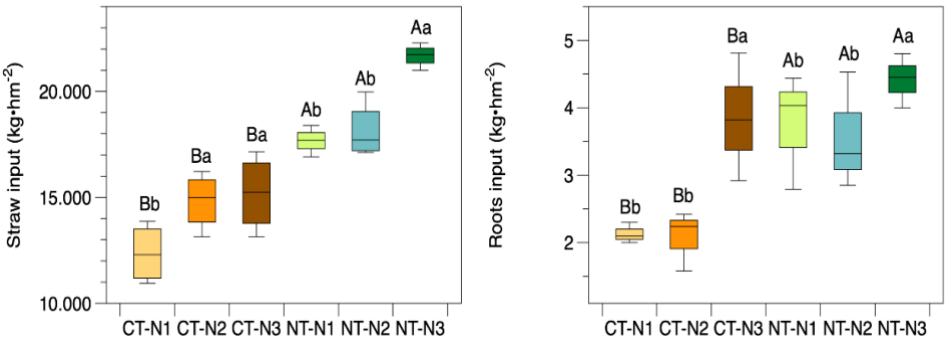

Fig. 3-S1 The straw input and roots input under long-term tillage and nitrogen application. Different capital letters indicate significant differences ($p < 0.05$) between two tillage treatments under the same nitrogen application rate; different lower-case letters indicate significant differences ($p < 0.05$) among nitrogen application rates under the same tillage treatment. N1, nitrogen application rate at 105 kg N ha^{-1}; N2, nitrogen application rate at 180 kg N ha^{-1}; N3, nitrogen application rate at 210 kg N ha^{-1}; CT, conventional tillage; NT, no-tillage.

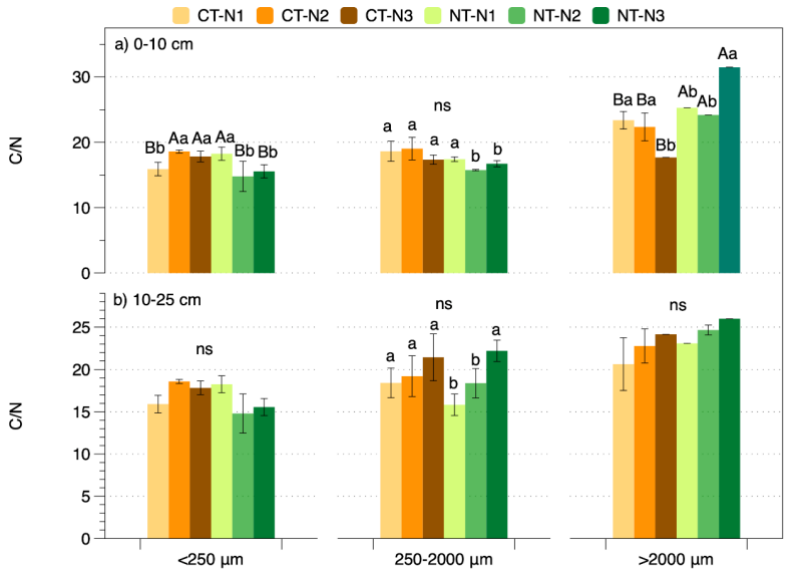

Fig. 3-S2 The ratio of carbon and nitrogen within soil aggregates under long-term tillage and nitrogen application. Vertical bars indicate the standard error of means (n = 3). Different capital letters indicate significant differences ($p < 0.05$) between two tillage treatments under the same nitrogen application rate; different lower-case letters indicate significant differences ($p < 0.05$) among nitrogen application rates under the same tillage treatment. N1, nitrogen application rate at 105 kg N ha^{-1}; N2, nitrogen application rate at 180 kg N ha^{-1}; N3, nitrogen application rate at 210 kg N ha^{-1}; CT, conventional tillage; NT, no-tillage.

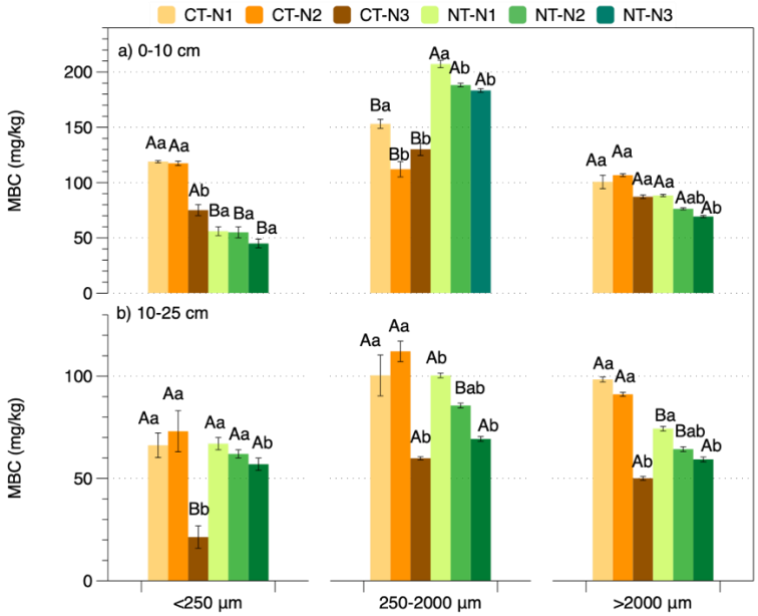

Fig. 3-S3 The MBC within soil aggregates under long-term tillage and nitrogen application. Vertical bars indicate the standard error of means (n = 3). Different capital letters indicate significant differences ($p < 0.05$) between two tillage treatments under the same nitrogen application rate; different lower-case letters indicate significant differences ($p < 0.05$) among nitrogen application rates under the same tillage treatment. MBC, soil microbial biomass carbon; N1, nitrogen application rate at 105 kg N ha^{-1}; N2, nitrogen application rate at 180 kg N ha^{-1}; N3, nitrogen application rate at 210 kg N ha^{-1}; CT, conventional tillage; NT, no-tillage.

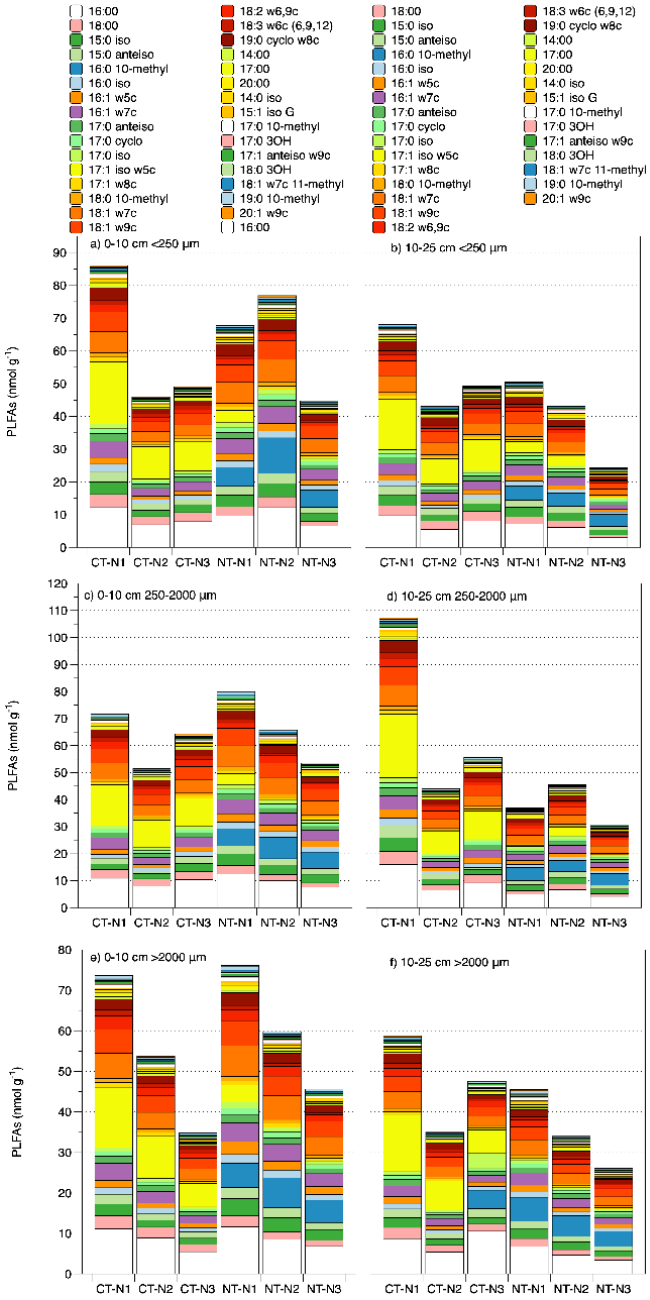

Fig. 3-S4 phospholipid fatty acids profiles within soil aggregates under long-term tillage and nitrogen application. N1, nitrogen application rate at 105 kg N ha^{-1}; N2, nitrogen application rate at 180 kg N ha^{-1}; N3, nitrogen application rate at 210 kg N ha^{-1}; CT, conventional tillage; NT, no-tillage.

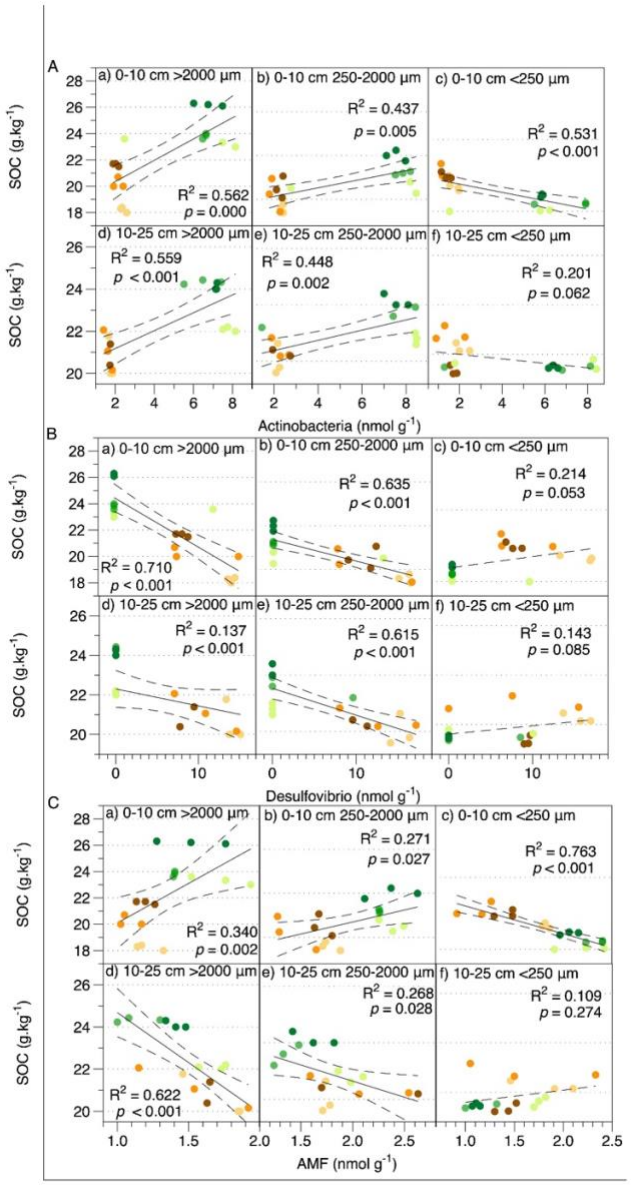

Fig. 3-S5 Relationships between Soil organic carbon and Actinomycetes, Desulfovibrio, and Arbuscular mycorrhizal fungi at different aggregate sizes and soil layers. Linear regression is shown as a black solid line. The black dashed line represents no significance. SOC, soil organic carbon; AMF, Arbuscular mycorrhizal fungi; N1,nitrogen application rate at 105 kg N ha^{-1}; N2, nitrogen application rate at 80 kg N ha^{-1}; N3, nitrogen application rate at 210 kg N ha^{-1}; CT, conventional tillage; NT, no-tillage.

Materials and methods

Straw mulching and root biomass

In 2019, Root samples were oven dried at 70°C for 72 hours and weighed to determine their dry mass. In 2018, after 20 days of natural air drying, straw samples were dried at 70°C for 72 hours and weighed to determine their dry mass.

Supplementary information for Chapter IV

Table 4-S1 Adonis test for the effect of tillage practices and soil depths on bacterial and fungal community composition based on all Bray-Curtis distances.

Table 4-S2 Primer sets and thermal procedures used in real-time quantitative PCR analysis

Fig. 4-S1 Porosity of the aggregates under long-term tillage and N application treatment.

Fig. 4-S2 Relationships between soil total porosity and irregular pores, elongated pores, regular pores under long-term tillage and N application treatment at 0-25 cm soil layers.

Fig. 4-S3 Relationships between soil pore connectivity and microbial network connectedness under long-term tillage and N application treatment at 0-25 cm soil layers (a,b).

Fig. 4-S4 Relationships between soil porosity (0-10μm) and fungal network connectedness under long-term tillage and N application treatment at 0-25 cm soil layers.

Fig. 4-S5 Relationships between soil porosity (10-30μm) and bacterial network connectedness under long-term tillage and N application treatment at 0-25 cm soil layers (a).

Table 4-S1 Adonis test for the effect of tillage practices and soil depths on bacterial and fungal community composition based on all Bray-Curtis distances.

Sample	bacteria		fungi	
	R^2	*p*	R^2	*p*
Tillage	0.3593	0.001	0.2234	0.036
Depth	0.1550	0.008	0.060	0.331

Table 4-S2 Primer sets and thermal procedures used in real-time quantitative PCR analysis

Gene	Primer	Primer sequence (5' - 3')	Citation
nifH	nifH-F	AAAGGYGGWATCGGYAARTCCACCAC	Rösch et al., 2002
	nifH-R	TTGTTSGCSGCRTACATSGCCATCAT	
AOA amoA	Arch-amoA 26F	GACTACATMTTCTAYACWGAYTGGGC	Park et al., 2008
	Arch-amoA 417R	GGKGTCATRTATGGWGGYAAYGTTGG	
cbbL	K2f	ACCAYCAAGCCSAAGCTSGG	Yuan at al., 2013
	V2r	GCCTTCSAGCTTGCCSACCRC	
cbbM	cbbM-F	TTCTGGCTGGGBGGHGAYTTYATYAARAARGACGA	Zhou at al., 2023
	cbbM-R	CCGTGRCCRGCVCGRTGGTARTG	

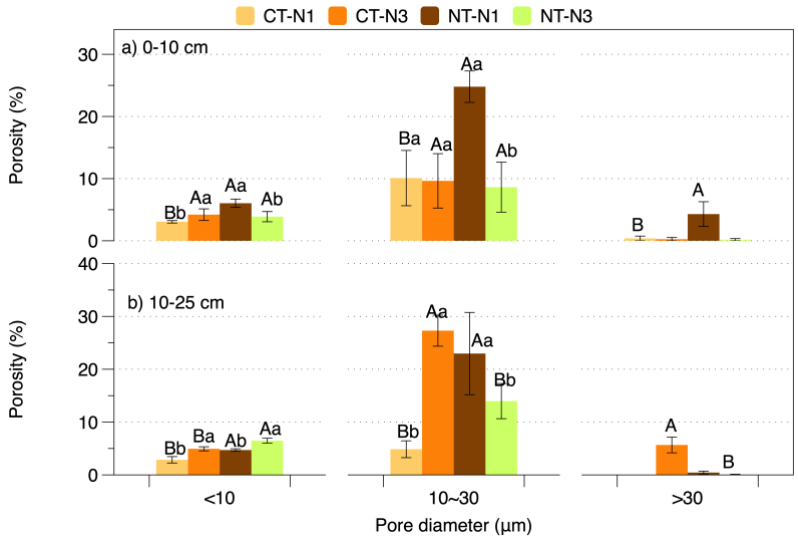

Fig. 4-S1 Porosity of the aggregates under long-term tillage and N application treatment. Vertical bars indicate the standard error of means (n = 3). Different capital letters indicate significant differences ($p < 0.05$) between two tillage treatments under the same N application rate; different lower-case letters indicate significant differences ($p < 0.05$) between two N application rates under the same tillage treatment. N1, N application rates at 105 kg N ha^{-1}; N3, N application rates at 210 kg N ha^{-1}; CT, conventional tillage; NT, no-tillage.

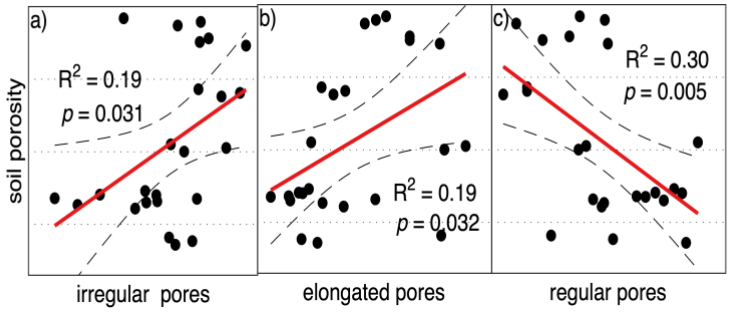

Fig. 4-S2 Relationships between soil total porosity and irregular pores, elongated pores, regular pores under long-term tillage and N application treatment at 0-25 cm soil layers. Linear regression is shown as a red solid line.

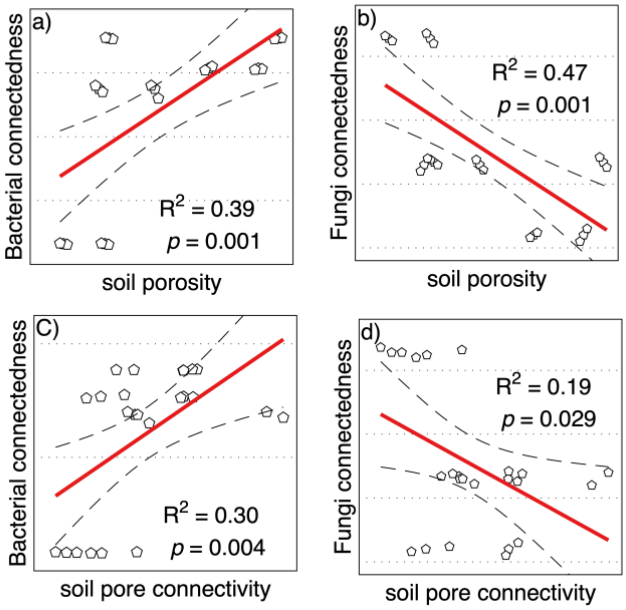

Fig. 4-S3 Relationships between soil pore connectivity and microbial network connectedness under long-term tillage and N application treatment at 0-25 cm soil layers (a,b). Relationships between soil porosity and microbial network connectedness under long-term tillage and N application treatment at 0-25 cm soil layers (c,d). Linear regression is shown as a red solid line.

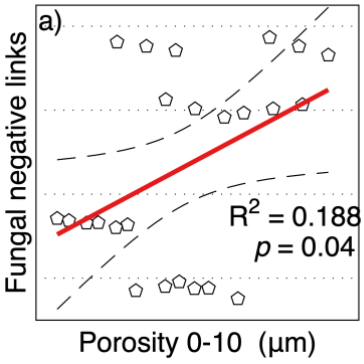

Fig. 4-S4 Relationships between soil porosity (0-10μm) and fungal network connectedness under long-term tillage and N application treatment at 0-25 cm soil layers. Linear regression is shown as a red solid line.

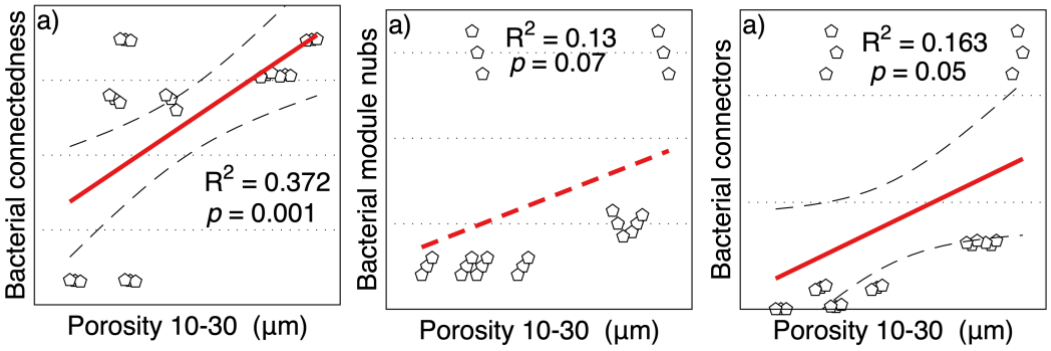

Fig. 4-S5 Relationships between soil porosity (10-30µm) and bacterial network connectedness under long-term tillage and N application treatment at 0-25 cm soil layers (a). Relationships between soil porosity (10-30µm) and bacterial keystone taxa of module nubs and connectors under long-term tillage and N application treatment at 0-25 cm soil layers (b, c). Linear regression is shown as a red solid line. The red dashed line represents no significance.