

## 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<sup>-1</sup>). Under no-tillage treatment, high nitrogen application rate increased SOC by 2.1–3.7 g·kg<sup>-1</sup> 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<sup>-1</sup> 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.

### 1. Introduction

There has been a dramatic increase recently 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 providing protection

for the sequestration of soil organic C (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)

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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 comprehending the interplay between microorganisms within aggregates, aggregate formation and SOC accumulation 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 (Solins 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  $\mu\text{m}$ ), macro-aggregates (250–2000  $\mu\text{m}$ ), and micro-aggregates (<250  $\mu\text{m}$ )] (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) (Amézqueta, 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., 2019a, 2019b, 2019c). 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 application rates (105, 180, and 210 kg N ha<sup>-1</sup>). 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., 2021a, 2021b). Numerous studies and meta-analyses consistently indicated that tillage intensity affected aggregate size distribution. In particular, conservation tillage had a greater potential to form macro-aggregates and increase mean weight diameter (MWD) (Kumar et al., 2019; Li et al., 2023; Liu et al., 2021a, 2021b). Furthermore, nitrogen fertilizer are 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 (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., 2019a, 2019b, 2019c). 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., 2017a, 2017b), specifically arbuscular mycorrhizal fungi (Dai et al., 2015) and actinomycetes (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 (Zhang et al., 2018), especially for arbuscular mycorrhizal fungi and actinomycetes biomarkers (Dai et al., 2015; Wang et al., 2017a, 2017b). 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 (Philipot 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 (Li et al., 2021). 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., 2019a, 2019b, 2019c), and the mean annual temperature is recorded at 7.4 °C (Li et al., 2021). 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 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 m<sup>2</sup> (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 (Li et al., 2021). 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<sup>-1</sup> for N1, N2, and N3, respectively. The average application rate of chemical fertilizer in China is 235 kg N ha<sup>-1</sup>, ranking it first worldwide as reported by Fao, Rome (1999). In the northern region of China, an application rate of 180 kg N ha<sup>-1</sup> 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<sup>-1</sup> according to a previous study (Wang et al., 2001). Therefore, 210 kg N ha<sup>-1</sup> 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 splitted aggregates samples into two groups. One portion was immediately freeze-dried and preserved in a refrigerator (-20 °C) for phospholipid fatty acid analysis (PLFAs) 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 16:1 $\omega$ 5c was assigned to arbuscular mycorrhizal fungi (Frostegård et al., 2011), whereas the i17:1 $\omega$ 5c 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 C (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 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 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. 1). By contrast, nitrogen application rates under no-tillage did not influence the aggregate distribution.

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

### 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 (Tables S4 and 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). With increasing nitrogen application, the SOC content within all aggregates increased significantly by 2.1 to 3.7 g·kg<sup>-1</sup> 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). With increasing nitrogen application, only the SOC within mega- and macro-aggregates under no-tillage was significantly increased.

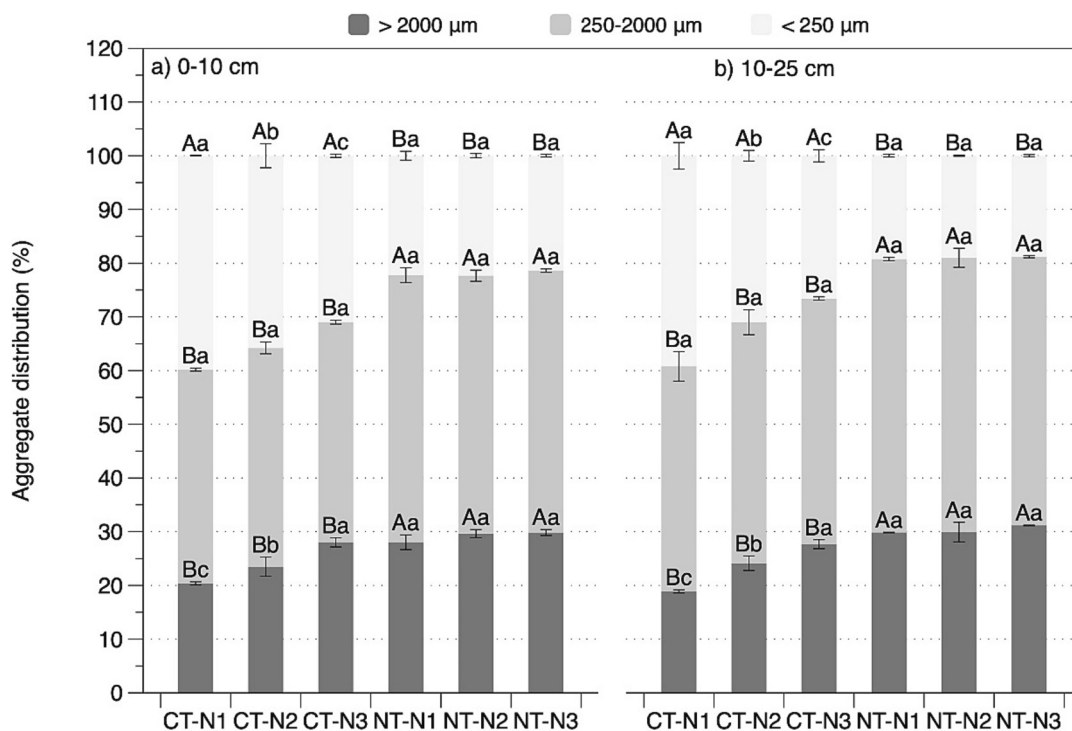


Fig. 1. Soil aggregate distribution under long-term tillage and nitrogen application. Vertical bars indicate the standard error of means ( $n = 3$ ). Different capital letters indicate significant differences ( $p < 0.05$ ) between two tillage treatments under the same nitrogen application rate; different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



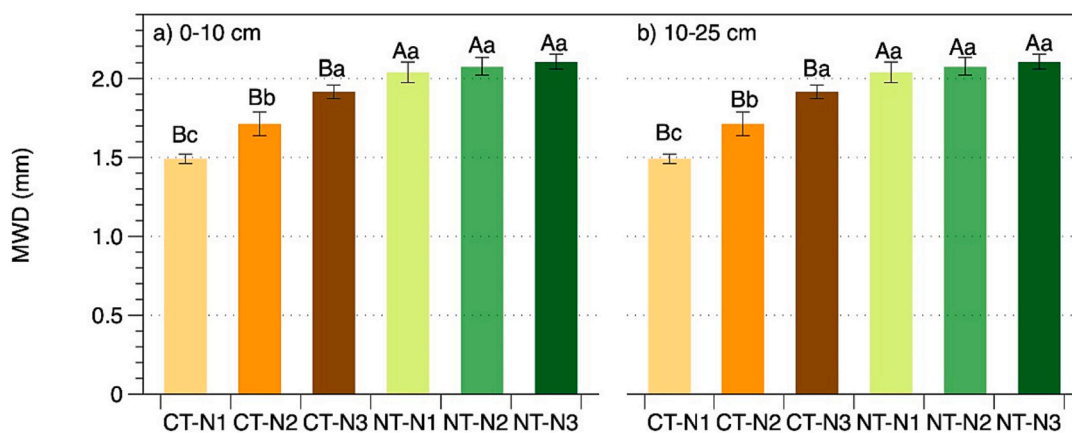


Fig. 2. The mean weight diameter under long-term tillage and nitrogen application. Vertical bars indicate the standard error of means ( $n = 3$ ). Different capital letters indicate significant differences ( $p < 0.05$ ) between two tillage treatments under the same nitrogen application rate; different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

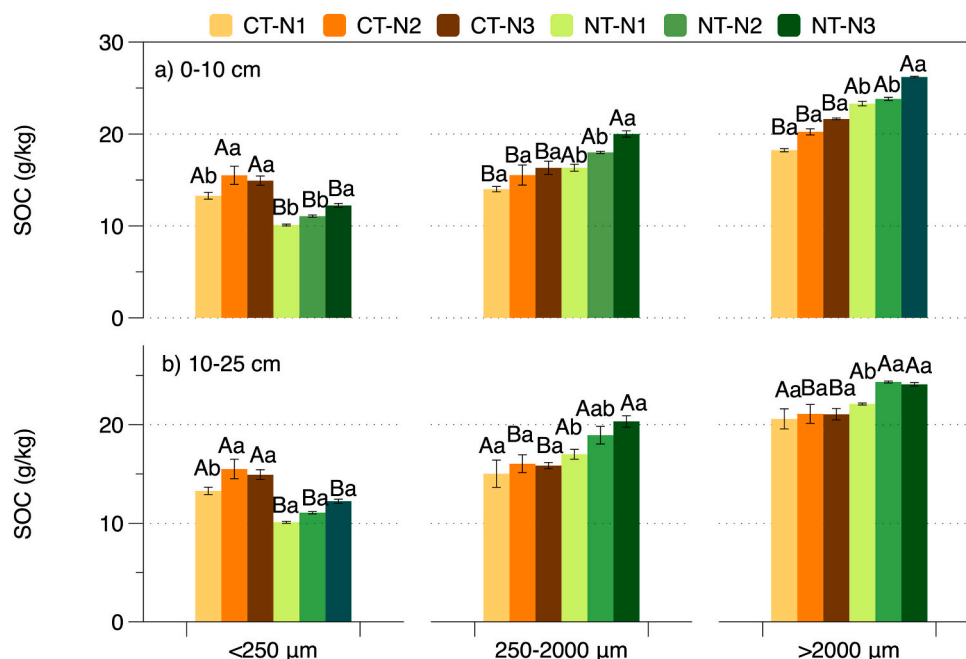


Fig. 3. The soil organic carbon content within soil aggregates under long-term tillage and nitrogen application. Vertical bars indicate the standard error of means ( $n = 3$ ). Different capital letters indicate significant differences ( $p < 0.05$ ) between two tillage treatments under the same nitrogen 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<sup>-1</sup>; N2, nitrogen application rate at 180 kg N ha<sup>-1</sup>; N3, nitrogen application rate at 210 kg N ha<sup>-1</sup>; CT, conventional tillage; NT, no-tillage.

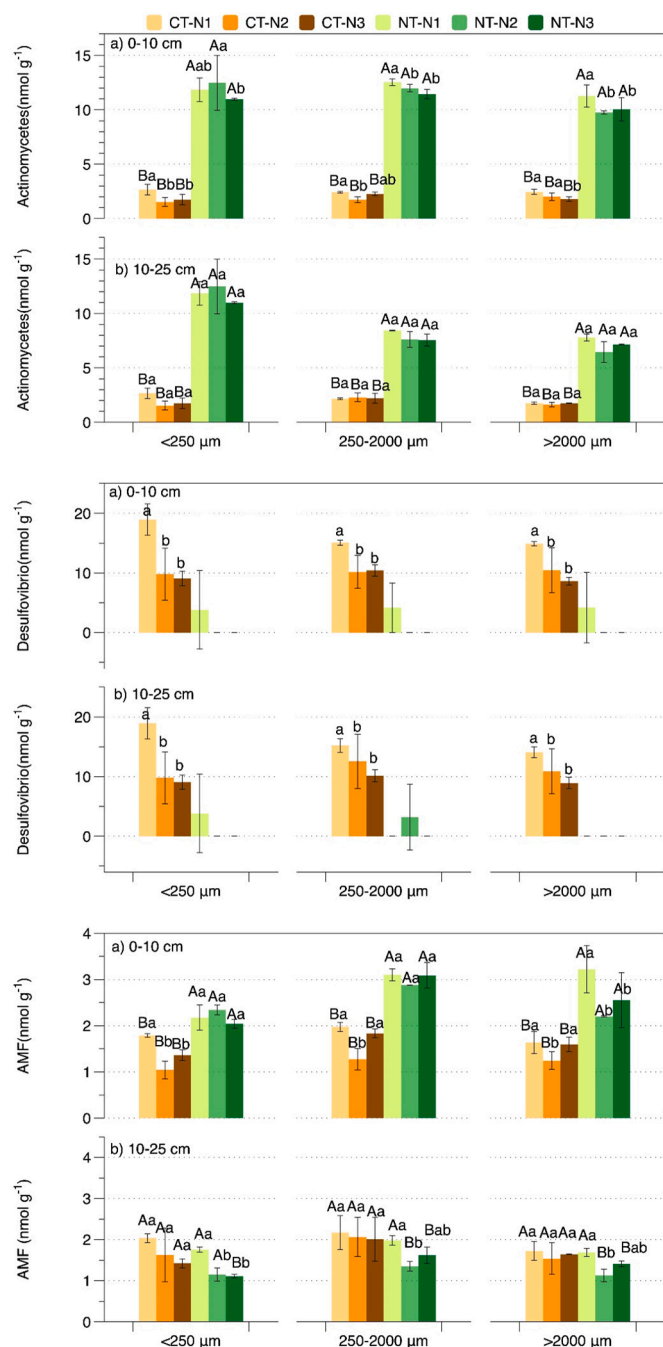
#### 4.3. Soil microbial community

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 S6).

For the 0–10 cm layer, bacterial, fungal, and total PLFAs were significantly impacted by tillage, nitrogen application rates, and their interaction (Table 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 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. 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<sup>-1</sup> (Fig. 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<sup>-1</sup>, 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 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 S8). Under no-tillage, increasing nitrogen application rates



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

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 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 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.

Moreover, under conventional tillage, increasing nitrogen application rates had little influence on the fungal, bacterial, and total PLFAs within all aggregates (Table 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 and nitrogen application 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. 1 and 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. 1). Secondly, compared with conventional tillage, more crop straw was invested under no-tillage (Fig. 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. 1). Under long-term conventional tillage, an increase in nitrogen application rates, even without straw input, resulted in an elevation of root biomass (Fig. S1) and root secretion, thereby augmenting the exudation of organic matter and fostering the formation of mega-aggregates (Figs. 1 and 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 serves 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), 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. 1). The negative impact of long-term conventional tillage on SOC sequestration was attributed to the decrease of mega- and macro-aggregates (Fig. 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. S3). A significantly higher concentration of MBC was found in micro-aggregates under conventional tillage versus no-tillage (Fig. 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). 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), 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 and nitrogen application 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 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 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 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; de 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; Zhang et al., 2018). Nitrogen application rates regulate the impact of long-term no-tillage on microbial biomass within

micro- and macro-aggregates (Table 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. 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, actinomycetes, desulfovibrio), and total microbial biomass within mega-aggregates under both tillage practices in 0–10 cm (Table S8). As the toxicity of urea, excessive nitrogen application has suppressed the soil microbial activity (Lian et al., 2018; Wang et al., 2018a, 2018b, 2018c). 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., 2018a, 2018b, 2018c). 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

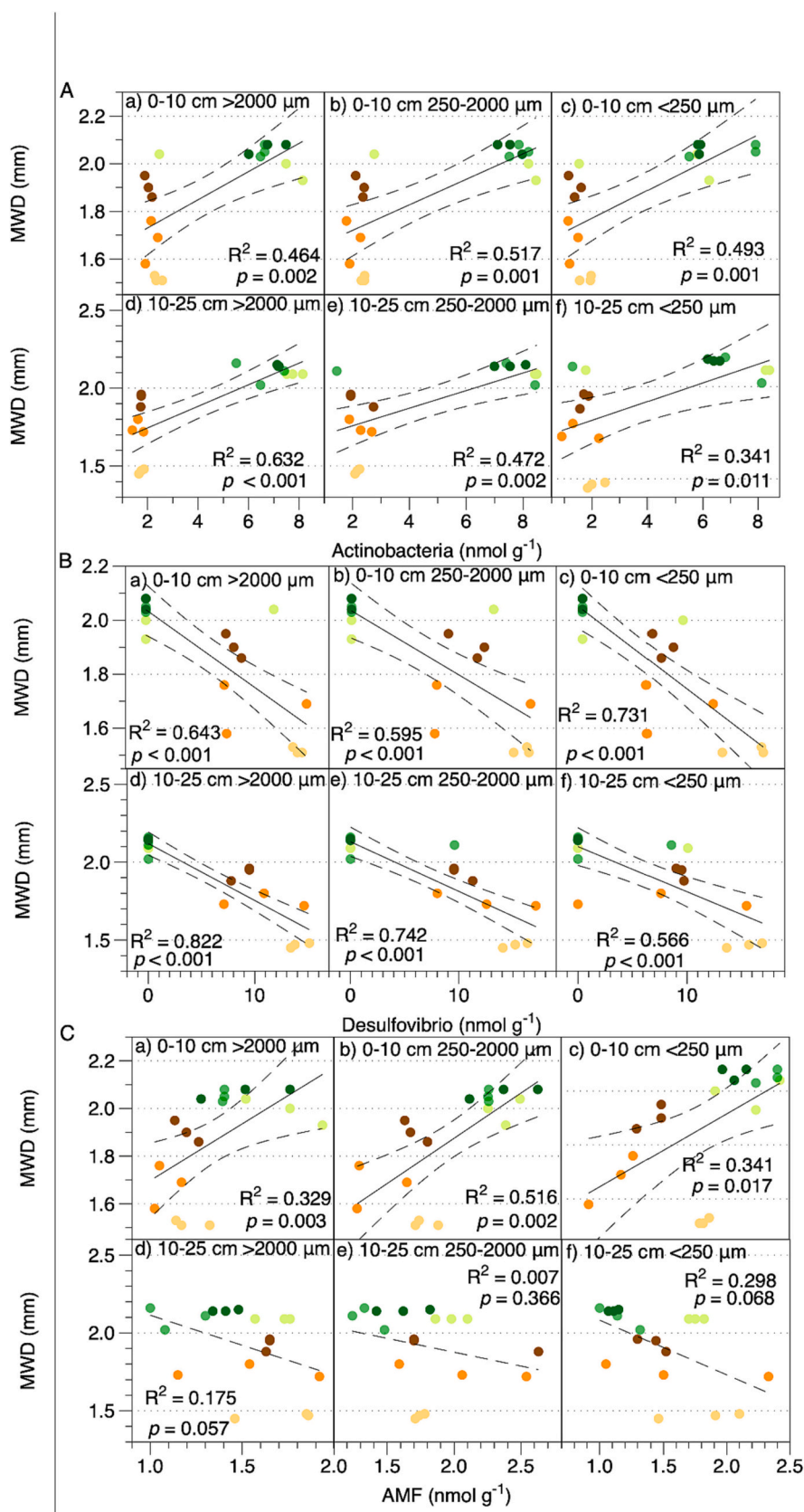
To investigate the microbial strategies that regulate the characteristics of soil aggregates, we have selected three scenarios from microbial communities significantly impacted by long-term tillage and nitrogen application for a thorough discussion.

### 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. 4), and a prior study also supported our finding (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-C degradation. Moreover, actinomycetes had a significant positive correlation with MWD (Fig. 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., 2020). These reformed 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 actinomycetes significantly increased SOC content within mega- and macro-aggregates (Fig. 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. 6).

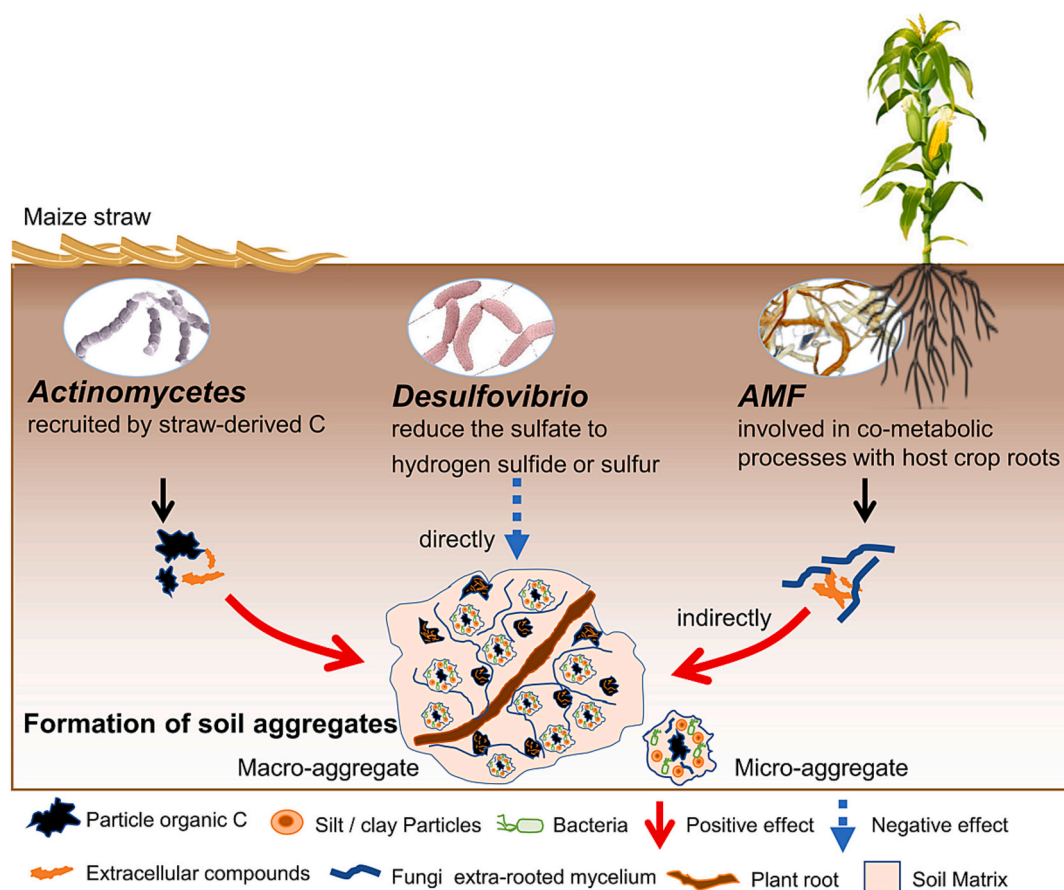
### 5.3.2. Desulfovibrio possibly limited the process of soil aggregate formation directly

Long-term conventional tillage significantly increased desulfovibrio biomass within all aggregates compared with long-term no-tillage (Fig. 4). As an anaerobic gram-negative bacterium, 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



**Fig. 5.** Relationships between Mean weight diameter and Actinomyces (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<sup>-1</sup>; N2, nitrogen application rate at 180 kg N ha<sup>-1</sup>; N3, nitrogen application rate at 210 kg N ha<sup>-1</sup>; CT, conventional tillage; NT, no-tillage.





**Fig. 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.

reduction directly inhibited the formation of aggregates under conventional tillage, leading to a decrease in soil aggregate stability (Fig. 5). However, increasing nitrogen application rates significantly reduced desulfovibrio biomass within all soil aggregate under conventional tillage (Fig. 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. 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. S5 and 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. 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. 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; de 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. 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. 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 OTU-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; Lehmann et al., 2017a, 2017b). 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 (Lehmann et al., 2017a, 2017b), they primarily participate in co-metabolic processes with the crop roots, which might indirectly contribute to the formation of soil aggregates (Fig. 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. 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., 2021a, 2021b) 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.

#### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.spc.2023.11.022>.

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