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RESEARCH ARTICLE

Functional Soil Organic Matter Fractions, Microbial Community, and Enzyme Activities in a Mollisol Under 35 Years Manure and Mineral Fertilization

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Abstract

Fertilization is a worldwide practice to maintain and increase crop productivity and improve soil quality in agricultural ecosystems. The interactive mechanisms of long-term fertilization affecting the functional soil organic matter (SOM) fractions, microbial community, and enzyme activities are unclear. We investigated the effects of manure and mineral fertilization on six SOM fractions (non-protected, physically, chemically, biochemically, physical-chemically, and physical-biochemically protected), microbial community structure, and enzyme activities based on a 35-year fertilization experiment. The combined application of manure and mineral fertilizers (NPKM) increased the soil organic carbon (SOC) and total nitrogen (TN) in the biochemically (28.6–43.9%) and physically (108–229%) protected fractions, compared to their content in the unfertilized soil (CK). The total phospholipid fatty acid content, Gram(−) bacteria, and actinomycetes, as well as the activities of α-1,4-glucosidase, β-1,4-N-acetylglucosaminidase, β-1,4-xylosidase, and cellobiohydrolase were highest under NPKM fertilization. The protected SOM fractions (physical, biochemical, physical-chemical, and physical-biochemical) were closely related to microbial community composition (accounting for 67.6% of the variance). Bacteria were sensitive to changes in the physically and biochemically protected fractions, whereas fungi responded more to the changes in the chemically protected fraction. In summary, long-term mineral and organic fertilization has a strong effect on microbial communities and activities through the changes in the functional SOM fractions.

Keywords Long-term fertilization · Soil organic matter fractions · Soil aggregation · Microbial community composition · Enzyme activities

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1 Introduction

Soil organic matter (SOM) is very important in maintaining fertility and in improving physical and biochemical soil properties (Zhao et al. 2016). Fertilization is a global management strategy to improve soil quality, and combined application of mineral and organic fertilizers helps to improve crop productivity. Numerous field experiments have demonstrated that long-term fertilization, especially the application of manure alone or in combination with mineral fertilizers, increases the SOM content (Wang et al. 2015; Tian et al. 2017). Microorganisms can regulate SOM cycling and carbon (C) sequestration (Balser and Firestone 2005). A better understanding of the mechanisms affecting SOM dynamics through stimulating microbial activities by long-term fertilization is critical for maintaining sustainable agroecosystems.

The total SOM is generally not very sensitive to changes in agricultural management practices owing to the presence of large background amounts of stable SOM (Zhao et al. 2016). For example, a few long-term agricultural management studies involving fertilization practices have no significant differences in the equilibrium SOM stocks, even at high C addition levels (Reicosky et al. 2002; Yin and Cai 2006). SOM consists of various functional fractions that differ in their intrinsic degradability and in the factors that control the decomposition rates (Stevenson 1994). Stewart et al. (2008) have proposed a conceptual model of SOM accumulation, dividing SOM fractions into free particulate organic matter (POM; unprotected), and microaggregate-associated (physically protected), silt- and clay-associated (chemically protected), and non-hydrolyzable (biochemically protected) fractions. For example, organic C mainly distributed within the microaggregate-protected particulate organic matter and within the hydrolyzable and non-hydrolyzable silt-sized fractions after long-term manure application (Tian et al. 2017).

Microbial community structure and activities are sensitive indicators of management-induced changes in both the soil organic carbon (SOC) and the biological properties of soil quality and health (Guillaume et al. 2016; Basanta et al. 2017). By comparing the effects of organic and mineral fertilization on microbial community structures, high microbial biomass in soils after manure application was observed (Tian et al. 2017). The changes in microbial communities can largely be explained by the labile SOM fractions (Cookson et al. 2005). Light fraction organic matter (LFOM) is an important C source for microorganisms (Cookson et al. 2005). Bacterial biomass has also been related to small-scale spatial variations in the particulate organic carbon (POC) (Sobczak et al. 1998). The abundance of total phospholipid-derived fatty acids (PLFAs), bacteria, fungi, and actinomycetes increased in soil after manure addition or in combination with mineral fertilizers (Dong et al. 2014; Tian et al. 2017).

The extracellular enzymes in soil are synthesized and secreted by microorganisms and roots and participate in SOM formation and decomposition (Burns et al. 2013). Mineral N has little influence on soil microbial biomass (Li et al. 2017). However, information on microbial communities and activities associated with functional SOM fraction dynamics after manure and mineral fertilizer amendment is particularly limited. Thus, further understanding of the interactive mechanisms involved in the effects of long-term organic and mineral fertilization on the microbial community structure and on the activities associated with functional SOM fractions is necessary.

Mollisols are widely distributed worldwide and also in northeast China and serves as a critical base of grain production (Ding et al. 2012). Therefore, sustainable management of black soils affects food security. Mollisols in northeast China have been severely degraded during the last decades, owing to extensive agricultural management patterns, such as intensive tillage (Ding et al. 2012). To investigate the effects of fertilization on soil quality, a long-term experiment was established in 1979. We examined the effects of mineral fertilizers and manure on functional SOM fractions, microbial community, and enzyme activities to understand the effects of long-term fertilization and provide a theoretical basis for sustainable management of black soils.

2 Materials and Methods

2.1 Site Description, Experimental Design, and Sampling

A 35-year long-term field experiment was initiated in 1979 in Haerbin, Heilongjiang Province (126° 35′ E, 45° 40′ N), northeast of China. This region is characterized by a temperate continental monsoon climate, with a mean annual temperature of 3.5 °C and annual precipitation of 533 mm. The soil was classified as black soil according to the Chinese classification system, a classification corresponding to Mollisol in the USDA soil taxonomy. The basic soil properties (0–20 cm) at the beginning of the field experiment were as follows: 26.7 g kg^{-1} SOC, 1.47 g kg^{-1} total N, 1.07 g kg^{-1} total phosphorus (P), 150 mg kg^{-1} available N, 51.0 mg kg^{-1} available P, 200 mg kg^{-1} available potassium, and a pH of 7.22.

Twenty-four treatments were laid out in a randomized complete block design with three field replicates. Each plot was 168 m^2 in size. In this study, five fertilization treatments were selected, including (1) control without fertilizer (CK), (2) N fertilization (N), (3) NPK fertilization (NPK), (4) manure application (M), and (5) combined manure and NPK fertilization (NPKM). The NPK fertilizers were applied as follows: 150 kg N ha^{-1} $year^{-1}$, 75 kg P_2O_5 ha^{-1} $year^{-1}$ (33.8 kg P ha^{-1} $year^{-1}$), and 75 kg K_2O ha^{-1} $year^{-1}$

(62.2 kg K ha^{-1} $year^{-1}$) for wheat and maize, and 75 kg N ha^{-1} $year^{-1}$, 150 kg P_2O_5 ha^{-1} $year^{-1}$ (67.6 kg P ha^{-1} $year^{-1}$), and 75 kg K_2O ha^{-1} $year^{-1}$ (62.2 kg K ha^{-1} $year^{-1}$) for soybean. Fresh horse manure, with 70.0% water content, was applied to the soil at an annual rate of 18.6 t ha^{-1} $year^{-1}$ fresh matter.

After the winter wheat harvest in May 2014, soil was sampled from each plot by collecting eight randomly selected cores (0–10 cm deep), which were mixed to yield one composite sample per plot. The samples were stored in sterile plastic bags, placed in a cooler box with ice at approximately 4 °C, and taken to the laboratory for further analysis. The leaves and root fragments were carefully removed, and the remaining soil samples were divided into three parts. One part was stored at − 80 °C and used for the analysis of microbial community, one part was stored at 4 °C and used for the measurement of enzyme activities, and the remaining portion was air-dried at room temperature and used for SOM fractionation analyses.

2.2 Soil SOM Fractionation

The soil was fractionated by using a combination of physical, chemical, and density methods described by Stewart et al. (2008, 2009) (Fig. 1). Firstly, three size fractions were obtained by partial dispersion and physical fractionation; these were coarse unprotected particulate organic matter (cPOM, > 250 mm), microaggregate fraction (μagg, 53–250 mm), and easily dispersed silt and clay fraction (dSilt and dClay, < 53 mm).

Further fractionation of the isolated microaggregate fraction was performed in the second step. Fine unprotected POM (fPOM) was primarily isolated by density flotation with 1.85 g cm^{-3} sodium polytungstate, followed by dispersal of the heavy fraction by shaking with 12 glass beads overnight and sieving (< 53 μm), separating the microaggregate-protected POM (> 53 μm in size, iPOM), and silt- and clay-sized fractions were derived from the microaggregate (μSilt and μClay).

The third step involved acid hydrolysis for the silt- and clay-sized fractions separated in the first two steps, as described by Plante et al. (2006a), by using both density floatation and initial dispersion and physical fractionation. Acid hydrolysis was performed by refluxing at 95 °C for 16 h in 6 mol L^{-1} HCl. After refluxing, the suspension was filtered and washed with deionized water over a glass fiber filter. The residues were dried at 60 °C and weighed. These samples represented the non-hydrolyzable fractions (NH-dSilt, NH-dClay, NH-μSilt, and NH-μClay). The hydrolyzable fractions (H-dSilt, H-dClay, H-μSilt, and H-μClay) were determined by the difference between the overall fractions and the non-hydrolyzable fractions.

All the fractions were divided into six functional SOM fractions on the basis of the assumed link between the isolated fractions and the protection mechanisms (Stewart et al. 2008). The unprotected fraction consisted of the cPOM and fPOM fractions. The physically protected fraction was iPOM. The chemically protected fraction was the hydrolyzable portion of the silt- and clay-sized fractions (H-dSilt and H-dClay). The biochemically protected fraction corresponded to the non-hydrolyzable portion remaining in the silt- and clay-sized

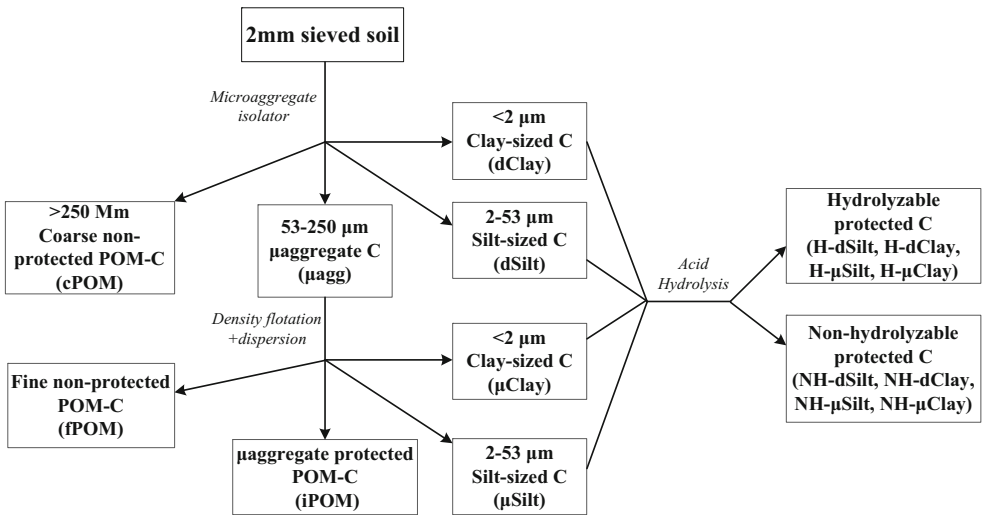

Fig. 1 Soil fractionation scheme to isolate six functional SOM fractions (modified from Stewart et al. 2008, 2009) cPOM coarse unprotected particulate organic matter, fPOM fine unprotected POM, iPOM microaggregate-protected POM, NH-μSilt non-hydrolyzable microaggregate-derived silt-sized fraction, NH-μClay non-hydrolyzable microaggregate-derived clay-sized fraction, H-μSilt hydrolyzable microaggregate-derived silt-sized fraction, H-μClay hydrolyzable microaggregate-derived clay-sized fraction, H-dSilt hydrolyzable easily dispersed silt-sized fraction, H-dClay hydrolyzable easily dispersed clay-sized fraction, NH-dSilt non-hydrolyzable easily dispersed silt-sized fraction, NH-dClay non-hydrolyzable easily dispersed clay-sized fraction

fractions after the acid hydrolysis (NH-dSilt and NH-dClay). Because of the various protection mechanisms within the physically protected fraction, it could be subdivided into three parts: a fraction with pure physical protection of POM (iPOM), physical-biochemically protected microaggregate-derived non-hydrolyzable silt and clay fractions (NH-μSilt and NH-μClay), and physical-chemically protected microaggregate-derived hydrolyzable silt and clay fractions (H-μSilt and H-μClay).

2.3 Analysis of Microbial Community Composition

The microbial community composition was analyzed on the basis of phospholipid fatty acid (PLFA) analysis (Frostegård et al. 1991). The fatty acids were extracted with 8 g of dry-weight-equivalent fresh soil by using a one-phase extraction mixture containing chloroform/methanol/phosphate buffer (1:2:0.5). The fatty acid methyl ester (FAME) content was analyzed by GC-MS (TRACE GC Ultra ISQ). The individual compounds were identified by comparing their relative retention times to commercially available 37 FAMEs (FAME 37 47885-U, Supelco, Inc.) and a mixture of 26 bacterial FAMEs (BAME 26 47080-U, Supelco, Inc.). The individual compounds were quantified on the basis of an internal standard.

The PLFAs have been used as biomarkers for microbial groups. In this study, we distinguished four microbial groups: Gram-positive [G(+)] bacteria (i14:0, i15:0, a15:0, a16:0, i16:0, a17:0, i17:0), Gram-negative [G(−)] bacteria (16:1ω7c, 18:1ω7c, cy17:0, cy19:0), fungi (18:2ω6c, 18:1ω9c), and actinomycetes (10Me16:0, 10Me17:0, 10Me18:0) (Frostegård et al. 1993; Zelles 1999).

2.4 Soil Extracellular Enzyme Activities

The activities of β-1,4-glucosidase (βG), α-1,4-glucosidase (αG), β-1,4-*N*-acetylglucosaminidase (NAG), L-leucine aminopeptidase (LAP), β-1,4-xylosidase (βX), and cellobiohydrolase (CBH) were measured using the method of Saiya-Cork et al. (2002). The enzyme substrates were used to assess the enzyme activities as follows: the 4-MUF-β-D-glucoside was for βG, 4-MUF-α-D-glucoside for αG, 4-MUF-N-acetyl-β-D-glucosaminide for NAG, L-leucine-7-amino-4-methylcoumarin for LAP, 4-MUF-β-D-xyloside for βX, and 4-MUF-β-D-cellobioside for CBH, respectively. The assays were conducted using 96-well microtiter plates, with eight replicate wells for each sample per assay. Eight replicate wells for each blank, a negative control, and a quench standard were also analyzed. The microplates were incubated at 20 °C for 4 h in the dark and then 10 μL of 1 M NaOH was added to each well to stop the reaction before the fluorescence was measured using a microplate fluorometer (Synergy^{H4}, BioTek) with 365-nm excitation and 450-nm emission filters. The enzyme activities are expressed in units of nmol g^{-1} h^{-1}, following corrections for negative controls and quenching.

2.5 Statistical Analysis

The data for SOM fractions, abundance of microbial groups, and enzyme activities depending on fertilization were analyzed using one-way analysis of variance (ANOVA). The differences were considered significant at $p < 0.05$, and a post hoc least significant difference (LSD) test was performed to compare the differences among the treatments.

The patterns of PLFAs were subjected to a principal component analysis (PCA). Redundancy analysis (RDA) was used to decipher the relationship between the functional SOM fractions and the overall microbial community structure. Variation partitioning analysis (VPA) was used to test the significance of the effects of different soil SOM fractions on the community structure. All the analyses, PCA, RDA, and VPA, were performed with CANOCO version 5.0.

3 Results

3.1 Organic Carbon and Total Nitrogen Content in SOM Functional Fractions

The SOC and total N (TN) contents in cPOM fraction decreased ($p < 0.05$) by 28.9% and 47.9% after NPKM application compared with CK, but that in fPOM fraction remained constant (Figs. 2 and 3). The SOC and TN contents in physically protected fractions (iPOM) increased by 35.8–229% after the combined mineral and manure application (NPKM), compared to the SOC and TN contents after other fertilizers application (Figs. 2 and 3; $p < 0.05$). The manure application alone or in combination with NPK fertilizer (NPKM) increased C and N contents in the biochemically protected NH-dSilt fractions (Figs. 2 and 3; $p < 0.05$). The C and N contents in H-μClay fraction decreased by 41.9% and 51.7% under NPKM addition, respectively (Figs. 2 and 3; $p < 0.05$).

3.2 Soil Microbial Community Structure and Enzyme Activities

The PCA analysis of the PLFA profiles indicated separation among the fertilizer amendments (Fig. 4, permutation test $p < 0.01$). PC1 and PC2 accounted for 80.3% and 11.4% of the total variance, respectively. The abundance of total PLFAs,

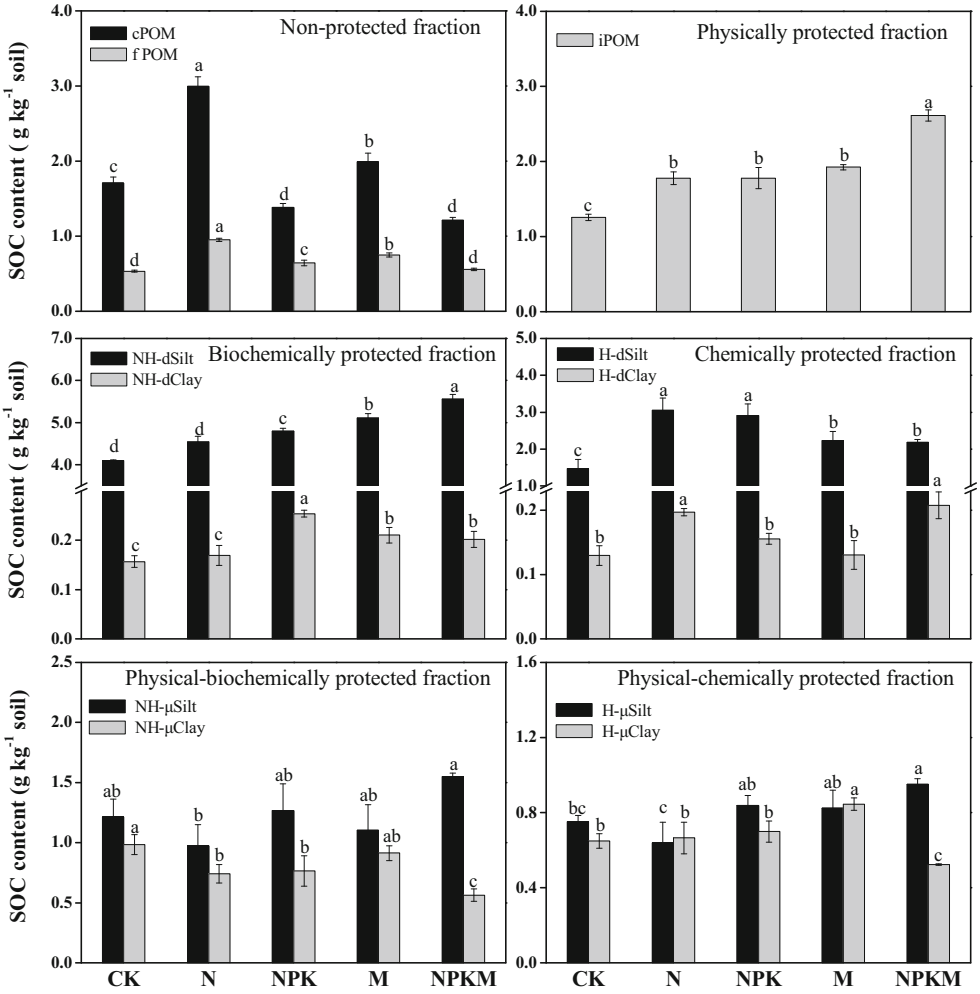

Fig. 2 Carbon (C) content in the functional SOM fractions under long-term fertilization. Letters indicate significant differences among the treatments ($p < 0.05$). Error bars are standard errors ($n = 3$). CK control without fertilizer, N N fertilization, NPK NPK fertilization, M manure application, NPKM combined manure and NPK fertilization

bacteria, fungi, G(+) bacteria, G(−) bacteria, and actinomycetes were respectively 2.05, 1.65, 4.75, 1.25, 2.73, and 7.86 times higher under NPKM than under CK (Table 1, $p < 0.05$). Both branched and monounsaturated PLFAs were highest under NPKM application (Table 1, $p < 0.05$). Compared with CK, the G(+)/G(−) ratio increased after the application of N fertilizer ($p < 0.05$), and declined under NPK, M, and NPKM addition (Table 1). The fungi/bacteria ratio under NPK was higher than that under CK, which varied from 0.075 to 0.12 (Table 1, $p < 0.05$). The ratio of monounsaturated/branched PLFAs (M/B) increased after long-term fertilization, though not statistically significant (Table 1).

The NPKM application led to the highest αG, NAG, and CBH activities (Fig. 5). The mineral N fertilization increased the αG activity (by 224%) compared with CK (Fig. 5; $p < 0.05$). The βG and βx activities remained stable after long-term fertilization (Fig. 5). Higher activities were observed for LAP and CBH under balanced mineral fertilizer (NPK) application, compared to the activities in the cases of individual applications of N and manure (Fig. 5; $p < 0.05$).

3.3 Relationship Between Functional SOM Fractions and Microbial Community Structure

The variation partitioning analysis showed that SOM fractions explained 79.4% of the variance in the microbial community structure (Fig. 6a). The total SOM (SOC, TN) and non-protected fraction (cPOM and iPOM) could only explain 5.5% and 0.4% of the variance, respectively. However, the protected fractions explained 67.6% of the variance (Fig. 6a). We also investigated the correlation between microbial groups and functional SOM fractions by using the RDA (Fig. 6a). The dominant PLFAs on axis1 were i17:0, a16:0, a17:0, 10Me16:0, and i16:0, whereas the dominant PLFAs on axis2 were 18:2ω6c, cy19:0, and 10Me18:0. Bacterial abundance closely correlated with SOC and TN contents in the physically and biochemically protected fractions ($p < 0.05$). The SOC content in the physically and biochemically protected fractions showed the longest projections on the first axis and the greatest effect on soil microbial community structure ($p < 0.01$). Both the SOC and TN contents in the physical-

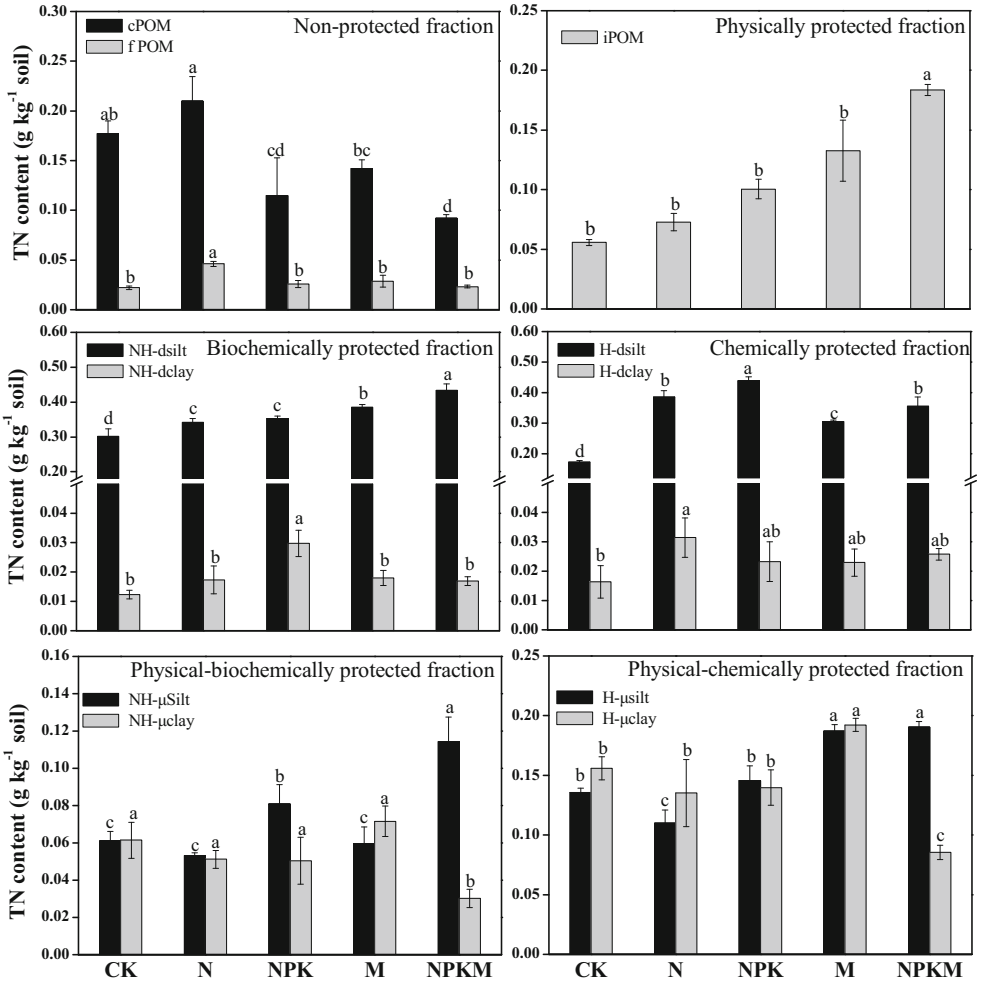

Fig. 3 Nitrogen content in functional SOM fractions under long-term fertilization. Letters indicate significant differences among the treatments ($p < 0.05$). Error bars are standard errors ($n = 3$). CK control without fertilizer, N N fertilization, NPK NPK fertilization, M manure application, NPKM combined manure and NPK fertilization

biochemically protected fractions and TN content in the physical-chemically protected fractions showed the most effect on the soil microbial community structure on axis 2 ($p < 0.01$) (Fig. 6b).

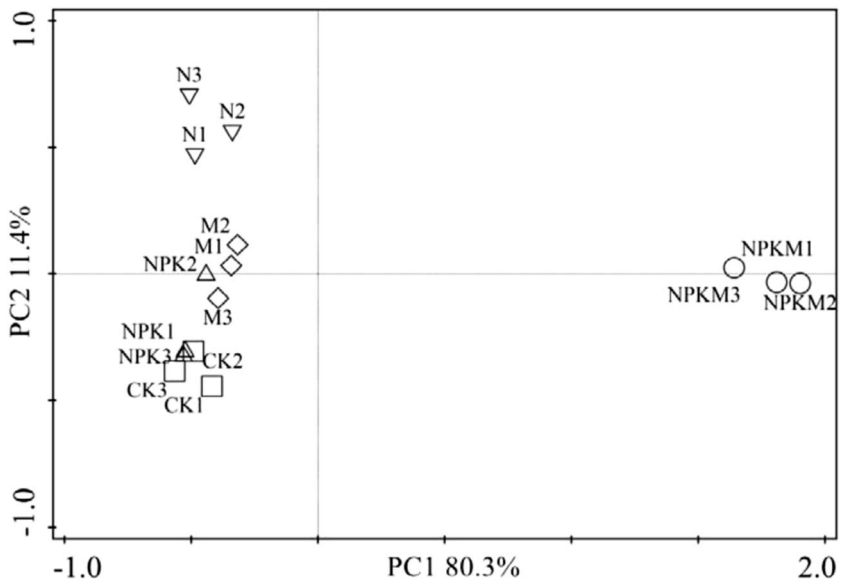

Fig. 4 Principal component analysis (PCA) of compositions of soil microbial communities after long-term fertilization. CK control without fertilizer, N N fertilization, NPK NPK fertilization, M manure application, NPKM combined manure and NPK fertilization

Table 1 Effects of long-term fertilization on the abundance of microbial groups (nmol g^{-1}) and their ratios

Abundances (nmol g^{-1})	CK	N	NPK	M	NPKM
Total PLFAs	1.75 (0.16)^{c}	2.25 (0.18)^{b}	2.18 (0.24)^{b}	2.43 (0.30)^{b}	3.59 (0.05)^{a}
Bacteria	1.61 (0.15)^{b}	1.76 (0.15)^{b}	1.84 (0.15)^{b}	1.93 (0.24)^{b}	2.66 (0.08)^{a}
Fungi	0.08 (0.01)^{b}	0.37 (0.04)^{a}	0.24 (0.10)^{a}	0.31 (0.07)^{a}	0.38 (0.01)^{a}
G(+)	1.15 (0.12)^{b}	1.34 (0.10)^{ab}	1.26 (0.08)^{ab}	1.24 (0.09)^{ab}	1.44 (0.05)^{a}
G(−)	0.45 (0.04)^{bc}	0.32 (0.08)^{c}	0.55 (0.10)^{bc}	0.69 (0.15)^{b}	1.23 (0.03)^{a}
Actinomycetes	0.07 (0.00)^{c}	0.12 (0.00)^{bc}	0.10 (0.00)^{c}	0.19 (0.03)^{b}	0.55 (0.08)^{a}
Monounsaturated PLFAs	0.25 (0.04)^{c}	0.49 (0.11)^{b}	0.34 (0.12)^{bc}	0.54 (0.15)^{b}	0.85 (0.00)^{a}
Branched PLFAs	0.64 (0.08)^{b}	0.71 (0.12)^{b}	0.72 (0.08)^{b}	0.82 (0.03)^{b}	1.27 (0.15)^{a}
F/B	0.05 (0.00)^{b}	0.08 (0.00)^{ab}	0.10 (0.02)^{a}	0.07 (0.01)^{ab}	0.06 (0.01)^{ab}
M/B	0.40 (0.03)^{a}	0.69 (0.17)^{a}	0.46 (0.12)^{a}	0.64 (0.16)^{a}	0.68 (0.07)^{a}
G(+)/G(−)	2.53 (0.05)^{b}	6.98 (1.78)^{a}	2.18 (0.14)^{b}	1.85 (0.27)^{b}	1.39 (0.25)^{b}
Sum (cy/ω7c)	1.60 (0.23)^{a}	0.22 (0.07)^{c}	1.43 (0.46)^{a}	0.72 (0.10)^{b}	0.86 (0.04)^{b}

Letters indicate significant differences among the treatments ($p < 0.05$). Values within the brackets are standard errors ($n = 3$)

CK control without fertilizer, *N* N fertilization, *NPK* NPK fertilization, *M* manure application, *NPKM* combined manure and NPK fertilization

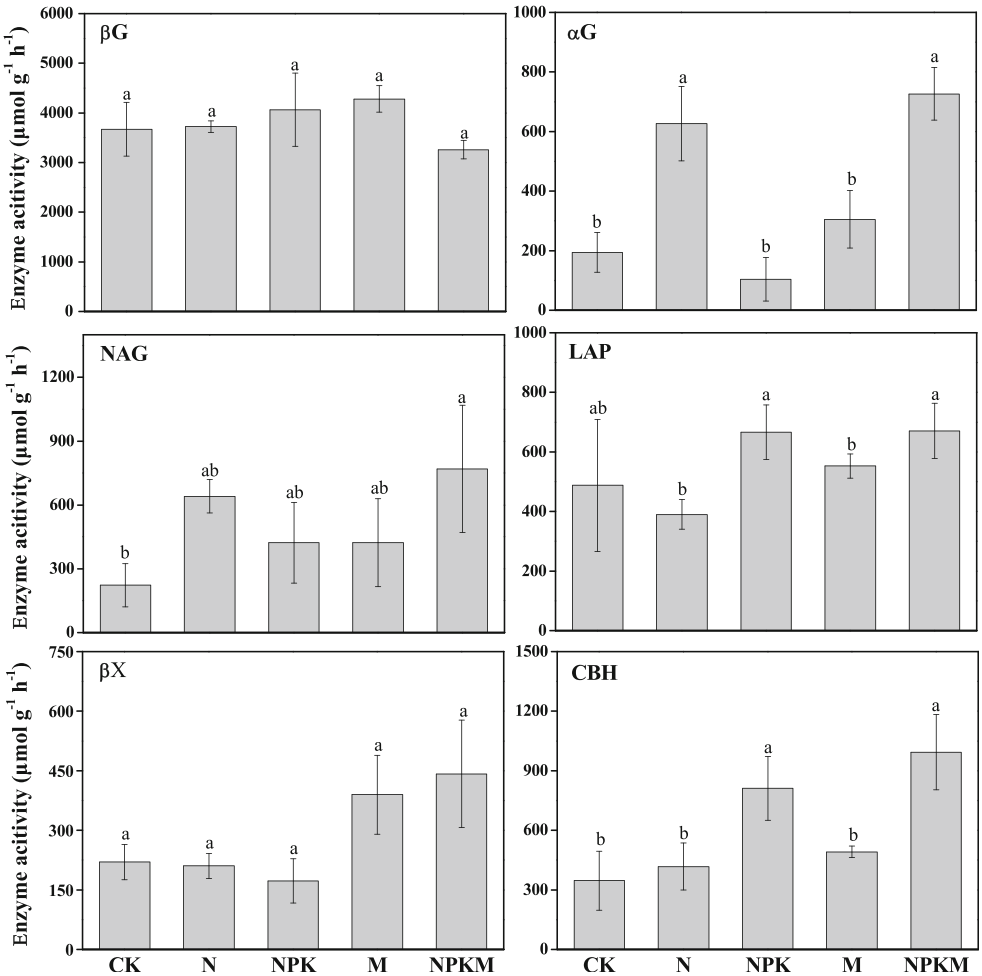

Fig. 5 Soil enzyme activities in response to long-term fertilization. Letters indicate significant differences among the treatments ($p < 0.05$). Error bars are standard errors ($n = 3$). CK control without fertilizer, N N fertilization, NPK NPK fertilization, M manure application, NPKM combined manure and NPK fertilization

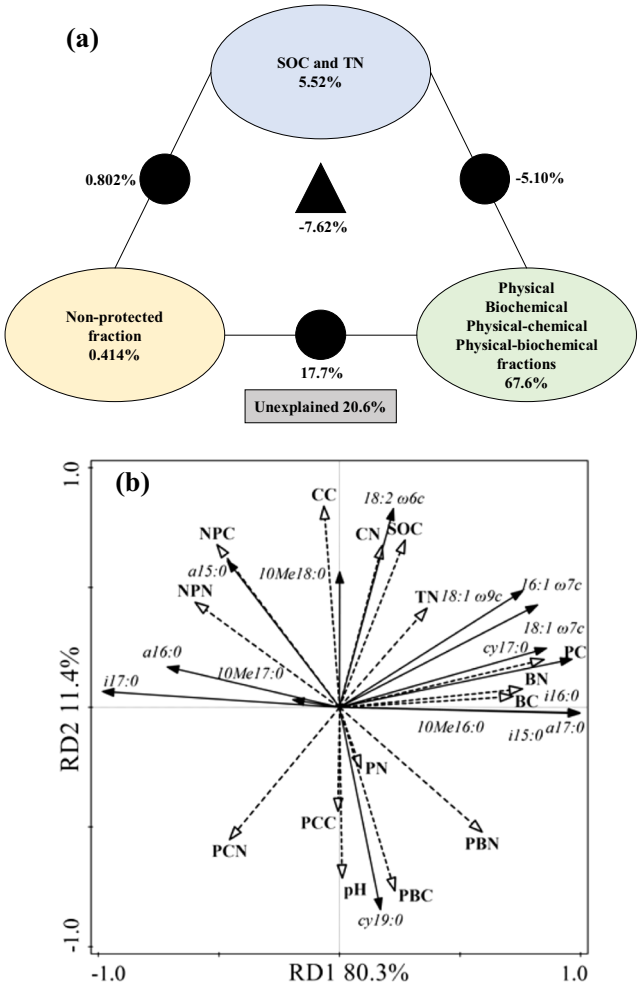

Fig. 6 **a** Variation partitioning analysis (VPA) of microbial community explained by functional SOM fractions and their interactions. **b** Redundancy analysis (RDA) of microbial community composition constrained by the functional SOM fractions after long-term fertilization. Values are percentages explained by the selected factors. SOC soil total organic carbon, TN soil total nitrogen, NPC non-protected soil organic carbon fraction, PC physically protected soil organic carbon fraction, PBC physical-biochemically protected soil organic carbon fraction, PCC physical-chemically protected soil organic carbon fraction, CC chemically protected soil organic carbon fraction, BC biochemically protected soil organic carbon fraction, NPN non-protected soil total nitrogen fraction, PN physically protected soil total nitrogen fraction, PBN physical-biochemically protected soil total nitrogen fraction, PCN physical-chemically protected soil total nitrogen fraction, CN chemically protected soil total nitrogen fraction, BN biochemically protected soil total nitrogen fraction

4 Discussion

4.1 Response of Soil Organic Matter Fractions and Enzyme Activities to Long-Term Fertilization

The combined application of mineral fertilizers and manure (NPKM) increased the SOC and TN contents of physically protected fractions ($p < 0.05$; Figs. 2 and 3). Long-term manure input improves microaggregation and enhances physical protection (Hai et al. 2010), consequently, leading to the decrease in the OM turnover and to the increase of organic carbon content in iPOM. Thus, the "microaggregate-within-macroaggregate fractions" (iPOM in this study) can be applied to diagnose the SOM storage, induced by agriculture management across climate conditions and soil types (Six et al. 2000). The biochemically protected SOM is characterized as a non-hydrolyzable fraction (Six et al. 2000). The SOC content in the biochemically protected fraction (NH-dSilt) increased after long-term application of manure alone or in combination with mineral fertilizers, thus confirming an increase in the stable SOM. In contrast, the SOC and TN contents in the non-protected cPOM fraction showed a declining trend under NPKM, indicating that organic residues decomposed faster after the NPKM application, in accordance with higher biological activity found after NPKM application (Joachim and Meike 2002). The non-protected fractions (cPOM and fPOM) were mainly composed of plant residues that are not closely associated with minerals and are not involved in organo-mineral complexes. The cPOM and fPOM fractions (especially cPOM) are relatively easily decomposable and are clearly depleted during cultivation (Solomon et al. 2002). Compared to microaggregates, cPOM is much more susceptible to mineralization (Six et al. 2002). These observations suggest that the physically and biochemically protected mechanisms play a dominant role in protecting SOM under manure and NPKM conditions. The SOC and TN contents in the biochemically protected fractions (NH-dSilt and NH-dClay) are strongly correlated with the total SOC and TN contents (Plante et al. 2006b). The SOC and TN contents associated with silt and clay particles in the biochemically protected fractions are expected to reach a saturation level, but before saturation, they best fit with a linear model (Stewart et al. 2008).

The SOC and TN contents affect enzyme activities by regulating the microbial community structure and functions. The activities of αG, NAG, and CBH were increased under the NPKM application compared to CK (Fig. 5), indicating that NPKM application mainly stimulated either the C- or N-cycling enzymes. These results are in agreement with the previous study showing that C-cycling enzymes are secreted to degrade the SOM and, thus, enable the microorganisms to acquire adequate C for growth under high N levels (Li et al. 2015). Long-term fertilization exhibited no significant influence on the βG and βx activities in our study (Fig. 5). Presumably, the manure would have provided so much carbon in different degradation stages that downregulation of the C enzymes would be expected. Therefore, this may result in different response of C cycling enzymes. The increased LAP and CBH activities, under balanced mineral fertilizer (NPK) application than under sole N and manure application, indicates that balanced mineral fertilization increased the need for enzyme production. Under low soil N levels, the LAP activity contributed to the acquisition of N by the degradation of

cellulose (Cusack et al. 2011). However, a high N level caused by N fertilization did not result in higher LAP activity (Fig. 5). In spite of this, combined application of mineral and manure fertilizers has been demonstrated to stimulate either the C- or N-cycling enzymes (Zhao et al. 2016).

4.2 Response of Microbial Communities to Long-Term Fertilization and Relationship Between Microbial Communities and Functional SOM Fractions

The effects of long-term NPK fertilization on microbial abundance and community structure were minimal as compared with those in CK, which is in line with the findings of Williams et al. (2013). However, NPKM fertilization influenced the soil microbial community structure and the abundance of G(−) bacteria and actinomycetes ($p < 0.05$; Table 1). This is mainly attributed to the fact that G(−) bacteria and actinomycetes play a critical role in the decomposition of lignocellulose of straw and largely depend on manure (Tang et al. 2014). The M and NPKM application provided adequate available C, leading to a lower ratio of G(+)/G(−) bacteria (Bird et al. 2011). The G(−) bacteria and eukaryotic microbial species are more active in the rhizosphere, but the G(+) bacteria are more abundant in the bulk soil (Lu et al. 2007), suggesting that the G(+) bacteria are well-adapted to soils with low substrate availability. The highest abundance of monounsaturated and branched PLFAs under NPKM suggested that the quantity of aerobic and anaerobic microbes was increased after NPKM addition. In contrast, the ratio of monounsaturated/branched (M/B) PLFAs, an indicator of the proportion of aerobic to anaerobic microorganisms, was independent of fertilization, indicating similar O_2 availability.

The microbial communities were dependent on the SOC and TN contents of the SOM fractions (Fig. 6a, b), which indicated the close relationship between SOM fractions and microorganisms. Generally, the nutrient content in the protected SOM fractions affected the distribution of microbial communities, accounting for 67.6% of the total variance ($p < 0.05$; Fig. 6a). The RDA showed that bacterial abundance labeled by i16:0, a17:0, 16:1 ω7c, cy17:0, and 18:1 ω7c were more sensitive to the physically and biochemically protected fractions (Fig. 6b). The fungal biomarkers (18:1 ω9c, 18:2 ω6c) were more sensitive to the chemically protected fraction. The more recalcitrant material in manure and SOM may increase the competitive ability of G(+) bacteria and actinomycetes, because they are well adapted to soils with low substrate availability (Zhang et al. 2015). Thus, changes in the microbial community structure can be driven by the C availability released by the SOM fractions.

5 Conclusions

Long-term fertilization influenced the functional soil organic matter fractions and microbial communities and activities. The soil organic carbon and total nitrogen contents in microaggregate-protected particulate organic matter (> 53 μm in size), non-hydrolyzable dispersed silt fractions, and non-hydrolyzable dispersed clay fractions were increased by 28.6–229% after long-term combined application of manure and mineral fertilizer addition compared with the content in unfertilized soil (control). The 35 years of fertilization increased the abundance of total phospholipid fatty acids, bacteria, actinomycetes, and soil enzyme activities (α-1,4-glucosidase, β-1,4-*N*-acetylglucosaminidase, β-1,4-xylosidase, cellobiohydrolase). The soil organic carbon content of physically and biochemically protected fractions was closely related to the variation in the bacterial abundance ($p < 0.01$), whereas the soil organic carbon and total nitrogen contents of chemically protected fraction had close relationship with fungal abundance, indicating that long-term fertilization has obvious effects on the soil organic matter functional fractions, which consequently affects the microbial communities.

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