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Temperature effects on soil organic carbon, soil labile organic carbon fractions, and soil enzyme activities under long-term fertilization regimes

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ABSTRACT

The effects of temperature changes on soil organic carbon (SOC), labile organic carbon fractions (microbial biomass carbon, MBC; dissolved organic carbon, DOC; particulate organic carbon, POC), and enzyme activities under long-term fertilization regimes as well as their relationships at different temperatures were investigated in this study. Soil samples were collected in the fluvo-aquic soil of a 26-year fertilizer trial in the North China Plain after maize harvest in 2012, and four treatments were selected: control of no fertilizer (CK), standard rate of mineral fertilizer treatment (SMF), standard rate of organic manure treatment with N input rate equal to SMF (SMA), and half-standard rate of organic manure plus half-standard rate of mineral fertilizer treatment (1/2(SMA + SMF)). We determined soil chemical properties and labile organic carbon fractions using standard methods and the activities of nine soil enzymes involved in C, N, and P cycling in a 21-day incubation experiment at different temperatures (5 °C, 15 °C, 25 °C, and 35 °C) by micro-plate fluorometric assay. Additionally, we investigated the relationships among them using redundancy analyses (RDA) at four temperatures. The results indicated that (1) temperature, fertilization, and their interaction had significant effects on SOC, MBC, DOC, POC, and most of the soil enzyme activities; (2) long-term organic manure treatments (SMA and 1/2 (SMA + SMF)) significantly improved SOC, MBC, DOC, and POC contents and seven hydrolytic enzyme activities (α-1,4-glucosidase, β-1,4-glucosidase, β-1,4-xylosidase, cellobiohydrolase, L-leucine amino-peptidase, β-1,4-*N*-acetylglucosaminidase, phosphatase) at different temperatures, compared with the mineral fertilized treatment (SMF) and CK. However, oxidoreductases (peroxidase and phenol oxidase) showed the opposite trend with hydrolytic enzyme activities and had higher values in SMF and CK treatments; (3) SOC, MBC, DOC, POC, and most of the soil enzyme activities decreased with increasing temperature; (4) RDA revealed that the dominant factors of SOC and soil labile organic carbon fractions affecting soil enzyme activities were POC and SOC at 5 °C, DOC and POC at 15 °C, DOC and SOC at 25 °C, and MBC, DOC, and SOC at 35 °C. In conclusion, temperature changes significantly altered soil enzyme activities by driving changes in the rates of SOC decomposition and the fractions of soil labile organic carbon. Our conclusions have clear implications for soil ecosystem and biogeochemical cycles under climate change.

1. Introduction

Soil enzymes with highly catalytic capacity are produced by soil microorganisms and are the main medium of controlling biochemical processes, such as soil organic carbon (SOC) decomposition and nutrient cycling (Dick, 1994; Allison and Jastrow, 2006). Changes in the activities of soil enzymes have been observed to be closely related to shifts in rates of SOC decomposition and patterns of turnover in organic carbon pools

Abbreviations: SOC, soil organic carbon; MBC, microbial biomass carbon; DOC, dissolved organic carbon; POC, particulate organic carbon; AG, α-1, 4-glucosidase; BG, β-1, 4-glucosidase; BXYL, β-1, 4-xylosidase; CBH, Cellobiohydrolase; LAP, L-leucine aminopeptidase; NAG, β-1, 4-N-acetylglucosaminidase; PHOS, phosphatase.

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(Bending et al., 2002; Sinsabaugh et al., 2005; Veres et al., 2015). For instance, a positive relationship between soil enzyme activities, such as soil phosphatase, β-glucosidase, cellulose and SOC increase has been found in agroecosystems (Böhme et al., 2005; Saha et al., 2008; Li et al., 2015); however, soil phenol oxidase activity showed negative correlation with SOC increase and the degree of soil humidification (Lin, 2010; Li et al., 2015). Soils with higher SOC content, especially higher labile organic carbon fractions such as microbial biomass carbon (MBC), potassium permanganate oxidizable carbon ($KMnO_4$ -C), and water-soluble carbohydrate carbon were also reported to have higher soil enzyme activities (Shi et al., 2006; Nayak et al., 2007; Bowles et al., 2014).

Both SOC including soil labile organic carbon fractions and soil enzyme activities were significantly affected by environmental factors, such as fertilization, tillage, irrigation, and temperature (Mandal et al., 2007; Lefevre et al., 2014). Many previous studies have demonstrated that long-term fertilization, especially organic manure input, could directly or indirectly influence SOC and soil enzyme activities in long-term experimental sites (Böhme et al., 2005). Liu et al. (2013) reported that SOC and labile organic carbon fractions [MBC, dissolved organic carbon (DOC), and particulate organic carbon (POC)] were significantly increased under long-term fertilization regimes, especially in treatment with organic manure application. Additionally, Saha et al. (2008) indicated that the application of organic manure over the long term could significantly increase soil cellulase, carbohydrate, acid and alkaline phosphatase, protease, and dehydrogenase activity; however, it had no effect on urease activity. Among environmental factors, climate change, particularly global warming, has been the major cause of accelerating global soil C losses (Knorr et al., 2005; Zhou et al., 2012), but, until now, the temperature sensitivity of SOC pools was still a topic for debate. Many researches revealed that soil labile organic carbon pools were more (Liski et al., 1999; Thornley and Cannell, 2001), less (Knorr et al., 2005) sensitive to warming than resistant organic carbon pools, or they responded similarly to global warming (Fang et al., 2005; Leifeld and Fuhrer, 2005). Recently, a range of studies have investigated the temperature sensitivity of soil enzyme activities in different soil ecosystems (Koch et al., 2007; Wallenstein et al., 2012; Stone et al., 2012). It was reported that the temperature sensitivity of soil enzyme activities decreased with increasing temperature (Stone et al., 2012), and these changes in the patterns of soil enzyme activities were demonstrated to be driven by changes in the patterns of soil labile organic carbon fractions under warming conditions (Zhou et al., 2013; Bowles et al., 2014). Zhou et al. (2013) indicated that a six-year warming trend increased the acid phosphatase and *N*-acetylglucosaminidase in surface soils but decreased β-glucosidase and acid phosphatase in the subsurface in temperate grassland due to changes in MBC.

All of these facts strengthen the idea that due to the strong effects of temperature on microbial decomposition of SOC pools, enzyme activities are sensitive to the changes in temperature (Zhou et al., 2013). However, temperature effects on changes in soil enzyme activities and soil labile organic carbon fractions under long-term fertilization regimes, especially regarding the mechanisms driving temperature changes on the patterns of soil enzyme activities, remain unclear. Therefore, an incubation experiment was conducted in a 26-year fertilizer trial, and the objectives of this research were to (1) investigate the responses of soil enzyme activities, SOC, and soil labile organic carbon fractions (MBC, DOC and POC) to increasing temperatures in different fertilizer treatments and (2) explore the dominant factors of SOC and soil labile organic carbon fractions controlling the patterns of soil enzyme activities under warming conditions. We hypothesize that temperature changes could regulate soil enzyme activities under long-term fertilization regimes by changing the decomposition processes of SOC, especially in regard to soil labile organic carbon fractions. These observations could contribute to the understanding of responses of soil enzyme activities to climate change, which is critical for predicting SOC decomposition rates and nutrient cycling and providing impetus for understanding the ways in which climate change may affect the biochemical functioning of soil ecosystems.

2. Materials and methods

2.1. Site description and experimental design

The experimental site was established in 1986 at the Dezhou Station of the Chinese Academy of Agricultural Sciences, Yucheng, Shandong, China (36° 50′ N, 116° 34′ E). The region surrounding the site has a warm, temperate semi-humid monsoon climate with an annual average temperature of 13.4 °C and mean annual precipitation of 569 mm. The soil is a fluvo-aquic light loam (clay 21.4%, silt 65.5%, sand 3.0%) and the initial properties of the soil (0–20 cm) in 1986 were as follows: total organic carbon of 3.93 g kg^{-1}; total N of 0.51 g kg^{-1}; available N, P, and K of 37.5, 7.5, and 73.0 mg kg^{-1}, respectively; cation exchange capacity of 15.84 cmol kg^{-1}; water-soluble salt content of 0.96 g kg^{-1}; and a pH value of 8.56. The cropping system was a typical winter wheat–summer maize rotation cropping system of the North China Plain.

This experiment was described in detail by Li et al. (2015); it consisted of six treatments with four replicates in a randomized complete block design, and the size of each plot was 28 m^2 (4 × 7 m). Paving slabs (0.8 m) separated each plot from the others. The treatments were (1) control of no fertilizer (CK); (2) standard rate of mineral fertilizer treatment that reflects the practice of local (SMF); (3) standard rate of organic manure treatment with N input rate equal to SMF (SMA); (4) half-standard rate of organic manure plus half-standard rate of mineral fertilizer treatment (1/2(SMA + SMF)); (5) double rates of standard organic manure treatment (DMA); and (6) double rates of mineral fertilizer treatment (DMF). The mineral fertilizers (N, P, and K) were urea, superphosphate, and potassium sulfate with standard input rates of 375–450 kg N ha^{-1}, 225–300 kg P_2O_5 ha^{-1}, and 150 kg K_2O ha^{-1} per year, respectively. The organic manure used was cattle manure, which originated from the nearby dairy industry and was completely composted for several months prior to application. Its application rate was based on organic manure total N content, and the range of nutrient contents in cattle manure were 1.00–1.84% N, 0.58–1.67% P_2O_5, and 0.98–1.98% K_2O, respectively.

Total mineral fertilizer was applied twice per year; half was applied in October before the sowing of winter wheat (Jimai 22), and the other half was applied in June before the sowing of summer maize (Zhengdan 958). For winter wheat, 40% N and 100% P_2O_5 and 100% K_2O of mineral fertilizer were applied before sowing, and 60% N was applied at the jointing stage of winter wheat. For summer maize, 40% N was applied before sowing, and 60% N was applied during the elongation stage of summer maize. Organic manure was applied once per year before winter wheat sowing. Mineral fertilizer and organic manure were uniformly broadcasted onto the topsoil before plowing the soil. The seeds were sown by hand, and the seeding rates of winter wheat and summer maize were 112.5 kg ha^{-1} and 67500 plants ha^{-1}, and their row spacings were 30 and 60 cm, respectively. The field management was in accordance with the practice of local farmers.

2.2. Soil sampling

To determine the effects of different fertilizer managements with long-term equal N input rate as the practice of local farmers

on soil labile organic carbon fractions (MBC, DOC, and POC) and enzyme activities, four fertilizer treatments (CK, SMA, SMF, 1/2 (SMA + SMF)) were selected for this research. Surface soil samples (0–20 cm) were collected randomly by auger after the maize harvest in 2012. Each sample was a bulk mix of 15 soil cores (5-cm diameter × 20 cm depth) from each plot and was immediately transported to the laboratory in an ice box. Soil moisture was determined as soon as we arrived in our laboratory, and then the soil sample was separated into two parts. One part was air-dried for the determination of total N, available P, available K, and pH value, and the other part was sieved to 2 mm and stored at 4 °C prior to the incubation experiment.

2.3. Incubation experiment

Temperature changes on SOC, soil labile organic carbon fractions, and enzyme activities were studied using an indoor cultivation method, according to Ai et al. (2012) and Lefevre et al. (2014). Soil moisture was adjusted to 60% of the field capacity with deionized water, and the fresh soil sample (equal to 100 g dry soil) was placed into a 500-mL wide-mouthed bottle that sealed with a rubber plug and was incubated in the dark for 21 days at 5 °C, 15 °C, 25 °C, or 35 °C. The bottle plug was opened for gas exchange at regular intervals, and the soil moisture was adjusted gravimetrically with deionized water to maintain 60% field capacity. Before incubation, soil samples from each treatment were collected to determine SOC, MBC, DOC, POC, and soil enzyme activities. After an incubation period of 21 days, a subsample of soil in each incubator was also collected. Each subsample was divided into two parts: one half was air dried at room temperature, sieved to 0.149 mm and 2 mm, and used for determination of SOC and POC, respectively, and the other half (also passed through the 2-mm sieve) was stored at 4 °C for later analysis of DOC, MBC, and soil enzyme activities.

2.4. Soil chemical analysis

The air-dried soil portion was analyzed for SOC by vitriol acid–potassium dichromate oxidation method, with 0.8000 mol L^{-1} 1/6 $K_2Cr_2O_7$ at 170–180 °C for 5 min, followed by titration of the digestates with $FeSO_4$; total N was analyzed by the Kjeldahl method, available P was analyzed by the $NaHCO_3$ method, available K was analyzed by flame photometer method, and soil pH was measured in a 1:2.5 (soil: water) mixture using the potentiometric method (Lu, 2000). The MBC was determined by the fumigation–extraction method (Vance et al., 1987), fresh soils were fumigated with alcohol-free chloroform for 24 h at 25 °C, extracted with 0.5 M K_2SO_4, and then filtered. The non-fumigated soil was similarly extracted prior to fumigation. The calculation equation was MBC = E_C/k_{EC}, where E_C = the differentials between organic carbon extracted from fumigated soils and organic carbon extracted from non-fumigated soils and k_{EC} was a conversion coefficient (=0.45). Soil DOC was extracted using 0.5 M K_2SO_4 (Jones and Willett, 2006). After shaking and centrifuging, the slurry was filtered using a 0.45-μm membrane filter for determination of DOC using an automated TOC analyzer (Multi N/C ®3100, analytikjena, Germany). Soil POC was dispersed using 5 g L^{-1} hexametaphosphate from the air-dried soil and placed on a reciprocating shaker (90 rev min^{-1}) for 18 h (Cambardella and Elliott, 1992). The slurry was poured over a 53-μm sieve using deionized water to separate. All of the material remaining on the sieve was transferred into a dry vessel, oven-dried at 60 °C for 48 h, and sieved to 0.149 mm for determination of the C content by the dichromate oxidation method, which is the method of SOC determination.

2.5. Soil enzyme activities analysis

In this study, we determined nine enzymes involved in carbon, nitrogen, and phosphorus cycling (Table 1) using a micro-plate enzyme assay, which can measure a large number of soil samples or enzymes in a short time (Marx et al., 2001). The activities of soil enzymes, except for phenol oxidase and peroxidase (non-fluorometric enzymes), were measured, with some buffer modification, by a fluorescent micro-plate enzyme assay (DeForest, 2009). Soil suspensions were prepared by adding 1.0 g dry mass of fresh soil into 125 mL deionized water as the buffer, mixing with an oscillator, and then 200 μL of each soil suspension was pipetted into a 96-well black plate with a multichannel pipette, continuously homogenized with a magnetic stirrer during the process. The assay well incorporated the 200 μL sample suspensions with 50 μL of substrate solution. The negative control well received 200 μL buffer and 50 μL substrate. The sample control well received 200 μL sample suspensions and 50 μL buffer. The reference standard well received 200 μL buffer and 50 μL standard (10 mM 4-MUB, or 7-amino-4-methyl-coumarin (MC) in the case of leucine aminopeptidase). The quench control well received 200 μL sample suspensions and 50 μL standard. There were eight replicate wells for each treatment (assay, negative control, sample control, reference standard, and quench control). The micro-plate was incubated at 25 °C for 4 h in the dark. We then used a Fluorescence Plate Reader (Scientific Fluoroskan Ascent FL, Thermo) at 365 nm excitation and 450 nm emission to determine the fluorescence. The activities (nmol h^{-1} g^{-1}) were calculated as nmol substrate converted per mL of sample.

Phenol oxidase and peroxidase activities were determined by using the substrate of L-DOPA in clear micro-plates (Saiya-Cork et al., 2002; DeForest, 2009). For peroxidase activity analysis, 0.3% H_2O_2 was required for all micro-plate wells. The black control well received 250 μL of buffer. The negative control well received 200 μL of buffer and 50 μL of 25 mM L-DOPA. The sample control

Table 1
Enzymes assayed in soils under long-term fertilization regimes, their corresponding substrates, their abbreviations used in this study, and their enzyme commission number (EC[a]), (4-MUB = 4-methylumbelliferyl, L-DOPA = L-3, 4-dihydroxyphenylalanine).

Enzyme	Substrate	Abbreviation	EC[a]
α-1,4-glucosidase	4-MUB-α-D-glucoside	AG	3.2.1.20
β-1,4-glucosidase	4-MUB-β-D-glucoside	BG	3.2.1.21
β-1,4-xylosidase	4-MUB-β-D-xyloside	BXYL	3.2.1.3
Cellobiohydrolase	4-MUB-β-D-cellobioside	CBH	3.2.1.91
L-Leucine aminopeptidase	L-Leucine-7-amino-4-methylcoumarin	LAP	3.4.11.1
β-1,4-*N*-acetylglucosaminidase	4-MUB-*N*-acetyl-β-D-glucosaminide	NAG	3.1.6.1
phosphatase	4-MUB-phosphate	PHOS	3.1.3.2
Peroxidase	L-DOPA	–	1.11.1.7
Phenol Oxidase	L-DOPA	–	1.10.3.2

[a] EC, Enzyme Commission number describing enzymatic function at increasing level of detail (there are four numbers, the first number distinguishes 1-oxireductases, 2-transferases, 3-hydrolases, 4-lyases, 5-isomerases, and 6-ligases).

Table 2
The properties and soil organic carbon (SOC), microbial biomass carbon (MBC), dissolved organic carbon (DOC), and particulate organic carbon (POC) contents of the soils under long-term fertilization regimes after the maize harvest in 2012. Long-term averages (mean ± SE) followed by the same letter in the same column are not significantly different (Duncan's test, $P < 0.05$) in different fertilizer treatments.

Treatment	Total N(g/kg)	Available P(mg/kg)	Available K(mg/kg)	pH value	SOC(g/kg)	MBC(mg/kg)	DOC(mg/kg)	POC(g/kg)
CK	0.83 ± 0.05 d	5.34 ± 1.72 d	94.73 ± 4.48 b	8.44 ± 0.04 a	7.34 ± 0.37 d	168.84 ± 16.88 c	77.86 ± 7.70 c	0.75 ± 0.08 d
SMA	1.77 ± 0.07 a	155.67 ± 28.50 a	130.46 ± 10.84 a	8.17 ± 0.04 b	15.68 ± 0.54 a	273.64 ± 11.21 a	186.86 ± 10.55 a	2.99 ± 0.31 a
SMF	1.04 ± 0.11 c	35.21 ± 13.25 c	96.81 ± 3.33 b	8.24 ± 0.07 b	8.57 ± 0.72 c	183.02 ± 30.98 c	82.57 ± 14.18 c	1.17 ± 0.24 c
1/2(SMA + SMF)	1.40 ± 0.13 b	94.71 ± 29.07 b	97.60 ± 10.60 b	8.21 ± 0.04 b	12.24 ± 1.22 b	242.32 ± 14.87 b	103.47 ± 16.85 b	1.87 ± 0.17 b

Note: CK, control of no fertilizer; SMF, standard rate of mineral fertilizer treatment; SMA, standard rate of organic manure treatment with N input rate equal to SMF; 1/2 (SMA + SMF), half-standard rate of organic manure plus half-standard rate of mineral fertilizer treatment.

Table 3
The enzyme activities of the tested soils under long-term fertilization regimes which were sampled after the maize harvest in 2012. Long-term averages (mean ± SE) followed by the same letter in the same column are not significantly different (Duncan's test, $P < 0.05$) in different fertilizer treatments. The enzyme activities were calculated in units of nmol h^{-1} g^{-1} except that peroxidase and phenol oxidase activities were calculated in units of μmol h^{-1} g^{-1}.

Treatment	AG	BG	BXYL	CBH	LAP	NAG	PHOS	Peroxidase	Phenol Oxidase
CK	14.19 ± 1.22 d	56.89 ± 3.39 c	24.25 ± 0.95 d	8.79 ± 0.53 b	321.06 ± 4.72 c	4.13 ± 0.14 d	197.00 ± 3.14 c	6.01 ± 0.22 d	9.12 ± 0.07 a
SMA	25.99 ± 1.04 a	85.40 ± 3.29 a	40.39 ± 2.82 a	20.89 ± 1.94 a	383.42 ± 10.13 a	7.36 ± 0.31 a	234.72 ± 3.70 a	7.68 ± 0.18 a	8.38 ± 0.04 c
SMF	16.46 ± 0.48 c	65.92 ± 2.77 b	27.62 ± 2.84 c	10.45 ± 0.83 b	286.11 ± 3.33 d	4.96 ± 0.43 c	180.48 ± 8.05 d	6.40 ± 0.15 c	8.73 ± 0.07 b
1/2(SMA + SMF)	23.38 ± 1.29 b	88.59 ± 4.57 a	37.51 ± 0.91 b	17.58 ± 3.78 a	332.56 ± 7.80 b	6.52 ± 0.55 b	207.80 ± 9.35 b	6.88 ± 0.04 b	8.23 ± 0.16 d

Note: CK, control of no fertilizer; SMF, standard rate of mineral fertilizer treatment; SMA, standard rate of organic manure treatment with N input rate equal to SMF; 1/2 (SMA + SMF), half-standard rate of organic manure plus half-standard rate of mineral fertilizer treatment. The soil enzyme abbreviations (AG, BG, BXYL, CBH, LAP, NAG and PHOS) were expressed in Table 1.

well received 200 μL of soil slurry and 50 μL of buffer. The assay well received 200 μL of soil slurry and 50 μL of 25 mM L-DOPA. The clear micro-plate was then incubated at 25 °C for 20 h in the dark. The activities were determined by measuring absorbance at 450 nm using the Fluorescence Plate Reader and calculated in units of μmol h^{-1} g^{-1}.

2.6. Statistical analysis

One-way analysis of variance (ANOVA) was used with the Duncan's test to evaluate significant difference ($P < 0.05$) in soil chemical properties (total N, available P, available K, pH values, and SOC), labile organic carbon fractions, and enzyme activities among different fertilizer treatments or different temperatures. Two-way ANOVA was performed to estimate the interaction effects of fertilizer treatments and temperatures on SOC, soil labile organic carbon fractions, and enzyme activities in SAS for Windows (Version 9.1). The polynomial fitting model was used to determine the temperature changes on soil enzyme activities with Origin version 8.5. RDA was used to analyze the relationship between SOC, soil labile organic carbon fractions, and enzyme activities with Canoco version 4.5.

3. Results

3.1. Effects of long-term fertilization regimes on the soil chemical properties, labile organic carbon fractions, and enzyme activities

Long-term fertilization regimes significantly increased soil total N, SOC, MBC, DOC, POC, available P, and available K contents with increasing application rate of organic manure; however pH values in different fertilizer treatments showed a negative trend (Table 2) after repeating application of fertilizers for 26 years in a winter wheat-summer maize cropping system in the North China Plain in 2012. The activities of hydrolytic enzymes (AG, BG, BXYL, CBH, LAP, NAG, PHOS) and peroxidase also increased significantly with the long-term application of organic manure (SMA, 1/2(SMA + SMF)); in contrast, phenol oxidase activity showed the opposite trend (Table 3).

3.2. Effects of temperature changes on SOC and soil labile organic carbon fractions under long-term fertilization regimes

Different fertilizer treatments showed significant effects on SOC and soil labile organic carbon fractions MBC, DOC, and POC (Table 4). It is observed that SOC, MBC, DOC, and POC contents showed the same trends among different fertilizer treatments, even at different temperatures, and increased significantly in the order of SMA > 1/2(SMA + SMF) > SMF > CK, except for the MBC at 5 °C and 35 °C (Table 4). In addition, we also found that SOC, MBC, DOC, and POC contents significantly responded to temperature changes and decreased with increasing temperature. SOC and labile organic carbon fractions under long-term fertilization regimes were significantly higher at 5 °C than those at 35 °C (Table 4). Compared with the initial contents before incubation, all of the contents of soil labile organic carbon fractions decreased significantly at the end of incubation, and in general, the decreasing rates of them increased with increasing temperature. Additionally, higher decreasing rate of them was found in SMA treatment than in other treatments no matter what temperature was (Fig. S1).

However, the interaction of fertilizer treatments and temperature had no significant impacts on SOC and MBC contents, but showed significant effects on DOC and POC contents (Table 4). MBC, DOC, and POC showed significant correlations with SOC separately, even at different temperatures, and all soil labile organic carbon fractions significantly increased with increasing SOC content (Fig. 1).

3.3. Effects of temperature changes on soil enzyme activities under long-term fertilization regimes

Different fertilizer treatments and temperatures significantly affected the soil enzyme activities, except in the case of phenol oxidase activity, on which no significant impact of temperature was found in this research. Two-way ANOVA showed that soil enzyme activities were significantly influenced by the interaction of fertilizer treatments and temperature (Table 5).

Table 4
Changes in soil organic carbon (SOC), microbial biomass carbon (MBC), dissolved organic carbon (DOC), and particulate organic carbon (POC) contents in soils under long-term fertilization regimes after 21 days of incubation at different temperatures (5, 15, 25, 35 °C) and two-way analysis of variance analysis of the interaction between temperature and treatments on SOC, MBC, DOC, and POC contents. Long-term averages (mean ± SE) followed by the same letter in the same column are not significantly different (Duncan's test, $P < 0.05$) in different fertilizer treatments at the same temperature, "-" indicates that SOC, MBC, DOC, and POC were not significantly affected by treatments, temperature, or their interaction at the $P < 0.05$ level.

Treatment		SOC(g/kg)	MBC(mg/kg)	DOC(mg/kg)	POC(g/kg)
5 °C	CK	7.28 ± 0.15 d	138.28 ± 16.42 c	36.02 ± 3.43 d	0.65 ± 0.03 d
	SMA	15.23 ± 0.13 a	230.78 ± 21.10 a	101.79 ± 3.57 a	2.67 ± 0.04 a
	SMF	8.32 ± 0.04 c	165.76 ± 7.70 c	45.41 ± 1.83 c	0.92 ± 0.10 c
	1/2(SMA + SMF)	12.56 ± 0.25 b	195.06 ± 26.14 b	83.69 ± 10.27 b	1.73 ± 0.04 b
15 °C	CK	7.06 ± 0.63 d	136.45 ± 20.31 d	33.23 ± 1.43 d	0.62 ± 0.08 d
	SMA	15.15 ± 0.17 a	217.96 ± 20.12 a	97.61 ± 1.74 a	2.71 ± 0.04 a
	SMF	8.31 ± 0.35 c	159.35 ± 10.40 c	42.11 ± 4.97 c	0.84 ± 0.13 c
	1/2(SMA + SMF)	12.66 ± 0.49 b	195.06 ± 21.65 b	83.17 ± 3.79b	1.67 ± 0.08 b
25 °C	CK	6.73 ± 0.09 d	119.97 ± 4.36 d	32.54 ± 1.04 d	0.55 ± 0.03 d
	SMA	15.34 ± 0.14 a	209.72 ± 16.95 a	95.35 ± 4.21 a	2.54 ± 0.13 a
	SMF	8.47 ± 0.30 c	153.39 ± 10.40 c	45.07 ± 2.23 c	0.79 ± 0.04 c
	1/2(SMA + SMF)	12.43 ± 0.05 b	191.40 ± 10.84 b	77.78 ± 2.44 b	1.74 ± 0.10 b
35 °C	CK	6.75 ± 0.22 d	117.68 ± 4.33 b	28.54 ± 2.34 d	0.44 ± 0.04 d
	SMA	15.04 ± 0.06 a	193.23 ± 16.28 a	92.05 ± 1.04 a	2.47 ± 0.09 a
	SMF	8.21 ± 0.18 c	128.67 ± 9.73 b	41.41 ± 2.87 c	0.79 ± 0.07 c
	1/2(SMA + SMF)	12.28 ± 0.21 b	192.77 ± 10.40 a	65.77 ± 1.21 b	1.79 ± 0.01 b
Two-way ANOVA	Treatment(Tr)	<0.0001	<0.0001	<0.0001	<0.0001
	Temperature (Temp)	0.0274	0.0004	<0.0001	<0.0001
	Tr × Temp	–	–	0.0073	0.0008

Note: CK, control of no fertilizer; SMF, standard rate of mineral fertilizer treatment; SMA, standard rate of organic manure treatment with N input rate equal to SMF; 1/2 (SMA + SMF), half-standard rate of organic manure plus half-standard rate of mineral fertilizer treatment.

In general, organic manure application significantly increased the activities of soil enzymes involved in C-cycling (AG, BG, BXYL, and CBH) under long-term fertilization regimes (Fig. 2). All of the fertilizer treatments significantly increased the AG activity in the order of SMA > 1/2(SMA + SMF) > CK > SMF at all four temperatures. BG, BXYL, and CBH showed the highest activities in 1/2(SMA + SMF) treatment, followed by SMA treatment and SMF treatment at 15 °C, 25 °C, and 35 °C. However, they were observed to be the lowest in 1/2(SMA + SMF) treatment except for BXYL activity at 5 °C. The PHOS activity, participating in the mineralization of soil organic phosphorus, showed a similar trend with BG and CBH activities (Fig. 2). After equal long-term N input, the application of organic manure (SMA and 1/2(SMA + SMF)) significantly increased the activities of NAG and LAP related to N-cycling relative to the SMF treatment, regardless of the temperature, and higher NAG and LAP activities were found in the combination of half-organic manure and half-mineral fertilizer treatment (1/2(SMA + SMF)) than those in SMA treatment (Fig. 2). Generally, the activities of peroxidase and phenol oxidase participating in the process of soil microbial oxidoreductase metabolism showed the opposite trend compared with other soil enzymes activities; these two enzyme activities in CK and SMF treatments were higher than those in organic manure treatments (SMA and 1/2(SMA + SMF)) at 5 °C, 15 °C, and 25 °C (Fig. 2).

In addition, we found that the seven hydrolytic enzyme activities (AG, BG, BXYL, CBH, LAP, NAG, and PHOS) in 1/2 (SMA + SMF) treatment were higher at 15 °C and 25 °C than those at 5 and 35 °C. However, these enzyme activities in other treatments (CK, SMF, and SMA) significantly decreased with increasing temperature (from 5 °C to 35 °C). In contrast, the oxidoreductase activities (peroxidase and phenol oxidase) showed the opposite trend (Fig. 2). A similar trend was found in Fig. S2, which generally suggested that these two oxidoreductase activities significantly increased after incubation for 21 days. However, the other seven hydrolytic enzyme activities decreased compared with those before incubation and the decreasing rates of hydrolase activities except for LAP were higher in SMA treatment than those in other treatments (Fig. S2).

3.4. Relationship between SOC, soil labile organic carbon fractions, and soil enzyme activities at different temperatures

RDA was used to explain the soil enzyme activities (AG, BG, BXYL, CBH, LAP, NAG, PHOS, peroxidase, and phenol oxidase) (response variables) using SOC, MBC, DOC, and POC (explanatory variables) under long-term fertilization regimes at four temperatures (Fig. 3). We found that all explanatory variables significantly affected soil enzyme activities (SOC, $P < 0.01$; MBC, $P < 0.05$; DOC, $P < 0.05$; and POC, $P < 0.05$) at all four temperatures (Fig. 3a–d).

At 5 °C (Fig. 3a), explanatory variables (SOC, MBC, DOC, and POC) accounted for 65.3% of the total variation in the model, and the first two axes accounted for 52.81% and 11.72%, respectively. RDA analysis showed that soil enzyme activities in organic manure-treated soils (SMA and 1/2(SMA + SMF)) were clearly separate from those in the other treatments (SMF and CK). These differences were mainly owing to the changes of POC and SOC, which explained 31% ($P < 0.01$) and 30% ($P < 0.01$) of total variation by the interactive forward selection of RDA. We observed that SMA with higher POC and SOC content was significantly different from the 1/2(SMA + SMF) treatment and they were significantly separate from each other (Fig. 3a). However, there was no difference between SMF and CK.

At 15 °C (Fig. 3b), explanatory variables accounted for 65.1% of the total variation in the model, and the eigenvalues of Axes 1 and 2 explained a variation of 53.27% and 9.17%, respectively. Forward selection results expressed that DOC and POC could explain 42.5% ($P < 0.01$) and 18.3% ($P < 0.01$) of the variation in soil enzyme activities, respectively, and were the better two subsets of the explanatory variables at 15 °C. SMA-treated soils were also clearly separated from the soils of the other three treatments, and the 1/2 (SMA + SMF) treatment showed a similar result to the SMA treatment. The CK treatment also had no clear separation from

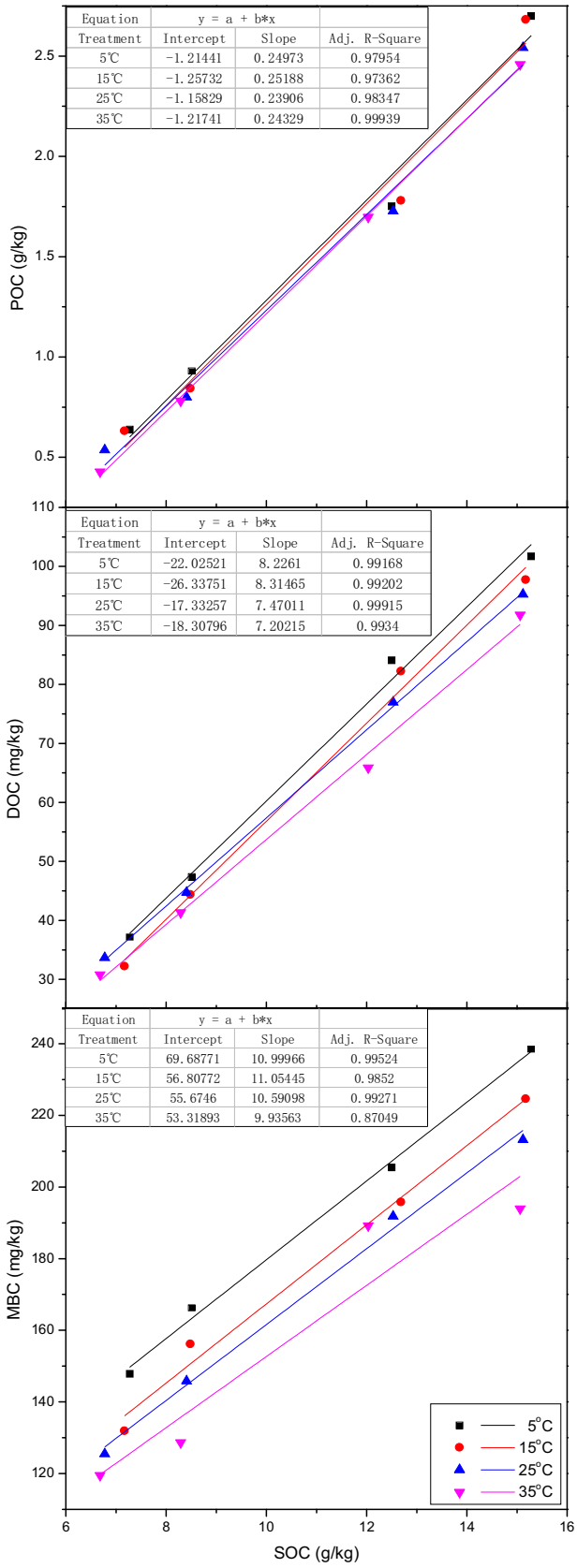

Fig. 1. Correlation between soil labile organic carbon fractions (microbial biomass carbon, MBC; dissolved organic carbon, DOC; particulate organic carbon, POC); and soil organic carbon (SOC) after 21 days of incubation at different temperatures (■—, ●—, ▲—, and ▼— indicates 5 °C, 15 °C, 25 °C, and 35 °C, respectively).

SMF at 15 °C, and the two treatments had negative values along Axis 1.

In the RDA model analyzing the relationship between soil enzyme activities and SOC, MBC, DOC, and POC at 25 °C, all explanatory variables accounted for 79.6% of the total variation, and the first two axes explained 75.14 and 2.58% of the total variation, respectively. DOC and SOC could explain 70.5% ($P < 0.01$) and 6.5% ($P < 0.05$) of the variation in soil enzyme activities and were found to play important roles in changing soil enzyme activities in the forward selection results presented (Fig. 3c). The organic manure treatments (SMA and 1/2(SMA + SMF)) had a clear separation from the mineral fertilizer treatments (CK and SMF) although CK and SMF, SMA and 1/2 (SMA + SMF) had no clear separation from each other at 25 °C.

At 35 °C (Fig. 3d), the model accounted for 68.3% of the total variation, and the eigenvalues of Axis 1 and Axis 2 explained 49.10% and 10.99% of the total variation, respectively. Forward selection showed that soil enzyme activities driven by MBC, SOC, and DOC explained 48.5% ($P < 0.01$), 8.4% ($P < 0.05$), and 9.4% ($P < 0.05$) of the variation, respectively, indicating that these were important factors affecting soil enzyme activities at 35 °C. There was also a clear and similar separation of the four treatments shown at 25 °C.

The fertilizer treatments showed different separations in the RDA models based on temperature warming (Fig. 3a–d). The organic manure treatments (SMA, 1/2(SMA + SMF)) were positively related to Axis 1, while the mineral fertilizer treatment (SMF) and CK showed negative values along Axis 1 at 15 °C, 25 °C, and 35 °C (Fig. 3b–d). At 5 °C, the organic manure-treated soils (SMA, 1/2 (SMA + SMF)) were significantly separated from SMF and CK along Axis 2, and SMA showed higher positive correlation with Axis 1 than did the other three treatments. Forward selection models showed that the key factors of SOC and soil labile organic carbon fractions driving changes in soil enzyme activities were POC and SOC at 5 °C, POC and DOC at 15 °C, DOC and SOC at 25 °C, and MBC, SOC, and DOC at 35 °C. The seven hydrolytic enzymes had positive correlations with SOC, MBC, DOC, and POC, except for LAP at 5 °C and 15 °C and PHOS at 5 °C, 25 °C, and 35 °C. In contrast, peroxidase and phenol oxidase had a negative correlation with SOC and soil labile organic carbon fractions at all four temperatures.

4. Discussion

4.1. Effects of temperature changes on SOC and soil labile organic carbon fractions under long-term fertilization regimes

MBC, DOC, and POC are all used to indicate the labile organic carbon fractions under different management practices (Haynes, 2005). In this study, SOC, MBC, DOC, and POC contents were significantly enhanced under long-term application of organic manure, regardless of whether it was combined with mineral fertilizer (SMA, 1/2(SMA + SMF)) at different temperatures, or whether it increased with an increasing input rate of organic manure (Table 4), indicating that application of organic manure played an important role in rising SOC and soil labile organic carbon fractions (Garcıa-Gil et al., 2000; Gong et al., 2009). This is because the application of organic manure not only increases overall inputs of organic carbon into the soil but also changes the rate of microbial decomposition. This in turn controls the rate of C inputs relative to C exports and accelerates plant residue deposition by increasing plant net primary productivity. As a result, the total soil carbon storage is enhanced (Rudrappa et al., 2006; Saha et al., 2008; Liu et al., 2014).

Soil labile organic carbon fractions (MBC, DOC, and POC) showed significant correlations with SOC regardless of temperature, and all fractions significantly increased with increasing SOC content (Fig. 1), supporting the findings of many previous studies

Table 5
Effects of different fertilizer treatments and temperature and the interaction between them on soil enzyme activities in the long-term fertilizer trial after 21 days incubation at different temperatures (5, 15, 25, 35 °C). "-" indicates that soil enzyme activities were not significantly affected by treatments, temperature or their interaction at the $P < 0.05$ level (Duncan's test).

Two-way ANOVA	AG	BG	BXYL	CBH	NAG	LAP	PHOS	Peroxidase	Phenol Oxidase
Treatment (Tr)	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Temperature (Temp)	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0002	–
Tr × Temp	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0001	0.0009

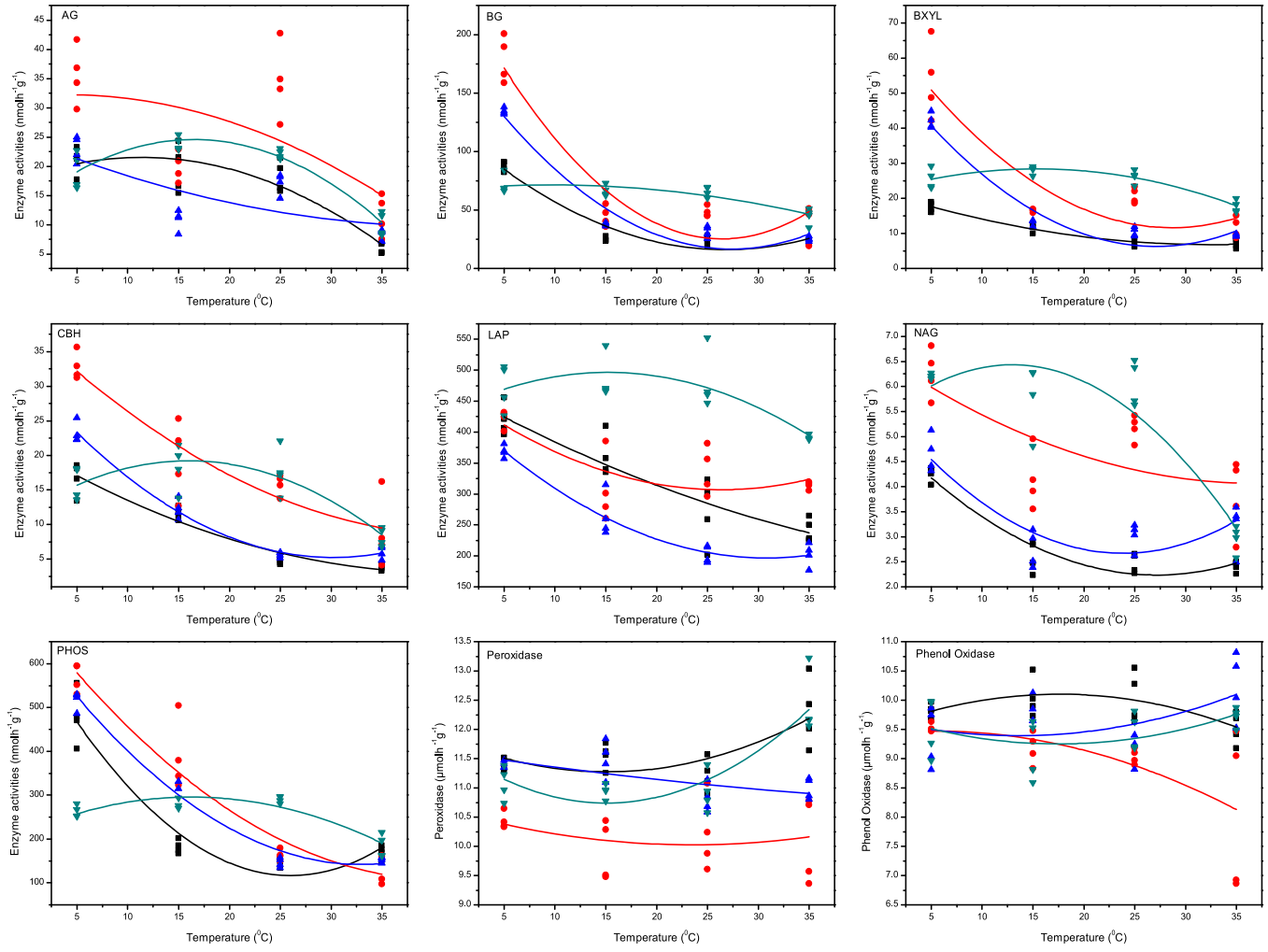

Fig. 2. Polynomial fitting of changes in soil enzyme activities in different fertilizer treatments after 21 days of incubation at different temperatures (■—, control of no fertilizer (CK); ▲—, standard rate of mineral fertilizer treatment (SMF); ●—, standard rate of organic manure treatment with N input rate equal to SMF (SMA); ▼—, half-standard rate of organic manure plus half-standard rate of mineral fertilizer treatment (1/2(SMA + SMF)).

(Liu et al., 2013, 2014). This result indicates that decrease of the labile organic carbon fractions also provides an early indication of a depletion in SOC (Liu et al., 2014). The slopes of the regression equations between soil labile organic carbon fractions and SOC increased in the order of POC, DOC, and finally MBC, indicating that MBC was the most sensitive index for reflecting the change in SOC under long-term fertilization regimes at different temperatures. This might be because of its smaller size range and highly labile nature (Janzen et al., 1992; Wander, 2004; Conant et al., 2011). Our report indicated that warming significantly decreased SOC and labile organic carbon fractions, which was in agreement with previous reports that soil warming accelerates SOC decay (Melillo et al., 2002; Fang et al., 2005), especially in SMA treatment (Fig. S1). This effect might have resulted from an increase in the rate of soil respiration and the total amount of mineralized carbon with increasing temperature and efficiency of soil microbes in using organic carbon (Allison et al., 2010; Wu et al., 2011; Lefevre et al., 2014; Hou et al., 2016).

4.2. Effects of temperature changes on soil enzyme activities under long-term fertilization regimes

In this research, temperature and fertilization had significant effects on most soil enzyme activities (Table 5), which was consistent with other findings (Mandal et al., 2007; Lin, 2010). Application of fertilizers, especially organic manure (SMA and 1/2 (SMA + SMF)), significantly enhanced the activities of soil hydrolytic enzymes (AG, BG, BXYL, CBH, LAP, NAG, and PHOS) under

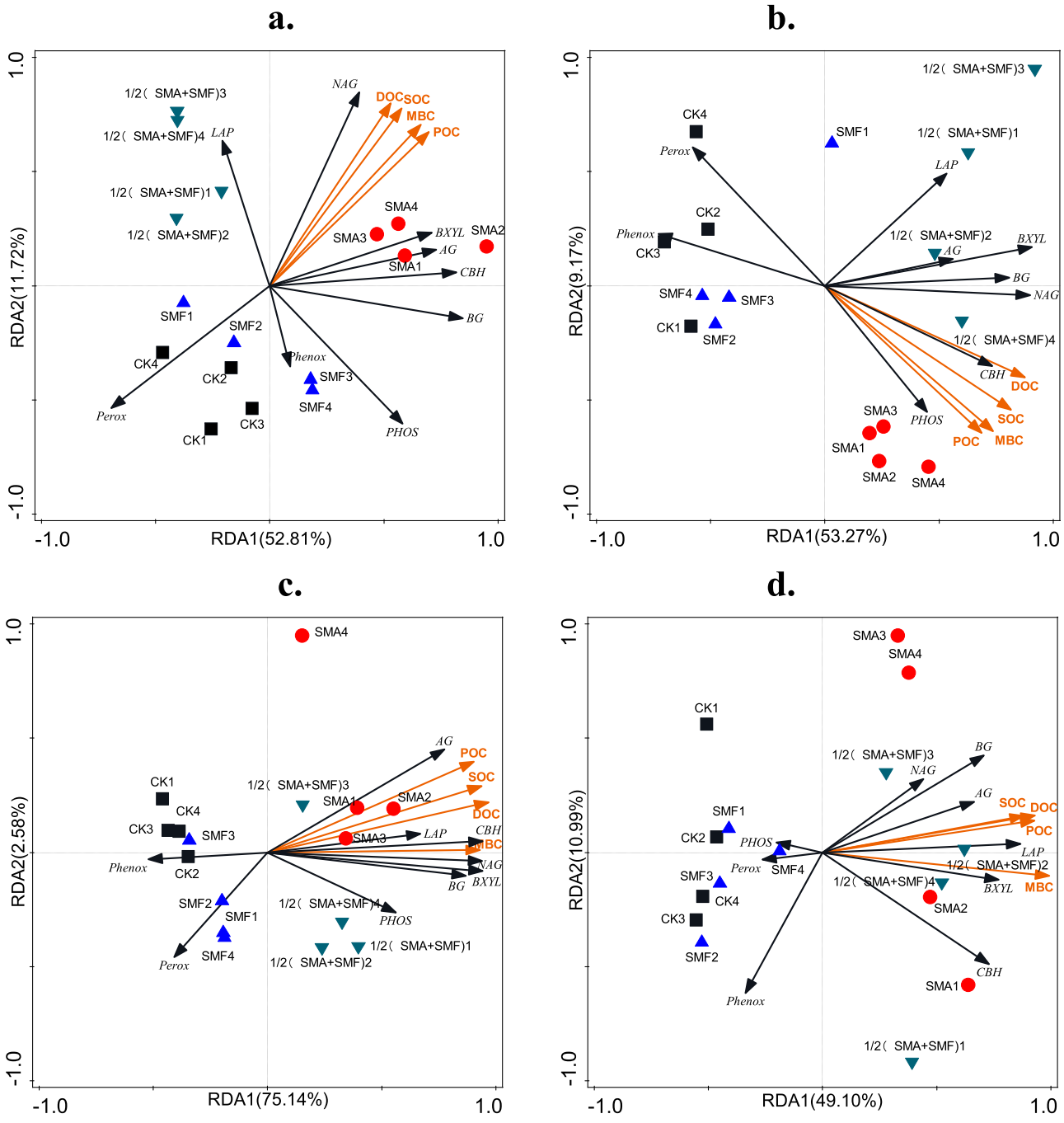

Fig. 3. Redundancy analysis of soil enzyme activities constrained by SOC and labile organic carbon fractions (MBC, DOC, and POC) in soils under long-term fertilization regimes after 21 days of incubation at (a) 5 °C, (b) 15 °C, (c) 25 °C, and (d) 35 °C. (■ CK: control of no fertilizer; ▲ SMF: standard rate of mineral fertilizer treatment; ● SMA: standard rate of organic manure treatment with N input rate equal to SMF; ▼ 1/2(SMA + SMF): half-standard rate of organic manure plus half-standard rate of mineral fertilizer treatment).

long-term regimes, and numerous previous studies have shown similar results (Chang et al., 2007; Saha et al., 2008; Li et al., 2015). It was also observed that soil enzyme activities changed more intensively during incubation in SMA treatment than did in other treatments (Fig. S2). This may be caused by enhanced microbial activity and diversity from years of organic manure application (Saha et al., 2008). Saha et al. (2008) indicated that application of mineral fertilizer decreased both acid and alkaline phosphatase activities. However, the activity of phosphatase under mineral fertilizer treatment determined in the current study remained higher than that under the non-fertilized control, regardless of temperature, which was in agreement with previous results (Böhme et al., 2005; Ai et al., 2012). The finding that mineral fertilizer application affected phosphatase activities differently may be due to different pH values and soil types (Shackle et al., 2000; Saha et al., 2008; Sinsabaugh, 2010). In our research, peroxidase and phenol oxidase activities negatively related to the activities of the other seven soil hydrolytic enzymes, a result was also supported by many previous studies (Saiya-Cork et al., 2002; Lin, 2010; Sinsabaugh, 2010). Sinsabaugh (2010) showed that

peroxidase and phenol oxidase activities generally increase with the increase of soil pH in ecosystems, which was consistent with our result because long-term application of organic manure in our experiment significantly decreased the soil pH value (Li et al., 2015). Sinsabaugh (2010) and Li et al. (2015) also showed that peroxidase and phenol oxidase activities increased with the loss of SOC, which was in agreement with our finding that the peroxidase and phenol oxidase activities were lower in SMA treatment than those in CK treatment, and they had a significantly negative correlation with SOC.

Additionally, soil hydrolytic enzyme activities decreased with increasing temperature in our study (Fig. 2), which was confirmed by the result that temperature warming dramatically decreased most enzyme activities in the subsurface (Zhou et al., 2013). Similar results were reported by Allison and Treseder (2008), who indicated that NAG decreased with increasing temperature in northern latitude ecosystems. Furthermore, the highest hydrolytic enzyme activities were found at 5 °C in this study, which may be attributed to suppressed enzyme production at higher temperatures, as previously reported (Wallenstein et al., 2012); Another primary reason is that soil carbon and nitrogen contents were demonstrated to be dominant factors in controlling enzyme production (Sinsabaugh et al., 2005), which was supported by the evidence from the highest carbon content in the incubated soils at 5 °C in our study.

4.3. Responses of soil enzyme activities to temperature warming depends on changes in SOC and soil labile organic carbon fractions

RDA models showed that the responses of soil enzyme activities to the organic manure fertilized treatments (SMA, 1/2(SMA + SMF)) with higher content of SOC and labile organic carbon fractions (MBC, DOC, and POC) were significantly different from those of the other two treatments (CK and SMF), regardless of temperature (Fig. 3a-d), indicating that application of organic manure altered soil enzyme activities by raising soil available C, especially in soil labile organic carbon fractions (Allison and Vitousek, 2004).

Most of the seven hydrolytic enzymes measured in this experiment had positive correlations with SOC and soil labile organic carbon fractions at different temperatures, whereas peroxidase and phenol oxidase activities showed an opposite trend, which was consistent with the findings of many previous studies (Saiya-Cork et al., 2002; Sinsabaugh et al., 2008; Sinsabaugh, 2010; Zhou et al., 2013; Xu et al., 2015). This was also supported by evidence from the peroxidase and phenol oxidase activities, which were significantly higher at warmer temperatures in the incubated soils but had lower soil labile carbon fractions (Fig. S1, Fig.S2). The forward selection results of RDA at the four temperatures indicated that the dominant factors of SOC and soil labile organic carbon fractions affecting soil enzyme activities altered with temperature increase as follows: POC and SOC at 5 °C, DOC and POC at 15 °C, DOC and SOC at 25 °C, and MBC, DOC, and SOC at 35 °C. Talbot et al. (2008) suggested that soil organic matter decomposition involved two processes with different soil enzymes and soil microbes. In the current study, the revealed temperature effect on soil enzyme activities is likely to be because of the changes in SOC and soil labile organic carbon fractions with increasing temperature, through controlling the rate of soil microbial decomposition and soil microbial mineral process of SOC mineralization (Shi et al., 2006; Schindlbacher et al., 2011). This confirms our hypothesis and indicates that temperature plays a major role in regulating the decomposition of different soil organic matter fractions, leading to soil microorganisms producing different soil enzymes that degrade soil organic matter. Many studies have showed similar results to these, which indicates that temperature warming induces lower enzyme activities, possibly because of the N-limitation of enzyme production by driving higher rates of decomposition of soil organic matter in the soil labile and resistant organic matter pools (Sinsabaugh et al., 2005; Conant et al., 2011).

5. Conclusions

Warming had a significant effect on SOC, soil labile organic carbon fractions (MBC, DOC, and POC) and enzyme activities (AG, BG, BXYL, CBH, LAP, NAG, PHOS, peroxidase, and phenol oxidase) under long-term fertilization regimes. Long-term organic manure treatments (SMA and 1/2(SMA + SMF)) significantly improved SOC, MBC, DOC, and POC contents and the seven measured hydrolytic enzyme activities (AG, BG, BXYL, CBH, LAP, NAG, PHOS) at different temperatures compared with mineral fertilized treatment (SMF) and CK. However, oxidoreductase (peroxidase and phenol oxidase) activities had the opposite trend compared with hydrolytic enzyme activities and had higher values in the SMF and CK treatments. RDA revealed that the dominant factors of SOC and soil labile organic carbon fractions affecting soil enzyme activities were POC and SOC at 5 °C, DOC and POC at 15 °C, DOC and SOC at 25 °C, and MBC, DOC, and SOC at 35 °C. This indicates that temperature regulated soil enzyme activities by changing the soil labile organic carbon fractions and driving higher rates of SOC decomposition. This result contributes to the improved understanding of the response of soil biochemical cycling systems to climate warming and may also be of use in future solutions to climate changes involving optimizing fertilization practices.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.apsoil.2016.02.004.

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