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Responses of soil bacterial community and enzyme activity to organic matter components under long-term fertilization on the Loess Plateau of China

Jun Wang [a,b,*], Xin Fu [b,c], Rajan Ghimire [d], Upendra M. Sainju [e], Yu Jia [f], Fazhu Zhao [a,b]

[a] State Key Laboratory of Soil Erosion and Dryland Farming on the Loess Plateau, Institute of Soil and Water Conservation, Chinese Academy of Sciences and Ministry of Water Resources, Yangling 712100, China
[b] Shaanxi Key Laboratory of Earth Surface System and Environmental Carrying Capacity, College of Urban and Environmental Science, Northwest University, Xi'an 710127, China
[c] College of Resource and Environmental Sciences, Hebei Agricultural University, Baoding 071001, China
[d] Agricultural Science Center, New Mexico State University, 2346 State Road 288, Clovis, NM 88101, USA
[e] USDA-ARS, Northern Plains Agricultural Research Laboratory, 1500 North Central Avenue, Sidney, MT 59270, USA
[f] Department of Natural Resource Sciences, Macdonald Campus of McGill University, Ste-Anne-de-Bellevue, Quebec H9X 3V9, Canada

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ABSTRACT

Soil bacterial community structure, enzyme activities and their relationships to soil carbon and nitrogen in response to long-term fertilization remain poorly understood. Therefore, the objective of this study was to evaluate soil carbon and nitrogen fractions, enzyme activity, and bacterial community structure at 0–15, 15–30, and 30–60 cm depths after 34-yr of continuous application of manure and inorganic fertilizers. The study had a randomized complete block design with six treatments and three replications. Treatments were inorganic nitrogen fertilizer only (N), nitrogen plus phosphrous fertilizers (NP), manure (M), nitrogen plus manure (NM), nitrogen plus phosphorus plus manure (NPM), and unfertilized control (CK) in a winter wheat (*Triticum aestivum* L.) monoculture system. Most soil carbon and nitrogen fractions at 0–15 and 15–30 cm were greater with M, NM, and NPM, and winter wheat yield was greater with NPM than other treatments. The NPM increased β-glucosidase, β-xylosidase, and β-*N*-acetylglucosidase compared to other treatments at all depths. Soil bacterial Shannon index was similar among treatments at 0–15 and 15–30 cm and lower in N and NP than other treatments at 30–60 cm. Compared to CK, inorganic and manure fertilization increased relative abundances of *Gemmatimonadetes* and *Bacteroidetes* but decreased those of *Nitrospirae*, *Planctomycetes,* and *Latescibacteria*. Increases in soil enzyme activities and bacterial communities after long-term application of inorganic N and P fertilizers and manure was related to increased substrate availability. Overall, a combination of chemical fertilizers and manure can enhance soil health and quality through increased soil organic matter component, enzyme activity, and bacterial abundance.

1. Introduction

Inorganic fertilizers have been widely used to improve soil fertility and crop yields across the world. However, long-term fertilizer application has become a significant environmental challenge because it increased soil acidity, nutrient runoff and leaching, and greenhouse gas emissions (Liu et al., 2013; Dou et al., 2016). The overuse of inorganic fertilizers in intensive agriculture is often associated with low nutrient use efficiency and high off-site environmental impacts (Fan et al., 2012). Studies show that excess nitrogen fertilizer that can not be retained in soil or utilized by a crop is lost to the atmosphere as NH_3, N_2O, NO_x, and N_2, or lost through leaching and runoff as NO_3^--N (Robertson et al., 2013). Although inorganic fertilizers increase the labile fractions of soil organic matter in short-term (Brar et al., 2013; Sainju et al., 2000), long-term soil carbon storage may decrease due to the increased microbial activity and carbon mineralization (Manna et al., 2007; Jiang et al.,

* Corresponding author at: State Key Laboratory of Soil Erosion and Dryland Farming on the Loess Plateau, Institute of Soil and Water Conservation, Chinese Academy of Sciences and Ministry of Water Resources, Yangling 712100, China.
E-mail address: wangj@nwu.edu.cn (J. Wang).

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2014). Studies also reported neutral or adverse effects of inorganic fertilizers on microbial biomass and functional diversity (Lupwayi et al., 2012; Wang et al., 2019).

Compared to inorganic fertilizers, manure and compost applications can increase soil carbon and nitrogen sequestration (Guo et al., 2015) and lead to increased microbial biomass, bacterial abundance, and enzyme activities (Böhme et al., 2005; Ge et al., 2010; Pauline et al., 2011; Zhang et al., 2015; Gai et al., 2018). The addition of organic residues such as green manure, compost, and biosolids application maintained a higher soil carbon level than a control system that involved no organic residue addition (Ghimire et al., 2018; Wuest and Gollany, 2013). Compost addition also increased the microbial biomass, abundance of cultivable microorganisms, soil respiration, and enzyme activities (Zhen et al., 2014). Using manure and compost alone may not produce sustainable high crop yields. Therefore, in subsistence agriculture in developing countries that relies on small landholding and involves crop-livestock integrated systems, inorganic fertilizers are often applied to supplement the compost. Combining organic and inorganic fertilizers improve soil fertility and increase crop yields and soil extracellular enzyme activities compared to inorganic fertilizer alone (Cai and Qin, 2006; Miao et al., 2019). However, there is inconsistency in response to fertilizer and compost application because of inherent variability in the nutrient content of composts.

Long-term fertilization experiments are established to evaluate the agronomic and environmental benefits of fertilizer management. For example, a long-term N fertilization study reported the benefits of N addition on nutrient use and conservation, soil C accumulation, and crop production (Khan et al., 2007). According to Brar et al. (2013), the addition of manure and inorganic fertilizers can enhance soil C fractions and microbial community up to a 60 cm depth. Crop root depth can exceed 1 m, and root residue and exudates can enhance microbial community structure and functions throughout the soil profile. However, most of the studies discussing the influence of fertility management practices on soil microbial community, enzyme activities, and nutrient cycling are limited to the surface soil layer (Coonan et al., 2019). Comparing soil carobon and nitrogen in surface and sub-surface soils, Ghimire et al. (2018) highlighted the role of subsoil fertility in improving crop yields. Information on the impact of fertilization strategy on microbial dynamics at subsurface layers will further advance our understanding of subsoil carbon and nutrients cycling.

This study aimed to evaluate the impacts of long-term fertilization on soil health, specifically, soil bacterial community responses, enzyme activities, and organic matter components on the Loess Plateau of China. We measured soil carbon and nitrogen fractions, enzyme activities, and bacterial community composition at 0–15, 15–30, and 30–60 cm soil depths after 34-yr annual application of inorganic fertilizers, organic manure, and the combination of them under winter wheat monoculture system. A mixture of manure and inorganic fertilizers was expected to enhance soil carbon and nitrogen fractions, enzyme activity, and bacterial abundance compared to those under inorganic fertilizers alone or the control without fertilization.

2. Materials and methods

2.1. Site description

A long-term field experiment was established in 1984 at the Shaanxi Changwu Agro-Ecological Station of China (107°44.70′E, 35°12.79 N), aiming to test the effects of fertilization and rotation on soil fertility and crop production under dryland cropping systems (Guo et al., 2008). The site has a sub-humid temperate climate, and the mean annual temperature and precipitation were 9.1 °C and 584 mm, respectively. The soil is Heilutu silt loam (Calcarid Regosol, *FAO World Reference Base Soil Classification System*, n.d.) with sand, silt, and clay contents of 4.5%, 65.6%, and 30.9%, respectively, a pH (H_2O) of 8.4 and a bulk density of 1.3 g cm^{-3}. The contents of organic carbon, total nitrogen, and $CaCO_3$ at 0–30 cm depth were 6.5 g kg^{-1}, 0.80 g kg^{-1}, and 105 g kg^{-1}, respectively, at the beginning of the experiment in 1984.

2.2. Experimental design and treatments

Details of the long-term field experiment were described by Guo et al. (2008). In this study, six treatments as (1) CK, the control with no fertilizer; (2) N, inorganic nitrogen fertilizer; (3) NP, inorganic nitrogen + inorganic phosphorusfertilizers; (4) M, organic manure; (5) NM, inorganic nitrogen fertilizer + organic manure; (6) NPM, inorganic nitrogen + inorganic phosphorus fertilizers + organic manure) under winter monoculture system were arranged in a randomized block design and replicated three times. The plot size was 10.3 × 6.5 m with a strip of 0.5 m. Urea (46% N) was applied to winter wheat at a 120 kg N ha^{-1} rate for the treatments that included inorganic nitrogen fertilizer and triple superphosphate (20% P_2O_5) of 17 kg P ha^{-1} for those included inorganic phosphorus fertilizer. Cattle manure (2205 kg ha^{-1}) with 800 kg C ha^{-1}, 87 kg N ha^{-1}, and 44 kg P ha^{-1} was applied each year for manure treatments. All inorganic fertilizers and organic manure were surface broadcast and plowed into the soil at a depth of 15 cm at wheat planting in late September each year. No potassic fertilizer was added due to high soil potassium content. Winter wheat was sown at a rate of 180 kg seeds ha^{-1} with 20 cm row-spacing. Weed control was done by hand-weeding as needed. Wheat was harvested in late June each year.

2.3. Soil sampling

Soil samples were collected from 0 to 15, 15–30, and 30–60 cm depths after wheat harvest in June 2018. Ten cores with a diameter of 2.5 cm were sampled randomly from each plot, separated into three depths, composited by the depth, and brought to the laboratory in a cooler on ice. In the laboratory, all the root pieces, rocks, and other foreign materials were removed, soil samples were sieved through a 2.0-mm mesh. Approximately 200 g air-dried subsamples were separated for soil carbon and nitrogen fractions analyses. The rest of the samples were stored in a -80 °C freezer for soil enzyme and microbial analysis.

2.4. Soil carbon and nitrogen fraction analyses

Soil organic carbon (SOC) and total nitrogen (STN) were measured by dry combustion method using an element analyzer (Euro Vector EA3000, Manzoni, Italy). Soil samples SOC and STN analysis were ground to < 0.15 mm and pretreated with 1.0 mol L^{-1} HCl to remove inorganic carbon. Particulate organic carbon and nitrogen (POC and PON) were measured by Cambardella and Elliott (1992) method by dispersing 10 g soil with 30 mL of 5 g L^{-1} sodium hexametaphosphate and passing through a 0.053 mm sieve. The solution and particles that passed through the sieve were oven-dried at 50 °C for 3–4 d. The POC or PON concentrations were calculated by the difference between SOC or STN concentrations in the whole-soil and the particles that passed through the sieve after sand correction.

The potential carbon and nitrogen mineralization (PCM and PNM) were measured using the method modified by Haney et al. (2004). Briefly, two 10 g soil samples were moistened to 50% field capacity with water, then placed in a 1 L jar containing beakers with 4 mL of 0.5 mol L^{-1} NaOH to trap evolved CO_2 and 20 mL water to maintain humidity. Soil samples in the jar were incubated at 21 °C for 10 d. The beaker containing NaOH was taken out from the jar after 10 d, and CO_2 absorbed in NaOH was measured by reverse titration with 1.5 mol L^{-1} $BaCl_2$ and 0.1 mol L^{-1} HCl. One beaker with the soil was taken out and extracted with 50 mL of 2 mol L^{-1} KCl for one hour. Using the modified Griess–Illosvay method, the concentrations of NH_4^+-N and NO_3^--N in the extract were determined with an autoanalyzer (CleverChem380, DeChem-Tech, Hamburg, Germany). The difference between the sum of NH_4^+-N and NO_3^--N concentrations before and after incubation was calculated as the PNM. The other container with moist soil was used for

measuring microbial biomass carbon and nitrogen (MBC and MBN) concentrations (Franzluebbers et al., 1995). The moist soil was fumigated with ethanol-free chloroform for 24 h and incubated for 10 d at 21 °C followed by incubation as for PCM measurement. The amount of CO_2-C absorbed in NaOH was divided by a factor of 0.41 without subtracting the values from the non-fumigated control to calculate the MBC concentration. The concentrations of NH_4^+-N and NO_3^--Nin the soil were measured using the autoanalyzer as above. The difference between the sum of NH_4^+-N and NO_3^--N concentrations in the sample before and after fumigation–incubation was divided by a factor of 0.41 to calculate MBN. The NH_4^+-N and NO_3^--N concentrations measured in the unfumigated-unincubated samples were considered as available nitrogen fractions.

2.5. Soil enzyme and microbial analyses

Frozen soil samples were incubated at 4 °C for one week to measure soil enzyme activities and microbial community structures. Activities of four enzymes (β-glucosidase, β-xylosidase, cellobiohydrolase, and β-*N*-acetylglucosaminidase) were measured as described by Saiya-Cork et al. (2002). Briefly, 1 g of soil was suspended in 125 mL sodium acetate buffer at a pH of 5.0 and blended at the highest speed for 1 min to make a slurry. About 200 L soil slurries were added to 96-well microplates, and then 50 μL of 200 μmol L^{-1} specific fluorometric substrate proxies were added to each sample well. Each sample was assayed in three replicate wells. Three replicated wells for blank, a negative control, and a quench standard were also included in the assay. The microplates were incubated in the dark at 25 °C for 4 h, then a 10 μL aliquot of 1 mol L^{-1} NaOH was added to each well to stop the reaction. Fluorescence was measured using a microplate fluorimeter (SynergyH4 Hybrid Reader, SynergyH4 BioTek, USA) with 365-nm excitation and 450-nm emission filters. The unit of enzyme activities was corrected as μmol h^{-1} g^{-1} dry soil.

Soil total DNA was extracted in triplicate from 0.5 g of frozen-dried soil using the Power Soil DNA Isolation Kit (MP Biomedical, Carlsbad, CA, USA) as described by the manufacturers' protocol. The quality and size of the extracted DNAs were checked by electrophoresis on a 1.0% agarose. The extracted soil DNA was stored at −80 °C until PCR amplification and analysis.The relative abundance of bacterial 16rRNA genes was quantified using the method reported by Caporaso et al. (2010). Using primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3′) and 907R (5'-GGACTACVSGGGTATCTAAT-3′), the V3-V4 fragment of bacterial 16rRNA genes were amplified. PCRs were conducted in the solution with 0.4 μL of two primers, 0.4 μL of FastPfu polymerase, and 10 ng of 1.25 μL template DNA. The thermal program, 98 °C for 2 min, 25 cycles of denaturation at 98 °C for 15 s, annealing at 55 °C for 30 s, and 72 °C for 30 s, followed by a final extension at 72 °C for 5 min was used to amplify samples. Each sample was amplified three times, and the amplicons were mixed to provide a final PCR product. PCR amplicons were extracted from 2% agarose gels and purified using the Power Soil DNA Isolation Kit (MP Biomedicals, Santa Ana, CA, USA).

The sequencing data were demultiplexed, quality-filtered, and processed using the Quantitative Insights into Microbial Ecology (QIME, v1.8.0) pipeline (Caporaso et al., 2010). Raw flowgrams were filtered and eliminated noise using UCHIME (v5.2.236), and the operation taxonomic units (OTUs) were clustered with less than 3% dissimilarity to remove low quality and polyclonal sequences (Edgar et al., 2011). Bacterial community Alpha diversity indices was calculated as estimated community diversity by the Shannon index using the Mothur software (v.1.30.1). The raw sequences were deposited in the Sequence Read Archive Database of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) with an accession number of SRP291266.

2.6. Statistical analysis

Data on soil carbon and nitrogen fractions, enzyme activities, bacterial community, and winter wheat yield were tested for distribution using the Shapiro-Wilk test, and all the variables were in a normal distribution. Differences in each variable among fertilization treatment were analyzed using a two-way analysis of variance (SPAA Statistics). Fertilization (main-plot treatment) and soil depth (split-plot treatment) were considered fixed effects for analysis of soil variables, and fertilization and year were fixed effects for the analysis of winter wheat yield data. The replication was a random effect in both analyses. Duncan's multiple range test was used to separate means when treatment and interaction effects were significant at $P \leq 0.05$ levels. Based on the relative abundance, principal coordinate analysis (PCoA) was used to explore the soil bacterial community composition. Correlations between soil enzyme activity and carbon and nitrogen fractions were analyzed using correlation analysis. Using CANOCO software, the redundancy analysis (RDA) was conducted to determine the multiple variations of correlation between enzyme activities, bacterial community, and soil carbon and nitrogen fractions (Braak and ter Smilauer, 2002).

3. Results

3.1. Soil carbon and nitrogen fractions and crop yield

Soil carbon and nitrogen fractions were significant with treatments, soil depth, and treatment × depth interaction. At 0–15 cm, SOC, MBC, PCM, STN, PNM, and NH_4^+-N were greater with M, NM, and NPM than CK and N (Table 1). The POC and PON were greater with M than NP and CK. The MBN was greater with NM and NPM than other treatments, and NO_3^--N was greater with NP than N and CK. At 15–30 cm, SOC, POC, PCM, STN, PON, and NO_3^--N were greater with NM and NPM than most other treatments. The MBC was greater with M and NPM than other treatments, and MBN and NH_4^+-N were greater with NPM than NP. At 30–60 cm, SOC, POC, and MBN were greater with NPM than CK; and MBC and PON were greater with M, NM, and NP than CK and N.

Wheat grain and aboveground biomass yields varied with treatments and year significantly, with a range of 1.50–4.28 and 3.45–9.20 Mg ha^{-1}, respectively. Average across years, grain and aboveground yields were greater with NPM and NM than other treatments, and with NP than M (Data not shown). The CK and N treatments had lower grain and aboveground biomass yields than other treatments.

3.2. Soil enzyme activities and their responses to C and N fractions

Similar to soil C and N fractions, soil enzyme activity also varied with treatments, soil depths, and their interaction (Table 2). At 0–15 cm, activities of β-glucosidase and β-xylosidase were greater with NPM than other treatments. The activity of Cellobiohydrolase was greater with NP and NM, and that of β-*N*-acetylglucosidase greater with NM than other treatments. At 15–30 cm, activities of β-glucosidase, β-xylosidase, and Cellobiohydrolase were greater with NPM than other treatments, and that of β-*N*-acetylglucosidase greater with NP, NM, and NPM than other treatments. At 30–60 cm, β-glucosidase, β-xylosidase, and β-*N*-acetylglucosidase activities were greater with NPM, M, and NM than other treatments. Cellobiohydrolase activity was greater with CK, N, and M.

The redundancy analysis showed that enzyme activities had significant relationships with soil carbon and nitrogen fractions at all depths (Fig. 1). At 0–15 cm, β-glucosidase and β-*N*-acetylglucosidase activities were positively related to all fractions, except POC, PON, and NO_3^--N, and Cellobiohydrolase activity was positively related to NO_3^--N ($P < 0.05$). At 15–30 cm, β-glucosidase, β-xylosidase, and Cellobiohydrolase activities increased with all carbon and nitrogen fractions except PNM. The β-*N*-acetylglucosidase activity increased with increasing PCM and NO_3^--N but decreased with increasing PNM. The β-*N*-acetylglucosidase activity was positively related to PON and MBC but negatively to PNM and PCM. At 30–60 cm, β-glucosidase was positively related to POC and MBN, β-xylosidase to MBN and PON, and β-*N*-acetylglucosidase to PON and MBC. However, activities of β-glucosidase, β-xylosidase, and β-*N*-acetylglucosidase were negatively related to NO_3^--N.

Table 1
Soilcarbon and nitrogen fractions under different fertilizer treatments at 0–15, 15–30, and 30–60 cm soil depths.

Soil depth	Treatment[a]	Soil C fractions[b]				Soil N fractions[b]					
		SOC	POC	PCM	MBC	STN	PON	PNM	MBN	NO_3^--N	NH_4^+-N
		g kg^{-1}		mg kg^{-1}		g kg^{-1}		mg kg^{-1}			
0–15 cm	CK	7.20c[c]	2.09b	184b	223c	1.01bc	0.327b	18.0c	7.86c	1.83bc	12.2b
	N	7.62c	3.54ab	216b	246c	0.910c	0.468ab	19.7bc	10.9b	1.58c	12.7b
	NP	9.07b	1.09c	292a	259c	1.07b	0.334b	21.9ab	12.8b	2.14a	12.5b
	M	10.6a	3.18a	310a	355ab	1.30a	0.593a	23.6a	12.2b	1.94ab	15.1a
	NM	11.4a	2.64ab	320a	327b	1.38a	0.510ab	23.7a	18.0a	1.99ab	15.3a
	NPM	11.4a	3.12a	300a	386a	1.33a	0.505ab	24.0a	18.3a	1.96ab	16.1a
15–30 cm	CK	5.76c	0.807b	194c	189c	0.703c	0.350c	20.8a	12.7c	1.39c	10.8b
	N	5.93c	0.879b	182c	189c	0.780c	0.371bc	20.8a	16.6abc	1.63b	13.5ab
	NP	6.58bc	0.891b	238b	183c	0.900b	0.417abc	19.6a	14.3bc	1.76b	12.7b
	M	7.71ab	1.86a	247b	293a	0.937b	0.452ab	19.7a	14.5bc	1.69b	13.6ab
	NM	8.51a	2.01a	281a	224b	1.11a	0.484a	20.8a	18.9ab	2.00a	13.4ab
	NPM	8.47a	1.62a	259ab	276a	1.10a	0.475a	19.7a	19.7a	1.98a	16.0a
30–60 cm	CK	4.52b	0.083b	257a	181b	0.577b	0.130b	20.9a	18.1d	1.83a	10.7a
	N	5.16a	0.094b	258a	157b	0.657ab	0.162b	20.8a	18.1d	1.78a	9.85a
	NP	4.96ab	0.146b	261a	222a	0.560b	0.304a	21.7a	18.7cd	1.78a	10.3a
	M	5.24a	0.224b	228a	241a	0.737a	0.311a	19.2ab	21.2b	1.69a	11.3a
	NM	4.83ab	0.186b	228a	260a	0.723a	0.350a	17.8b	20.2bc	1.83a	10.2a
	NPM	5.19a	0.586a	252a	257a	0.720a	0.349a	20.4ab	24.0a	1.56a	10.6a

[a] Treatments are CK – control, N - nitrogen fertilizer, NP - nitrogen plus phosphorus fertilizer, M – manure, NM - nitrogen fertilizer plus manure, and NPM - nitrogen, phosphorus fertilizer plus manure.
[b] Soil carbon and nitrogen fractions are SOC - soil organic C, POC - particulate organic carbon, PCM - potential C mineralization, MBC - microbial biomass carbon, STN - soil total nitrogen, PON - particulate organic nitrogen, PNM - potential N mineralization, MBN - microbial biomass nitrogen.
[c] Values within a column and soil depth followed by the same letter are not significantly different at $P \leq 0.05$.

Table 2
Soil enzyme activities under different fertilizer treatments at 0–15, 15–30, and 30–60 cm soil depths.

Soil depth	Treatment[a]	β-Glucosidase	Cellobiohydro-lase	β-xylosidase	β-*N*-acetylgluco-sidase
		μmol h^{-1} g^{-1} dry soil			
0–15 cm	CK	1.56e[b]	262cd	698d	56.4d
	N	2.15d	228d	981bc	54.1d
	NP	2.92b	370a	904bc	66.9d
	M	2.97b	300b	848cd	105c
	NM	2.52c	337a	1030b	151a
	NPM	4.19a	271bc	1420a	129b
15–30 cm	CK	0.75e	40.2e	266e	33.4c
	N	1.15d	61.9de	331e	34.4c
	NP	1.27d	82.1cd	765c	92.6a
	M	1.77c	103c	473d	52.9b
	NM	2.43b	142b	860b	78.3a
	NPM	2.78a	211a	986a	79.9a
30–60 cm	CK	0.0604c	78.6a	26.8d	6.38b
	N	0.0685c	82.8a	40.1c	9.19b
	NP	0.0752c	44.6b	52.7b	8.83b
	M	0.120b	84.5a	62.1a	7.28b
	NM	0.122b	29.5b	46.7bc	2.26a
	NPM	2.52a	44.3b	62.4a	2.22a

[a] Treatments are CK – control, N - nitrogen fertilizer, NP - nitrogen plus phosphorus fertilizer, M – manure, NM - nitrogen fertilizer plus manure, and NPM - nitrogen, phosphorus fertilizer plus manure.
[b] Values within a column and soil depth followed by the same letter are not significantly different at $P \leq 0.05$.

3.3. Soil bacterial communities and their responses to C and N fractions

Overall, 3,842,739 quality sequences of 430 to 444 bp length based on OTUs were obtained across all soil samples. Shannon index showed no significant difference in bacterial α-diversity among treatments at 0–15 and 15-30 cm depths (Fig. 2). However, N and NP treatments had lower bacterial α-diversity than other treatments at 30–60 cm depth. The principal coordinate analysis (Fig. 3) showed significant variations in soil bacterial communities among treatments and soil depths. At 0–15 cm, bacterial community structure was similar for all manure applied treatments (M, NM, and NPM), which was distinctly separated from N, NP, and CK (Fig. 3a). At 15–30 cm, the bacterial community was similar for N, NP, NM, and NPM, but separated from N and CK (Fig. 3b), while at 30–60 cm, the bacterial communities for NM and M were separated from NPM, N, and CK (Fig. 3c). Soil bacterial community was similar among soil depths at 0–15 and 15–30 cm but distinct at 30–60 cm (Fig. 3d).

The most dominant bacterial phyla were *Proteobacteria*, *Acidobacteria,* and *Actinobacteria* in all soil depths, accounting for 18.8–35.6%, 16.5–20.5%, 12.5–17.4% of the total bacterial sequence, respectively. Chloroflexi, Gemmatimonadetes, and *Nitrospirae* followed these with contributions of 9–16%, 8–12%, and 3–9%, respectively. The relative abundance of bacterial phyla varied with treatment × soil depth interaction (Table 3). At 0–15 cm, the relative abundance of *Proteobacteria* was greater with NP than CK. The *Chloroflexi* and *Firmicutes* were greater with N than M and *Gemmatimonadetes* greater with M than CK, N, and NP, while *Nitrospirae*, *Latescibacteria, GAL15*, and *Planctomycetes* were higher with CK than NP, NM, and NPM. At 15–30 cm, the relative abundance of *Proteobacteria* was greater with NPM than CK and

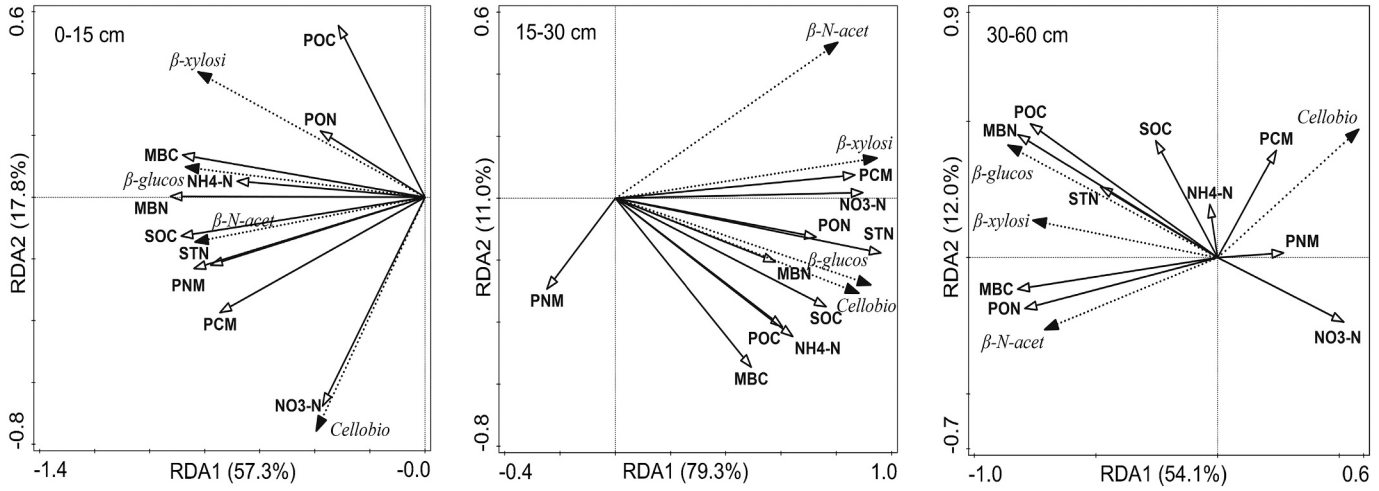

Fig. 1. Redundancy analysis (RDA) for relationships among the soil enzyme activities (dotted lines) and soil carbon and nitrogen fractions (solid lines). Soil enzyme activities are *β-glucos* - *β-glucosidase*, *Cellobio* - *cellobiohydrolase*, *β-xylosi* - β-xylosidase, and *β-N-acet* - *β-N-acetylglucosidase*. Soil carbon and nitrogen fractions are SOC - soil organic carbon, POC - particulate organic carbon, PCM - potential carbon mineralization, MBC - microbial biomass carbon, STN - soil total nitrogen, PON - particulate organic nitrogen, PNM - potential N mineralization, MBN - microbial biomass nitrogen.

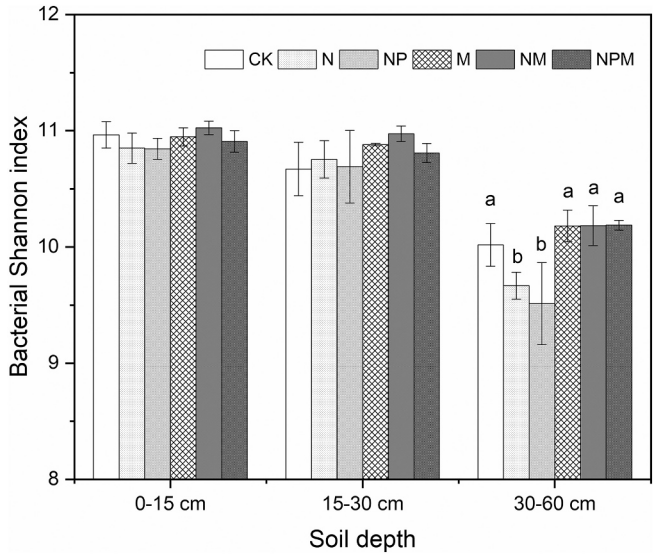

Fig. 2. Soil bacterial diversity (Shannon index) after 34-yr fertilizer application.

N, *Actinobacteria* greater with NM than NP and M, and *Chloroflexi, Nitrospirae*, *Latescibacteria, GAL15, and Firmicutes* greater with CK than N, M, NP, NM, or NPM. The *Gemmatimonadetes* and *Planctomycetes* were greater with M than CK, NM, or NPM. At 30–60 cm, the abundances of *Proteobacteria* and *Bacteroidetes* were greater with NP and NPM than CK. The *Acidobacteria, Nitrospirae*, *Planctomycetes,* and *Latescibacteria* were greater with CK than NP, NPM, M, or NM, *Actinobacteria* and *Firmicutes* greater with NM than NP, *Gemmatimonadetes* greater with M, NM, and NPM than other treatments, and *GAL15* greater with N than M, NM, and NPM.

Redundancy analysis showed that relative abundances of *Proteobacteria, Acidobacteria, Gemmatimonadetes*, and *Bacteroidetes* were positively, and those of Actinobacteria, Chloroflexi, Nitrospirae, *Planctomycetes*, *Latescibacteria,* and *GAL15* were negatively related to most soil carbon and nitrogen fractions at 0–15 cm (Fig. 4). At 15–30 cm, the relative abundances of *Proteobacteria*, *Actinobacteria*, *Gemmatimonadetes,* and *Bacteroidetes* were related to all carbon and nitrogen fractions positively, except PNM. In contrast, the relative abundances of other bacterial phyla were negatively related to soil carbon and nitrogen fractions. At 30–60 cm, the relative abundances of *Proteobacteria*, *Actinobacteria*, *Gemmatimonadetes*, *Bacteroidetes,* and *Firmicutes* were related to all carbon and nitrogen fractions positively, except PCM, PNM, and NO_3^--N.

4. Discussion

4.1. Long-term fertilization effect on soil organic carbon and nitrogen

Maintenance of soil organic carbon and nutrients in agroecosystems depends on carbon and nitrogen inputs and soil biogeochemical cycling. This study revealed an increase in soil carbon and nitrogen storage with manure application because they enhance SOC, STN, and POC. Manure alone or in combination with inorganic fertilizers increased soil carbon and nitrogen fractions compared to inorganic fertilizer alone, possibly due to increased organic matter inputs from organic manure. Organic manures have several macro and micronutrients needed for plant and microbial communities regulating soil carbon and nitrogen cycling. The absence of nutrient inputs through chemical fertilizers and manure, in contrast, reduced soil carbon and nitrogen fractions (Table 1). The exceptions were NO_3^--N at 0–15 cm, PNM at 15–30 cm, and PCM, PNM, NO_3^--N, and NH_4^+-N at 30–60 cm with inconsistent responses to chemical and organic sources of nutrients. The differences in the response of these parameters may be associated with the relative rate of nutrient release from manure and inorganic fertilizers. Application of inorganic fertilizers enhanced some labile fractions, such as PNM, MBC, PON, and NO_3^--N. Studies found an increase in MBC and MBN (Geisseler and Scow, 2014; Zhang et al., 2017), as well as total soil carbon and nitrogen storage, with organic manure compared to inorganic fertilizers or the no fertilizer control (Giacometti et al., 2013; Gai et al., 2018). However, this does not apply when carbon and nitrogen loss from soil or in crop harvest is not compensated by the inputs from crop residues, soil amendments, and nutrients. Inorganic fertilizer application can reduce SOC and STN compared to the control (e.g., Belay et al., 2002; Qiu et al., 2016). Regardless of treatments, the decrease in soil carbon and nitrogen fractions with depth was probably due to reduced root growth and lower organic matter inputs from the root residue in lower depths. Manure was applied in topsoils. The bulk of root biomass (>50%) in cereal crops is also concentrated in 0–20 cm depth (Fan et al., 2016), contributing to organic matter accumulation in surface soils.

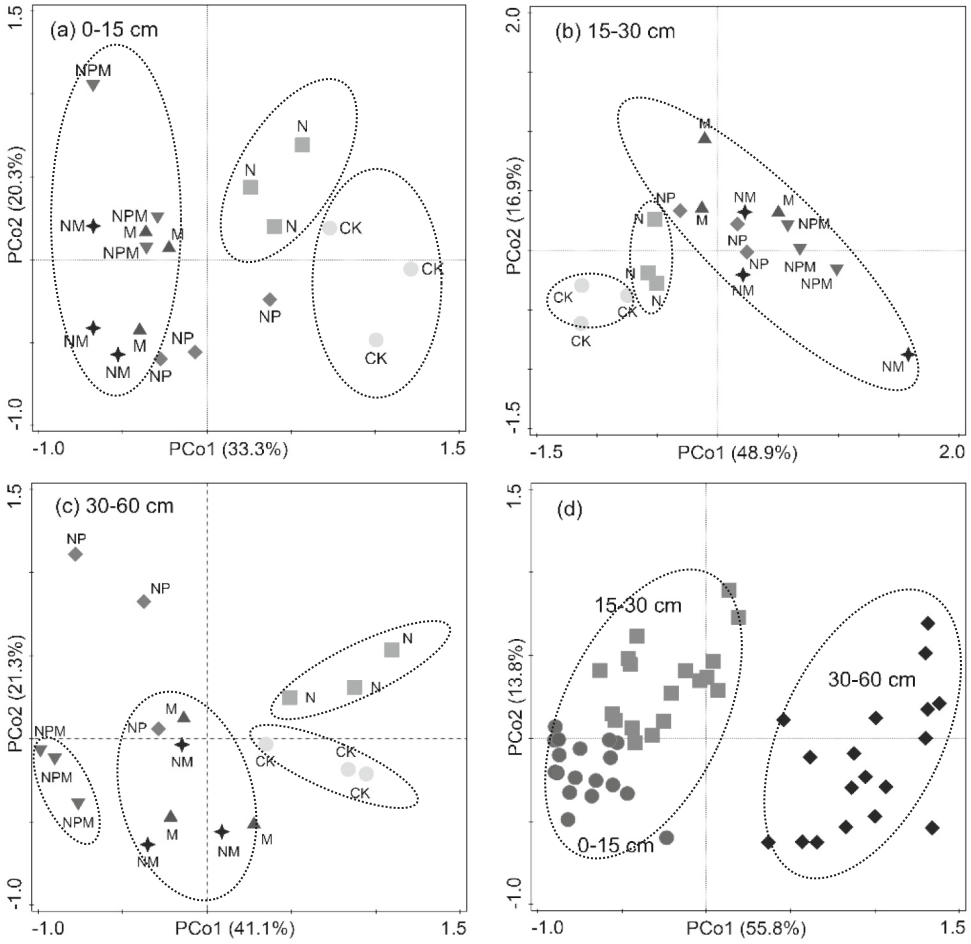

Fig. 3. Principal coordinates analysis (PCoA) of soil bacterial community composition under different fertilizer treatments based on Bray-Curtis distances at the depths of 0–15 cm (a), 15–30 cm (b), 30–60 cm (c), and across soil depths (d). Treatments are CK – control, N - nitrogen fertilizer, NP - nitrogen plus phosphorus fertilizer, M – manure, NM - nitrogen fertilizer plus manure, and NPM - nitrogen, phosphorus fertilizer plus manure.

4.2. Soil extracellular enzyme activities varied with soil organic matter inputs

Soil extracellular enzyme activities are good indicators of soil organic matter decomposition (Sinsabaugh et al., 2014; Jian et al., 2016). Most enzyme activities were greater with fertilization than without, and with maure or maure puls chemical fertilizers increased enzyme activities to a greater magnitude than chemical fertilizer alone (Table 2). This indicates that increased C and N inputs from manure alone or combined with chemical fertilizers favored β-glucosidase, cellobiohydrolase, β-xylosidase, and β-*N*-acetylglucosidase activities. In a recent meta-analysis study, Miao et al. (2019) reported a consistent increase in different enzyme activities in manure plus chemical fertilizer treatments compared to inorganic fertilizer alone or no fertilization in croplands of China. Manure application increases substrate availability and supports microbial growth, leading to enhanced enzyme activities compared to inorganic fertilizers or no fertilizer application (Böhme and Böhme, 2006; Tang et al., 2017).

Complex substrates, such as polysaccharides, are decomposed by hydrolytic soil enzymes, such as β-glucosidase, Cellobiohydrolase, and β-xylosidase (Jian et al., 2016). Therefore, the increase in SOC with manure and other organic inputs could increase β-glucosidase and β-*N*-acetylglucosidase activities (Sinsabaugh et al., 2014). In this study, the activities of β-glucosidase, cellobiohydrolase, and β-xylosidase were positively correlated to most carbon fractions at 0–30 cm and those of β-glucosidase and β-xylosidase to POC and MBC (Fig. 1). However, β-*N*-acetylglucosidase, associated with microbial nitrogen acquisition (Jian et al., 2016), was positively related to STN and PNM at 0–15 cm but negatively to PNM at 15–30 cm and NO_3^+-N at 30–60 cm. We did not fully understand the role of this enzyme in nitrogen cycling. Since soil nitrogen fractions are the products of many pathways, including different substrates and enzymes, enzymatic response to nitrogen fractions may vary between surface and subsurface soils. In low fertility soils, such as Alfisols and Aridsoils, the tight nitrogen demand may stimulate nitrogen acquisition enzymes (Sinsabaugh et al., 2008).

4.3. Relationships between fertilizer inputs, soil bacterial community, and organic matter components

Distinct separations of bacterial community composition with various treatments (Fig. 2) suggest that manure and manure plus nitrogen and phosporus fertilizers promoted certain groups of bacteria while nitrogen fertilizer and control treatments promoted another group of bacteria at 0–15 and 15–30 cm. At 30–60 cm, an additional group of bacteria was developed with the combination of manure and chemical fertilizers. This is probably a result of variations in carbon, nitrogen, and phosphorus concentrations in different soil layers. Specifically, the difference was clearly visible between bacterial communities associated with manure and inorganic fertilizers at 0–15 and 15–30 cm and their composition at 30–60 cm (Fig. 3). Variations in relative abundances of various bacterial phyla with treatments showed that bacterial growth responded differently to chemical fertilizers and manure applications.

Table 3
Relative abundances of bacterial phyla under different fertilizer treatments at 0–15, 15–30, and 30–60 cm soil depths.

Soil depth	Phylum	Treatment[a]					
		CK	N	NP	M	NM	NPM
0–15 cm	*Proteobacteria*	30.9b[b]	32.4ab	35.6a	33.5ab	33.8ab	32.8ab
	Acidobacteria	19.0a	19.6a	18.7a	19.5a	18.7a	20.5a
	Actinobacteria	17.4a	16.4a	15.0a	15.1a	14.9a	14.9a
	Chloroflexi	9.78ab	10.4a	9.69ab	9.04b	9.95ab	9.97ab
	Gemmatimonadetes	9.11b	9.67b	9.06b	11.0a	10.1ab	11.2a
	Nitrospirae	4.18a	3.74b	3.25cd	3.41bc	2.91d	2.95d
	Planctomycetes	2.95a	1.60b	1.93b	1.90b	1.86b	1.30b
	Latescibacteria	0.827a	0.589ab	0.491b	0.598ab	0.401b	0.435b
	Bacteroidetes	2.01a	2.11a	2.42a	2.35a	2.84a	2.40a
	GAL15	0.048a	0.022b	0.032ab	0.026ab	0.009b	0.019b
	Firmicutes	0.495ab	0.554a	0.408b	0.406b	0.439ab	0.526ab
15–30 cm	*Proteobacteria*	29.6b	30.2b	32.4ab	31.4ab	32.4ab	34.6a
	Acidobacteria	19.7a	18.9a	20.0a	19.8a	18.4a	18.5a
	Actinobacteria	13.6ab	13.8ab	12.6b	12.9b	15.3a	13.9ab
	Chloroflexi	13.1a	13.0a	11.5ab	10.7b	10.6b	10.6b
	Gemmatimonadetes	8.47b	9.68ab	9.90ab	10.9a	9.86ab	10.1ab
	Nitrospirae	6.03a	5.17a	4.66c	4.88bc	4.33bc	4.11c
	Planctomycetes	2.78ab	2.77ab	2.42ab	3.06a	2.22b	2.13b
	Latescibacteria	2.58a	2.18b	1.81c	1.44d	0.925e	0.857e
	Bacteroidetes	1.16a	1.44a	1.76a	1.82a	2.18a	2.10a
	GAL15	0.209a	0.226a	0.180a	0.056b	0.035b	0.036b
	Firmicutes	0.770a	0.594ab	0.535b	0.503b	0.654ab	0.510b
30–60 cm	*Proteobacteria*	18.8b	21.7b	30.1a	21.6b	21.3b	27.6a
	Acidobacteria	19.1a	19.0a	16.5b	17.9ab	17.7ab	16.9b
	Actinobacteria	16.7ab	16.2ab	14.7b	15.6ab	17.1a	16.5ab
	Chloroflexi	15.6a	14.8a	12.7a	14.6a	15.1a	13.6a
	Gemmatimonadetes	7.89b	7.59b	8.35b	12.6a	10.8a	11.8a
	Nitrospirae	9.39a	8.24a	6.11bc	6.57b	6.42b	4.91c
	Planctomycetes	1.95a	1.57ab	1.26bc	1.61ab	1.30bc	1.04c
	Latescibacteria	3.34a	3.01ab	2.52bc	2.98ab	2.45c	1.77d
	Bacteroidetes	0.431b	0.582ab	1.06a	0.923ab	0.973ab	1.14a
	GAL15	4.04ab	4.33a	3.70abc	2.65cd	2.87bcd	1.97d
	Firmicutes	1.37b	1.67ab	1.27b	1.67ab	2.43a	1.46b

[a] Treatments are CK – control, N - nitrogen fertilizer, NP - nitrogen plus phosphorus fertilizer, M – manure, NM - nitrogen fertilizer plus manure, and NPM - nitrogen, phosphorus fertilizer plus manure.
[b] Values within a column and soil depth followed by the same letter are not significantly different at $P \leq 0.05$.

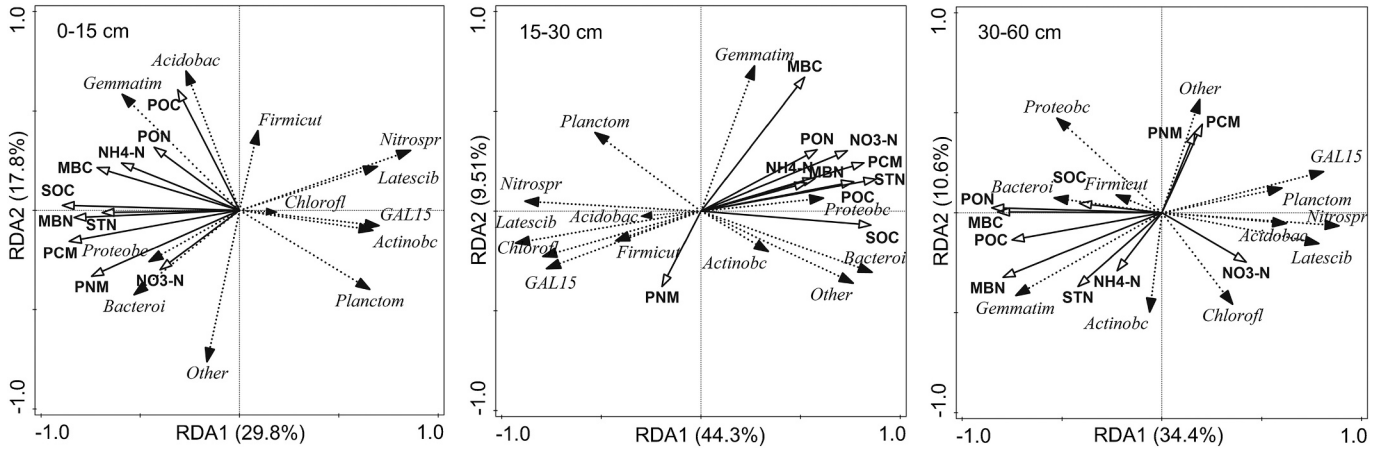

Fig. 4. Eigenvectors from redundancy analysis (RDA) to show the relations among the soil enzyme activities (blue arrows) and soil carbon and nitrogen fractions (red arrows) in the first two axes. Bacterial communities are *Acidobac - Acidobacteria*, *Actinobc - Actinobacteria*, *Chlorofl - Chloroflexi*, *Becteroi -Bacteroidetes*, *Firmicut – Firmicutes*, *Gemmatim - Gemmatimonadetes*, *Latescib - Latescibacteria*, *Nitrospr - Nitrospirae*, *Planctom - Planctomycetes*, *Proteobc - Proteobacteria*. Soil carbon and nitrogen fractions: SOC - soil organic carbon, POC - particulate organic carbon, PCM - potential carbon mineralization, MBC - microbial biomass carbon, STN - soil total nitrogen, PON - particulate organic nitrogen, PNM - potential N mineralization, and MBN - microbial biomass nitrogen. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

While *Gemmatimonadetes* and *Bacteroidetes* responded positively with applications of manure alone or manure plus chemical fertilizers, others, such as *Chloroflexi*, *Nitrospirae, Planctomycetes, Latescibacteria*, and *GAL15,* enhanced with inorganic fertilizers or the control without fertilization. Increased carbon availability for energy and nutrients, such as nitrogen and phosphorus, from manure and manure plus inorganic fertilizers, may have promoted the growth of *Gemmatimonadetes,* and *Bacteroidetes*. In contrast, the application of inorganic fertilizers or the control without fertilizers and manure enhanced the growth of *Chloroflexi*, *Nitrospirae, Planctomycetes, Latescibacteria*, and *GAL15*, suggesting

that these bacteria rely their growth mostly on nutrient availability, but less on carbon as the source of energy. Copitrophic bacteria, such as *Proteobacteria* and *Actinobacteria,* grow favorably in carbon and nutrient-rich environments over oligotrophic bacteria, such as *Chloroflexi, Nitrospirae, and Acidobacteria* (Trivedi et al., 2015; Zhao et al., 2018). In inorganic fertilizer only treatments, reduced carbon sources may have reduced the growth of *Chloroflexi* and *Planctomycetes.* Imbalanced fertilizers application for a long-time can reduce the growth of these bacteria (Eo and Park, 2016; Bei et al., 2018). Similarly, manure application decreased the growth of *Actinobacteria* compared to that of inorganic fertilizers (Peacock et al., 2001).

Bacterial communities associated with carbon and nitrogen cycling can be described by their relations with soil carbon and nitrogen fractions. Positive associations between *Proteobacteria*, *Acidobacteria*, *Gemmatimonadetes,* and *Bacteroidetes* and most soil carbon and nitrogen fractions at 0–30 cm suggest their contribution to carbon and nitrogen cycling, soil carbon sequestration, and nitrogen availability in agricultural soils (Fig. 4). The relationship was weaker at 30–60 cm when carbon and nitrogen concentrations decreased. Ghimire et al. (2018) demonstrated a decrease in yield with soil carbon and nitrogen loss from the subsoil. It appears, the complex interaction of nutrients and the microbial community at surface and subsurface soils affect nutrient cycling and thereby crop production. The response of *Actinobacteria* and *Firmicutes* to treatments varied at different depths. Further study of microbial community dynamics in the deeper soil profile may improve our understanding of carbon and nutrient cycling in the soil profile and their significance to crop production. This study highlights the role of subsoil microbial communities in carbon and nitrogen cycling in surface and subsoil under diverse fertility management systems. *Actinobacteria*, *Chloroflexi*, *Nitrospirae*, *Planctomycetes*, *Latescibacteria,* and *GAL15* were not or negatively related to carbon and nitrogen fractions. These bacterial may have a minimal impact on carbon and nitrogen cycling.

5. Conclusions

This study evaluating soil carbon and nitrogen fractions, enzyme activity, and bacterial community structure in long-term fertility management plots reinforced the importance of organic manure and integrated nutrient management in maintaining carbon and nitrogen in the soil profile and microbial growth. Long-term application of chemical fertilizers decreased most of soil carbon and nitrogen fractions, extracellular enzyme activities, and soil bacterial abundance. Manure alone or manure plus chemical fertilizers enhanced soil carbon and nitrogen fractions and enzyme activities. Enzyme activities and relative abundance of the dominant bacterial communities were positively related to carbon and nitrogen fractions, especially at 0–15 and 15–30 cm soil layers. The role of subsoil microbial communities should not be discounted in long-term fertility studies because they may alter carbon and nitrogen cycling process in the subsoil, affecting carbon and nitrogen storage in the soil profile and thereby crop production. Manure shoule be included in the fertilization practices to enhance soil health and quality in the Loess Plateau of China.

Declaration of competing interest

We state that all authors in the manuscript have agreed to submit to your journal, and there is no conflict of interest for all authors.

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