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Spatio-temporal patterns of enzyme activities after manure application reflect mechanisms of niche differentiation between plants and microorganisms

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ABSTRACT

Manure is an important source of nutrients for plants and stimulates a wide range of enzyme-mediated microbial processes. Such stimulation, however, depends on manure distribution and the duration of its decomposition in soil. For the first time, we investigated the spatio-temporal patterns of enzyme activities as affected by manure application strategies: 1) Localized manure: manure application as a layer in the upper soil; 2) Homogenized manure: mixing manure throughout the soil; and 3) Control without manure. Tibetan barley was planted on soil managed with yak manure from the Tibetan Plateau. Soil zymography was used to visualize the two-dimensional distribution and dynamics of the activities of three enzymes responsible for cycling of carbon (β-glucosidase), nitrogen (N-acetylglucosaminidase) and phosphorus (phosphomonoesterase) over 45 days. The manure detritusphere increased enzyme activities relative to the control (which had only the rhizosphere effect of barley) and this stimulation lasted less than 45 days. Enzyme activities in the manure-induced hotspots were higher than on the barley rhizoplane, indicating that the detritusphere stimulated microbial activities more strongly than roots. Homogenized manure led to 3−29% higher enzyme activities than localized manure, but shoot and root biomass was respectively 3.1 and 6.7 times higher with localized manure application. Nutrients released by high enzyme activities within the whole soil volume will be efficiently trapped by microorganisms. In contrast, nutrients released from manure locally are in excess for microbial uptake and remain available for roots. Consequently, microorganisms were successful competitors for nutrients from homogeneous manure application, while plants benefited more from localized manure application. We conclude that localized manure application decreases competition for nutrients between the microbial community of manure and the roots, and thereby increases plant performance.

1. Introduction

Livestock manure application has been widely accepted as a sustainable management practice in agriculture, providing environmentally and agronomically sound outcomes (Risse et al., 2006; Brandjes et al., 1996; Scotti et al., 2015). Manure incorporation into soil forms a detritusphere abundant in organic carbon (OC) and

nutrients (Moore et al., 2004). It is beneficial for improvement of soil quality and crop production (Butler et al., 2013; Calleja-Cervantes et al., 2015; Zaller and Köpke, 2004).

The application strategy is an important aspect of manure management (Webb et al., 2010; Thomsen, 2005). It affects soil-plant-microbial interactions by determining the locations of nutrients or altering soil properties (moisture, O_2 diffusion, bulk density) (Acosta-Martínez and Waldrip, 2014; Zhu et al., 2015). As a consequence, responses of plants and microorganisms vary depending on the manure application strategy. For instance, mixing of manure into soil increased soil microbial biomass (Lovell and

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Jarvis, 1996; Malik et al., 2013), but no response of soil microbial biomass was observed when manure pats were placed on the soil surface (Lovell and Jarvis, 1996; Cai et al., 2014). Although remarkable increases in plant production have been reported after either incorporating manure into soil (Malik et al., 2013) or broadcasting manure on the soil surface (Aarons et al., 2009; Matilla, 2006), a direct comparison of plant production under various manure application strategies is still lacking.

Enzymes, excreted by both plants and microbes, are early indicators of soil quality and the main mediators of organic matter decomposition (Nannipieri et al., 2007; Sinsabaugh et al., 2008). Assays of enzyme activities have been widely used to investigate the influence of manure application on soil nutrient cycling and microbial activities. Most studies observed significantly increased enzyme activities in soils amended with livestock manures (Liang et al., 2014; Calleja-Cervantes et al., 2015; Bell et al., 2006). However, the study of spatial and temporal responses of enzyme activities requires advanced visualization technology (Acosta-Martínez and Waldrip, 2014).

On the Tibetan Plateau, yaks (Bos grunniens) are one of the main species of livestock, and around 40% of their manure is used as fertilizer for cropland and pastures (FAO, 2003; Wang, 2009). However, the impact of yak manure application strategies on the growth of Tibetan barley — a staple crop — and on soil enzyme activities remains unknown. Such knowledge could lead to better manure application strategies. We used soil from the Tibetan Plateau for better consideration of local nutrient conditions and soil properties, and in the context of prevalent ecosystem degradation (Babel et al., 2014; Hafner et al., 2012).

Here we used direct soil zymography (Razavi et al., 2016) to investigate the impact of different yak manure application strategies on the growth of Tibetan barley (Hordeum vulgare L.) and on the temporal and spatial patterns of enzyme activities in Tibetan soil. We compared manure application strategies using three treatments (Fig. 1): 1) Localized manure: manure application as a layer in the upper soil; 2) Homogenized manure: mixing manure throughout the soil; and 3) No manure: a control without manure application. Our objectives were to investigate the effects of manure application strategy on plant shoot and root biomass and on the spatial and temporal patterns of soil enzyme activities. Direct soil zymography was used to visualize and quantify the spatial and temporal distribution of enzyme activity for the three enzymes: β-glucosidase, phosphomonoesterase and N-acetylglucosaminidase. β-glucosidase is responsible for catalyzing the hydrolysis of terminal 1,4-linked β-D-glucose residues from β-D-glucosides (German et al., 2011) and is

involved in the carbon (C) cycle. Phosphomonoesterase, which catalyzes the hydrolysis of organic phosphorus (P) compounds to inorganic P (Eivazi and Tabatabai, 1977; Malcolm, 1983), is involved in the P cycle. N-acetylglucosaminidase (chitinase), which accomplishes the decomposition of chitin to yield low molecular weight chitooligomers (Hamid et al., 2013), is responsible for C- and nitrogen (N) -acquisition.

The considerable addition of labile organic compounds and nutrients in manure are expected to greatly influence plant and microorganism activities, and therefore soil enzyme activities. We hypothesized - H1: weaker enzyme activities at the root-soil interface as compared with a strong increase of enzyme activities in the manure-induced detritusphere; H2: stronger stimulation of plant growth by the homogenized manure application strategy.

2. Materials and methods

2.1. Soil and yak dung sampling

Soil was sampled at the research station "*Kobresia* Ecosystem Monitoring Area" (KEMA) (31°16′45″N 92°59′37″E, 4410 m a.s.l.), which was established by Prof. Georg Miehe with the support of the VW foundation, and which now belongs to the Tibet University and the Institute of Tibetan Plateau Research in Nagqu. The soil was classified as a *Stagnic Eutric Cambisol* (*Humic*) (WRB, 2014) with a texture of 50% sand, 33% silt and 17% clay. The pH value (H_2O) was 6.9 ± 0.03 and soil bulk density was 0.92 g cm^{-3}. Yak dung was collected from Nangqian town, Yu Shu Prefecture (32°04′N, 96°31′E, 3600 m a.s.l.). Before being sampled, dung was piled and composted in the field.

In total, 10 soil core samples (25 cm deep, 5 cm diameter) were taken within an area of ca. 100 m^2. All the samples were hand-mixed and roots and stones were removed. The composite soil and composted yak dung samples were stored in ziplock bags at 4 °C, transported to the laboratory of the University of Göttingen and passed through a 2 mm sieve in preparation for incubation. Daily mean temperature during the sampling month ranged from 3.2 °C to 21.3 °C, so the temperature used for transportation was not uncommon and would not strongly affect soil and manure characteristics.

Additional soil and dung samples were oven-dried at 60 °C for 48 h to measure carbon (C) and nitrogen (N) content. Initial water content was measured by oven-drying samples at 105 °C. Soil C and N contents were $3.4 \pm 0.11\%$ and $0.3 \pm 0.01\%$, respectively. The yak dung contained $37 \pm 0.3\%$ C and $1.3 \pm 0.04\%$ N.
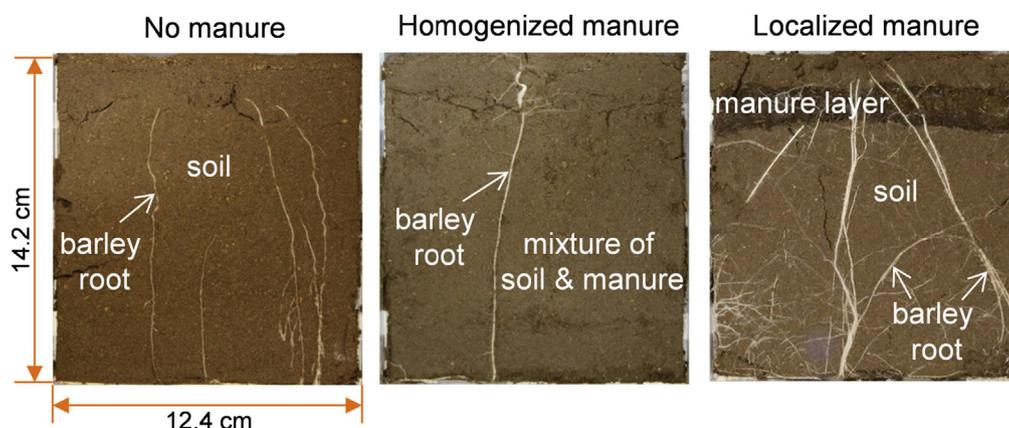

Fig. 1. Rhizoboxes with barley growing under three manure application strategies: No manure (left), manure homogenized with the whole soil (middle), and manure localized in the soil layer between 1.0 and 2.5 cm below the soil surface (right).

2.2. Experimental set-up

Experimental samples were prepared to simulate the following manure applications (Fig. 1): 1) Localized manure: manure application as a layer buried in the upper soil; 2) Homogenized manure: mixing manure into the soil. Homogenized manure application was comparable to fertilizer broadcasting and plowing; and 3) No manure: a control without manure application. For the localized manure application, 110 g fresh soil (water content: 10%) was first added to a rhizobox (14.2 × 12.4 × 1.0 cm) and then 5 g of composted yak dung (water content: 94%) was evenly spread across the soil surface in a 1.5 cm layer. A small quantity of soil was then spread above the manure to form a shallow soil layer (~1 cm) to ensure plant growth. For the homogenized manure strategy, 110 g fresh soil and 5 g composted yak dung were mixed homogeneously and placed in rhizoboxes (mixture: 4.5% C and 0.3% N; water content: 11%). The third treatment only included 110 g soil and was the control ("No manure"). Each application strategy had three replicates, so a total of nine rhizoboxes were prepared. Tibetan barley seeds (Hordeum vulgare L.) were germinated on filter paper for 72 h to ensure plant growth, to avoid fungal contamination and errors caused by seedling difference. One seedling was planted in each rhizobox at a depth of 5 mm. Yak dung (5 g) was added to 110 g soil to meet the optimal C/N ratio for barley growth, taking into consideration the low plant density used in this study (i.e. one seedling for each rhizobox) (Aarons et al., 2009; Liu et al., 2013). The rhizoboxes were placed in an incubation chamber set to 20 °C, with photosynthetically active radiation intensity of 300 μmol m^{-2} s^{-1} and 14 h daytime, which is within the range of the field conditions during the growing season.

Plants grew for 45 days, after which the roots completely occupied the rhizoboxes. During growth, the rhizoboxes were kept inclined at an angle of 45°, so that the roots grew along the lower wall of the rhizoboxes. The soil water content was maintained at 65% of the water holding capacity by maintaining the rhizobox at constant weight with distilled water.

After the incubation was stopped at day 45, the barley plants were destructively sampled. All visible roots were also picked out from the soil. The roots were washed with distilled water to remove soil particles and plant biomass was oven-dried at 60 °C for 48 h. Shoot and root biomass were then weighed.

2.3. Soil zymography and imaging procedure

Zymography was performed after 5, 25 and 45 days as an in situ non-destructive technique to study the spatial and temporal patterns of enzyme activities as affected by manure applications. We made the first zymogramms at day 5 (at early stage of growth) to avoid strong effects of roots and physical disturbances. We followed the protocol improved by Razavi et al. (2016). Membranes saturated with 4-methylumbelliferone (MUF) substrates were used for visualization of enzyme activities. The substrates become fluorescent when enzymatically hydrolyzed by the corresponding enzyme (Dong et al., 2007). 4-Methylumbelliferyl-β-D-glucoside (MUF-G) was used as substrate to detect β-glucosidase activity; 4-methylumbelliferyl-phosphate (MUF-P) to detect phosphomonoesterase activity; and 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide (MUF-C) to detect chitinase activity. Each of these substrates was separately dissolved to a concentration of 10 mM in universal buffer (MES buffer, pH: 6.7) (Koch et al., 2007) (Sigma-Aldrich, Germany). Polyamide membrane filters (Tao Yuan, China) with a diameter of 20 cm and a pore size of 0.45 μm were cut to fit the rhizoboxes. Membranes were saturated with the substrate solution for each enzyme. The rhizoboxes were opened from the lower, rooted side and the saturated membranes were applied

directly to the soil surface (Razavi et al., 2016). After incubation for 1 h, the membranes were carefully lifted off the soil surface and any attached soil particles were gently removed using tweezers. One hour of incubation time was selected based on preliminary experiments and previous studies (Hoang et al., 2016).

To quantify the zymogram images, a standard calibration that relates the activities of various enzymes to zymogram fluorescence (i.e. fluorescence of the saturated membrane) is required. The calibration was based on zymography of 2 × 2 cm membranes soaked in a solution of MUF — the fluorescent tag attached to each substrate proxy — with concentrations of 0.01, 0.05, 0.1, 0.5, 1, 3, 6, 8, 10 mM. The amount of MUF on an area basis was calculated from the solution volume taken up by the membrane and its size. The
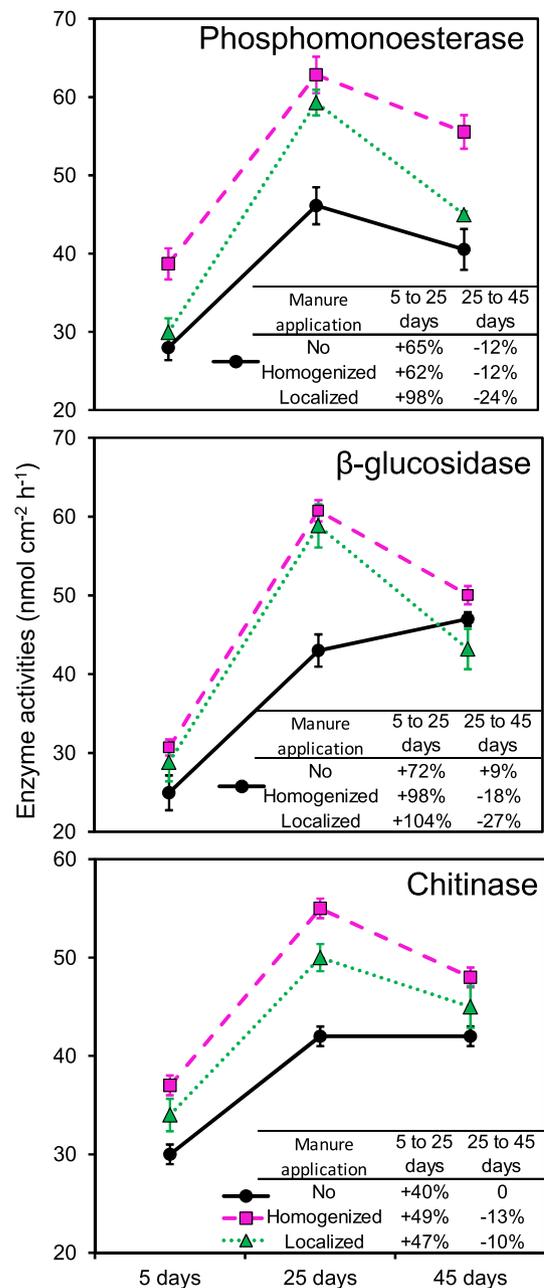

Fig. 2. Response of phosphomonoesterase (top), β-glucosidase (middle) and chitinase (bottom) activities to manure application strategies over time. The embedded tables show relative changes of enzyme activities between 5 and 25 days, and 25 and 45 days. Error bars represent standard deviations (±SD).

membranes used for calibration were photographed (EOS 5D, Canon) under UV light and analyzed in the same way as for the samples (Razavi et al., 2016).

2.4. Image processing and analysis

Fluorescence of the zymograms under UV light shows the areas where substrate has been enzymatically degraded. The intensity of fluorescence is proportional to the activity of the enzyme. To get quantitative information, we processed the zymogram images in Matlab, according to Razavi et al. (2016). Briefly, zymograms were transformed to 16-bit grayscale images as matrices and corrected for light variations and camera noise (Menon et al., 2007; Zarebanadkouki et al., 2012). Then, all zymograms were referenced based on the grayvalue of a reference object embedded in all the zymograms. The scaled black flat field identical in all images was considered as a background (reference object) during the whole image processing. We used the grayvalue obtained from these black sides of the sample as the referencing point. After referencing the zymograms, we calculated an average background grayvalue through the zymograms of calibration lines at concentration of zero and subtracted this value from all the zymograms. The grayvalue of each zymography pixel was converted to enzyme activity using the calibration function obtained for each enzyme (Razavi et al., 2016).

The processed 16-bit grayscale images were used for further analysis. To assess the response of plant roots to the manure application strategies in the context of enzyme activities, the average enzyme activities of the rhizoplane were compared with the activities of soil hotspots. Entirely visible, non-overlapping roots at the soil surface were selected. Hotspots were considered to be areas in which the grayvalues of five adjacent pixels (each equals 0.1×0.1 mm) all exceeded the average greyvalue of the whole image (i.e. >0.7, represented by red in Fig. S1). Twenty squares (each 50×50 pixels) were randomly arranged on these hotspots and the average grayvalue of these squares (G_H) was calculated to represent the average for soil hotspots. The roots distinguishable on a zymogram were segmented and the average grayvalues of the segments (G_R) were calculated. G_R and G_H were then converted to respective enzyme activities: E_R and E_H. The ratio
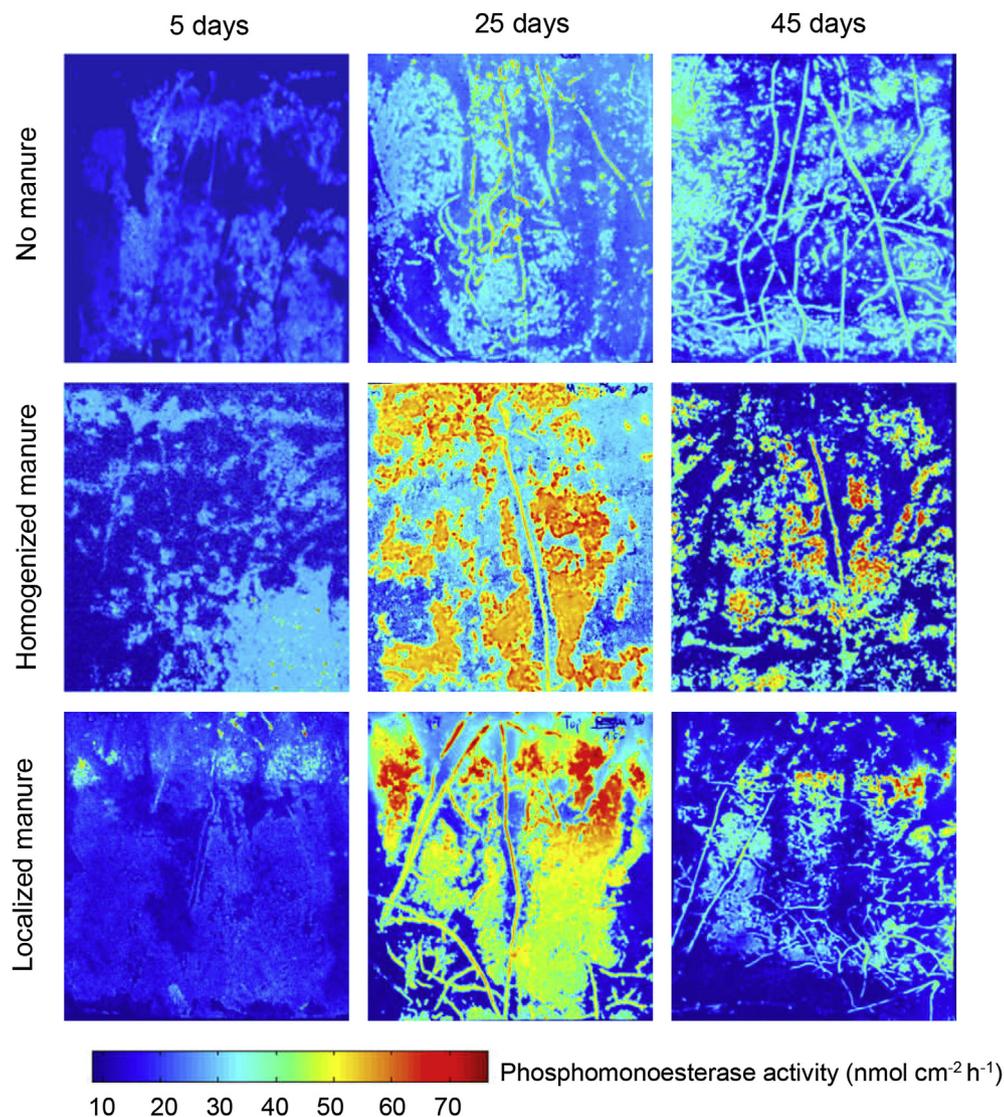

Fig. 3. Examples of zymograms for phosphomonoesterase activities. Three rows represent response of activities to three manure application strategies: 1) No manure, 2) Homogenized manure and 3) Localized manure. Figures from left to right are the measurements at days 5, 25 and 45. The color bar corresponds to phosphomonoesterase activity (nmol cm^{-2} h^{-1}).

of E_R to E_H was used to compare enzyme activities on the rhizoplane with that in the soil hotspots. Only the ratios at days 25 and 45 were considered in this study, as plant roots were not recognizable for most images at day 5.

To represent the vertical extension of enzyme activities in the localized manure application, the standardized grayvalues were plotted against the depth (cm) from the top of the rhizobox. Briefly, a segmented vertical line was drawn through the image from the top to bottom, and the grayvalues of all the pixels on this line were extracted. All grayvalues were then standardized to the maximal grayvalue. Consequently, all the values ranged from 0 to 1.0. In total, 20 separate vertical lines were randomly selected from each image and the average standardized grayvalues of these replicates were plotted against depth. Five-parameter Weibull regression in Sigmaplot (v. 12.5) was used to correlate the relative units with depth. The depth from the manure application to the constant level of the regression curve was considered as the detritusphere extension of enzyme activity.

Effects of manure application on enzyme activities were quantified as effect sizes:

$$\text{Effect size} = (E_M - E_{CON}) / E_{CON} \tag{1}$$

where E_M is the enzyme activity with manure application (homogenized or localized), and E_{CON} is the enzyme activity of the control. An effect size greater than zero indicates that the manure application strategy had a positive effect on enzyme activity.

To confirm the boundaries of categories of enzyme activities during soil hotspots consideration, one-way analysis of variance (ANOVA) was applied to assess significant differences between independent variables (mean values of five adjacent pixels, i.e. equal to 0.1 mm). The significant results were then considered as a boundary of each category (from very low activity to hotspot) (Fig. S1). Differences in enzyme activity and plant biomass between the three treatments (no manure, homogenized manure and localized manure) were also tested with ANOVA, where $p < 0.05$ from Tukey's HSD test indicated significance. Normality of the values and homogeneity of variance were tested using Shapiro-Wilk's W test and Levene's test. When data did not meet the normality requirement (e.g. shoot biomass data), the data were transformed by logarithm or square root. All these analyses were performed in STATISTICA 12.0 (StatSoft Inc.).

3. Results

3.1. Manure application strategies affected temporal patterns of enzyme activity

Enzyme activities increased from day 5 to day 25 after manure application, but decreased after 25 days (Figs. 2 and 3, S2 and S3). Phosphomonoesterase, β-glucosidase and chitinase activities were 47–104% higher on day 25 than on day 5. However, their activities had decreased 10–27% by day 45 relative to the activities on day 25. In the control, these enzyme activities had increased 40–72% by day 25 compared with their activities on day 5, but showed no significant changes between days 25 and 45 (-12% - $+9\%$, $p > 0.05$).

All enzyme activities increased with manure application relative to the control (Fig. 4). Homogenized manure generally induced larger increases than localized manure. Homogenized manure increased phosphomonoesterase, β-glucosidase and chitinase activity by 6–41% in comparison with the control (Fig. 4). In contrast, localized manure induced an increase of phosphomonoesterase and chitinase activities by 7–29% compared with the control. Localized manure also increased β-glucosidase activity by 16 and 37% on days 5 and 25, but its activity was 8% lower than the control
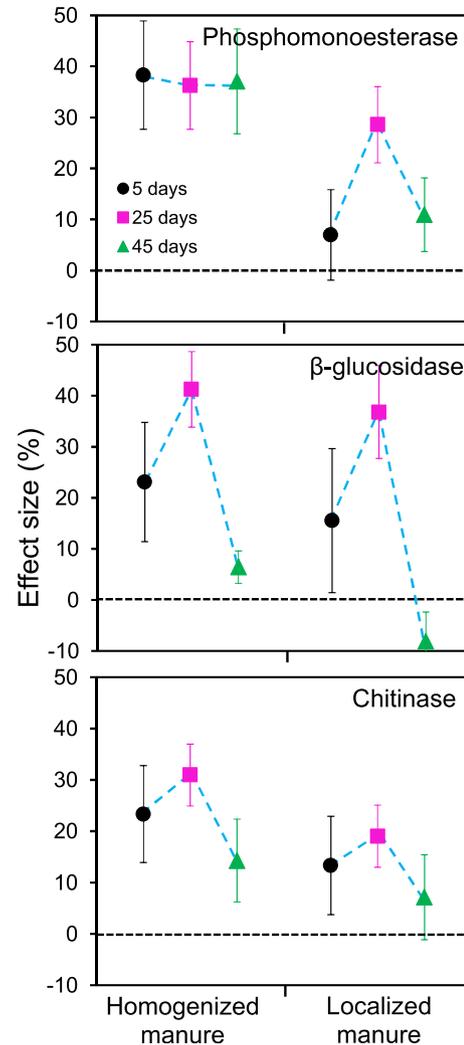

Fig. 4. Effects of manure application strategies on phosphomonoesterase (top), β-glucosidase (middle) and chitinase (bottom) activities in the whole soil. The effect size (Eq. (1)) shows the change of enzyme activities in soil with homogenized or localized manure addition compared to the control. Error bars represent standard deviations (±SD).

on day 45 ($p > 0.05$).

3.2. Detritusphere extension of enzyme activities

A clear downward extension of enzyme activities from the manure layer into the underlying soil was observed with localized manure application (Fig. 5). This extension was enzyme-specific: for example, phosphomonoesterase activity extended from 3.1 cm on day 5 to 9.2 cm on day 25 and finally exceeded 10 cm depth on day 45. In comparison, the extension of β-glucosidase activity was less (3.1 cm on day 5, 4.7 cm on day 25 and 7.0 cm on day 45). Such extension was not seen for chitinase (data not shown). All distances were measured from the top of the rhizobox and included the depth of the manure layer.

3.3. Response of plants to manure application strategies

The ratio of E_R to E_H (enzyme activities on the rhizoplane to that in soil hotspots) were all below 1.0 following manure application (Fig. 6, $p < 0.05$), indicating that average enzyme activities on the rhizoplane were lower than the activities in manure-induced soil
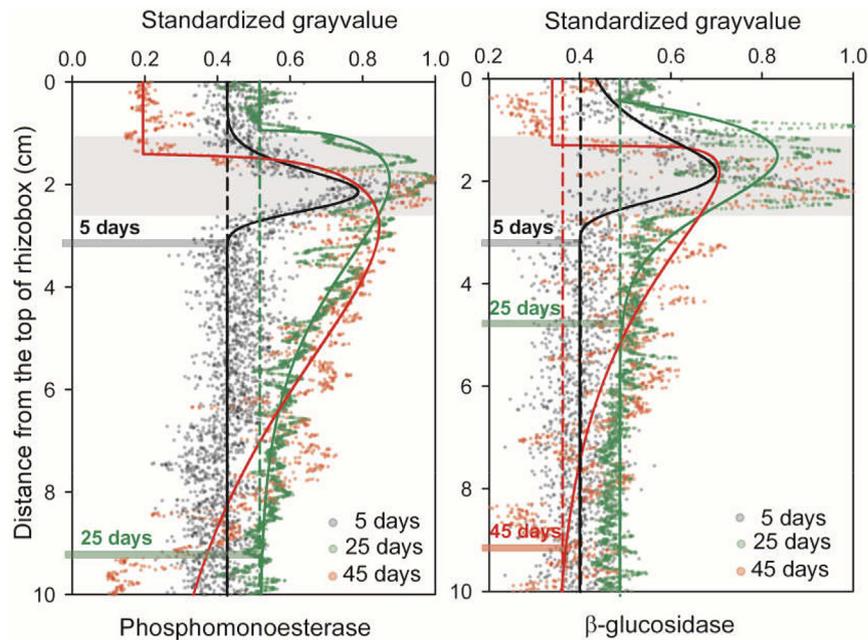
Fig. 5. The detritusphere extension for phosphomonoesterase (left) and β-glucosidase activities (right) from the initial manure layer at the top (presented as the shaded area between 1.0 and 2.5 cm) over time. The depth from the manure application to the constant level of the regression curve was considered as the detritusphere extension of enzyme activity. This distance at days 5 and 25 was marked by semitransparent strips (black for 5 days and green for 25 days). Due to the limited rhizobox size, the roots started to grow laterally once they reached the bottom, after around 10–15 days of growth, inducing very high root densities at the bottom (ca. 2–3 cm). To avoid artefacts from high root densities, we used only the upper 10 cm of the membrane. According to the regression, the depth at day 45 already exceeded the membrane boundary (>10 cm) and thus was not presented. Five-parameter Weibull regression was used to fit enzyme activities with the distance from the top of the rhizobox. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

hotspots. This ratio did not change over time for homogenized manure application. Phosphomonoesterase and β-glucosidase activities on the rhizoplane were both around 10% lower than that in the soil hotspots, while chitinase activity was 15% lower. In contrast to the homogenized manure, when localized manure was applied, the E_R to E_H ratio decreased from day 25 to day 45. For instance, the ratio of phosphomonoesterase activities decreased from 0.89 to 0.74, while that of β-glucosidase decreased from 0.79 to 0.68. For chitinase, this ratio had the highest change (from 0.98 to 0.75). For the control, this ratio was always around 1.0, except for that of phosphomonoesterase at day 25 (~1.13).

Manure application strategy had a significant impact on shoot and root biomass of barley (Fig. 7). Localized manure produced the highest shoot and root biomass (respectively, 3.1 and 6.7 times higher than for homogenized manure, $p < 0.05$). Localized manure significantly decreased the shoot/root ratio from 2.7 to 1.1 ($p < 0.05$), indicating that manure application strategies modified the trade-off between shoot and root biomass.

4. Discussion

4.1. Temporal response of enzyme activities to manure application strategy

The capability of manure to regulate soil biological processes was controlled by the manure application strategy. Homogenized manure induced higher activities of C-, N-, and P-acquisition enzymes than localized manure (Fig. 4). Three mechanisms drove these differences in response to manure application strategies. The first two mechanisms were manure-induced changes to soil physico-chemical properties (Haynes and Naidu, 1998; Dungait et al., 2009). First, by mixing into soil, manure directly loosened the soil, decreased the bulk density and increased the soil porosity

(Celik et al., 2004). Second, labile organic compounds and nutrients in the manure were also sufficiently mixed with soil following homogenized manure application. The third mechanism was the loading of indigenous enzymes and microbes from manure into the soil (Dinesh et al., 1998; Criquet et al., 2007; Tiquia, 2002). These mechanisms provided a favorable environment for soil microbial proliferation and activity in the rhizoboxes with homogenized manure. In contrast, localized manure affected biological processes through gradual leaching of soluble organic substances and mineral nutrients into the soil (Dickinson et al., 1981). Transport of indigenous enzymes from the manure layer into the soil was negligible due to strong adsorption by soil particles (Poll et al., 2006). Consequently, the combined effects of abiotic (e.g. loose soil structure) and biotic factors (e.g. organic carbon, nutrients, enzymes and microbes) induced higher enzyme activities in the rhizoboxes with homogenized manure.

Activities of all tested enzymes demonstrated a consistent pattern over time for both manure application strategies: i) All enzyme activities increased in the first 25 days. Most enzyme activities in the homogenized and localized manure applications were higher than in the control (Fig. 2). ii) Enzyme activities decreased from day 25 to day 45 in the homogenized and localized manure applications. In contrast, all enzyme activities in the control remained stable during this period. This indicated that the heightened enzyme activities in the homogenized and localized manure applications were mainly caused by the manure-induced detritusphere. Indeed, manure added quite substantial amounts of labile organic substances to the soil, thereby increasing microbial activity and thus nutrient demand and enzyme expression. Over time, these substances were completely decomposed, resulting in lower microbial activity and thus reductions in enzyme activity. Similarly, studies based on destructive methods demonstrated such short-term acceleration of microbial processes induced by sewage
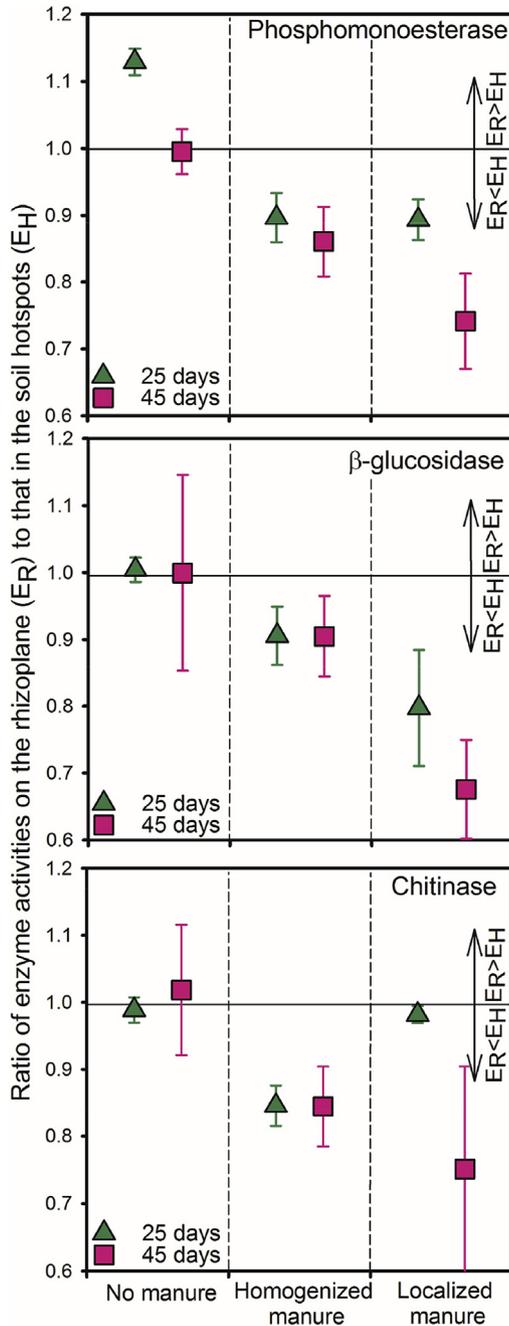
Fig. 6. Ratio of E_R to E_H for phosphomonoesterase (top), β-glucosidase (middle) and chitinase (bottom). E_R and E_H are the average enzyme activities on the rhizoplane and in the soil hotspots, respectively. The values above 1.0 reflect higher enzyme activities around the roots than in hotspots in root-free soil areas. Error bars represent standard deviations (±SD).

Fig. 7. Plant biomass and shoot/root ratio under three manure application strategies: 1) No manure, 2) Homogenized manure and 3) Localized manure. The capital and lower-case letters show significant differences between application strategies ($p < 0.05$). Error bars represent standard deviations (±SD).

4.2. Spatial response of enzyme activities to manure application strategies

The spatial distribution of enzyme activities was noticeably affected by manure application strategy. In localized manure application, enzyme activities in the top manure layer were higher than in the control and in the soil below this top manure layer (Fig. 3, S2 and S3), which was in accordance with our first hypothesis (H1) about the strong increase of enzyme activities in the detritusphere compared with the root-soil interface below the manure layer. This means that manure itself had higher enzyme and microbial activities, in support of the mechanism that manure-derived enzymes or microorganisms contributed to the increased enzyme and microbial activities (Calleja-Cervantes et al., 2015; Dinesh et al., 1998). Though indigenous enzymes of manure were all concentrated in the localized manure layer, the nutrients in this layer could be leached downward. Leaching of available nutrients and available organics from the manure layer stimulated microorganisms, and consequently enzyme activities, in the soil below the layer (Kang et al., 2009). Therefore, enzyme activities extended downwards over time because of the redistribution of nutrients and organics (Fig. 5). Such extension indicated a gradual influence of manure on soil biochemical processes and this influence was also enzyme-specific. For instance, phosphomonoesterase exhibited deeper and faster downward extension (Fig. 5). In contrast, this extension was narrower for β-glucosidase. The significantly higher ratio of E_R to E_H for phosphomonoesterase (~1.13) in the control also demonstrated that phosphomonoesterase activity on the rhizoplane was 13% higher than in the soil hotspots, suggesting that the soil was P-deficient (Ren et al., 2016) and thus the plant secreted

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sludge (Criquet et al., 2007; Pascual et al., 2002).

Furthermore, the stable enzyme activities from day 25 to day 45 in the control (with only the rhizosphere effect of barley, Fig. 8) demonstrated that the duration of hot moments in the rhizosphere was at least 20 days. This was much longer than the lifetime (only a few days) of hotspots initiated by single releases of root exudate, as evidenced by time-resolved ^{14}C imaging after $^{14}CO_2$ pulse labeling of *Lolium perenne* (Pausch and Kuzyakov, 2011). Therefore, we conclude that continuous inputs of labile organics due to root growth prolonged the duration of hot moments in the rhizosphere.
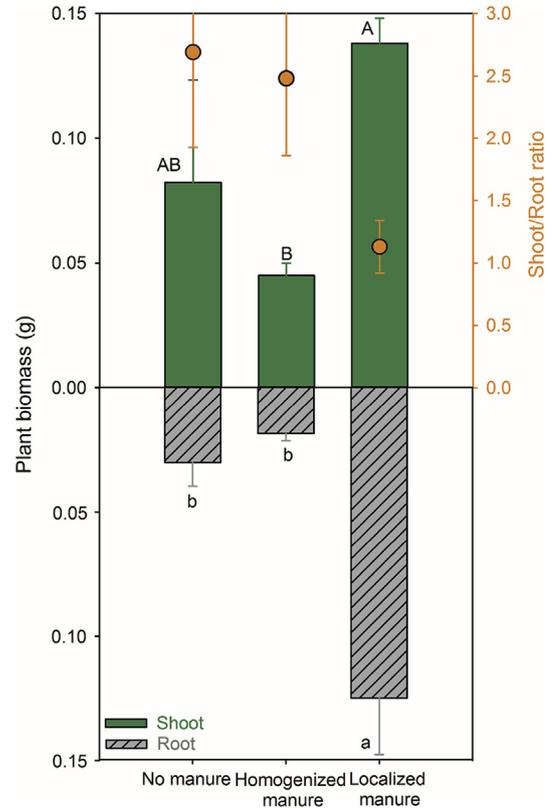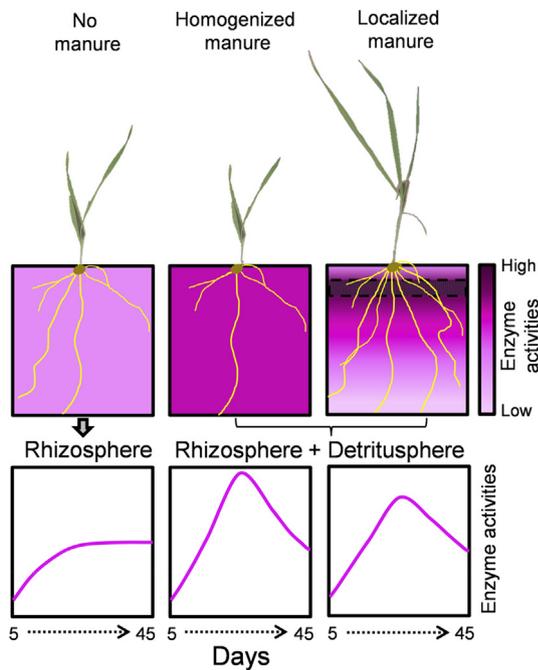
Fig. 8. General responses and localization of soil enzyme activities to manure application strategies over time. A clear detritusphere extension of enzyme activities was observed below the localized manure. The manure-induced detritusphere stimulated larger increases of enzyme activities than the "No manure" treatment (i.e. only the rhizosphere effect of barley), although the increase lasted less than 45 days. Homogenized manure elevated enzyme activities more than localized manure, while localized manure induced higher shoot and root biomass than homogenized manure.

more phosphomonoesterase to obtain inorganic P for its growth (Hunter et al., 2014). In comparison, β-glucosidase, which is mostly involved in the degradation of cellulose, showed a narrower extension. The wider extension of phosphomonoesterase compared to β-glucosidase activities has also been observed in the rhizosphere (Razavi et al., 2016). In the present study, the detritusphere extension of enzyme activities was much wider than the rhizosphere extension observed in other studies (e.g. Razavi et al., 2016; Tarafdar and Jungk, 1987; Sauer et al., 2006). This is explained by the direction of water fluxes: to the roots in the rhizosphere, but from the manure layer downwards. Consequently, extension of enzyme activities was much faster and wider in the detritusphere than in the rhizosphere, due to vertical diffusion and leaching processes.

4.3. Response of plants to manure application strategies

Following manure application, enzyme activities in the manure-induced detritusphere were higher than on root surfaces. This — in accordance with our first hypothesis (H1) - indicated that the detritusphere became more attractive to microbes than the rhizosphere, because high microbial activities tended to be in the hotspots of bulk soil instead of being balanced between the rhizoplane and soil hotspots, as in the control ($E_R \approx E_H$). Furthermore, though enzyme activities on the rhizoplane were lower, the reason for this may differ between homogenized and localized manure applications. For the homogenized manure application, tough competition for inorganic and organic nutrients between microbes and roots was initiated as soon as manure-derived microbes and labile substrates were introduced by mixing manure into the soil (Kuzyakov and Blagodatskaya, 2015; Malik et al., 2013; Xu et al., 2006, 2011). This may temporarily reduce plant nutrient availability, depress

root growth and explain the lower enzyme activities on the root surface for homogenized manure application. In contrast, with localized manure application, the pre-existing and newly mineralized nutrients were easily leached downward and competition between microbes and plant roots within the localized manure layer was weaker than for the homogenized manure application. This spatial niche differentiation for the manure microbial community and roots decreased their competition for nutrients and simultaneously increased nutrient uptake, and so, the plant biomass. Both situations were also reflected in the shoot and root biomass at day 45: shoot and root biomass with localized manure application were respectively 3.1 and 6.7 times higher than for homogenized manure application, and so our second hypothesis (H2) was rejected. Compared to the control, the relatively low plant biomass in the homogenized manure application also indicated that strong competition between microbes and roots existed when manure was homogenized with soil. This significant difference demonstrated that localized manure was more advantageous for barley growth than homogenized manure.

This is especially important on the Tibetan Plateau, because soils have been very seriously degraded in the last 30–50 years due to intensive human activities (e.g. overgrazing) and climate change (Chen et al., 2013). This has induced large soil organic carbon and nutrient losses and thus considerably decreased soil fertility. Localized manure application has been found to increase soil ammonium and nitrate concentrations in soils of the Tibetan Plateau (Cai et al., 2014; He et al., 2009). However, manure application at the soil surface leads to ammonia volatilization, involving significant nitrogen losses and negative effects on the environment. This is especially important on the Tibetan Plateau, because the solar radiation is much higher compared with other regions around the world (Liu et al., 2012), which increases the temperature of manure and accelerates the ammonia volatilization. Alternatively, homogenized manure application may reduce nitrogen losses by avoiding the impact of solar radiation. Therefore, to thoroughly investigate the impact of both manure application strategies, the effects on nitrogen emissions and leaching should also be considered.

5. Conclusions

For the first time, we elucidated and visualized the impacts of different manure application strategies on enzyme activities in soil *in situ*, spatially and temporally. The manure-induced detritusphere increased enzyme activities more than the rhizosphere effect of barley alone. Manure-induced hotspots also showed higher enzyme activities than the rhizoplane. Together, these findings demonstrate that microbial activities in the detritusphere are much more stimulated than on the root-soil interface (i.e. rhizosphere and rhizoplane). The detritusphere's vertical extension of phosphomonoesterase activity from the localized manure application was much faster than that of β-glucosidase activity. Overall, homogenized manure increased enzyme activities more than localized manure. However, localized manure induced 3.1 and 6.7 times higher shoot and root biomass, respectively. We conclude that localized manure application decreases competition for nutrients between microorganisms and roots and simultaneously increases plant performance.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2017.05.006.

References

Aarons, S.R., O'Connor, C.R., Hosseini, H.M., Gourley, C.J.P., 2009. Dung pads increase pasture production, soil nutrients and microbial biomass carbon in grazed dairy systems. Nutrient Cycling in Agroecosystems 84, 81–92. http://dx.doi.org/10.1007/s10705-008-9228-5.

Acosta-Martínez, V., Waldrip, H.M., 2014. Soil enzyme activities as affected by manure types, application rates, and management practices. In: He, Z., Zhang, H. (Eds.), Applied Manure and Nutrient Chemistry for Sustainable Agriculture and Environment. Springer Netherlands, pp. 99–122. http://dx.doi.org/10.1007/978-94-017-8807-6_6.

Babel, W., Biermann, T., Coners, H., Falge, E., Seeber, E., Ingrisch, J., Schleuß, P.M., Gerken, T., Leonbacher, J., Leipold, T., Willinghöfer, S., Schützenmeister, K., Shibistova, O., Becker, L., Hafner, S., Spielvogel, S., Li, X., Xu, X., Sun, Y., Zhang, L., Yang, Y., Ma, Y., Wesche, K., Graf, H.F., Leuschner, C., Guggenberger, G., Kuzyakov, Y., Miehe, G., Foken, T., 2014. Pasture degradation modifies the water and carbon cycles of the Tibetan highlands. Biogeosciences 11, 6633–6656. http://dx.doi.org/10.5194/bg-11-6633-2014.

Bell, J.M., Robinson, C.A., Schwartz, R.C., 2006. Changes in soil properties and enzymatic activities following manure applications to a rangeland. Rangeland Ecology & Management 59, 314–320. http://dx.doi.org/10.2111/05-172R1.1.

Brandjes, P.J., de Wit, J., van der Meer, H.G., 1996. Environmental Impact of Animal Manure Management. Food and Agriculture Organization of the United Nations. Available at: (2017/1/3). http://www.fao.org/wairdocs/lead/x6113e/x6113e00.htm#Contents.

Butler, T.J., Weindorf, D.C., Han, K.J., Muir, J.P., 2013. Dairy manure compost quality effects on corn silage and soil properties. Compost Science & Utilization 17 (1), 18–24. http://dx.doi.org/10.1080/1065657X.2009.10702395.

Cai, Y.J., Wang, X.D., Tian, L.L., Zhao, H., Lu, X.Y., Yan, Y., 2014. The impact of excretal returns from yak and Tibetan sheep dung on nitrous oxide emissions in an alpine steppe on the Qinghai-Tibetan Plateau. Soil Biology & Biochemistry 76, 90–99. http://dx.doi.org/10.1016/j.soilbio.2014.05.008.

Calleja-Cervantes, M.E., Fernández-González, A.J., Irigoyen, I., Fernández-López, M., Aparicio-Tejo, P.M., Menéndez, S., 2015. Thirteen years of continued application of composted organic wastes in a vineyard modify soil quality characteristics. Soil Biology & Biochemistry 90, 241–254. http://dx.doi.org/10.1016/j.soilbio.2015.07.002.

Celik, I., Ortas, I., Kilic, S., 2004. Effects of compost, mycorrhiza, manure and fertilizer on some physical properties of a Chromoxerert soil. Soil & Tillage Research 78, 59–67. http://dx.doi.org/10.1016/j.still.2004.02.012.

Chen, H., Zhu, Q., Peng, C., Wu, N., Wang, Y., Fang, X., Gao, Y., Zhu, D., Yang, G., Tian, J., Kang, X., Piao, S., Ouyang, H., Xiang, W., Luo, Z., Jiang, H., Song, X., Zhang, Y., Yu, G., Zhao, X., Gong, P., Yao, T., Wu, J., 2013. The impacts of climate change and human activities on biogeochemical cycles on the Qinghai-Tibetan Plateau. Global Change Biology 19, 2940–2955. http://dx.doi.org/10.1111/gcb.12277.

Criquet, S., Braud, A., Nèble, S., 2007. Short-term effects of sewage sludge application on phosphatase activities and available P fractions in Mediterranean soils. Soil Biology & Biochemistry 39, 921–929. http://dx.doi.org/10.1016/j.soilbio.2006.11.002.

Dickinson, C.H., Underhay, V.S.H., Ross, V., 1981. Effect of season, soil fauna and water content on the decomposition of cattle dung pats. New Phytologist 88, 129–141. http://dx.doi.org/10.1111/j.1469-8137.1981.tb04576.x.

Dinesh, R., Dubey, R.P., Prasad, G.S., 1998. Soil microbial biomass and enzyme activities as influenced by organic manure incorporation into soils of a rice-rice system. Journal of Agronomy and Crop Science 181 (3), 173–178. http://dx.doi.org/10.1111/j.1439-037X.1998.tb00414.x.

Dong, S.F., Brooks, D., Jones, M.D., Grayston, S.J., 2007. A method for linking in situ activities of hydrolytic enzymes to associated organisms in forest soils. Soil Biology & Biochemistry 39 (9), 2414–2419. http://dx.doi.org/10.1016/j.soilbio.2007.03.030.

Dungait, J.A.J., Bol, R., Bull, I.D., Evershed, R.P., 2009. Tracking the fate of dung-derived carbohydrates in a temperate grassland soil using compound-specific stable isotope analysis. Organic Geochemistry 40, 1210–1218. http://dx.doi.org/10.1016/j.orggeochem.2009.08.001.

Eivazi, F., Tabatabai, M.A., 1977. Phosphatases in soils. Soil Biology & Biochemistry 9, 167e172. http://dx.doi.org/10.1016/0038-0717(77)90070-0.

FAO, 2003. The Yak, second ed. Available at: (2017/1/3). http://www.fao.org/docrep/006/ad347e/ad347e0l.htm#bm21.9.

German, D.P., Weintraub, M.N., Grandy, A.S., Lauber, C.L., Rinkes, Z.L., Allison, S.D., 2011. Optimization of hydrolytic and oxidative enzyme methods for ecosystem studies. Soil Biology & Biochemistry 43, 1387–1397. http://dx.doi.org/10.1016/j.soilbio.2011.03.017.

Hafner, S., Unteregelsbacher, S., Seeber, E., Xu, X., Li, X., Guggenberger, G., Miehe, G., Kuzyakov, Y., 2012. Effect of grazing on carbon stocks and assimilate partitioning in Tibetan montane pasture revealed by $^{13}CO_2$ pulse labeling. Global Change Biology 18 (2), 528–538. http://dx.doi.org/10.1111/j.1365-2486.2011.02557.x.

Hamid, R., Khan, M.A., Ahmad, M., Ahmad, M.M., Abdin, M.Z., Javed, S., 2013. Chitinase: an update. Journal of Pharmacy & BioAllied Sciences 5 (1), 21–29. http://dx.doi.org/10.4103/0975-7406.106559.

Haynes, R.J., Naidu, R., 1998. Influence of lime, fertilizer and manure applications on soil organic matter content and soil physical conditions: a review. Nutrient Cycling in Agroecosystems 51, 123–137. http://dx.doi.org/10.1023/A:1009738307837.

He, Y.X., Sun, G., Liu, L., Luo, P., Wu, N., Luo, G.R., 2009. Effect of yak dung on high-frigid meadow soil nutrition in northwestern Sichuan, China. Chinese Journal of Applied and Environmental Biology 15, 666–671 (in Chinese with English abstract).

Hoang, D.T.T., Razavi, B.S., Kuzyakov, Y., Blagodatskaya, E., 2016. Earthworm burrows: kinetics and spatial distribution of enzymes of C-, N- and P- cycles. Soil Biology & Biochemistry 99, 94–103. http://dx.doi.org/10.1016/j.soilbio.2016.04.021.

Hunter, P.J., Teakle, G.R., Bending, G.D., 2014. Root traits and microbial community interactions in relation to phosphorus availability and acquisition, with particular reference to Brassica. Frontiers in Plant Science 5, 27. http://dx.doi.org/10.3389/fpls.2014.00027.

Kang, H., Kang, S., Lee, D., 2009. Variations of soil enzyme activities in a temperate forest soil. Ecological Research 24, 1137–1143. http://dx.doi.org/10.1007/s11284-009-0594-5.

Koch, O., Tscherko, D., Kandeler, E., 2007. Temperature sensitivity of microbial respiration, nitrogen mineralization, and potential soil enzyme activities in organic alpine soils: temperature sensitivity in alpine soils. Global Biogeochemical Cycles 21 (4), GB4017. http://dx.doi.org/10.1029/2007GB002983.

Kuzyakov, Y., Blagodatskaya, E., 2015. Microbial hotspots and hot moments in soil: concept & review. Soil Biology & Biochemistry 83, 184–199. http://dx.doi.org/10.1016/j.soilbio.2015.01.025.

Liang, Q., Chen, H., Gong, Y., Yang, H., Fan, M., Kuzyakov, Y., 2014. Effects of 15 years of manure and mineral fertilizers on enzyme activities in particle-size fractions in a North China Plain soil. European Journal of Soil Biology 60, 112–119. http://dx.doi.org/10.1016/j.ejsobi.2013.11.009.

Liu, G.Y., Nima, Z.X., Nima, Z.X., Song, G.Y., 2013. The study on barley production under different nitrogen levels. Tibet Journal of Agricultural Sciences 35, 17–20 (in Chinese with English abstract).

Liu, J.D., Liu, J.M., Linderholm, H.W., Chen, D.L., Yu, Q., Wu, D.R., Haginoya, S., 2012. Observation and calculation of the solar radiation on the Tibetan Plateau. Energy Conversion and Management 57, 23–32. http://dx.doi.org/10.1016/j.enconman.2011.12.007.

Lovell, R.D., Jarvis, S.C., 1996. Effect of cattle dung on soil microbial biomass C and N in a permanent pasture soil. Soil Biology & Biochemistry 28 (3), 291–299. http://dx.doi.org/10.1016/0038-0717(95)00140-9.

Malcolm, R.E., 1983. Assessment of phosphatase activity in soils. Soil Biology & Biochemistry 15, 403e408. http://dx.doi.org/10.1016/0038-0717(83)90003-2.

Malik, M.A., Khan, K.S., Marschner, P., Fayyaz-ul-Hassean, 2013. Microbial biomass, nutrient availability and nutrient uptake by wheat in two soils with organic amendments. Journal of Soil Science and Plant Nutrition 13 (4), 955–966. http://dx.doi.org/10.4067/S0718-95162013005000075.

Matilla, P.K., 2006. Ammonia Emissions from Pig and Cattle Slurry in the Field and Utilization of Slurry Nitrogen in Crop Production. Doctoral Dissertation. Agri-food Research Reports 87. Available at: (2017/1/3). http://www.mtt.fi/met/pdf/met87.pdf.

Menon, M., Robinson, B., Oswald, S.E., Kaestner, A., Abbaspour, K.C., Lehmann, E., Schulin, R., 2007. Visualization of root growth in heterogeneously contaminated soil using neutron radiography. European Journal of Soil Science 58, 802–810. http://dx.doi.org/10.1111/j.1365-2389.2006.00870.x.

Moore, J.C., Berlow, E.L., Coleman, D.C., de Ruiter, P.C., Dong, Q., Hastings, A., Johnson, N.C., McCann, K.S., Melville, K., Morin, P.J., Nadelhoffer, K., Rosemond, A.D., Post, D.M., Sabo, J.L., Scow, K.M., Vanni, M.J., Wall, D.H., 2004. Detritus, trophic dynamics and biodiversity. Ecology Letters 7, 584–600. http://dx.doi.org/10.1111/j.1461-0248.2004.00606.x.

Nannipieri, P., Ascher, J., Ceccherini, M.T., Landi, L., Pietramellara, G., Renella, G., Valori, F., 2007. Microbial diversity and microbial activity in the rhizosphere. Ciencia del suelo 25, 89e97.

Pascual, J.A., Moreno, J.L., Hernández, T., Garcıa, C., 2002. Persistence of immobilized and total urease and phosphatase activities in a soil amended with organic wastes. Bioresource Technology 82 (1), 73–78. http://dx.doi.org/10.1016/S0960-8524(01)00127-4.

Pausch, J., Kuzyakov, Y., 2011. Photoassimilate allocation and dynamics of hotspots in roots visualized by ^{14}C phosphor imaging. Journal of Plant Nutrition and Soil Science 174, 12–19. http://dx.doi.org/10.1002/jpln.200900271.

Poll, C., Ingwersen, J., Stemmer, M., Gerzabek, M.H., Kandeler, E., 2006. Mechanisms of solute transport affect small-scale abundance and function of soil microorganisms in the detritusphere. European Journal of Soil Science 57, 583–595.

http://dx.doi.org/10.1111/j.1365-2389.2006.00835.x.

Razavi, B.S., Zarebanadkouki, M., Blagodatskaya, E., Kuzyakov, Y., 2016. Rhizosphere shape of lentil and maize: spatial distribution of enzyme activities. Soil Biology & Biochemistry 96, 229–237. http://dx.doi.org/10.1016/j.soilbio.2016.02.020.

Ren, F., Yang, X.X., Zhou, H.K., Zhu, W.Y., Zhang, Z.H., Chen, L.T., Cao, G.M., He, J.S., 2016. Contrasting effects of nitrogen and phosphorus addition on soil respiration in an alpine grassland on the Qinghai-Tibetan Plateau. Scientific Resports 6, 34786. http://dx.doi.org/10.1038/srep34786.

Risse, L.M., Cabrera, M.L., Franzluebbers, A.J., Gaskin, J.W., Gilley, J.E., Killorn, R., Radcliffe, D.E., Tollner, W.E., Zhang, H., 2006. Land application of manure for beneficial reuse. In: Rice, J.M., Caldwell, D.F., Humenik, F.J. (Eds.), Animal Agriculture and the Environment: National Center for Manure and Animal Waste Management White Papers. American Society of Agricultural and Biological Engineers, St. Joseph, pp. 283–316.

Sauer, D., Kuzyakov, Y., Stahr, K., 2006. Spatial distribution of root exudates of five plant species as assessed by ^{14}C labelling. Journal of Plant Nutrition and Soil Science 169, 360–362. http://dx.doi.org/10.1002/jpln.200621974.

Scotti, R., Bonanomi, G., Scelza, R., Zoina, A., Rao, M.A., 2015. Organic amendments as sustainable tool to recovery fertility in intensive agricultural systems. Journal of Soil Science and Plant Nutrition 15 (2), 333–352. http://dx.doi.org/10.4067/S0718-95162015005000031.

Sinsabaugh, R.L., Lauber, C.L., Weintraub, M.N., Ahmed, B., Allison, S.D., Crenshaw, C., Contosta, A.R., Cusack, D., Frey, S., Gallo, M.E., Gartner, T.B., Hobbie, S.E., Holland, K., Keeler, B.L., Powers, J.S., Stursova, M., Takacs-Vesbach, C., Waldrop, M.P., Wallenstein, M.D., Zak, D.R., Zeglin, L.H., 2008. Stoichiometry of soil enzyme activity at global scale. Ecology Letters 11, 1252–1264. http://dx.doi.org/10.1111/j.1461-0248.2008.01245.x.

Tarafdar, J.C., Jungk, A., 1987. Phosphatase activity in the rhizosphere and its relation to the depletion of soil organic phosphorus. Biology and Fertility of Soils 3, 199–204. http://dx.doi.org/10.1007/BF00640630.

Thomsen, I.K., 2005. Crop N utilization and leaching losses as affected by time and method of application of farmyard manure. European Journal of Agronomy 22 (1), 1–9. http://dx.doi.org/10.1016/j.eja.2003.10.008.

Tiquia, S.M., 2002. Evolution of extracellular enzyme activities during manure composting. Journal of Applied Microbiology 92, 764–775. http://dx.doi.org/10.1046/j.1365-2672.2002.01582.x.

Wang, Q., 2009. Prevention of Tibetan eco-environmental degradation caused by traditional use of biomass. Renewable and Sustainable Energy Reviews 13, 2562–2570. http://dx.doi.org/10.1016/j.rser.2009.06.013.

Webb, J., Pain, B., Bittman, S., Morgan, J., 2010. The impacts of manure application methods on emissions of ammonia, nitrous oxide and on crop response-A review. Agriculture. Ecosystems and Environment 137, 39–46. http://dx.doi.org/10.1016/j.agee.2010.01.001.

WRB, 2014. World Reference Base for Soil Resources. FAO, Rome. World Soil Resources Reports 106.

Xu, X., Ouyang, H., Kuzyakov, Y., Richter, A., Wanek, W., 2006. Significance of organic nitrogen acquisition for dominant species in an alpine meadow on the Tibet plateau, China. Plant and Soil 285, 221–231. http://dx.doi.org/10.1007/s11104-006-9007-5.

Xu, X., Ouyang, H., Richter, A., Wanek, W., Cao, G., Kuzyakov, Y., 2011. Spatio-temporal variations determine plant–microbe competition for inorganic nitrogen in an alpine meadow. Journal of Ecology 99, 563–571. http://dx.doi.org/10.1111/j.1365-2745.2010.01789.x.

Zaller, J.G., Köpke, U., 2004. Effects of traditional and biodynamic farmyard manure amendment on yields, soil chemical, biochemical and biological properties in a long-term field experiment. Biology and Fertility of Soils 40, 222–229. http://dx.doi.org/10.1007/s00374-004-0772-0.

Zarebanadkouki, M., Kim, Y.X., Moradi, A.B., Vogel, H.J., Kaestner, A., Carminati, A., 2012. Quantification and modeling of local root water uptake using neutron radiography and deuterated water. Vadose Zone Journal 11. http://dx.doi.org/10.2136/vzj2011.0196.

Zhu, K., Bruun, S., Larsen, M., Glud, R.N., Jensen, L.S., 2015. Heterogeneity of O_2 dynamics in soil amended with animal manure and implications for greenhouse gas emissions. Soil Biology & Biochemistry 84, 96–106. http://dx.doi.org/10.1016/j.soilbio.2015.02.012.