

# No temperature acclimation of soil extracellular enzymes to experimental warming in an alpine grassland ecosystem on the Tibetan Plateau

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Received: 12 September 2012 / Accepted: 28 March 2013  
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**Abstract** Alpine grassland soils store large amounts of soil organic carbon (SOC) and are susceptible to rising air temperature. Soil extracellular enzymes catalyze the rate-limiting step in SOC decomposition and their catalysis, production and degradation rates are regulated by temperature. Therefore, the responses of these enzymes to warming could have a profound impact on carbon cycling in the alpine grassland ecosystems. This study was conducted to measure the responses of soil extracellular enzyme activity and temperature sensitivity ( $Q_{10}$ ) to experimental warming in samples from an alpine grassland ecosystem on the

Tibetan Plateau. A free air-temperature enhancement system was set up in May 2006. We measured soil microbial biomass, nutrient availability and the activity of five extracellular enzymes in 2009 and 2010. The  $Q_{10}$  of each enzyme was calculated using a simple first-order exponential equation. We found that warming had no significant effects on soil microbial biomass C, the labile C or N content, or nutrient availability. Significant differences in the activity of most extracellular enzymes among sampling dates were found, with typically higher enzyme activity during the warm period of the year. The effects of warming on the activity of the five extracellular enzymes at 20 °C were not significant. Enzyme activity in vitro strongly increased with temperature up to 27 °C or over 30 °C (optimum temperature;  $T_{opt}$ ). Seasonal variations in the  $Q_{10}$  were found, but the

**Electronic supplementary material** The online version of this article (doi:10.1007/s10533-013-9844-2) contains supplementary material, which is available to authorized users.

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effects of warming on  $Q_{10}$  were not significant. We conclude that soil extracellular enzymes adapted to seasonal temperature variations, but did not acclimate to the field experimental warming.

**Keywords** Global warming · Temperature acclimation · Soil extracellular enzyme activity ·  $Q_{10}$  · Alpine grassland · Tibetan plateau

## Introduction

Alpine grassland soils on the Tibetan Plateau, like those in high-latitude ecosystems, store large amounts of organic carbon (Yang et al. 2008; Shi et al. 2012), and the decomposition rates of these C pools may increase due to rising global temperatures (Kirschbaum 1995). In fact, the Tibetan Plateau has experienced striking warming during the last half-century (Guo and Wang 2011; Liu and Chen 2000; You et al. 2008; Niu et al. 2004) and is considered to be one of the world's most sensitive areas to global climate change (Liu and Chen 2000; Tang et al. 2009). Therefore, the alpine grassland soils on the plateau provide a unique opportunity to explore the feedback between the soil organic carbon (SOC) stock and climate change (Yang et al. 2008).

Soil extracellular enzymes involved in the decomposition of polymeric SOC to dissolved organic carbon (DOC), have been studied to improve the understanding of the physiological underpinnings of the soil C cycle and help to refine models of the soil-C responses to global warming (Allison et al. 2010; Schimel and Weintraub 2003). However, the fundamental role of temperature in regulating enzyme activity under field conditions has been examined in relatively few studies (Wallenstein et al. 2011). In fact, changes in temperature not only affect enzyme catalysis rates but also affect enzyme production and degradation rates in the environment (Wallenstein et al. 2011). Shifts in enzyme activity can in turn lead to changes in decomposition, SOC storage, and other ecosystem processes (Sinsabaugh et al. 2002; Waldrop et al. 2004). Therefore, predicting the responses of these enzymes to elevated temperature is essential for understanding the underlying mechanisms of how climate change will affect soil and ecosystem functioning.

Several studies suggested that increasing temperature may amplify C release from soils to the atmosphere and provide a positive feedback for

increasing temperature (Davidson et al. 2000; Cox et al. 2000). However, the biological effects of warming on soil microbes, which have a major influence on the net C balance of terrestrial ecosystems, remains unclear. For example, previous studies found that the warming-induced increase in soil  $CO_2$  fluxes to the atmosphere tends to decline over time and eventually results in no or small differences between soils at different temperatures (Luo et al. 2001; Melillo et al. 2002; Rustad et al. 2001; Bronson et al. 2008; Eliasson et al. 2005). This attenuation response could result from the acclimation of soil microbial physiology to increasing temperature (Allison et al. 2010; Zogg et al. 1997; Bradford et al. 2008; Bradford et al. 2010; Davidson and Janssens 2006; Luo et al. 2001). An alternative explanation for such a response is depletion of labile pools of soil organic matter (SOM) (Hartley et al. 2007; Eliasson et al. 2005; Knorr et al. 2005; Tingey et al. 2006). While differentiating between these two explanations in the field is difficult (Rinnan et al. 2011), insights into the physiological responses of microbial communities to warming may provide a mechanistic understanding of soil C flux in changing environments (Allison et al. 2010).

Changes in the temperature sensitivity of  $V_{max}$  and  $K_m$  [ $V_{max}$  is the maximum reaction rate and  $K_m$  is the Michaelis–Menten constant (Michaelis and Menten 1913)], commonly referred to as ‘temperature acclimation’, could offset temperature-induced increases in respiratory activity (Davidson and Janssens 2006). However, experimental evidence of the temperature acclimation of soil extracellular enzymes to experimental warming in the lab and/or in the field is rare. The lack of experimental evidence reduces our ability to predict feedback between the terrestrial C cycle and climate warming. Meanwhile, most of the predictions on feedback between microbial decomposition and temperature do not take into account the kinetics of extracellular enzymes (Allison et al. 2010). Yet, long-term field experiments (Bardgett et al. 2008; Davidson and Janssens 2006) and carbon cycle models (Luo 2007) must consider all possible/potential changes in soil enzyme activity (Davidson and Janssens 2006; Trasar-Cepeda et al. 2007). Therefore, more studies are needed to clarify the biological effects of warming on soil microbes through changes in soil extracellular enzymatic properties.

Determining the activity of an enzyme at different temperatures not only provides knowledge on the optimal temperature range for its activity, but also

allows the estimation of the value of the temperature coefficient, or  $Q_{10}$  (increase in activity per 10 °C increases in temperature) (Trasar-Cepeda et al. 2007). However, existing studies of extracellular enzymes mostly focus on spatial or temporal patterns in potential activity, which is typically measured at a single reference temperature in lab assays (Henry 2012; Wallenstein et al. 2011). This approach neglects the fundamental role of temperature in regulating enzyme activity under field conditions (Wallenstein and Weintraub 2008). Therefore, it is necessary to measure enzyme activity over a range of temperatures to calculate  $Q_{10}$  values, which is an important property of in situ enzymatic activity (Fenner et al. 2005; Koch et al. 2007; Trasar-Cepeda et al. 2007; Wallenstein et al. 2009). To the best of our knowledge, no published studies have examined the effects of warming on the  $Q_{10}$  of soil extracellular enzymes in the alpine grassland ecosystem on the Tibetan Plateau. Moreover, most reports on the effects of warming on soil enzyme activity refer to hydrolases, and little is known about the temperature sensitivity of oxidoreductases (Trasar-Cepeda et al. 2007), the activity of which is often correlated with the mass loss rate of particulate organic matter at the ecosystem scale (Sinsabaugh 2010). More than 60 % of SOC is stored in the heavy fraction on the Tibetan Plateau (Tian et al. 2009), but the effects of warming on oxidoreductases in this unique ecosystem remain largely unknown.

Therefore, we set out to determine the effects of warming on soil microbial activity over the fourth and fifth years of experimental warming during a five-year field warming experiment. Specifically, we investigated the effects of warming on microbial biomass, nutrient availability and potential activity of soil extracellular enzymes, including phenol oxidase (POX),  $\beta$ -1,4-glucosidase (BG),  $\beta$ -D-cellobiohydrolase (CB),  $\beta$ -1,4-xylosidase (BX), and  $\alpha$ -1,4-glucosidase (AG). These soil extracellular enzymes can be broadly divided into two groups: (1) oxidative enzymes which degrade poor-quality, chemically complex compounds such as lignin in co-metabolic acquisition of nutrients; and (2) hydrolytic enzymes responsible for the acquisition of C to support primary metabolism (Sinsabaugh and Moorhead 1994; Sylvia et al. 2004; Sinsabaugh 2010) (Table 1). Warming has the potential to affect the rapidly and slowly cycling C pools differently. For example, an increase in the

relative complexity of soil C compounds could result from reduced oxidative enzyme activity (Cusack et al. 2010). In addition, over the duration of the warming experiment we expected that the relative abundance of recalcitrant soil C pools would increase, which may lead to increases in the activity of oxidative enzymes; meanwhile we expected that the relative abundance of labile soil C pools would decrease, potentially leading to decreases in the activity of hydrolytic enzymes. Therefore, we hypothesized that: (1) these two groups of enzymes would respond differently to experimental warming. In particular, we predicted that the oxidative enzyme activity would increase while the hydrolytic enzyme activity would decrease under the fourth and fifth years of experimental warming; (2) soil extracellular enzyme would acclimate to experimental warming, and thus offset temperature-induced increases in respiratory activity we previously observed at this experimental site during the growing season in 2006, 2007 and 2008 (Lin et al. 2011). More specifically, we predicted that soil extracellular enzyme would acclimate to elevated temperature with reduced  $Q_{10}$  in warmed soils.

## Materials and methods

### Experimental site

We designed a field warming manipulation experiment at the Haibei Alpine Grassland Ecosystem Research Station (37°36'N, 101°19'E, 3,215 m a.s.l.). The mean annual temperature is  $-1.7$  °C (maximum of 27.6 °C and minimum  $-37.1$  °C; (Zhao and Zhou 1999). The mean annual precipitation ranges from 426 to 860 mm, over 80 % of which falls in the growing season from May to September (Zhao and Zhou 1999). The mean temperature and total rainfall during the growing seasons from 1 May to 30 September in 2009 and 2010 were 7.7 and 8.3 °C, and 308.5 and 437.4 mm, respectively (He et al. unpublished data). The plant community at the experimental site is dominated by *Kobresia humilis*, *Festuca ovina*, *Elymus nutans*, *Poa pratensis*, *Carex scabrivostris*, *Scripus distigmaticus*, *Gentiana straminea*, *Gentiana farreri*, *Leontopodium odiumnanum*, *Blvsmsus sinocompressus*, *Potentilla nivea* and *Dasiphora fruticosa* (Luo et al. 2009). The soil developed in this

**Table 1** Soil extracellular enzymes assayed in the current study, with their enzyme commission number (EC), abbreviation, function and corresponding substrate

Enzyme	EC	Abbreviation	Function	Substrate
phenol oxidase	1.10.3.2	POX	Degrades lignocellulose	L-DOPA
$\beta$ -1,4-glucosidase	3.2.1.21	BG	Releases glucose from cellulose	4-MUB- $\beta$ -D-glucoside
$\beta$ -D-cellobiohydrolase	3.2.1.91	CB	Releases disaccharides from cellulose	4-MUB- $\beta$ -D-cellobioside
$\beta$ -1,4-xylosidase	3.2.1.37	BX	Degrades hemicellulose	4-MUB- $\beta$ -D-xyloside
$\alpha$ -1,4-glucosidase	3.2.1.20	AG	Releases glucose from soluble saccharides	4-MUB- $\alpha$ -D-glucoside

*DOPA* L-3,4-dihydroxyphenylalanine, *4-MUB* 4-methylumbelliferyl

ecosystem is Mat-Gryic Cambisol (Chinese Soil Taxonomy Research Group 1995).

#### Controlled warming-grazing experiment

The infrared warming system was established in May 2006 and was described previously by Kimball et al. (2008). Eight hexagonal arrays of Mor FTE infrared heaters (1,000 W, 240 V) were deployed over the vegetation canopy, with eight dummy arrays over control plots. The heaters were controlled using the proportional integral derivative output (PID) control system to ensure constant warming in warmed and control plots. The set-point differences of the vegetation canopy temperature between warmed and corresponding control plots were 1.2 °C during the daytime and 1.7 °C at night during the growing season (from May to September). The canopy temperatures were scanned every second by the sensors, and the heaters were modulated at 1-s intervals, although the time-constant of the ceramic heating elements is ~6 min (Kimball et al. 2008). Fifteen-min averages were output by the data loggers. The power output of the heaters was manually set at 1,500 W plot<sup>-1</sup> during winter (from October to April) because some infrared thermometers were not working.

The experiment plots used a factorial design with warming and grazing treatments and were replicated four times within each treatment combination: no-warming with no-grazing (control), no-warming with grazing, warming with no-grazing (warming), and warming with grazing. In total, 16 plots of 3-m diameter were used in a complete randomized block design in the field. Mean seasonal soil temperature increases in the warmed plots above the control plots ranged from 1.1 to 1.8 °C at 20-cm soil depth during the growing seasons in 2006, 2007 and 2008 (Luo et al.

2010; Lin et al. 2011) and by 1.9 °C at 10-cm soil depth from 2006 to 2010 (Wang et al. 2012). Soil moisture decreases by 15.6, 19.1 and 17.8 % at 10-cm soil depth in 2008, 2009 and 2010 (Wang et al. 2012). In this study, we only used soil samples from non-grazing plots.

#### Soil sampling

In each plot, we removed surface litter and collected three soil cores (16 mm in diameter) randomly from the surface soil (0–10 cm) and sub-layer soil (10–20 cm) on six sampling dates (Table S1). Our samples were representative of the below-ground biomass since previous studies showed that the biomass at 0–10 cm accounts for 85–95 % of the total biomass at 0–40 cm (Zhou 2001). Samples from the same depth were pooled, packed in polyethylene bags, immediately stored in a portable refrigerator, and transported to the laboratory. The composite samples were passed through a 2-mm sieve, and then all the visible plant materials were manually removed from the sieved soil. One-half of each processed sample was divided into two sub-samples, one stored at four and the other at –20 °C for no more than 1 week, and subsequently used for measurements of microbial biomass nutrients (C and N concentrations) and extracellular enzyme activity, respectively. The remaining soil samples were used to measure the gravimetric soil water content by drying at 105 °C for 48 h.

#### Nutrient availability measurement

In this study, we only measured soil nutrient availability during the growing season of 2010 (Table 2) by using ion-exchange resin bags (Allison et al. 2008).

Ion-exchange resins have been widely used to measure soil N availability (Allison et al. 2008; Natali et al. 2012; Giblin et al. 1994). In our experiment, each nylon mesh bag was filled with 5 g anion or cation exchange resin, soaked in 0.5 M HCl for 20 min, rinsed with deionized water, and washed with 2 M NaCl. We placed two anion and two cation bags in each warmed or control plot at 5 cm below the soil surface. The bags were retrieved about 6 weeks later, rinsed in deionized water and extracted with 60 mL 2 M KCl on a shaker table for 60 min. We determined  $\text{NH}_4^+$  and  $\text{NO}_3^-$  by colorimetric analysis with an FIAstar 5000 flow injection analyzer (FOSS Tecator, Höganäs, Sweden). Measurements from two anion or cation bags in each plot were averaged to represent the nutrient availability in that plot.  $\text{NH}_4^+$  and  $\text{NO}_3^-$  availability was expressed as  $\text{ng N g}^{-1}$  resin  $\text{day}^{-1}$ .

#### Microbial biomass measurement

We used the chloroform fumigation extraction method (Wu et al. 1990; Brookes et al. 1985; Vance et al. 1987) to measure microbial biomass C (MBC) and N (MBN) on August 5, and September 16, 2009. Six-gram soil samples were first fumigated with ethanol-free chloroform for 24 h and then extracted in 0.5 M  $\text{K}_2\text{SO}_4$  for 30 min. Meanwhile, six-gram subsamples were directly extracted in a similar manner without being fumigated. Both fumigated and non-fumigated extracts were filtered and frozen until analysis of DOC and dissolved organic nitrogen (DON). DOC concentration in the extracts was measured by a TOC analyzer (Multi N/C 3100, Analytik Jena, Germany). Colorimetric analysis by a flow injection auto analyzer (AutoAnalyzer 3, Seal Analytical, German) was used to determine DON concentration. The difference in concentration between the fumigated and non-

fumigated extracts is an estimate of MBC and MBN. To account for incomplete extractability, we used an extraction efficiency factor of 0.45 for microbial C (Vance et al. 1987; Wu et al. 1990) and 0.54 for microbial N (Brookes et al. 1985).

#### Soil extracellular enzyme assays

Phenol oxidase activity was measured on four sampling dates (May 3, June 14, August 9 and September 16 of 2010), BG and CB activity were measured on six sampling dates (August 5, September 16 of 2009 and May 3, June 14, August 9 and September 16 of 2010) and BX and AG were measured on two sampling dates (August 5, September 16 of 2009). In order to estimate potential enzyme activities in our soil samples (pH 6.4; 2 M KCl), enzyme assays were performed in soil slurries made with sodium acetate buffer (pH 5.5). We acknowledge that sodium acetate/acetic acid buffer has a pH range of 3.6–5.6 and would be inappropriate for samples with pH > 5.6 (German et al. 2011), but the questions being asked in this study were not influenced by the assay pH.

We measured POX activity (Table 1) following a modified version of the method described by German et al. (2011) and Saiya-Cork et al. (2002) using L-3,4-dihydroxyphenylalanine (DOPA) (Table 1) as the substrate. We homogenized 1 g of soil in 100 ml of 50 mM sodium acetate buffer (pH 5.5) in a Waring blender for 2 min. The slurries were then added to 96-well microplates. Fifty microliters of 5 mM DOPA was added to each well. The negative control contained 200  $\mu\text{l}$  of acetate buffer and 50  $\mu\text{l}$  of DOPA in each well. The blank contained 200  $\mu\text{l}$  sample suspension and 50  $\mu\text{l}$  of acetate buffer in each well. We used six analytical replicates for each soil sample, blank and control. The microplates were incubated in

**Table 2** Mean nutrient availabilities in the control and warmed soils in 2010

	Date	Control	Warming	<i>P</i> value
Resin $\text{NH}_4^+$ ( $\text{ng N g}^{-1}$ resin $\text{day}^{-1}$ )	2 May to 13 June	485 $\pm$ 90	557 $\pm$ 125	0.77
	13 June to 8 August	439 $\pm$ 129	496 $\pm$ 83	0.52
	8 August to 14 September	145 $\pm$ 16	211 $\pm$ 38	0.13
Resin $\text{NO}_3^-$ ( $\text{ng N g}^{-1}$ resin $\text{day}^{-1}$ )	2 May to 13 June	381 $\pm$ 42	432 $\pm$ 154	0.44
	13 June to 8 August	356 $\pm$ 104	384 $\pm$ 129	0.83
	8 August to 14 September	44 $\pm$ 18	77 $\pm$ 50	0.54

Values are means  $\pm$  SE ( $n = 4$ )



the dark at 4, 10, 15, 20, 25 or 30 °C for 18 h. Activity was quantified using a fluorescence spectrometer (Spectramax M2, Molecular Devices, USA) by measuring the absorbance at 450 nm. Enzyme activity was expressed as micromoles per gram dry weight per hour.

We measured the activity of four hydrolytic enzymes (Table 1) following a modified version of the method described by Saiya-Cork et al. (Saiya-Cork et al. 2002) and Steinweg et al. (2012) using fluorometric techniques, which included the construction of unique seven-point calibration curves for each soil sample to provide better correction for non-linear fluorescence quenching dynamics than the usual single-point correction (Weedon et al. 2012). We homogenized 2.75 g of soil in 91 ml of 50 mM sodium acetate buffer (pH 5.5) in a Waring blender for 2 min. Slurries of 200 µl were then added to 96-well microplates. Fifty microliters of 200 µM fluorometric substrate (saturating concentration) were added to each well. We used six analytical replicates for each soil sample. The microplates were incubated in the dark at 4, 10, 15, 20, 25 or 30 °C for 24 h. The amount of fluorescence was determined using a fluorescence spectrometer (Spectramax M2, Molecular Devices, USA) set to 365 nm for excitation and 450 nm for emission. Enzyme activity was expressed as nanomoles per gram dry weight per hour.

Optimum temperature, estimated as the temperature at which the maximum value of enzyme activity occurred was obtained using the Johnson and Lewin model (Johnson and Lewin 1946) (Table S2).  $T_{\text{opt}}$  generally ranged from 27 to 30 °C, and in some cases, it was > 30 °C. Considering that the  $T_{\text{opt}}$  of some enzymes was < 30 °C (Table S2), we limited our investigation to five temperature steps (4, 10, 15, 20 and 25 °C) for all the enzymes. This permitted the comparison of  $Q_{10}$  on different sampling dates with those obtained by fitting the simple first-order exponential equation,

$$f(T) = A \cdot \exp(B \cdot T) \quad (1)$$

where  $A$  is the exponential constant, or activity at 0 °C,  $B$  is the exponential parameter of the equation and  $T$  is temperature in degrees Celsius (°C). We fitted Eq. 1 by ordinary least-squares regression of log-transformed enzyme activity on log-transformed temperature. Following Wallenstein et al. (2009), regression

coefficients (slopes) represent the temperature sensitivity as  $Q_{10}$  values according to:

$$Q_{10} = \exp(\text{slope} \times 10) \quad (2)$$

### Statistical analyses

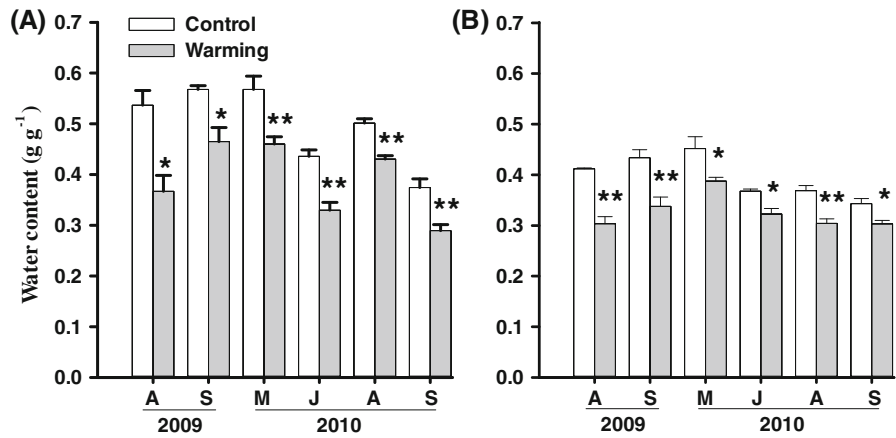
Repeated measure analysis of variance (ANOVA) was used to assess the effects of warming and sampling time on soil water content, nutrient availability, soil microbial biomass, labile C and N content, enzyme activity and the apparent  $Q_{10}$  of enzyme activity with block as a random factor and warming as a fixed effect. If the interactive effects of warming and sampling time were significant, the effects of warming within each date were tested using a post hoc paired  $t$  test. Soil water content and soil microbial biomass, labile C and N content, nutrient availability, enzyme activity and  $Q_{10}$  values were log-transformed to improve normality. Two-way ANOVA was used to test the effects of treatment (warming, control), and incubation temperature (4, 10, 15, 20, 25 and 30 °C) on enzyme activity (Table S3). A significance level of < 0.05 was used for all analyses except where noted. All statistical analyses were performed in R 2.3.0 (R Development Core Team 2011).

## Results

### Soil water content, nutrient availability and microbial biomass

Soil water content declined significantly in the warmed plot at 0–10 cm and 10–20 cm (Fig. 1). There were no interactions between warming and sampling date at 0–10 cm ( $P = 0.174$ ) and at 10–20 cm ( $P = 0.072$ ).  $\text{NH}_4^+$ -N was the dominant form of inorganic nitrogen in the alpine grassland ecosystem (Table 2). On average,  $\text{NH}_4^+$  availability was 1.4 times higher than the  $\text{NO}_3^-$  availability. The main effect of warming was not significant for either  $\text{NH}_4^+$  ( $P = 0.468$ ) or  $\text{NO}_3^-$  availability ( $P = 0.876$ ).  $\text{NH}_4^+$  and  $\text{NO}_3^-$  availability showed similar seasonal patterns, and were highest in the early-growing season and lowest in the late-growing season of 2010. The main effect of warming was not significant for MBC at 0–10 cm and 10–20 cm soil depth (Table 3). No significant effects of warming on MBN were detected except in August 2009 at 10–20 cm soil depth

**Fig. 1** Soil water content in the warmed and control plots at 0–10 cm (A) and 10–20 cm (B). Bars show mean and 1 SE on each date ( $n = 4$ ). Asterisk and double asterisk denote significance level ( $P < 0.05$  or  $P < 0.001$ ) for warming effects on the soil water content within the sampling date. A August, S September, M May, J June



( $P = 0.017$ ). Labile C and N content showed no significant response to warming on either of the two sampling dates (Table 3).

#### Soil extracellular enzyme activity

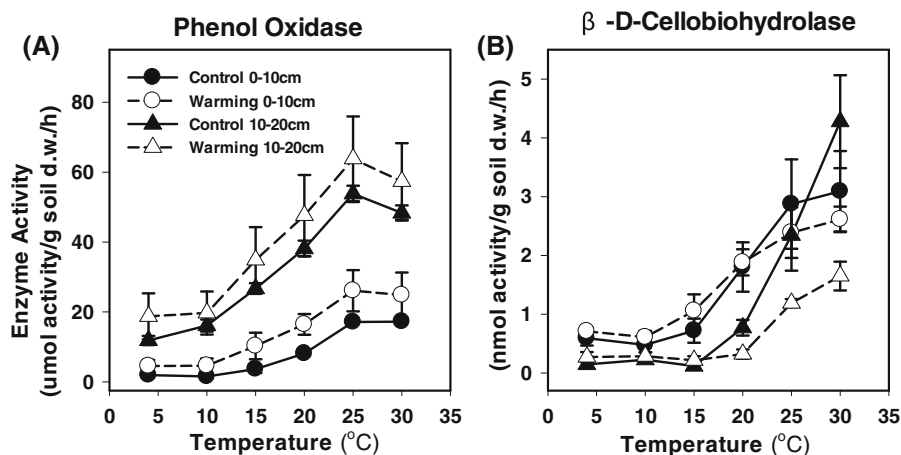
The activity of all extracellular enzymes increased with higher incubation temperature (e.g. Fig. 2). We found main effects of incubation temperature on the activity of all five enzyme throughout the sampling

dates at 0–10 cm ( $P < 0.001$ ) and 10–20 cm soil depth ( $P < 0.050$ ) (Table S3). However, the treatment (experimental warming) effects were only significant in 12 out of 40 cases (Table S3; see Fig. 2a 10–20 cm). The interactions between incubation temperature and treatment were only significant in 1 out of 40 cases ( $P = 0.021$ ), which was for CB in May, 2010 at 10–20 cm (Fig. 2b). Thus, we chose the potential activity at 20 °C to assess the effects of warming and sampling date and their interactions (Tables 4, S3,

**Table 3** Microbial biomass C (MBC), N (MBN) and  $K_2SO_4$  extracted C ( $C_{KSO}$ ) and N ( $N_{KSO}$ ) in the control and warmed soils on August 5, and September 16, 2009. Values are means  $\pm$  SE ( $n = 4$ )

	Date	Depth (cm)	Control	Warming	<i>P</i> value
MBC (mg C kg <sup>-1</sup> dry soil)	Aug.	0–10	1807 $\pm$ 242	1600 $\pm$ 235	0.260
		10–20	1307 $\pm$ 193	1005 $\pm$ 226	0.330
	Sept.	0–10	2121 $\pm$ 148	1756 $\pm$ 98	0.078**
		10–20	1003 $\pm$ 165	1608 $\pm$ 419	0.260
MBN (mg N kg <sup>-1</sup> dry soil)	Aug.	0–10	214 $\pm$ 26	197 $\pm$ 19	0.300
		10–20	122 $\pm$ 6	78 $\pm$ 13	0.017*
	Sept.	0–10	275 $\pm$ 22	305 $\pm$ 21	0.280
		10–20	133 $\pm$ 7	147 $\pm$ 9	0.300
$C_{KSO}$ (mg C kg <sup>-1</sup> dry soil)	Aug.	0–10	341 $\pm$ 91	340 $\pm$ 54	0.840
		10–20	180 $\pm$ 54	243 $\pm$ 26	0.260
	Sept.	0–10	520 $\pm$ 21	606 $\pm$ 40	0.081**
		10–20	488 $\pm$ 80	455 $\pm$ 125	0.840
$N_{KSO}$ (mg N kg <sup>-1</sup> dry soil)	Aug.	0–10	68 $\pm$ 6	41 $\pm$ 7	0.370
		10–20	32 $\pm$ 3	27 $\pm$ 3	0.240
	Sept.	0–10	98 $\pm$ 10	92 $\pm$ 2	0.650
		10–20	62 $\pm$ 7	54 $\pm$ 5	0.370

\* Indicates  $P < 0.05$ ; \*\* indicates  $P < 0.10$



**Fig. 2** Temperature dependence of enzyme activity at 4, 10, 15, 20, 25 and 30 °C (mean  $\pm$  1 SE,  $n = 4$ ). **A** Rates of POX activity in August 2010. **B** Rates of CB activity in May 2010

S4). In general, we found no significant effects of warming on the activity of the oxidative enzyme or the four hydrolytic enzymes (Fig. 3; Table 4). The seasonal patterns of enzyme activity differed for most enzymes (Fig. 3; Table 4). POX, BG, CB and BX showed the highest activity in June or August corresponding to the early- to mid-growing season (Table S1). No significant interactions between warming and sampling date were found for any of the five enzymes (Table 4). POX activity was much lower in the 0–10 cm surface soil than the 10–20 cm soil (Fig. 3), and this pattern was different from the other four hydrolytic enzymes.

#### Temperature sensitivity of soil extracellular enzymes

In general, no significant warming effect was detected in the  $Q_{10}$  of the oxidative enzyme and the four hydrolytic enzymes (Table 4). Only BG in May, 2010 at 10–20 cm and CB in June, 2010 at 10–20 cm were significantly lower and CB in September, 2009 at 0–10 cm was significantly higher in warmed than control plot soils (Fig. 4). Seasonal patterns of  $Q_{10}$  differed for most of the enzymes (Fig. 4; Table 4). There were no significant interactions between warming and sampling date for any of the enzymes (Table 4). The  $Q_{10}$  of POX was relatively invariant on the sampling dates at 10–20 cm (Fig. 4). This pattern was different from the seasonal pattern of the  $Q_{10}$  of POX at 0–10 cm.

## Discussion

### Soil extracellular enzyme activity

Warming had little effect on the potential activity of the five oxidative and hydrolytic enzymes. Our results are consistent with most previous field experiments showing that enzyme activity is insensitive to small increases in soil temperature (Bell et al. 2010; Bell and Henry 2011; Sardans et al. 2007). Among all of these studies, the effects of warming were too small relative to the seasonal temperature variation to elicit a response (Bell et al. 2010). Furthermore, our results did not support the primary hypothesis that oxidative and hydrolytic enzyme activity would respond differently to experimental warming. Our results suggested that experimental warming has no impacts on the relative depolymerization rates of soil C fractions of different chemical qualities.

This weak impact of warming on enzyme activity may be due to the reduction of soil moisture. Microbes may down-regulate enzyme production, especially substrate or other resources become limiting (Allison et al. 2011). No significant or only small effects of warming on MBC, MBN,  $C_{KSO}$ , and  $N_{KSO}$  may indirectly indicate the regulation of enzyme production. In addition, as field soil moisture decreases, the extant pool of functional enzymes may not be able to interact freely with substrates due to the constraints of diffusion. In this study, we found that the soil water content was significantly lower in warmed plots at all



**Table 4** Results of the repeated measure ANOVA for the interactive effects of treatment (warmed and control) and sampling date on enzyme activity at 20 °C and their  $Q_{10}$ 

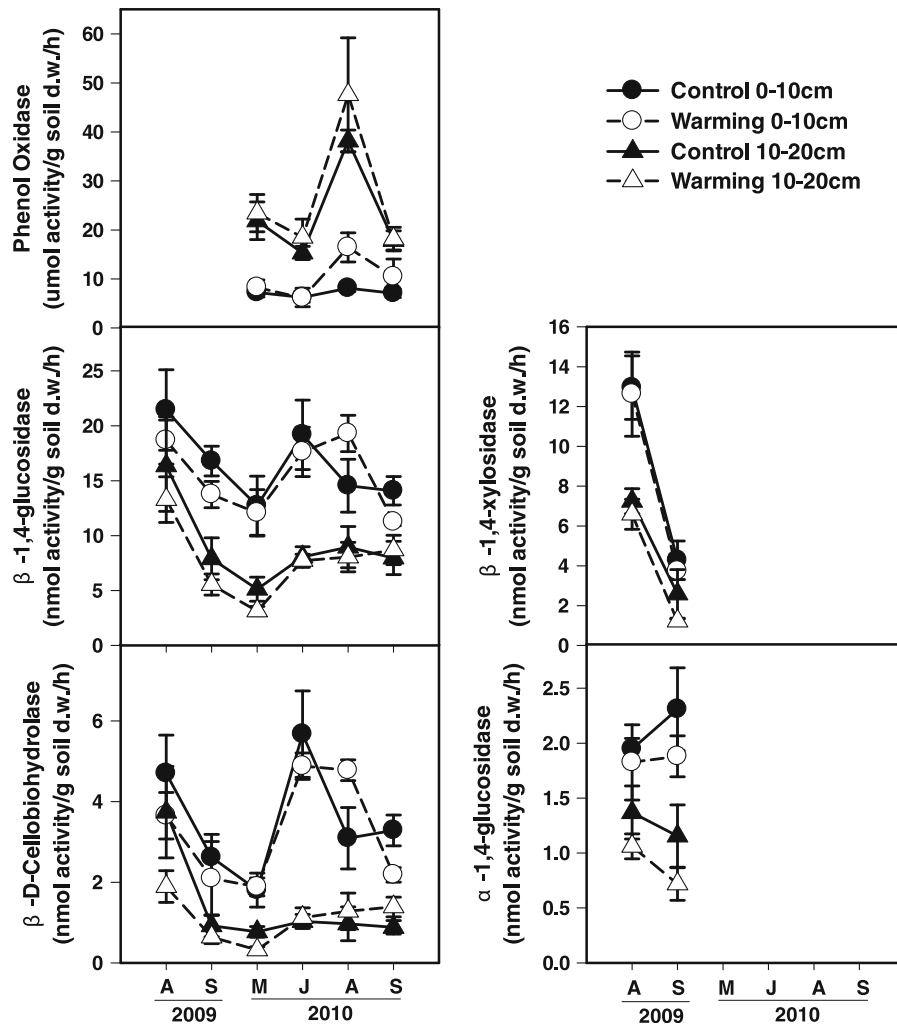
Enzyme	df	Enzyme activity						$Q_{10}$					
		0–10 cm			10–20 cm			0–10 cm			10–20 cm		
		MS	F	P	MS	F	P	MS	F	P	MS	F	P
<i>POX</i>													
Treat.	1	0.536	2.381	0.138	0.053	0.846	0.368	0.055	0.703	0.411	0.000	0.006	0.940
Date	3	0.660	2.928	<b>0.057</b>	1.374	22.080	<b>0.000</b>	0.391	4.985	<b>0.009</b>	0.027	0.864	0.475
Date × Treat.	3	0.153	0.680	0.574	0.007	0.109	0.954	0.027	0.340	0.797	0.002	0.069	0.976
<i>BG</i>													
Treat.	1	0.033	0.396	0.534	0.226	1.309	0.261	0.051	1.429	0.241	0.003	0.100	0.754
Date	5	0.300	3.556	<b>0.011</b>	1.385	8.010	<b>0.000</b>	0.101	2.816	<b>0.032</b>	0.038	1.278	0.297
Date × Treat.	5	0.077	0.917	0.483	0.070	0.407	0.840	0.003	0.073	0.996	0.069	2.352	<b>0.062</b>
<i>CB</i>													
Treat.	1	0.140	0.544	0.466	0.049	0.087	0.769	0.147	2.219	0.147	0.2046	1.808	0.192
Date	5	1.543	6.020	<b>0.000</b>	2.489	4.434	<b>0.004</b>	0.666	10.061	<b>0.000</b>	0.762	6.738	<b>0.001</b>
Date × Treat.	5	0.324	1.263	0.304	0.722	1.285	0.295	0.218	3.297	<b>0.017</b>	0.301	2.659	<b>0.049</b>
<i>BX</i>													
Treat.	1	0.022	0.158	0.701	0.238	0.851	0.380	0.008	0.249	0.631	0.005	0.657	0.439
Date	1	4.994	35.567	<b>0.000</b>	9.490	33.929	<b>0.000</b>	0.177	5.326	<b>0.050</b>	0.276	35.770	<b>0.000</b>
Date × Treat.	1	0.000	0.002	0.965	0.080	0.288	0.605	0.060	1.806	0.216	0.003	0.397	0.544
<i>AG</i>													
Treat.	1	0.070	0.622	0.453	0.369	2.203	0.172	0.019	0.097	0.764	0.059	0.380	0.553
Date	1	0.022	0.197	0.669	0.461	2.752	0.131	0.086	0.448	0.522	0.082	0.528	0.486
Date × Treat.	1	0.006	0.056	0.819	0.033	0.197	0.667	0.016	0.081	0.783	0.183	1.174	0.307

The abbreviations of enzymes are as in Table 1. Bold indicates  $P < 0.10$

the sampling dates with reductions up to 32 % (Fig. 1). Our results are consistent with those of Allison & Treseder (2008), who reported that warming results in a 22 % decrease of soil moisture, accompanied by little effect on soil enzyme activity, except for a weak trend toward lower activity of *N*-acetylglucosaminidase. Thus, the reduction in soil moisture due to warming likely contributed to the absence of responses by soil extracellular enzymes.

We used the potential activity of soil extracellular enzymes under standard laboratory conditions (20 °C) to examine the long-term responses of microbial communities to experimental warming. However, the short-term temperature responses of the extant enzyme pool to warming should not be ignored (Fig. 2), because such a short-term increase of temperature might increase enzyme activity without changing the enzyme pools. Bell et al. (2010) reported that the potential activity of hydrolase enzymes is ~20 times higher in soil slurries incubated at 22 °C

than those incubated at 1 °C. Baldrian et al. (2012) also reported that enzyme activity in vitro strongly increases with temperature up to the highest temperature recorded in situ (20–25 °C). Positive relationships between temperature and enzyme activity are consistently found (Wallenstein et al. 2009; Stone et al. 2012; Trasar-Cepeda et al. 2007; German et al. 2012; Koch et al. 2007; Khalili et al. 2011). It is important to consider short-term temperature responses to understand the role of temperature in regulating enzyme activity, because the potential activity measured under standard laboratory conditions might not represent the in situ experimental warming effects. Indeed, the in situ temperatures are different between the control and warmed plots under long-term experimental warming in the study; such a difference might induce an increase in the activity of soil extracellular enzyme from the warmed soils as shown in Fig. 2. Therefore, more information is required to link the potential activity of soil



**Fig. 3** Potential activity of POX, BG, CB, BX and AG measured on different sampling dates (August 5, September 16 of 2009 and May 3, June 14, August 9 and September 16 of

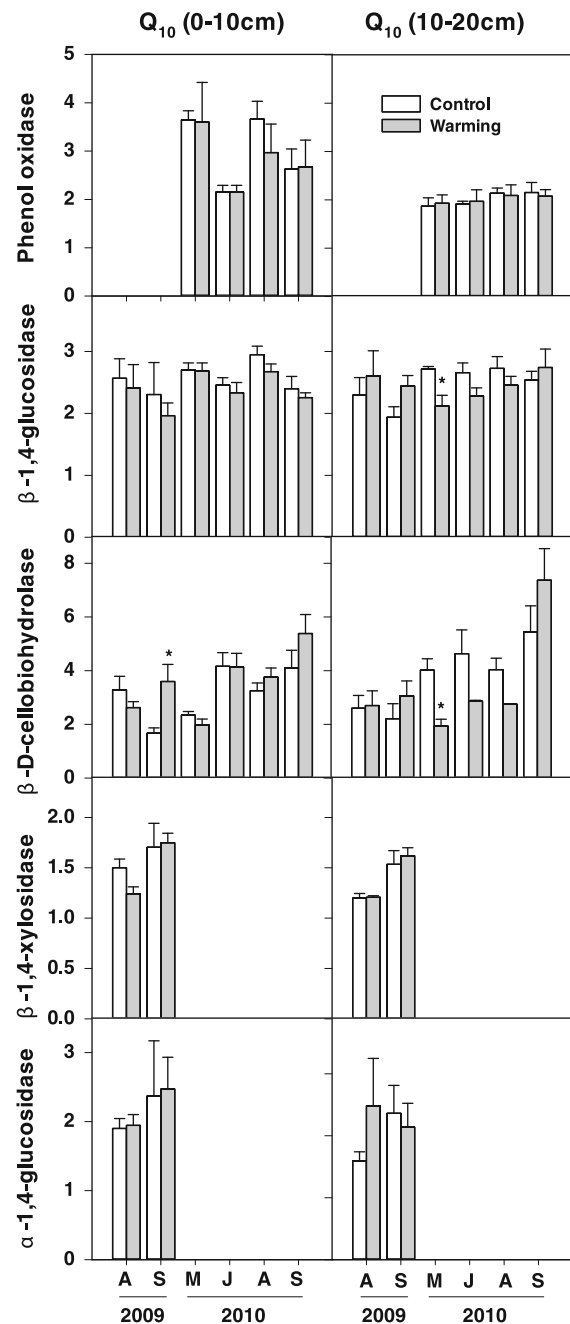
2010) under the standard laboratory condition (20 °C) at 0–10 cm and 10–20 cm (mean  $\pm$  1 SE,  $n = 4$ )

extracellular enzyme measured in the lab with in situ rates (Wallenstein and Weintraub 2008).

Although warming had little effect on the potential activity of the five oxidative and hydrolytic enzymes, seasonal variation in soil extracellular enzyme activity is important to understand microbial adaptation to long-term climate change. In this study, N-availability might be the key driver regulating seasonal patterns of enzyme activity in the alpine grassland ecosystem. Although the potential activity of most soil extracellular enzymes was highest in the mid-growing season of 2010, the  $\text{NH}_4^+$  and  $\text{NO}_3^-$  availability was lowest during the same period. Our results are consistent with

some studies, such as Weintraub and Schimel (2005), who found that potential protease activity peaks as nutrient pools crash in the mid-growing season. In contrast, Wallenstein et al. (2009) showed that most of the potential enzyme activity in Arctic tundra soils are highest before the soils thaw and decline during summer, and concluded that N-limitation may explain the declines in overall enzyme activity. Substrate availability may be one of the keys to understand seasonality in enzyme activity (Boerner et al. 2005). For example, Kaiser et al. (2010) reported that soil enzyme activity reaches peak values in spring or autumn, due to root exudates or input of fresh litter. In

this study, we found that the increased enzyme production was associated with decreased levels of soluble N in the environment, which could be a



**Fig. 4**  $Q_{10}$  of POX, BG, CB, BX and AG on different sampling dates (August 5, September 16 of 2009 and May 3, June 14, August 9 and September 16 of 2010) at 0–10 cm and 10–20 cm (mean  $\pm$  1 SE,  $n = 4$ ). Asterisk indicates  $P < 0.05$ . A August, S September, M May; J June

microbial strategy to avoid N-limitation by increasing production of C- and N-acquiring enzymes during periods of reduced soil mineral N.

In addition, seasonal variations in soil enzyme activity have been attributed to variations in temperature and/or moisture (Boerner et al. 2005; Fenner et al. 2005). At our study site, the mean annual temperature is  $-1.7$  °C, so temperature might a major controlling factor that drive seasonal patterns of soil extracellular enzyme activity because the enzyme-producing activity of microbes is likely limited by temperature in the early- or late-growing seasons, while this limitation does not persist during the mid-growing season. For example, Wittmann et al. (2004) and Baldrian et al. (2012) reported that potential enzyme activity is predicted to account for 7–32 or 14–54 % of total annual activity in the cold period. Several studies have reported similar results that maximum microbial enzyme activity occurs in summer (Matinizadeh et al. 2008; Baldrian et al. 2012; Kang and Freeman 1999). Here, we showed that in the alpine grassland, temperature also strongly affects the seasonal patterns of enzyme activity, leading to higher activity in the warm season. However, seasonal temperature cycles are confounded with changes in plant activity, soil moisture and microbial community composition (Curiel Yuste et al. 2007; Björk et al. 2008). Therefore, temperature should not be viewed in isolation (Davidson et al. 2000) and temperature control of enzyme activity need to be further explored in other biomes (Wallenstein et al. 2011).

We found that POX activity was much lower in the 0–10 cm surface soil than the 10–20 cm soil. Within the soil profile, enzyme activity generally declines with soil depth. However, there are exceptions (Brockett et al. 2012). Qiu et al. (2005) found that POX activity showed a ‘wave’ distribution, in which activity peaked at 15 cm. In addition, in soils vegetated with gamagrass, POX activity increases with depth and with decreasing aggregate size (Jintaridith et al. 2009). The aggregate size decreased with soil depth at our study site; the proportion of aggregate  $<0.002$  mm was 20.8 % at 0–10 cm and 25.0 % at 10–20 cm (Cao 2010). Therefore, this may account for the increase in POX activity with soil depth. In addition, the soil developed in this ecosystem is Mat-Gryic Cambisol, and most dead and live roots are distributed in the upper layer (0–8 cm) (Cao 2010). Thus, this labile C pool is likely the primary substrate

used by plants and microbes to support primary metabolism, while the lignin and secondary compounds are likely the priority substrates at 8–20 cm.

#### Temperature sensitivity of soil extracellular enzymes

The  $Q_{10}$  values of individual enzymes obtained in our study ranged between 1.47 and 3.48, roughly corresponding to the values for tropical to boreal soils (1.5–3.0) (Wallenstein et al. 2009; Stone et al. 2012; German et al. 2012). The mean  $Q_{10}$  value of CB was highest (3.48) among the five enzymes. This indicates that there would be a larger change in the activity of CB than those of the other enzymes with the same temperature increase. Very few studies have examined the  $Q_{10}$  of POX (Trasar-Cepeda et al. 2007), for the methods of assaying its activity are not well resolved (Sinsabaugh 2010; German et al. 2011) and POX assays do not generally follow Michaelis–Menten kinetics (German et al. 2012). In this study, we found that POX activity followed a simple first-order exponential equation at temperatures below 25 °C. The mean  $Q_{10}$  of POX was 2.47, which is close to the overall mean value of 2.41 for all five enzymes. This indicates that the changes in rates of oxidative enzyme reaction would not result in disproportionately higher decomposition rates of chemically complex compounds (e.g. lignin) in this alpine grassland ecosystem. Therefore the results did not support the primary hypothesis that oxidative and hydrolytic enzyme activity would respond differently to experimental warming. In addition, our results were not consistent with those of Wang et al. (2011), who reported that the  $Q_{10}$  of POX is significantly higher than those of other soil enzymes in a subtropical forest, due to the quantity and quality of POX substrates (lignin). Because of the range and complexity of biological and physiochemical factors that affect enzyme temperature-sensitivity (Wallenstein et al. 2011), we do not yet have a mechanistic understanding of the drivers of  $Q_{10}$  of POX from the findings in this study.

We found no significant effects of warming on the  $Q_{10}$  of the oxidative enzyme and the four hydrolytic enzymes. Wallenstein et al. (2009) reported that the  $Q_{10}$  of most enzymes is greatest in frozen soils. Such adaptation of microbial extracellular enzymes to temperature has also been reported by German et al.

(2012), who reported that some microbial extracellular enzymes from higher latitudes are more sensitive to temperature increases than those from lower latitudes. In fact, cold-adapted microorganisms tend to be more responsive to increasing temperature than warm-adapted microorganisms (Koch et al. 2007; Dong and Somero 2009; Brzostek and Finzi 2012), which increase the catalytic potential of enzyme (Bradford et al. 2010; Siddiqui and Cavicchioli 2006) and substrate affinity (Fenner et al. 2005) to offset the decrease in enzyme activity at lower temperatures (Georlette et al. 2004). However, our results did not support our assumption that enzymes from control soils would be more responsive to field warming. One of the reasons could be that the effects of warming were too small relative to diurnal or seasonal temperature variation. Another reason may be soil moisture limitation, which has been reported by Suseela et al. (2012), who found that warming does not affect the apparent  $Q_{10}$  of heterotrophic respiration due to limited soil moisture.

We found that the  $Q_{10}$  of soil extracellular enzymes varied among different sampling dates (Fig. 4). In fact, several studies have demonstrated that the temperature sensitivity of extracellular enzymes changes seasonally (Brzostek and Finzi 2012; Wallenstein et al. 2009; Fenner et al. 2005; Koch et al. 2007; Trasar-Cepeda et al. 2007). Changes in isoenzyme pools might be the main reason for the variation in enzyme activity on different sampling dates (Wallenstein et al. 2009, 2011), but the drivers of seasonal patterns of  $Q_{10}$  are not fully understood. We found that the  $Q_{10}$  of POX varied with soil depth (Fig. 4). At 10–20 cm, a relatively invariant  $Q_{10}$  of POX was found on the sampling dates. This pattern differed from the seasonal pattern of the  $Q_{10}$  of POX at 0–10 cm, and indicated that POX at different soil depths is likely to have different patterns of seasonality. The different seasonal patterns of POX might result from soil temperature change across the soil profile (Luo et al. 2009), such the temperature change with depth may vary from diel to seasonal timescales. Thus the choice of the soil depth used for inferring the  $Q_{10}$  of POX may strongly influence the shape of the temperature response curve. In addition, the seasonal patterns are more likely to result from changes in isoenzyme pools of POX at different depths, because we found the activity of POX increased over the soil profile at 0–10 and 10–20 cm.

## Absence of temperature acclimation of soil extracellular enzymes to experimental warming

In our study, experimental warming did not change the  $Q_{10}$  of most soil extracellular enzymes, whereas soil respiration significantly decreased during the growing seasons in 2009 and 2010 (He et al., unpublished data). The decrease of soil respiration in 2009 and 2010 were not from the effects of warming on the activity or  $Q_{10}$  of soil extracellular enzymes. Davidson and Janssens (2006) suggested that the  $K_m$  and  $V_{max}$  of most enzymes increases with temperature, and the  $Q_{10}$  of  $K_m$  and  $V_{max}$  can neutralize each other, which could offset temperature-induced increases in respiratory activity. In this study, we assumed that the enzyme substrates were abundant, and thus  $K_m$  becomes insignificant, and the temperature response of  $V_{max}$  determines that of the respiratory activity. Our results showed that there were no significant effects of warming on the activity and  $Q_{10}$  of most soil extracellular enzymes, which suggest that the effects of warming on the activity of these enzymes cannot directly result in the decrease of soil respiration. Thus, our primary prediction that soil extracellular enzymes would acclimate to elevated temperature with reduced  $Q_{10}$  in warmed soils was not supported. However, substrate quality, temperature, and temporal and spatial differences in substrate availability can also contribute to the large variability in  $Q_{10}$  observed in natural ecosystems (Davidson and Janssens 2006). Hartley et al. (2007, 2008) reported that substrate availability, not microbial acclimation to the higher temperature, plays a dominant role in determining the response of heterotrophic soil respiration to warming. Thus, the changes of  $K_m$  with temperature could explain the lack of warming response in this study. In addition, it is likely that reduced soil moisture played a role in the negative effects of warming on soil respiration, because reduced soil moisture may cause substrate limitation and suppress decomposition rates. Thus, the indirect effects of our experimental warming on microbial substrates and soil moisture probably contributed to the negative responses of soil respiration.

Our study used a specific experimental setup to simultaneously examine the effects of warming on soil nutrient availability, microbial biomass, and the potential activity and  $Q_{10}$  of soil extracellular enzymes. We found no significant warming effects on these parameters in the alpine grassland ecosystem. Soil respiration may be more strongly constrained by declining soil

moisture than by temperature. Our results indicate that rising temperatures in high altitude ecosystems may cause a negative feedback on the soil C cycle, particularly in alpine grassland ecosystems with drier soils. One of the most important reasons for understanding the temperature sensitivity of soil enzymatic reactions is to improve predictions of soil respiration responses to temperature (Wallenstein et al. 2011). Although absence of microbial acclimation to experimental warming at the level of soil extracellular enzymes was found in our study, the drivers of soil respiration at the molecular level need further investigation. In addition, because of the uniqueness in geography, climate, and soil characteristics, concurrent investigation of the effects of warming on enzyme activity and temperature sensitivity will generate further insights into the effects of global warming on terrestrial C balance and will improve our ability to predict how soil biogeochemical cycles will respond to changes in the environment. Therefore, additional studies of soil extracellular enzymes responses to long-term temperature change and climate gradients are essential for accurately predicting soil C turnover in a changing climate.

**Acknowledgments** We thank Donovan German, Steven Allison, Matthew Wallenstein, Meg Steinweg and Shawna McMahon for help with lab protocols and advice, Lei Liu for access of microplate reader, Wenjing Chang and Zhengxia Chen for assistance in soil microbial biomass analysis, Xuemei Zhao and Jianjing Ma for assistance with enzyme assays, and Nan Cong and Samraat Pawar for statistical analysis. The authors are grateful to Biao Zhu, Chao Song, Yan Geng, Yue Shi, Iain Bruce and three anonymous referees for valuable and constructive comments that substantially improved this manuscript. This study was supported by the National Program on Key Basic Research Project (Grant No. 2010CB950602), the National Natural Science Foundation of China (Grants No. 31025005 and 31021001), and the Program of “One Hundred Talented People” of the Chinese Academy of Sciences (Grant No. KSCX2-YW-Z-0806). HC is supported by the Research Fellowship for International Young Scientists of Chinese Academy of Sciences. SW is partially supported by the National Natural Science Foundation of China (Grant No. 41230750) and the National Basic Research Program (2010CB833502). We thank USDA Grant to SSI (2010) at Colorado State University that allowed XJ to learn enzyme assays.

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