

Microbial Communities and Associated Enzyme Activities in Alpine Wetlands with Increasing Altitude on the Tibetan Plateau

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Abstract Microbial communities, enzyme activities and soil physiochemical characteristics were investigated in alpine wetlands with altitude changes in the Nyainqentanglha Mountains on the Tibetan plateau. The results showed that with an increasing altitude gradient, soil physicochemical properties such as total organic carbon (TOC), total nitrogen (TN), water-soluble organic carbon (WSOC), water-soluble organic nitrogen (WSON) and pH decreased coincident with a decrease in mean annual temperature (MAT). Bacteria biomass, fungal biomass and actinobacterial biomass all decreased with an increase in altitude gradient. The ratio of Gram-positive bacteria to Gram-negative bacteria and the ratio of cyclopropyl to precursor fatty acids all indicate that the level of environmental harshness intensified with an increase in altitude. We also found that soil enzyme activities such as phenol oxidase, peroxidase, L-asparaginase, protease, urease and alkaline phosphatase all consistently decreased with an increase in altitude gradient. Additionally, the activities of peroxidase, protease and alkaline phosphatase declined, mainly due to a reduction in enzyme activities with temperature rather than the reduction of associated microbial biomass. Statistical analysis showed that both microbial biomass and enzyme activities were significantly influenced by mean

annual temperature, suggesting that temperature is a key factor that affects microbial communities and soil enzyme activities in alpine wetlands on the Tibetan Plateau and indicating that climate warming may significantly impact these areas.

Keywords Microbial community · Enzyme activity · Altitude gradient · Alpine wetlands

Introduction

To better understand the impact of global climate change on a variety of ecosystems, knowledge of altitudinal diversity patterns is needed (Lomolino 2001; Rahbek 2005). Soil microorganisms play an important role in soil organic turnover and biogeochemical cycling. Additionally, soil microbial community composition and activity are influenced by biotic and abiotic factors. Many recent studies have begun to focus on latitude distribution of microbial communities and activities (Fierer and Jackson 2006; Griffiths et al. 2011; Rousk et al. 2010; Margesin et al. 2009), whereas little information on microbial composition or diversity patterns is available for altitudinal gradients. Previous studies showed that fungal biomass and diversity decreased with an increase in altitude in the Austrian Central Alps (Margesin et al. 2009; Schinner and Gstraunthaler 1981). Similarly, a decrease in the bacteria population with an increase in altitude was observed in the cold temperate Kalasi Lake and a tropical dry deciduous forest (Giri et al. 2007; Ma et al. 2004). In contrast, Mannisto et al. (2007) observed that microbial communities were affected by soil pH rather than altitude in the Arctic fjelds of Finnish Lapland. Shen et al. (2013) also observed that soil pH was the key factor that drove the spatial distribution of microbial communities along an altitudinal gradient at Changbai Mountain. Moreover, Djukic et al. (2010) suggested that microbial community

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structure was controlled by decomposition conditions and vegetation composition along an altitudinal gradient in the Austrian Limestone Alps.

Studies on soil microbial communities in wetlands along altitude gradients are still very limited. Wetlands are special ecosystems at the interface between terrestrial and aquatic ecosystems, providing a unique habitat for a diverse range of microbial species. However, wetlands are very sensitive to global climate change (Erwin 2009) and respond to it through alteration of energy, hydrological or carbon patterns. Wetlands also play an important role in the regulation of global climate change by sequestering and releasing a major proportion of atmospheric carbon in the biosphere (Limpens et al. 2008; Ma et al. 2016). Therefore, a better understanding of the changes in wetland microbial communities and their associated activities in response to altitudinal gradients and underlying mechanisms is of scientific and economic importance, especially in the context of potential climate change.

The Tibetan Plateau, known as “the third pole” of the world, is located in the middle of the Asian continent with a total area of over 2.5 million square kilometres. Because of its extreme cold and semiarid-arid environmental features, the Tibetan Plateau is very sensitive to climate change (Yao et al. 2000). The total wetland area of the Tibetan Plateau is very large and has important ecological functions such as climatic regulation and water storage. The Nyainqentanglha Mountains, marked by a wide distribution of extremely tall mountains, are located in the middle and south of the Tibetan Plateau. There are multiple wetlands in the Nyainqentanglha Mountains ranging from 4200 m to 5100 m, accompanied by changes in geographic factors. Therefore, the Tibetan Plateau provides an ideal place for the study of the structure of microbial communities along an elevation gradient.

Phospholipid fatty acid (PLFA) technology uses the cellular membrane lipids of microorganisms as biomarkers for specific organisms, and thus is commonly used to create the fingerprint of studied microbial communities or indicators of environmental stress (Guckert et al. 1986). The total concentration of PLFA is used to measure viable microbial biomass, since phospholipids are readily degraded after cells die (Zelles 1997). Soil enzyme activities play a very important role in many soil functions such as nutrient cycling, decomposition and organic matter decomposition. Moreover, soil enzyme activities are related to soil physiochemical characters (Amador et al. 1997), the structure of microbial community (Kourtev et al. 2002; Waldrop et al. 2000), vegetation type (Kourtev et al. 2002; Sinsabaugh et al. 2002), disturbance (Boerner et al. 2000; Bolton et al. 1993; Garcia and Hernandez 1997), and successional stage (Tscherko et al. 2003). We integrated these two methods to study microbial activities with an increase of elevation. Also, we want to decipher whether the change of microbial activities is caused by the change of microbial biomass or the response of enzymes to a decrease of temperature.

Accordingly, the aims of our research are (1) to examine the soil microbial community structure and soil enzyme activities with altitude changes in the Nyainqentanglha mountains, and (2) to study the relationship between community structure and soil enzyme activities and their driving factors.

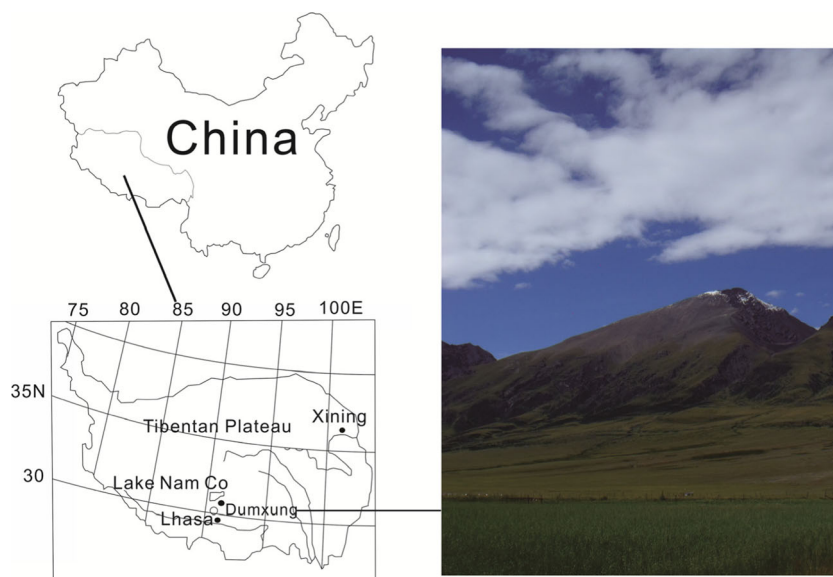
Materials and Methods

This study was conducted along the south-facing slope of the Nyainqentanglha Mountains in the central Tibetan Plateau (30°29′–30°43′N, 91°05′–91°08′E). To study the impact of environmental factors on microbial activities, we chose alpine wetlands located along Dangqu river in an altitudinal gradient in Damxung County (Fig. 1). With the increase of altitude, environmental factors, such as temperature, precipitation and radiation, change coincidentally. To minimize potential influences of precipitation, alpine wetlands were chosen because soils in alpine wetlands are immersed with large amount of water so that precipitation may not strongly affect soil water content. There are many alpine wetlands located in Damxung County with a range of altitude between 4200 m and 5000 m a.s.l. (30°29′–30°43′N, 91°05′–91°08′E). Seven alpine wetlands lied in different altitudes were chosen (Table 1). According to the meteorological observation between 1963 and 2008 at Damxung station (4288 m a.s.l.), the mean annual air temperature (MAT) in January and July was 1.8 °C and 10.9 °C, respectively. Also, with an increase of additional 100-m above sea level, the temperature dropped more than 0.5 °C (Table 1). The annual precipitation was 479 mm (290–700 mm, with >85% rainfall occurring in the growing season between May and September), which was much less than the annual pan evaporation of 1726 mm (Wang et al. 2013). The major vegetation types include alpine meadow and sedge, with *Oxytropis ochrocephala* and *Kobresia* as dominant species. No specific permissions were required for the field survey which was done in a small plot in a pastureland which is not privately-owned or protected in any way. The field studies did not involve endangered or protected species.

Study Sites and Sample Collections

Soil samples were taken from 0 to 10 cm depth on hummocks in wetlands in August, 2011. Details of the location, vegetation, climate and soil characteristics are provided in Table 1. We selected representative types of wetland to determine final sample sites with minimum amount of disturbance. In order to minimize spatial heterogeneity, seven soil cores were collected along a diagonal line across each plot, and were subsequently mixed to form one composite sample. Four replicates samples were taken per wetland at each altitude. Soils were sieved to remove plant roots and stones (sieve mesh 2 mm) at sampling sites, and the subsamples were then packed in ice

Fig. 1 The location of the altitudinal transect along south-facing slope on Nyaiqentanglha Mountain at Dumxung country. The photo was taken from an elevation of 4300 m, and the view is straight up the slope transect



blocks and sent back to the laboratory. Each sample was divided into two subsamples, with one part stored at $-80\text{ }^{\circ}\text{C}$ for microbial analysis and the rest preserved at $4\text{ }^{\circ}\text{C}$ for physico-chemical analysis.

Soil Physicochemical Analysis

Air-dried soil samples were used to measure soil pH, electrical conductivity, particle size, total C, N and P. Gravimetric soil moisture was calculated on 5 g fresh subsamples after drying in a $105\text{ }^{\circ}\text{C}$ oven for 12 h. Soil pH was detected in a 1:2.5 mixture of soil and deionized water using a sartorius PB-10 digital pH meter. Soil electrical conductivity was measured in a 1:5 mixture of soil and deionized water using DDBJ-350 conductivity meter (Precision and Scientific Corp. Shanghai, China). Soil texture (Clay/silt/sand percentage) was determined with a Malvern Mastersizer 2000 with a measurement range of 0.02–2000 μm . Soil total organic C was measured using a Shimadzu TOC–TN analyzer (Shimadzu Corp., Kyoto, Japan). 0.5 g air-dried soil which was sieved by 100 meshes was digested 1.5 h by 1.5 g mixed catalyst and 4 mL concentrated sulphuric acid, and then dilute with deionized water to 50 mL to analyze total N and P. Total N was quantified using a continuous flow analyzer. Total P in soils was detected by ultraviolet spectrophotometer.

Soil water-soluble carbon (WSOC) and nitrogen (WSON) were measured by the method of Jones and Willett (2006). Briefly, 2.5 g of fresh soil was shaken with 25 mL of distilled water (1:10 w/v soil-to-solution ratio) for 15 min in 50 mL polypropylene bottles on a reciprocating shaker at a speed of 200 rpm. The soil extracts were then centrifuged at $8000\times g$ for 10 min and the supernatant was recovered and stored in polypropylene bottles in a $4\text{ }^{\circ}\text{C}$ freezer prior to analysis. Concentrations of WSOC and WSON were determined with a Shimadzu TOC–TN analyzer (Shimadzu Corp., Kyoto,

Japan). Water extracted of Cl^- , NO_3^- , SO_4^{2-} from soil were measured using ion chromatograph (Binex ICS900).

Soil Enzyme Assays

Polyphenol oxidase (PPO) (EC 1.10.3.2) and peroxidase (EC 1.11.1.7) activities were measured spectrophotometrically using pyrogallol (PG) as a substrate (Allison and Jastrow 2006). β -glucosidase activity was assayed by the method of Eivazi and Tabatabai (1988), using the substrate analogue para-nitrophenyl- β -D-glucopyranoside (pNPG). Invertase (EC 3.2.1.26) activity was assayed by incubating 1.00 g sieved ($\sim 2\text{ mm}$) fresh soil in a 100 mL Erlenmeyer flask mixed with 15 mL 2 M acetate buffer and 15 mL 1.2% sucrose substrate solution for $50\text{ }^{\circ}\text{C}$ for 3 h. A modified assay for urease (EC 3.5.1.5) activity based on that of Kandeler and Gerber (1988) was used. The activities of L-asparaginase were determined using the method previously reported with a few modifications: fresh soil (1.0 g) was placed in a 50 mL volumetric flask was treated with 0.2 mL toluene and 9 mL TRIS buffer (0.1 M, pH 10.0). The 50 mL flask was swirled for a few seconds to mix the contents, 1 mL 0.05 M L-asparagine solution was added, and the flask was swirled again for a few seconds. Then the flask was stopped and placed in an incubator at $37\text{ }^{\circ}\text{C}$ for 2 h. Protease activity was determined as reported by Ladd and Butler (1972). All enzymes activities were analyzed used colorimetric method by spectrophotometer. Measuring wavelengths were: Polyphenol oxidase–490 nm, Peroxidase–294 nm, β -glucosidase–400 nm, Invertase–508 nm, Urease–578 nm, L-asparagine–500 nm and Protease–500 nm.

PLFAs Extraction and Separation

PLFA lipids were extracted from lyophilized soil biomass using a single-phase Bligh and Dyer method (White et al.

Table 1 Soil physicochemical parameters of all samples

Elevation (m)	MAT (°C) ^A	MAP (mm) ^A	Predominant species	Moisture (%)	pH	TOC (g kg ⁻¹)	TN (g kg ⁻¹)	C/N	TP (g kg ⁻¹)	WSOC (mg kg ⁻¹)	WSON (mg kg ⁻¹)	Chloride (mg kg ⁻¹)	Nitrate (mg kg ⁻¹)	Sulfate (mg kg ⁻¹)
4267	1.2	360	<i>Kobresia pygmaea</i> , <i>Elymus dahuricus</i>	80.0 (7.3)ab	7.9 (0.0)a	86.4 (12.9)ab	5.2 (0.8)ab	16.5 (0.2)ab	0.9 (0.1)b	555.4 (83.4)a	11.2 (0.94)a	41.5 (4.8)a	16.6 (3.8)a	39.0 (2.8)a
4352	0.9	265	<i>Kobresia pygmaea</i>	111.3 (18.8)ab	7.7 (0.1)a	112.4 (26.9)a	6.9 (1.5)a	16.0 (0.9)ab	0.6 (0.1)c	485.2 (63.4)a	38.3 (8.1)ab	29.2 (5.7)b	12.6 (2.4)a	37.2 (5.7)a
4500	0.2	211	<i>Kobresia pygmaea</i> , <i>sedge</i>	90.4 (9.8)a	7.2 (0.2)b	92.9 (7.7)a	5.6 (0.7)ab	17.0 (1.1)ab	0.6 (0.1)c	228.1 (37.1)b	22.5 (7.3)bc	18.9 (2.1)c	5.8 (1.1)b	34.1 (4.1)ab
4567	-0.9	221	<i>Kobresia pygmaea</i> , <i>Oxytropis ochrocephala</i>	41.2 (9.4)b	6.9 (0.1)bc	37.9 (8.7)b	2.19 (0.5)b	17.1 (0.7)ab	0.6 (0.1)c	127.2 (6.2)b	14.2 (1.55)c	11.4 (2.0)cd	6.5 (1.9)b	13.1 (2.0)c
4737	-2.2	303	<i>Kobresia pygmaea</i>	80.4 (17.5)ab	6.3 (0.2)d	104.7 (22.0)a	6.2 (1.3)a	17.1 (1.4)ab	1.1 (0.1)a	205.7 (40.2)b	22.9 (2.91)bc	14.1 (2.0)cd	3.6 (1.3)b	15.9 (2.4)c
4820	-2.8	353	<i>Kobresia pygmaea</i>	41.0 (5.7)b	6.6 (0.1)cd	36.5 (9.8)b	1.9 (0.5)b	18.9 (1.2)a	0.6 (0.0)c	171.5 (24.5)b	15.4 (1.8)c	9.1 (1.2)d	1.8 (0.2)b	20.2 (6.2)c
4956	-3.8	417	<i>Kobresia pygmaea</i>	53.8 (4.2)b	6.9 (0.1)bc	43.8 (5.3)b	3.0 (0.2)b	14.6 (1.9)b	0.7 (0.0)bc	146.6 (5.9)b	13.6 (1.4)b	11.8 (1.4)cd	2.6 (0.4)b	22.3 (5.1)bc

A—Data was mean value of thirty years from 1980 to 2010. Data in brackets indicate standard deviation, different letters indicate significant difference among the treatments at $P < 0.05$ with LSD
 MAP mean annual precipitation, MAT mean annual temperature, MAP mean annual precipitation, TOC total organic carbon, TN total nitrogen, TP total phosphorus, WSOC water-soluble organic carbon, WSON water-soluble organic nitrogen

1979) with minor modifications. Briefly, 5 g ($n = 4$) mixed soil sample was extracted twice with one phase mixture of chloroform–methanol–phosphate buffer (1:2:0.8, v/v/v, pH 7.4) for a minimum 2 h by vigorously shaking. The solutions were split into two phases by adding one volume of water and chloroform. The organic phase was recovered and then fractionated into neutral lipids, glycolipids and phospholipids on a silica acid (Unisil, Clarkson Chemical Co., Williamsport, Pa.) column by consecutive elution with chloroform, acetone and methanol, respectively. The phospholipids were subjected to a mild alkaline methanolysis, which yields the methylesters of ester-linked fatty acids (FAMES), and then PLFAs samples were preserved in -20 °C until analysis. FAMES were analysed by gas chromatography (GC) and identified using the Sherlock Microbial Identification System. It consisted of a 6890A Series GC (Agilent Technologies, Palo Alto, CA), a flame ionization detector equipped with an Ultra 2 column (30 m, 0.2 mm ID, 0.33 μ m film) and a computer and associated software (Sherlock Pattern Recognition Software, MIDI, Newark, DE).

Statistical Analysis

All samples were measured in quadruplicate. One-way analysis of variance (ANOVA) was used to assess differences in soil factors and microbial properties along the altitude; LSD Multiple Range Test was performed on significant results using the SPSS software. Differences in PLFA profiles, soil enzyme activities and physicochemical characteristics of soils along the altitude gradient were analyzed using Canonical Correspondence Analysis (CCA) in the CANOCO 4.5 program, which is a constrained ordination method, for which ordination axes are constrained to be linear combinations of environmental factors. All statistical analyses were statistically significant at $P = 0.05$ level.

Results

Soil Physicochemical Properties with Altitudinal Changes

Soil physicochemical properties mainly displayed a decreasing trend with increasing altitude (Table 1). From 4267 to 4956 m a.s.l, WSOC and WSON decreased sharply, from 555.41 mg kg⁻¹ to 146.58 mg kg⁻¹ and from 50.83 mg kg⁻¹ to 13.57 mg kg⁻¹, respectively. Soil TOC and TN were highest at 4352 m a.s.l (TOC = 112.4 g kg⁻¹ soil and TN = 6.9 g kg⁻¹ soil, respectively) and, in general, declined with increasing elevation. A similar decreasing trend was found for nitrate, nitrite and ammonium with increasing altitude. The pH significantly decreased from 7.88 to 6.87 with increasing elevation. All sample pH values were near neutral. Total phosphorus,

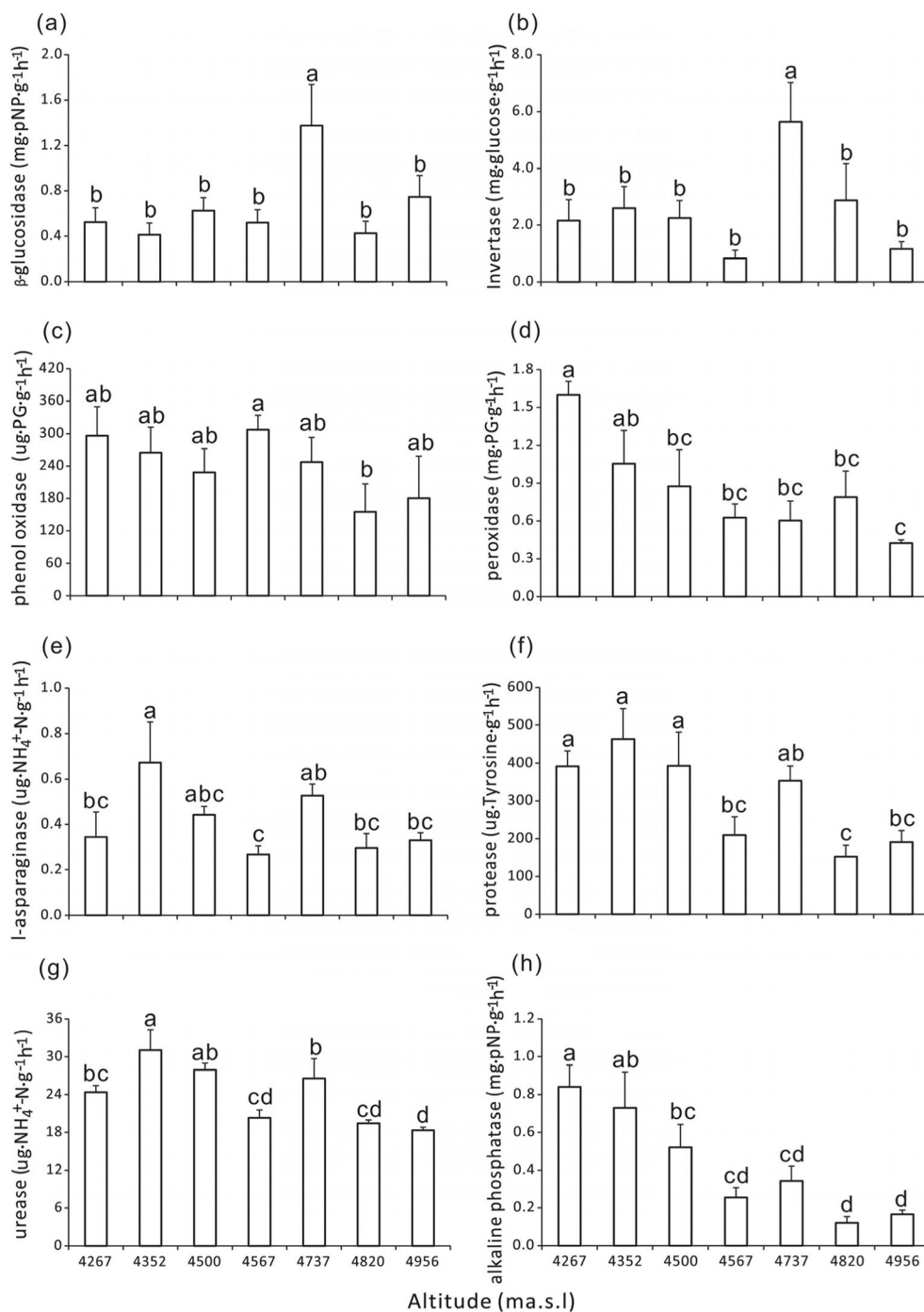
moisture and sulphate showed no significant change along the altitudinal gradient.

Enzyme Activities along the Altitudinal Gradient

Most enzyme activities also showed a trend similar to that of soil physicochemical properties, which decreased with increasing altitude (Fig. 2). Peroxidase, phenol oxidase and alkaline phosphatase had the highest activities at 4267 m, which were 1598.24 mg PG g⁻¹ h⁻¹ for peroxidase, 296.35 µg PG

g⁻¹ h⁻¹ for phenol oxidase and 840.81 mg pNP g⁻¹ h⁻¹ for alkaline phosphatase. Peroxidase, phenol oxidase and alkaline phosphatase activities decreased sharply to 423.44 mg PG g⁻¹ h⁻¹, 180.26 µg PG g⁻¹ h⁻¹ and 166.38 mg pNP g⁻¹ h⁻¹ at the highest altitude of 4956 m, respectively. L-asparaginase, urease and protease had their highest activities at 4352 m, which were 0.67 µg NH₄⁺-N g⁻¹ h⁻¹ for L-asparaginase, 31.03 µg NH₄⁺-N g⁻¹ h⁻¹ for urease and 463.43 µg Tyrosine g⁻¹ h⁻¹ for protease. L-asparaginase, urease and protease activities also quickly decreased to 0.33 µg NH₄⁺-

Fig. 2 Soil enzyme activities of soil samples change along the altitude gradients. Soil (a) β-glucosidase, (b) invertase, (c) phenol oxidase, (d) peroxidase, (e) L-asparaginase, (f) protease, (g) urease, (h) alkaline phosphatase. Values are means ± SD



$\text{N g}^{-1} \text{h}^{-1}$, $18.33 \mu\text{g NH}_4^+-\text{N g}^{-1} \text{h}^{-1}$ and $190.36 \mu\text{g Tyrosine g}^{-1} \text{h}^{-1}$ at the highest altitude of 4956 m. All enzyme activities had a significant decreasing trend with increasing altitude except for β -glucosidase and invertase. Both of their highest activities were at 4737 m. The β -glucosidase level was $1.38 \text{ mg PNP g}^{-1} \text{h}^{-1}$, and invertase was $5.65 \text{ mg glucose g}^{-1} \text{h}^{-1}$. The activities of β -glucosidase and invertase had no significant changes at other altitude gradients other than 4737 m.

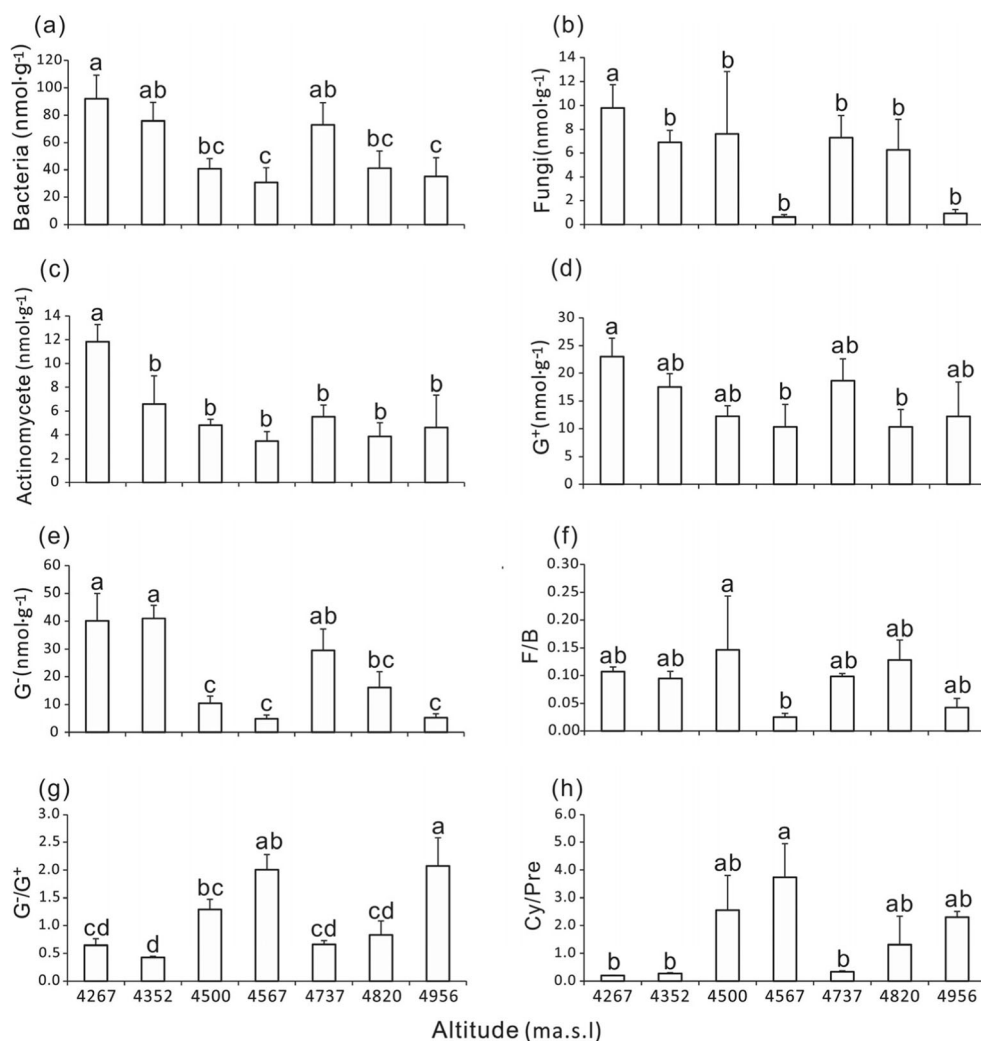
Community Composition (PLFAs) and Biomass along the Altitudinal Gradient

The microbial biomass, expressed as total PLFAs, significantly decreased with increasing altitude (Fig. 3). Along the altitude gradient from 4267 m to 4956 m, total microbial biomass per gram of soil was highest in stands with the lowest altitude of 4267 m and was lowest in stands with the highest altitude of 4956 m. Overall, the biomass of bacteria (i14:0, i15:0, a15:0, C15:1, i16:0, 16:1w9c, 16:w7c, 16:1w5c, 16:1w3c, i17:0, a17:0, C17:1, cy17:0, C18:1, i19:0, cy19:0, (Federle et al.

1986; Frostegard et al. 1993; Tunlid et al. 1989)), actinomycetes (10Me16:0, 10Me17:0, 10Me18:0, (Zelles 1997)), Gram-positive bacteria (i14:0, i15:0, a15:0, i16:0, i17:0, a17:0, i19:0, 10Me16:0, 10Me17:0, 10Me18:0, (Zogg et al. 1997)), Gram-negative bacteria (cy17:0, cy19:0, 15:1, 16:1w9, 16:1w7, 16:1w5, 18:1w7, 19:1, (Mutabaruka et al. 2007)) and fungi (C18:2, 18:1w9c, (Federle et al. 1986)) all decreased along the altitude gradient. From low altitude to high altitude, bacteria biomass quickly fell from $91.99 \text{ nmol g}^{-1}$ to $35.22 \text{ nmol g}^{-1}$ and fungal biomass decreased from 9.78 nmol g^{-1} to 0.90 nmol g^{-1} , respectively. Actinomycetes, Gram-positive bacteria and Gram-negative bacteria decreased 50%, 50% and 85% from 4267 ma.s.l. to 4956 ma.s.l., respectively.

The microbial community structure also showed significant changes with increasing altitude. The fungus to bacteria ratio (F/B) was used as a broad taxonomic microbial grouping, and the ratio of Gram-positive bacteria to Gram-negative bacteria (G^+/G^-) was used in conjunction with the ratio of the sum of cyclopropyl PLFAs to the sum of their monoenoic

Fig. 3 Soil microbial community structure and community-relation ratios change along the altitude gradients. G^+ —gram-positive bacteria, G^- —gram negative bacteria, F/B—fungi/bacteria, G^+/G^- —gram positive bacteria/g negative bacteria, cy/pre—cyclopropyl/precursor. Soil (a) bacteria, (b) fungi, (c) actinomycete, (d) gram positive bacteria, (e) gram negative bacteria, (f) fungi/bacteria ratio, (g) gram positive bacteria/g negative bacteria ratio, (h) cyclopropyl/precursor ratio. Values are means \pm SD



precursors (cy17:0 + cy19:0)/(16:1w7c + 18:1w7c; abbreviated as cy/pre) as indicators of environmental stress in bacteria communities (Kieft et al. 1997). The ratio of fungi/bacteria, the ratio of Gram-positive bacteria/Gram-negative bacteria and the ratio of cyclopropyl/precursor increased with altitude, indicating that environmental stress was increasing and that the soil microbial community was changing with increasing altitude. With increasing altitude, the cy/pre ratio ranged from 0.2 to 2.3. Likewise, the F/B ratio and the G⁺/G⁻ ratio ranged from 0.1 to 0.13 and 0.6 to 2.1, respectively.

Multivariate Analysis of Soil Physicochemical and Microbial Properties

In mountain areas, temperature decreases with increasing altitude. Meanwhile, soil physicochemical and microbial properties can change along altitude gradients. Pearson correlation showed a significantly positive relationship between MAT and many soil physicochemical variables, and MAT might be the key factor that affects soil physicochemical properties (Table 2). WSON and WSOC were significantly related to MAT ($r = 0.738, P < 0.001; r = 0.673, P < 0.001$, respectively). In addition, pH was positively correlated with MAT ($r = 0.764, P < 0.001$). A similar positive relationship was found between MAT/ TOC and TN ($r = 0.443, P < 0.01; r = 0.442, P < 0.01$).

Soil physicochemical properties showed a significant correlation with MAT, and soil microbial variables also showed the same trend. Phenol oxidase ($r = 0.386, P < 0.05, n = 28$), peroxidase ($r = 0.629, P < 0.001, n = 28$), urease ($r = 0.539, P < 0.01, n = 28$), protease ($r = 0.588, P < 0.001, n = 28$) and alkaline phosphatase ($r = 0.759, P < 0.001, n = 28$) had a significant positive correlation with MAT (Table 3). MAT was significantly correlated with bacteria biomass ($r = 0.438, P < 0.01, n = 28$), fungal biomass ($r = 0.338, P < 0.05,$

$n = 28$), actinomycete biomass ($r = 0.467, P < 0.01, n = 28$), Gram-positive bacteria biomass ($r = 0.338, P < 0.05, n = 28$) and Gram-negative bacteria biomass ($r = 0.537, P < 0.01, n = 28$) (Table 4). There were many soil physicochemical properties affected by soil microbial variables besides MAT, including moisture, pH, TOC, TN, WSOC and WSON. This is why all of the variables significantly decreased with altitude. In particular, phenol oxidase activity was affected by MAT, WSOC, WSON, Cl⁻ and nitrate. Peroxidase, urease, protease and alkaline phosphatase activities were affected by MAT, moisture, pH, TOC, TN, WSOC, WSON, Cl⁻, nitrate and sulphate. Invertase and glucosidase activities were affected by moisture, pH, TOC, TN and TP. Asparaginase activity was only affected by moisture and TN. Additionally, bacteria, Gram-positive bacteria and Gram-negative bacteria biomass were affected by MAT, moisture, pH, TOC, TN, WSOC and WSON. Similarly, actinomycete biomass was affected by MAT, pH, TN, WSOC, WSON, Cl⁻ and nitrate. Fungal biomass was affected by MAT, TOC, TN and Cl⁻.

Using a Canonical Correspondence Analysis, we retained four components. The first two components (CCA1 and CCA2) explained 67.2% and 11.6% of the total variance, respectively, while the other two components explained 5.4% and 4.6%, respectively. CCA1 and CCA2 were chosen to draw a biplot (Fig. 4), as they together explained 78.8% of the total variance. Notably, CCA 1 was mainly weighted by physicochemical characteristics (sulphate, TOC, TN, moisture, Cl⁻, WSOC and WSON) and biological (invertase, L-asparaginase, protease and urease) variables together with mean annual temperature (MAT). TOC, pH, L-asparaginase, TN, moisture, urease, protease, chloride ion, WSOC and WSON exhibited strong positive correlations with MAT. The relationships between MAT and soil parameters indicated that soil enzyme activities and soil physicochemical properties corresponded to variation in MAT. In comparison with CCA1,

Table 2 Correlation coefficients (r) between mean annual temperature and soil physicochemical properties

Parameter	MAT	Moisture	TOC	TN	TP	WSOC	WSON	pH	Silt	Clay	Sand	Chloride	Nitrate	Sulfate
MAT	1.000													
Moisture	0.491**	1.000												
TOC	0.443**	0.850***	1.000											
TN	0.442**	0.834***	0.978***	1.000										
TP	-0.066	0.160	0.405*	0.426*	1.000									
WSOC	0.738***	0.545***	0.582***	0.569***	0.176	1.000								
WSON	0.673***	0.449**	0.562***	0.593***	0.375*	0.898***	1.000							
pH	0.764***	0.351*	0.226	0.299	-0.157	0.716***	0.706***	1.000						
Silt	0.025	0.453**	0.335*	0.378*	-0.165	0.202	0.125	0.223	1.000					
Clay	-0.535**	0.025	0.031	0.087	0.092	-0.401*	-0.405*	-0.391*	0.419*	1.000				
Sand	-0.001	-0.446**	-0.330*	-0.375*	0.158	-0.181	-0.105	-0.202	-0.999***	-0.999***	1.000			
Chloride	0.759***	0.444**	0.499**	0.514**	0.264	0.897***	0.888***	0.742***	0.147	0.147	-0.126	1.000		
Nitrate	0.764***	0.369*	0.432*	0.472**	0.197	0.844***	0.905***	0.787***	0.102	0.102	-0.082	0.895***	1.000	
Sulfate	0.572***	0.535**	0.530**	0.529**	-0.029	0.711***	0.624***	0.648***	0.278	0.278	-0.261	0.714***	0.580***	1.000

TOC total organic carbon, TN total nitrogen, TP total phosphor, WSOC water-soluble organic carbon, WSON water soluble organic nitrogen

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001, n = 28$

Table 3 Correlation coefficients (r) between soil physicochemical properties and soil enzyme activities

Parameter	Phenol oxidase	Peroxidase	Urease	Protease	Invertase	Glucosidase	Asparaginase	Alkaline phosphatase
MAT	0.386*	0.629***	0.539**	0.588***	-0.061	-0.268	0.239	0.759***
Moisture	0.194	0.438**	0.953***	0.865***	0.446**	0.345*	0.639***	0.704***
pH	0.259	0.517**	0.307*	0.433*	-0.328*	-0.474**	0.127	0.664***
TOC	0.227	0.343*	0.841***	0.794***	0.534**	0.474**	0.497**	0.713***
TN	0.219	0.327*	0.812***	0.816***	0.465**	0.453**	0.484**	0.738***
C/N	0.040	0.134	0.103	-0.042	0.305	0.091	0.06	-0.099
TP	0.195	0.080	0.109	0.288	0.435**	0.510**	0.108	0.235
WSOC	0.382*	0.619***	0.485**	0.563***	0.082	-0.155	0.195	0.803***
WSON	0.436**	0.517**	0.378*	0.626***	0.019	-0.083	0.098	0.846***
Chloride	0.399*	0.625***	0.360*	0.527**	-0.061	-0.206	0.145	0.809***
Nitrate	0.456**	0.598***	0.320*	0.529**	-0.143	-0.246	0.013	0.776***
Sulfate	0.090	0.507**	0.446**	0.531**	0.068	-0.133	0.249	0.660***

TOC total organic carbon, TN total nitrogen, TP total phosphor, WSOC water soluble organic carbon, WSON water soluble organic nitrogen

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, $n = 28$

CCA2 was weighted by only a few variables, and β -glucosidase and phenol oxidase exhibited a more powerful influence on discrimination of soil samples. Additionally, pH, peroxidase, nitrate, WSON, TP and WSOC also showed an important influence on the second axis. The samples at 4267 and 4352 m were apparently discriminated from other altitude samples, as more than half of the variables clustered near the low-altitude samples.

Discussion

In this study, we examined soil microbial community structure and enzyme activities along an altitude gradient in the

Nyainqentanglha Mountains on the Tibetan Plateau. We found that most soil microbial biomass and enzyme activities decreased with an increase in altitude. In contrast, Djukic et al. (2010) and Wagai et al. (2011) observed that soil microbial communities did not display a consistent altitudinal change along altitude gradients. One possible explanation for the apparent disparity lies in the difference in site selection. For example, Djukic et al. (2010) and Wagai et al. (2011) collected samples from forests and shrubland, whereas we analysed microbial communities in a wetland ecosystem. Compared with forests, wetland ecosystems are more sensitive to temperature (Erwin 2009), in terms of changes in associated vegetation and soil physicochemical properties with changes in temperature.

Table 4 Correlation coefficients (r) between soil physicochemical properties and soil microbial communities

Parameter	Bacteria	Fungi	Actinomycetes	G ⁺	G ⁻	F/B	G ⁺ /G ⁻	cy/pre
MAT	0.438**	0.338*	0.467**	0.338*	0.537**	0.136	-0.401**	-0.195
Moisture	0.439**	0.288	0.284	0.320*	0.514**	0.120	-0.386*	-0.322*
pH	0.317*	0.070	0.533**	0.286	0.379*	-0.083	-0.177	-0.115
TOC	0.431*	0.326*	0.259	0.298	0.506**	0.185	-0.457**	-0.376*
TN	0.445**	0.328*	0.315*	0.324*	0.507**	0.181	-0.422**	-0.373*
C/N	-0.201	0.060	-0.415*	-0.333*	-0.032	0.124	-0.397*	0.088
TP	0.346*	0.141	0.290	0.288	0.304	-0.033	-0.234	-0.245
WSOC	0.530**	0.281	0.527**	0.408*	0.666***	0.071	-0.507**	-0.410*
WSON	0.527**	0.245	0.592***	0.413*	0.617***	0.015	-0.457**	-0.370*
Chloride	0.545***	0.420*	0.593***	0.429*	0.631***	0.213	-0.446**	-0.346*
Nitrate	0.405**	0.182	0.528**	0.301	0.502**	-0.020	-0.329*	-0.182
Sulfate	0.159	0.251	0.201	0.056	0.284	0.265	-0.351*	-0.157

TOC total organic carbon, TN total nitrogen, TP total phosphor, WSOC water soluble organic carbon, WSON water soluble organic nitrogen, G⁺ Gram positive bacteria, G⁻ Gram negative bacteria, Cy Cyclopropyl PLFA, Pre Precursor PLFA

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, $n = 28$

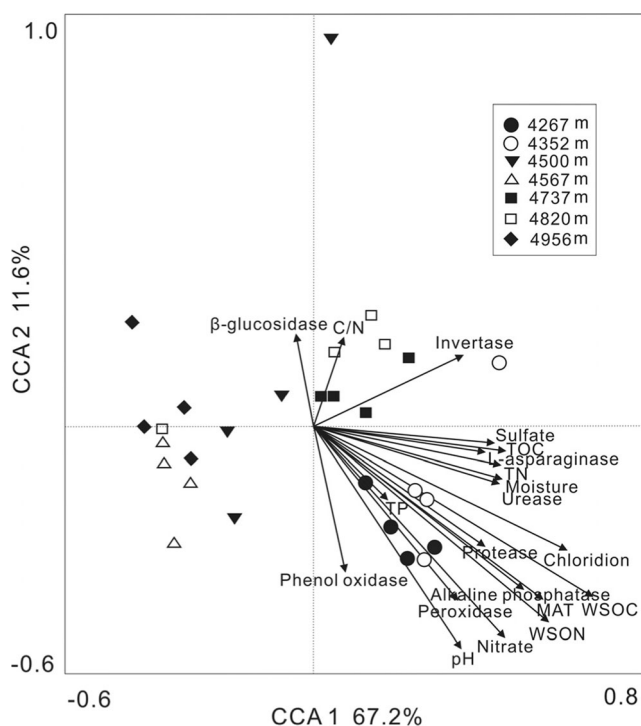


Fig. 4 CCA-ordination biplot (CCA1 vs CCA2) of soil PLFAs and 19 soil microbial and physicochemical factors. WSOC–water-soluble organic carbon, WSON–water-soluble organic nitrogen, TOC–total organic carbon, TN–total nitrogen, TP–total phosphor

Higher-altitude regions are characterized by very cold temperature and frequent frost and ice events. Overall, temperature represents a key factor that is closely correlated to environmental harshness, i.e., cold climate, low levels of nutrients and sparse vegetation, which in turn influence microbial activities and community structure, as shown in this study. Low-altitude soils had higher levels of total nitrogen, soil organic matter and clay than high-altitude soils, which might explain the difference in enzyme activities and microbial biomass between low-altitude soils and high-altitude soils.

Lovell et al. (1995) suggested that differences in soil microbial biomass were associated with changes in community structure, such as a change in the relative proportion of bacteria and fungi. In our study, we interpreted a difference in soil microbial biomass (bacteria, actinomycetes, fungi, Gram-positive bacteria and Gram-negative bacteria) and community structure along the altitude gradient as a result of soil physicochemical properties including nutrients that were affected by temperature decreases. There was a significant decrease in bacteria biomass, as well as a decrease of actinomycete and Gram-negative bacteria biomass in high-altitude soils compared with low-altitude soils, whereas the ratio of Gram-positive to Gram-negative bacteria increased with the altitude gradient. Gram-negative bacteria appeared to be more competitive in low-altitude soils (with suitable temperature and high nutrient content) because they grow under substrate-rich

conditions, while Gram-positive bacteria are dominant in resource-limited areas (Atlas and Bartha 1997). However, the fraction of Gram-positive bacteria also decreased with increasing altitude, mainly caused by low nutrient content. However, these organisms were more adapted to harsh conditions than Gram-negative bacteria, which could help explain why the ratio of Gram-positive to Gram-negative bacteria increased with altitude. The fraction of fungal biomass and the ratio of bacteria to fungi showed no significant change along the altitude gradient. In cold environments, fungi are characterized by lower optimum and maximum temperatures for growth compared with bacteria (Margesin et al. 2003) and are thus well adapted to cold climates. The ratio of cyclopropyl/precursor could be used to indicate the level of environmental harshness. Cyclopropyl fatty acid increases when environmental harshness increases, which explains why the ratio of Cy/Pre increased with increasing altitude.

Soil enzyme activities reveal microbial metabolic requirements and available soil nutrients (Caldwell 2005). Bacteria, fungi and plant roots can synthesize enzymes and release them into soils. Soil enzyme activities are very stable and can retain their functions for more than a year (Alkorta et al. 2003). Bacteria can be a major source of peroxidase, alkaline phosphatase and protease (Bach and Munch 2000; Kamimura and Hayano 2000), whereas plants can be a major source of urease and β -glucosidases. Fungi (Halaouli et al. 2006), bacteria (Faccio et al. 2012) and plants (Mayer 2006) are sources of phenol oxidase, which is a “latch” on a global carbon store (Freeman et al. 2001). The tested β -glucosidase, invertase, phenol oxidase, peroxidase, L-asparaginase, protease, urease and alkaline phosphatase in the present study play an essential role associated with C, N, and P cycles in soils.

In mountain areas, temperature decreases with increasing altitude; elevation gradients thus represent powerful natural experiments in which soil enzyme activities respond to variation in temperature. Most soil enzyme activities clearly decreased with increased elevation. Similar results were found by Margesin et al. (2009), who showed that soil enzyme activities decreased with increasing elevation in alpine and sub-alpine soils. Invertase, β -glucosidases, phenol oxidase and peroxidase are closely related to carbon cycling in soil. Invertase is an important enzyme in the carbon conversion cycle via the hydrolysis of carbohydrates. The inhibition of most β -glucosidases by glucose significantly contributes to the need for high cellulase loads (Qing et al. 2010). Both invertase and β -glucosidases display similar patterns along an altitude gradient, with the highest activities detected at 4737 m a.s.l. The activities of invertase and β -glucosidases were generally recognized to be related to SOC, consistent with our results. We speculate that temperature might indirectly influence these two enzymes by influencing SOC and TP in soils. Phenol oxidase can promote phenol oxidation into 1,2-benzoquinone and 1,4-benzoquinone in aerobic conditions.

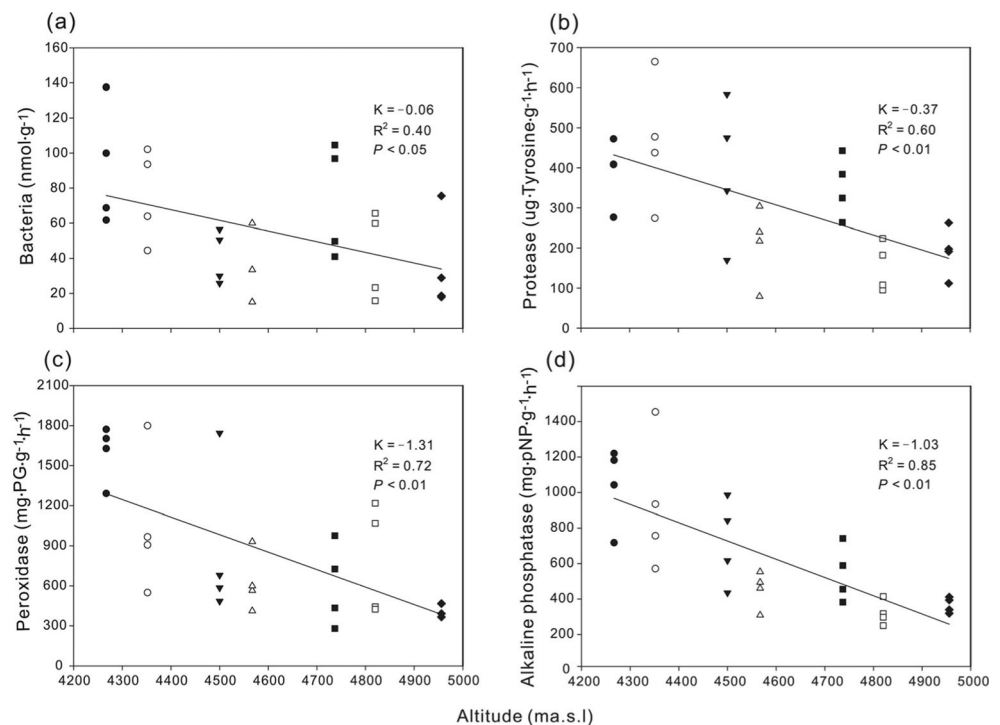
Quinones can inhibit microbial infection for self-protection. Moreover, phenol oxidase can degrade lignin and soluble phenolic compounds, thereby reducing carbon storage (Fenner et al. 2005). Peroxidase can oxidize substrates using hydrogen peroxide as the electron acceptor. The activities of phenol oxidase and peroxidase significantly decreased with an increase in altitude, suggesting that an increasingly anaerobic environment accompanies an increase in altitude, which led to decreased enzyme activity (Fenner et al. 2005; Freeman et al. 2001). Thus, wetland ecosystems at lower elevation in the Tibet Plateau play a more important role in global carbon cycles. L-asparaginase, protease and urease were correlated with the nitrogen cycle. All of these enzymes are hydrolytic enzymes. In particular, urease is a key enzyme for soil nitrogen transformation that can decompose urea into ammonia and carbon dioxide. Therefore, urease activity is a very important indicator of soil nitrogen status, which is closely related to TN, DON and ammonium. Urease activity significantly decreased with an increase in altitude, which coincides with the change in nitrogen content along the altitude gradient. The activities of the other two enzymes showed consistent changes along the altitude gradient. Enzyme activities also decreased with increasing altitude, which suggests that decomposition of soil organic matter might decrease with an increase in altitude, and lower elevations surely play larger roles in the global nitrogen cycle. Uchima et al. (2011) also observed a decrease in enzyme activities with an increase in altitude (i.e., a decrease in temperature). Alkaline phosphatase is responsible for phosphorus cycling in wetland soils, and alkaline phosphatase also decreased

with a decrease in temperature, indicating that low temperature is not favourable for phosphorus absorption from soil minerals.

An understanding of the patterns and mechanisms of soil enzyme activities along an altitudinal gradient would greatly enhance our understanding of the influence of elevation on microbial distribution, especially for soil enzymes that are mainly derived from soil microbes. Peroxidase, alkaline phosphatase and protease activities showed consistent changing patterns along the altitude gradient (Fig. 5). It is obvious that soil enzyme activities decreased faster than bacterial biomass with increasing altitude, which suggests that decreasing soil enzyme activities were not only caused by decreased bacterial biomass but were also affected by decreased temperature. In other words, the decreasing activities of peroxidase, protease and alkaline phosphatase were mainly caused by a decline in enzyme capacity with temperature, with the latter mainly affected by temperature ($r = 0.629$, $P < 0.001$; $r = 0.588$, $P = 0.001$, $r = 0.759$, $P < 0.001$, respectively).

In conclusion, our results showed that temperature affects microbial community structure and soil enzyme activities. With an increase in altitude, i.e., a decrease in temperature, soil physicochemical properties changed along the altitude gradient. Soil enzyme activities (phenol oxidase, peroxidase, L-asparaginase, protease, urease and alkaline phosphatase) and microbial biomass all decreased with increasing altitude. The declines in peroxidase, alkaline phosphatase and protease activities, all derived from soil microbes, were mainly caused by decreasing enzyme capacity with temperature rather than a decrease in microbial biomass.

Fig. 5 Downward trend of bacteria from all soil samples and three soil enzymes activities which derived from soil microbial. When significant ($P < 0.05$), the linear regression is drawn. Soil (a) bacteria, (b) peroxidase, (c) protease, (d) alkaline phosphatase



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