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Rolling in the deep: Priming effects in earthworm biopores in topsoil and subsoil

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ABSTRACT

Priming effect is the change of soil organic matter (SOM) decomposition due to the addition of labile carbon (C) sources. Earthworms incorporate organic matter into their burrow-linings thereby creating preferred habitats for microorganisms, but the roles of such burrows in priming effect initiation is unknown. Here we study the mechanisms driving SOM decomposition in top- and subsoil biopores and additionally in the rhizosphere. Given the topsoil was newly formed after ploughing 10 months prior to sampling, we hypothesized that (1) SOM accessibility, enzyme activities and efficiency of enzymatic reaction (K_a) are main drivers of different priming effect in biopores vs. bulk soil and rhizosphere, subsoil vs. topsoil and (2) the production of microbial enzymes in biopores depends on microbial community composition. To test these hypotheses, biopores formed by *Lumbricus terrestris* L. and bulk soil were sampled from topsoil (0–30 cm) and two subsoil depths (45–75 and 75–105 cm). Additionally, rhizosphere samples were taken from the topsoil. Total organic C (C_{org}), total N (TN), total P (TP) and enzyme activities involved in C-, N-, and P-cycling (cellobiohydrolase, β-glucosidase, xylanase, chitinase, leucine aminopeptidase and phosphatase) were measured. Priming effects were calculated as the difference in SOM-derived CO_2 from soil with or without ^{14}C-labeled glucose addition.

Enzyme activities (V_{max}) and the catalytic efficiency (K_a) were higher in biopores compared to bulk soil and the rhizosphere, indicating that the most active microbial community occurred at this site. Negative correlations between some enzymes and C:N ratio in bulk soil are explained by higher content of fresh organic C in the topsoil, and the corresponding C and nutrient limitations in the subsoil. The positive correlation between enzyme activities and Corg or TN in biopores, however, was associated with the decrease of C and TN with pore age in the subsoil. In the subsoil, priming effect in biopores was 2.5 times higher than bulk soil, resulting from the favorable conditions for microorganisms in biopores and the stimulation of microbial activities by earthworm mucus. We conclude that earthworm burrows provide not only the linkage between top- and subsoil for C and nutrients, but strongly increase microbial activities and accelerate SOM turnover in subsoil, contributing to nutrient mobilization for roots.

1. Introduction

The earthworm *Lumbricus Terrestris* L. is an anecic species inhabiting one single vertical burrow (drilosphere) throughout its entire life (Don et al., 2008), transporting fresh plant detritus from the soil surface downwards while mixing it with mineral soil particles (Lee, 1985; Brown et al., 2000). Earthworms alter soil structure (Lavelle, 1997), distribute litter carbon (C) throughout the entire soil profile (Jégou et al., 2000) and accelerate C turnover over longer time scale (Yavitt et al., 2015). Along burrows, the improved air circulation, enrichment of soil organic matter (SOM) and nutrients, as well as the water retention may reduce or even override the biogeochemical differences between top- and subsoil.

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Earthworm burrowing activities affect soil C stocks in the topsoil and subsoil altering microbial activities, for which enzyme activity is a sensitive indicator.

Metabolic enzymes are proteins produced by microbiota, plant roots and soil fauna to hydrolyze SOM. Thus, by stimulating microbial communities (Don et al., 2008), distributing extracellular enzymes (exoenzymes; Hoang et al., 2016a) and enhancing enzyme activities (Dempsey et al., 2013), earthworms indirectly and directly affect enzyme activities. Moreover, earthworm mucus, which is a water-soluble mixture of low molecular weight carbohydrates, acts as a primer for plant residue mineralization (Bityutskii et al., 2012). Similar to earthworms, also plant roots not only produce enzymes (Asmar et al., 1994) but also alter enzyme activities through modification of root morphology, exudation and interaction with microorganisms (Asmar et al., 1994; Fontaine et al., 2007; Razavi et al., 2016a). In order to understand the specificity of these biological processes (Bar-Even et al., 2011) and the sensitivity of enzymes to biotic effect (earthworms and roots) (German et al., 2012), kinetic enzyme parameters are approximated by the Michaelis-Menten equation. Other than the maximal catalytic reaction rate (V_{max}) and substrate affinity (K_m), the catalytic efficiency (K_a – determined as V_{max}/K_m) should be considered to reflect the association between historic catalytic properties of enzymes and microbial competition for available substrates (Kovarova-Kovar and Egli, 1998; Moscatelli et al., 2012; Tischer et al., 2015). The catalytic efficiency (V_{max}/K_m) represents the formation or dispersion of an enzyme-substrate complex in soil. Higher value of V_{max}/K_m suggests that dispersion of enzyme-substrate complex occurs faster than its formation, i.e. more SOM is decomposed by microorganisms (Gianfreda et al., 1995; Ekberli et al., 2006; Kizilkaya and Ekberli, 2008; Razavi et al., 2017). Thus, the catalytic efficiency indicates altered SOM decomposition in soil microhabitats as compared to bulk soil.

Priming effect is defined as a short-term change in SOM turnover caused by organic C addition (Kuzyakov et al., 2000). Priming effect can be divided into two processes: apparent priming effect and real priming effect. Apparent priming effect is connected to microbial turnover, while the real priming effect links to SOM turnover. These processes are differently regulated between topsoil and subsoil by the availability of fresh C inputs (De Graaff et al., 2014), physical accessibility of decomposer to substrates (Salomé et al., 2010; De Graaff et al., 2014) and different response of microbial community to SOM inputs (Sanaullah et al., 2016). However, the processes controlling priming in biopores have not been investigated at all (Brown, 1995; Kuzyakov, 2010). Labile C incorporated into the subsoil biopores may accelerate old C decomposition, inducing C turnover in this layer (Don et al., 2008). Meanwhile, Salomé et al. (2010) suggested exoenzyme access to substrate as a foundation of C turnover in the subsoil contrarily to topsoil.

While plant residues are the primary source of microorganism-stimulating C inputs to biopores, living roots also impact soil microorganisms *via* rhizodeposition and exudation of organic compounds (sugars, amino acids, organic acids) (Pausch et al., 2013a). Winter barley (*Hordeum vulgare* L.), for example, is characterized by a fibrous root system that transfers 17% of total assimilated C to belowground pools (roots, microorganisms, soil organic matter) (Kuzyakov and Domanski, 2000). 70% of the whole root system, however, is found in the upper 30 cm of the soil profile, where nutrient contents are highest (Lucas et al., 2000; Steingrobe et al., 2001). Despite the short root exudate lifetimes of no more than a few days (Pausch and Kuzyakov, 2011; Kuzyakov and Blagodatskaya, 2015), the rhizosphere is critically important microbial hotspot in soil (Blagodatskaya and Kuzyakov, 2008; Kuzyakov and Blagodatskaya, 2015). In contrast, earthworm burrows can exist for even longer than the lifetime of *Lumbricus Terrestris* L. itself (Tiunov and Scheu, 1999; Stromberger et al., 2012) and can be re-occupied by succeeding generations. The longevity of biopores and rhizosphere affects the stability of their corresponding microbial activities through microhabitat persistence, C content, and nutrient availability and, therefore, regulates SOM decomposition by microorganisms.

The goal of this study was to investigate the effects of earthworm activity in biopores (burrows) and of root activity on altered SOM decomposition (priming effect), and whether this priming effect is depth dependent. Based on this investigation, we compared SOM mineralization induced by glucose supply in biopores and rhizosphere. Accordingly, we hypothesized that (i) priming effects are more pronounced in biopores than in bulk soil due to more accessible SOM presented by earthworm activity; (ii) the production of microbial enzymes in biopores depends on microbial community composition; (iii) enzyme activities and efficiency of enzymatic reaction (K_a) play the main roles driving different priming effect in subsoil vs. topsoil, biopores vs. bulk soil. To this end, we measured enzyme activities and SOM-derived CO_2 associated with earthworm burrows, rhizosphere and bulk soil at different soil depths (0–30 cm, 45–75 cm and 75–105 cm), and assessed the role of biopores in the priming effect, especially in the subsoil.

2. Material and methods

2.1. Soil sampling and sample preparation

The study site belongs to the research station at Campus Klein-Altendorf (50° 37′ N, 6° 59’ E) south-west of Bonn, Germany. The topsoil was ploughed 10 months prior sampling to grow winter barley (*Hordeum vulgare* L.). Winter barley was sown at a density of 320 grains m^{-2} on 2nd October, 2014. The soil is classified as a Haplic Luvisol (WRB). The topsoil and subsoil characteristics are given in Vetterlein et al. (2013). Sampling of drilosphere, rhizosphere and bulk soil was carried out in April 2015 from three independent plots (each 2 m × 2 m). The season affects earthworm abundance and according to Spurgeon and Hopkin (1999), highest caches are found in spring and winter, and lowest in summer and autumn in an unmanaged grassland in England. Moreover, temperature variation alters soil egestion rates (Curry et al., 1995). We sampled soil in April as this was their most abundant time and also the right time to excavate the worm burrows (Curry et al., 1995).

Drilosphere was collected within the innermost part of burrows (Tiunov and Scheu, 1999) at 3 depths (0–30 cm, 45–75 cm, 75–105 cm). In order to implement field sampling, a soil pit was dug to 150 cm to expose a soil profile. We did not remove the plants before excavating earthworm burrows so as to prevent top-burrow destruction in the topsoil. Burrow pores were carefully opened on one side with a sharp knife to reveal the burrow walls, according to Hoang et al. (2016b). A micro-spoon (5 × 100 mm) was acquired to scratch the dark surface of cast along burrow walls within each 10 cm increment of soil depth. This layer of drilosphere is supposed to be within a few millimeters (Parkin and Berry, 1999) or up to 1 cm in thickness (Jégou et al., 2000). We therefore tried to sample soil materials in more or less 2 mm thickness of burrow walls. Rhizosphere soil adhering to roots was collected only from the topsoil (0–30 cm) (Grayston et al., 1998). Bulk soil was considered as soil at a distance greater than 2 cm from root or earthworm pores and was collected from 0 to 30, 45–75, and 75–105 cm depths. However, due to very high density of roots in the topsoil, bulk soil was partly affected by rhizosphere. Samples were stored field-fresh at 5 °C (<one month) until use. Before the main experiments started, root litter and plant debris had been removed with tweezers. Soil samples were divided into 3 subsamples for analyzing (i) water content, total C (TC), total N (TN) and total P (TP), (ii) enzyme

kinetics and (iii) incubation experiment. The soil water content was determined in 1 g of soil by drying at 105 °C, of which 35–50 mg dry soil was ground to powder in a ball mill for TC and TN content analysis by dry combustion (VarioMax, Elementar). For total P (TP), samples were extracted by hot acid digestion of 0.5 g dry soil in *aqua regia*. Phosphorus content of the extracts was determined by inductively coupled plasma optical-emission spectroscopy (Ultima 2 ICP-OES, HORIBA Jobin Yvon, Longjumeau, France).

2.2. Enzyme kinetics

The subsamples for enzyme analysis were pre-conditioned for 2 days at room temperature (20 °C) prior to measurement. To determine activities of six enzymes involved in organic matter decomposition, we used fluorescent substrates (Sigma-Aldrich, Germany) corresponding to the respective enzymes. Cellobiohydrolase (CBH) (EC 3.2.1.91), β-glucosidase (GLU) (EC 3.2.1.21) and xylanase (XYL) (EC 3.2.1.8), responsible for cellulose and hemicellulose hydrolysis, were analyzed using 4-methylumbelliferyl-β-D-cellobioside, 4-methylumbelliferyl-β-D-glucopyranoside, and 4-methylumbelliferyl-7-β-D-xylopyranoside, respectively. N-acetyl-β-glucosaminidase (NAG) (chitinase, EC 3.2.1.52) and leucine aminopeptidase (LAP) (EC 3.4.11.1), respectively catalyzing chitin degradation and protein and peptide hydrolysis, were assayed by 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide and L-leucine-7-amino-4-methylcoumarin. Activity of acid phosphatase (APT) (EC 3.1.3.2), which plays a role in organic phosphate decomposition, was measured with 4-methylumbelliferyl-phosphate. Half a gram of soil (dry weight equivalent) was suspended in 50 mL sterile water by shaking for 20 min and dispersing with an ultrasonic disaggregator for 2 min (Pausch et al., 2016). 51 μL of the soil suspension was pipetted into 96-well microplates (Brand pureGrade, black). Thereafter, 50 μL of buffer (MES or TRIZMA) and 100 μL of the corresponding substrates at concentrations of 20, 40, 60, 80, 100, 200 and 400 μmol substrate g^{-1} soil were added (Razavi et al., 2015). Immediately after substrate addition, the microplates were measured fluorometrically (excitation wavelength 360 nm, emission 450 nm) and the measurement was repeated after 30 min, 1 h, and 2 h (Wallac 1420, Perkin Elmer. Turku, Findland). The same process was performed for all enzymes. The results were calculated based on the Michaelis-Menten equation by comparison to standard curves prepared with separately purchased MUF (cellobiohydrolase, β-glucosidase, xylanase, N-acetyl-β-glucosaminidase, acid phosphatase) or AMC (leucine aminopeptidase).

2.3. Incubation

For the incubation experiment, 6 g of soil (dry weight equivalent) from each sample were equally subdivided into two vials (each 24 mL volume). In total, 42 vials were prepared: burrows (3 replicates, 3 depths), bulk soil (3 replicates, 3 depths), and rhizosphere (3 replicates, Ap horizon).

The samples were pre-incubated for 11 days at 21 ± 1 °C, until the daily basal respiration was constant, in order to stabilize microbial activities prior to substrate addition (Falchini et al., 2003). During the pre-incubation, the samples were allowed to dry to the desired water content (50% WHC). The CO_2 evolved from the soil was trapped in 300 μL of 1.0 M NaOH solution placed in small caps (1.5 mL) attached to the vial bottom. The incubation vials were kept air-tight with rubber septa, which were covered again with aluminum seal crimp caps. To substitute fresh NaOH for the NaOH with trapped CO_2, the vials were opened every 24 h and a syringe was used to withdraw the solution. Opening the vials also prevented the development of anaerobic conditions.

Uniformly labeled ^{14}C glucose was added to unlabeled glucose to make a stock solution of 12×10^4 DPM (disintegrations per minute) per vial, before being added to the soil. The total incubation period after glucose addition was 24 days. Glucose solution (100 μL) was applied with a fine needle to one of each pair of vials, while distilled water (100 μL) was added to the other (control). The amount of added glucose C corresponded to 50% of microbial biomass C in the drilosphere, rhizosphere and bulk soil (microbial biomass C of 0.82, 0.19 and 0.03 mg C g^{-1} soil, respectively) based on the results of a previous study at the same site (Hoang et al., 2016b). The implicit assumption here is that the priming effect is a direct function of microbial biomass present in the target soil (Xiao et al., 2015) but that may not always be true (Guenet et al., 2010; Shahzad et al., 2015). Moreover, the use of vastly different amount of added glucose in different soil compartments becomes more complicated. Priming effect is not a linear function external OM input but priming could be modeled effectively as a function of the response of microbial biomass to litter inputs (Xiao et al., 2015). We acknowledge that this approach may not cover all the aspects of arguments on mechanisms behind priming effect from biopores induced by glucose addition. However, to our knowledge this approach is the optimum to deal with soil material limitation as the addition of glucose at 50% microbial biomass can avoid an exponential decrease of priming effect and microorganisms are not limited in energy input (Blagodatskaya and Kuzyakov, 2008). After addition, the CO_2 evolved from the soil was trapped in 600 μL of 1.0 M NaOH. For the first two days, the NaOH solution was exchanged at 6, 10, 16, 24 and 36 h after glucose addition, daily for the rest of first week, and every 2 days for the following weeks. Based on this experimental design, we monitored: (1) total CO_2 and $^{14}CO_2$ evolution in glucose amended samples, and (2) unlabeled CO_2 originating from microbial respiration of soil organic matter pools. Four blanks were included (vials without soil) to correct for CO_2 trapped during sample handling. The ^{14}C activity of CO_2 was measured in 300 μL NaOH aliquots added to 2.5 mL of the scintillation cocktail Rotiszint Eco Plus (Carl Roth, Germany). Radioactivity was quantified counted using a liquid scintillation counter (1450 LSC MicroBeta TriLux, Perkin Elmer Inc., USA). The remaining 20 μL NaOH solution was used to measure total CO_2-C with a Shimadzu TOC-5050A Total Organic Carbon Analyzer.

2.4. Calculation and statistical analysis

Activities (V_{max}, nmol g^{-1} MUF or AMC dry soil h^{-1}) and kinetics (K_m, μmol substrate g^{-1} soil) of each enzyme were defined using non-linear regression (Michaelis-Menten kinetics) and fitted by OriginPro 8.5 (OriginLab, Massachusetts, USA). The Michaelis-Menten equation was applied to calculate kinetic parameters (V_{max} and K_m):

$$V = \frac{V_{max[S]}}{K_m + [S]}$$

where V_{max} is maximum reaction rate catalyzed by enzymes at a saturated substrate concentration and K_m represents the substrate concentration at a reaction rate equal to half maximal velocity ($\frac{V_{max}}{2}$). The catalytic efficiency of enzymatic reactions was determined by the V_{max}-to-K_m ratio.

The priming effect (μg C g^{-1} soil day^{-1}) was calculated as the difference in SOM-derived CO_2 from soil amended with glucose and control soil:

PE = SOM-derived CO_2 (with glucose) − SOM-derived CO_2 (without glucose)

SOM-derived CO_2 evolved from soil with glucose was calculated as the difference between total CO_2 and glucose-derived CO_2 (μg C

g^{-1} soil h^{-1}) (Blagodatskaya et al., 2007):

SOM-derived CO_2 (with glucose) = Total CO_2 (with glucose) − Glucose-derived CO_2

The glucose-derived CO_2 was determined from the ^{14}C activity of NaOH traps.

Cumulative CO_2 results were converted to μg C g^{-1} soil. The ^{14}C efflux rate is provided as μg C g^{-1} soil h^{-1}.

Based on ^{14}C-CO_2 pattern which demonstrated glucose-originated CO_2 we can estimate priming effect mechanisms: parent and real priming effects. If this value is apparently high and emerge shortly within hours after glucose addition, then we can assume the occurrence of apparent priming effect (Kuzyakov et al., 2000). If this value decreases to some extent over time and the cumulative priming effect shows positive value, then the period is considered as real priming effect.

The standard error of the mean (SE) for CO_2, enzyme activities and enzyme kinetics is presented in the figures. The effects of soil depth and soil type (biopore, rhizosphere or bulk soil) were assessed by two-way ANOVA using STATISTICA 12 (StatSoft Inc., USA), considering each as independent factors. A *t*-test was applied to test for significant deviation of the priming effect from zero. P-values less than 0.05 were considered to indicate significance.

3. Results

3.1. Total organic C, total N, P and C/N ratio

The soil pH was 6.5 at 0–30 cm and 6.9–7.1 below 45 cm, while the $CaCO_3$ content was <1 mg g^{-1} soil for the whole soil profile (Kautz et al., 2014). Therefore, we considered total C as total soil organic C (C_{org}). Total organic C was higher in biopores (7.64–13.4 mg C g^{-1} soil) than in bulk soil (3.2–9.5 mg C g^{-1} soil) (Fig. 1a), which was similar to the pattern observed for total N (Fig. 1b). C:N ratios were higher in biopores than bulk soil, being unchanged in biopores with depth but significantly decreased in bulk soil (Appendix 1). The rhizosphere soil had similar total organic C and total N contents to bulk soil at 0–30 cm. For biopores and bulk soil, total organic C and total N contents decreased with depth, but did not differ between the two subsoil layers (45–75 cm and 75–105 cm). In contrast to C and N, total P was highest in the rhizosphere (Fig. 1c).

3.2. Enzyme activity and catalytic efficiency

Most enzyme activities were highly correlated with total organic C and total N ($P < 0.01^{**}$, $P < 0.05^{*}$) with $R^2 > 0.6$ (except for cellobiohydrolase and β-glucosidase in bulk soil) (Fig. 2). At all soil depths, enzyme activities were positively correlated with total organic C and total N in biopores, but negatively correlated or uncorrelated (Fig. 2a and b) in bulk soil. Acid phosphatase activities did not show any correlation with total phosphorus in both biopores and bulk soil. In the topsoil, activities of all enzymes were generally higher in biopores compared to rhizosphere and bulk soil. In the subsoil, higher cellobiohydrolase, β-glucosidase and leucine aminopeptidase activities (Fig. 2a, b, f) were associated with higher contents of total organic C and total N (Fig. 1) in biopores than in bulk soil, while activities of xylanase and chitinase (Fig. 2c, d, e) decreased by up to six fold in biopores. Most enzyme activities in biopores were 1.6–8 times higher in topsoil compared to subsoil.

Catalytic efficiency (K_a) was calculated by dividing K_m by V_{max} for each enzyme. In the topsoil, catalytic efficiency for most enzymes was up to 8 times higher in biopores than in rhizosphere or bulk soil, with the exception of leucine aminopeptidase (Fig. 3). The catalytic efficiency was similar between rhizosphere and bulk soil in the topsoil. In subsoil, the catalytic efficiency of enzymes involved in cellulose, chitin and leucine hydrolysis were higher in biopores than in bulk soil at 45–75 cm, but were similar between biopores and bulk soil at 75–105 cm. Importantly, catalytic efficiency increased with depth in bulk soil but decreased in biopores at lower depths.

In general, much higher activities of most enzymes were detected in biopores vs. bulk soil and in top biopores vs. sub biopores but in sub-bulk soil vs. top-bulk soil. However, enzyme activities followed opposite trends associated with total organic C and total N, and C:N ratios along soil depths. In the topsoil, the catalytic efficiency of all enzymes was higher in biopores than in bulk soil or rhizosphere, but in subsoil no clear pattern was discerned.

3.3. CO_2 efflux from soil and priming effect induced by labile C input

$^{14}CO_2$ efflux in the first 6 h of incubation reflected 2.7 to 4.14 times faster decomposition of glucose in biopores compared to bulk soil and rhizosphere at 0–30 cm (Fig. 4). There was a time lag in peak ^{14}C efflux at greater soil depths, i.e. $^{14}CO_2$ evolution rate in the subsoil peaked later than in the topsoil for both biopores and bulk soil.

Considering cumulative SOM-derived CO_2, the difference between samples with and without glucose amendment indicated an increase in SOM decomposition (positive priming effect), which started at the 16th hour in biopores regardless of soil depths (Fig. 5a). Compared to topsoil biopores, the priming effect in subsoil biopores was more intense (Fig. 5b). T-test results demonstrated a p-value >0.05 for absolute priming of SOM decomposition in rhizosphere and bulk soil, which indicated no extra SOM degradation due to glucose addition. Meanwhile, the priming effect was positive in biopores at all depths, but much lower in topsoil than subsoil (Figs. 5b and 6). The priming effect in biopores was generally higher than in bulk soil and rhizosphere, but was only significant at 75–105 cm. Similarly, only at 75–105 cm was a positive priming of SOM decomposition observed in bulk soil. Normalizing the volume of priming effect with the amount of added glucose demonstrated that glucose addition induced more than 100% priming of SOM decomposition in the bulk subsoil, but only 15% in the subsoil biopores (Supplementary 1).

4. Discussion

4.1. Total organic C, total N and P

Despite the small soil volume of biopores (0.2–2% of total soil volume) (Ehlers et al., 1983), they largely contribute to plant litter incorporation in soil profile. Total organic C and total N contents were higher in biopores than in bulk soil (Hoang et al., 2016b). Earthworms accumulate C in biopores through their burrowing activities, delivering a mixture of plant debris and mineral particles downwards through the soil profile. Along with C accumulation, N was also incorporated into biopores. Indeed, low C:N litter fractions are a favorable food for earthworms (Hendriksen, 1990; Amador et al., 2003; Svensson and Friberg, 2007). The decrease of total organic C and total N with depth in both biopores and bulk soil suggests less C and nutrient inputs in the subsoil than topsoil (Kautz et al., 2013).

The soil was ploughed to 30 cm 10 months before sampling, so topsoil biopores recently incorporated fresh organic matter compared to subsoil biopores. Total organic C and total N decreased with depth 1.4–1.7 times faster in bulk soil than in biopores, suggesting that earthworms reduced the C-input gap between the top- and subsoil. The similarity of total P content in biopores and bulk soil

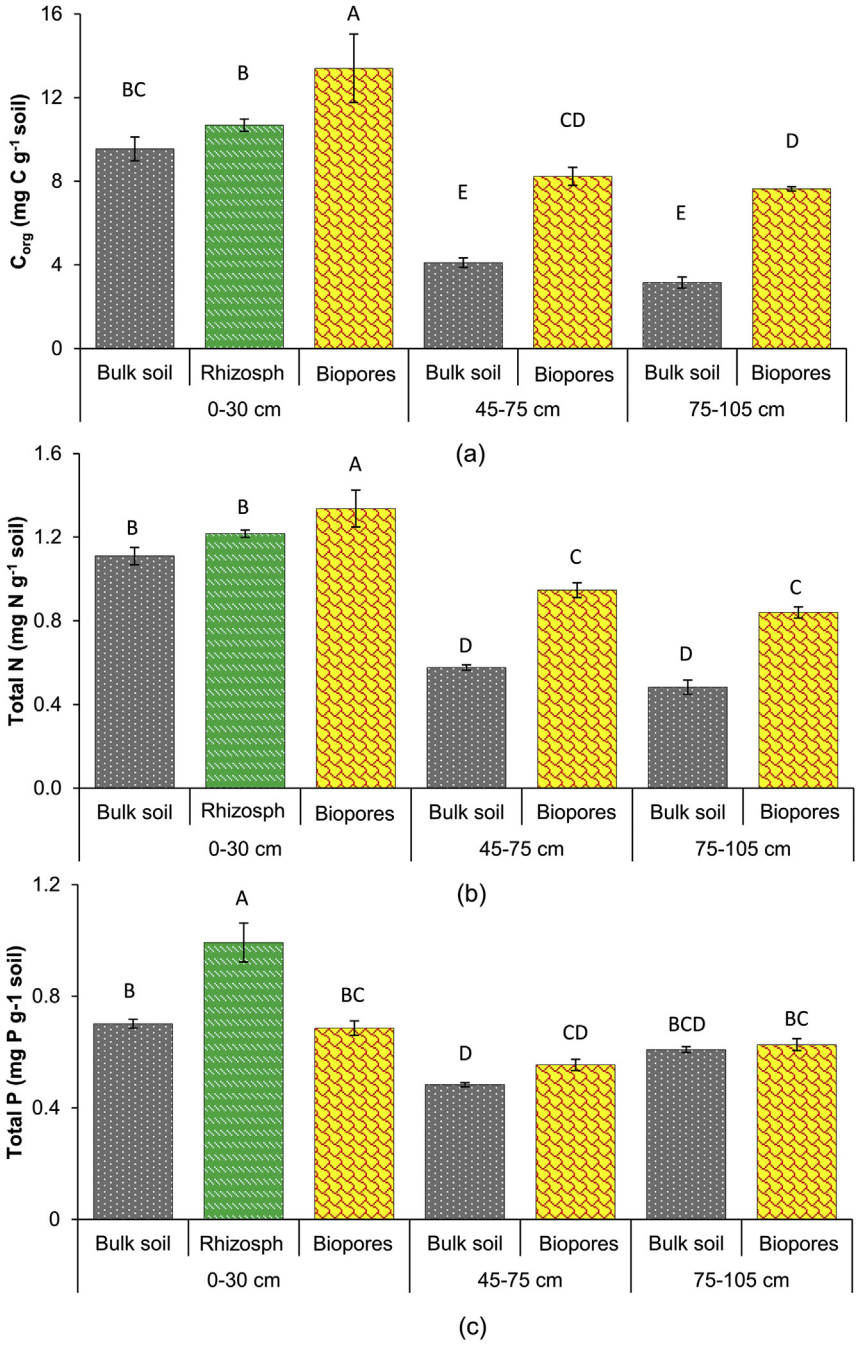

Fig. 1. Total organic C, total N and total P in earthworm burrows compared to rhizosphere and bulk soil. Earthworms increased C_{org} and total N contents in biopores ($P < 0.05$) by 1.2–2.4 times compared to bulk soil. The differences in C_{org} and total N between biopores and bulk soil in the subsoil was up to 1.7 times greater than in topsoil, suggesting a very important role of earthworms for organic matter accumulation in the subsoil. Rhizosphere (rhizosph) has a minor effect on C_{org} and total N in the topsoil. In contrast, total P was higher in rhizosphere ($P < 0.05$) than biopores and bulk soil. This parameter was similar between biopores and bulk soil for the whole soil profile. Bars labeled with uppercase letters indicate significant differences ($P < 0.05$).

demonstrated the persistence of this parameter under worm effect.

4.2. Correlation of enzyme activities with total organic C, total N and total P

The variation of enzyme activities in biopores was explained well by total organic C and total N content (except for acid phosphatase) ($P < 0.01$). On the contrary, either the negative correlation or nonlinear correlation between enzyme activities and these parameters in bulk soil along soil depths was unexpected. These opposite trends are consistent with enzyme synthesis by microorganisms to acquire energy (C) and nutrients (N or P) from suitable soil organic matter pools. The production of enzymes depends on the nutrient content of organic matter (Allison et al., 2007), microbial community composition (Sinsabaugh et al., 2005; Allison et al., 2007), and nutrient limitation (Allison and Vitousek, 2005). Earthworms seem to homogenize SOM quality through soil profile indicated by stable variation of C:N ratio with depth (Appendix 1) but the rate of microbial decomposition is SOM quality independent as the enzyme activities exponentially decreased with depth.

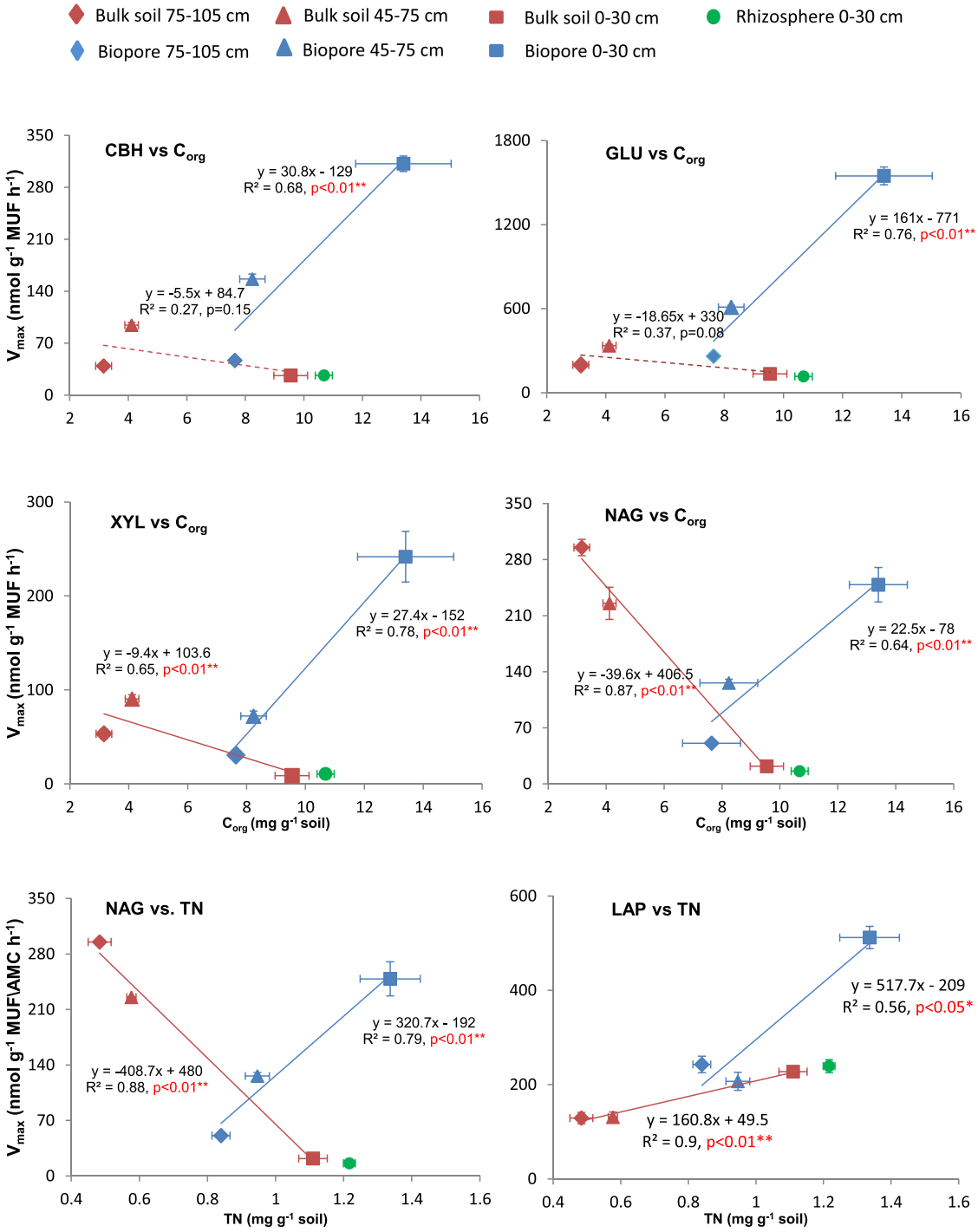

Fig. 2. Positive correlations between enzyme activities and C_{org} and TN in biopores vs. negative correlations in bulk soil along soil profile. Activities of most enzymes are highly correlated to C_{org} and total N ($P < 0.05$) (except CBH, GLU in bulk soil). The regression lines are based on all replicates, but only means are presented. Non-significant relation is indicated with dashed line.

This result, in turn, supports our assumption that microbial community composition is different between topsoil biopores and subsoil biopores. Enzyme production could be influenced by the presence of different microbial groups which produce exoenzymes with different nutrient affinity or require different nutrient concentration (Allison et al., 2007). By contrast, in bulk soil the increases of enzyme activities were associated with the decrease of total C and N in lower depths (Fig. 1) proportional to the increasing mineral composition. This result is reasonable as Allison et al. (2007) found that the activity of enzymes regulating C and N mineralization declined in organic but not mineral soil. Obviously, the decrease of C:N ratio in bulk soil is due to the decrease of plant-derived C in the agricultural subsoil, leading to the lack of C-input for microbial demands. In addition, the occlusion of SOM within soil aggregates (Rumpel and Kögel-Knabner, 2011), the mineral stabilization enhancement and the reduction of enzyme turnover rate in deep soil (Stone et al., 2014) may explain the increasing enzyme activity in the bulk soil.

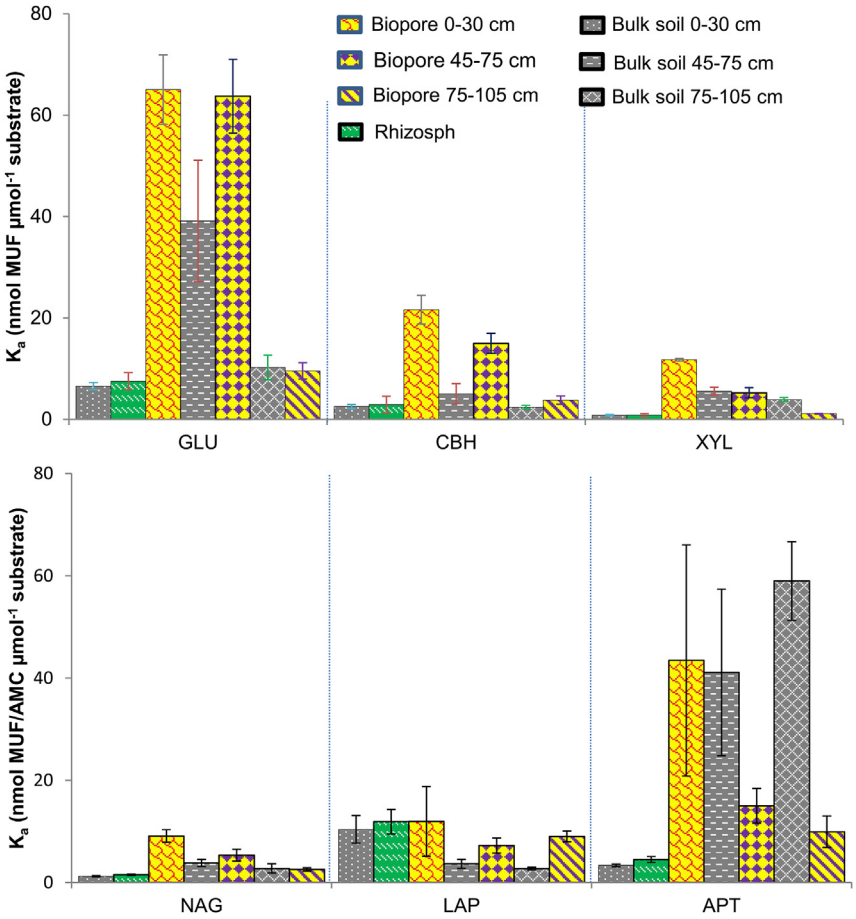

Fig. 3. Catalytic efficiency (K_a) is enzyme-specific and generally decreased with soil depth (except for APT). In the topsoil, enzyme efficiency (K_a) was up to 8 times higher in biopores compared to rhizosphere and bulk soil for most enzymes (except LAP). In the subsoil, enzyme activities involved in mining cellulose, chitin and leucine were significantly lower ($P < 0.05$) in biopores in comparison to bulk soil at 45–75 cm. Acid phosphatase was higher in biopores than in bulk soil at 0–30 cm but lower in two below depths.

We found a sharp increase of acid phosphatase activity in bulk soil with depth but the ratios of C-acquiring enzymes/P-acquiring enzymes and N-acquiring enzymes/P-acquiring enzymes decreased minimum 4 times through soil profile. This result suggested a strategy of microbial community to invest in the synthesis of P-acquiring enzymes. Combining with the slight variation of total P (Fig. 1) in bulk soil, the increase of acid phosphatase in bulk subsoil implied a microbial-P limitation. Nevertheless, acid phosphatase in biopores which was one order of magnitude higher than bulk soil in the topsoil (Appendix 2) may result from the enrichment of SOM input into biopores (Fig. 1). This result was consistent with findings by Le Bayon and Binet (2006). However, two to nine times lower acid phosphatase in biopores than bulk soil in the subsoil in this study was also detected by Hoang et al. (2016b). These contrasting trends reveal differential mechanisms involved in mediating acid phosphatase activity in topsoil and subsoil. In our study, biopores in topsoil were newly formed after plowing so the enhancement of acid phosphatase may result from microbial stimulation by fresh wormcast and directly by the worm's own enzymes (Satchell and Martin, 1984). Phosphatase production and activity are linked to P microbial demand and inorganic P availability (Olander and Vitousek, 2000). Hence, in the subsoil, higher acid phosphatase activity in bulk soil than biopores indicated a severe limitation of P availability, which suggested elevated enzyme production by microorganisms. Moreover, SOM in subbulk soil has more aged residence time (Rumpel and Kögel-Knabner, 2011) which is supposed to be relevant to the fact that activity of enzyme involved in P mineralization increases with site age (Allison et al., 2007). Acid phosphatase activities were expected to be higher in the rhizosphere than the bulk soil, as this enzyme is also produced by plant roots (McLachlan, 1980; Bais et al., 2004). However, similar acid phosphatase activities coupled with higher total P content in the rhizosphere than in bulk topsoil suggested more stable organic P compounds in the bulk soil.

4.3. Catalytic efficiency (K_a)

The kinetic parameters (V_{max} and K_m) express the "quantity" (V_{max}) and the substrate affinity of an enzyme, which are useful for assessing the changes in microbial activities (Masciandaro et al., 2000; Razavi et al., 2016b). The ratio of these two parameters (V_{max}/K_m) is termed the catalytic efficiency (K_a). K_a reveals changes in microbial community composition via alteration in soil enzymes (Kujur and Patel, 2013). The higher K_a in biopores compared to both bulk soil and rhizosphere for most enzymes in the topsoil (except for leucine aminopeptidase), implies enhanced organic C availability under earthworm effect, which induces microbial respiration (Kujur and Patel, 2013). Furthermore, catalytic efficiency is linked to enzyme-substrate formation. Accordingly, higher K_a in biopores than other soils demonstrated the accelerated dissociation of enzyme-substrate complexes (Tabatabai, 1973; Kizilkaya and Ekberli, 2008) in biopores than in bulk soil and the rhizosphere, resulting in a high flux of substrate to product (Albery and Knowles, 1976) and implying that the most active microbial community

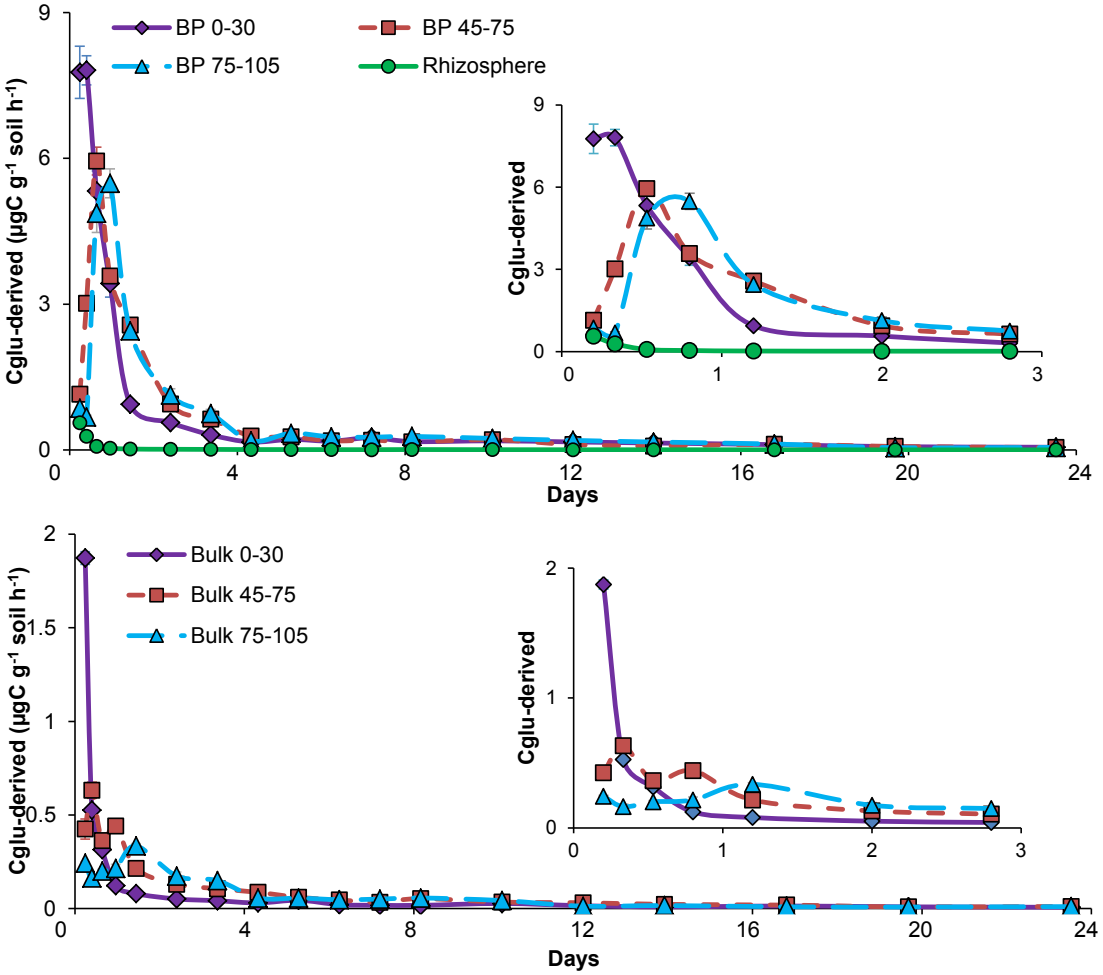

Fig. 4. $^{14}CO_2$ efflux rate after glucose addition. $^{14}CO_2$ evolution rate was 2.7–4.14 times higher in biopores than in the bulk soil after the first 6 h of incubation, but in rhizosphere soil it was lower than in bulk soil at 0–30 cm. There was a time lag in peak $^{14}CO_2$ at greater soil depth, being later at the lower depths than for the uppermost layer.

resides in biopores. Leucine aminopeptidase catalytic efficiency in biopores was similar to bulk soil and rhizosphere. This was also the case for xylanase and β-glucosidase in biopores at 45–75 cm and 75–105 cm. The catalytic efficiency of all enzymes in the rhizosphere of topsoil was similar to bulk soil. The very well developed root system of barley occupies the entire Ap horizon, and consequently transforms the bulk soil into a rooted soil more comparable to rhizosphere conditions. Acid phosphatase catalytic efficiency in the topsoil was higher in biopores than in the bulk soil and rhizosphere, but was up to 6-fold lower in the subsoil. As describing previously, earthworms did not affect total P, but they may convert immobilized organic P to microorganism-available forms in their biopores by gut passage. The enhanced availability of P in biopores reduced acid phosphatase syntheses by microorganisms (Chaoui et al., 2003; Le Bayon and Binet, 2006; Spohn and Kuzyakov, 2013). An efficient enzyme will mediate a high flux of substrate to product (Albery and Knowles, 1976), thus, the difference in K_a between topsoil and subsoil supports our argument regarding the occurrence of different microbial communities in the topsoil vs. subsoil.

4.4. $^{14}CO_2$ efflux rate and priming effect in biopores

The $^{14}CO_2$ efflux rate 6 h after glucose amendment was higher in biopores than in bulk soil at all depths (Fig. 4) and was 14 times lower in rhizosphere than in biopores. This means that glucose increased CO_2 efflux from biopores more strongly than from rhizosphere or bulk soil. Microbial activities in the rhizosphere are stimulated by glucose-containing root exudates (Kuzyakov, 2002; Shi et al., 2011; Derrien et al., 2004). Other than sugars, additional compounds such as amino acids and organic acids may be the main stimulators of microbial activity and the rhizosphere priming effect (Scott-Denton et al., 2005; Kuzyakov et al., 2007; Bird et al., 2011). Moreover, glucose is the simplest of the components of root exudates (Kuzyakov, 2002) and may not induce PE (Hamer and Marschner, 2002; Blagodatskaya et al., 2009) in topsoils, as the topsoils are already receiving abundant levels of labile C (Salomé et al., 2010), resulting in partial nutrient saturation for the microorganisms residing there. Hence, additional glucose caused only minor effects on already boosted microbial activities in the rhizosphere. High microbial populations in biopores (Don et al., 2008; Hoang et al., 2016b) or the limitations in nutrient and energy-rich C compounds in bulk soil (indicated by less total organic C and total N) accelerated glucose decomposition as an available energy source for microorganisms.

The time lag between the maximum $^{14}CO_2$ efflux rate in subsoil biopores vs. topsoil biopores illustrates a shift in the microbial community in response to glucose amendment and native SOM decomposition (Fig. 4). The higher proportion of fast-growing microorganisms (r-strategist) residing in the topsoil (Fontaine et al.,

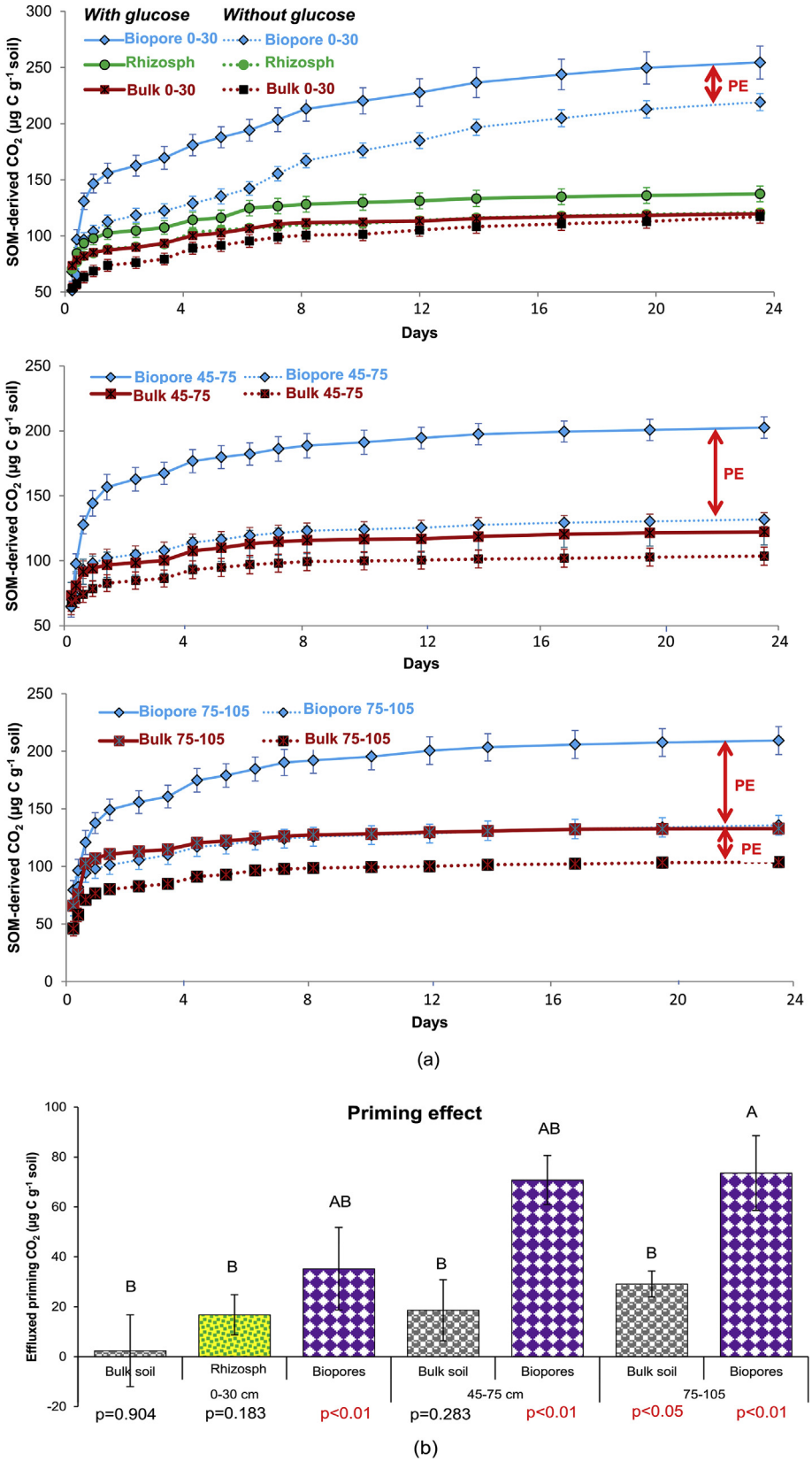

Fig. 5. Absolute priming of SOM decomposition in biopores (a) Priming effect dynamics: The difference in cumulative CO_2 from SOM with glucose amendment and without glucose was significantly larger in biopores than rhizosphere and bulk soil; (b) Cumulative priming effects at day 24 of the experiment: The stronger effect of earthworms on priming effect in subsoil than topsoil was attributable to the limitation of labile C in the subsoil and newly formed substances in the topsoil. Rhizosphere and bulk soil in the two upper horizons were not subject to priming (*t*-test, $p > 0.05$).

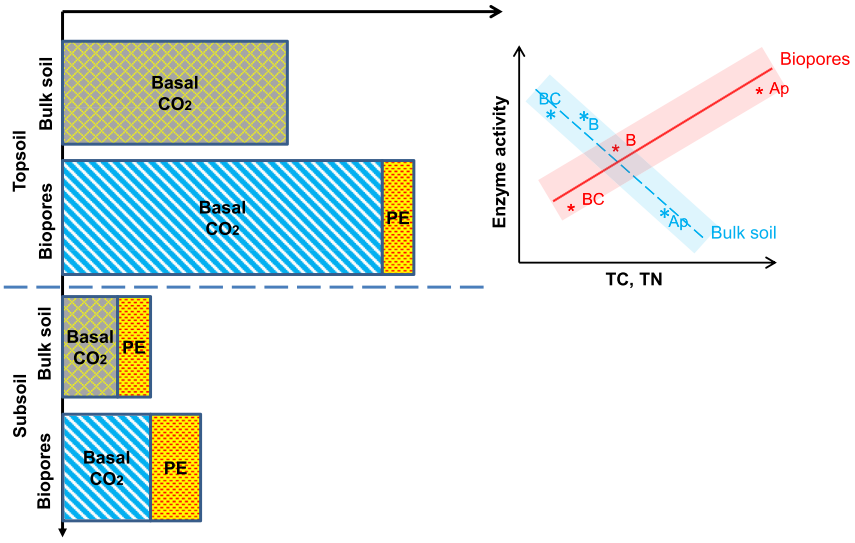

Fig. 6. Priming of soil organic matter decomposition was seen in biopores over the whole soil profile, but only observed in bulk soil in the subsoil. The far higher microbial biomass in biopores than in bulk soil boosts soil organic matter decomposition after glucose addition, while N starvation of microorganisms in subsoil is the main driver for higher priming effects over topsoil. Across different depths, negative correlations between enzyme activity and total C (TC) and total N (TN) in bulk soil contrast with positive correlations in biopores. Shading indicates the range of enzyme activities as a function of TC and TN. A, B and BC indicated soil horizons from topsoil to subsoil, respectively.

2007) quickly assimilated C-glucose to meet their energetic demands. The dominance of sugar-feeding populations has been proposed to occur during the first stage of litter decomposition (Alexander, 1964). Therefore, elevated-CO_2 efflux throughout the first day of incubation originated from microbial glucose degradation rather than SOM decomposition (Fontanine et al., 2003). Similarly, microorganisms in biopores quickly decomposed glucose to CO_2, as demonstrated by the pronounced peaks, particularly in the subsoil. Hence, microorganisms in biopore walls showed a quick response to resource amendments (Tiunov and Scheu, 1999).

Top-bulk soil was enriched in labile C sources derived from plant litter whereas the sub-bulk soil was devoid of such sources. Consequently, the topsoil microbes were far less C limited than their subsoil counterparts, and thus, they respond less intensively to labile C availability than subsoil microbes. Such activated microorganisms may induce the decomposition of native SOM, causing a positive priming effect in the sub-bulk soil. Despite a high $^{14}CO_2$ efflux rate due to the quick C assimilation in the topsoil, $^{14}CO_2$ evolution was exhausted sharply by time (Fig. 4). However, $^{14}CO_2$ evolution decreased more gradually in the subsoil of biopores and bulk soil during the incubation. These $^{14}CO_2$ evolutions were originated from glucose and emerged shortly (within 1 day) after this substrate addition. Therefore, according to Kuzyakov et al. (2000) CO_2 priming during this period (Fig. 5a) was governed by apparent priming effect. In contrast, $^{14}CO_2$ effluxes from day 4 to the end of incubation in biopores, bulk soil and rhizosphere were very low, even approximately 0 at some point of time (Fig. 4). This means that the longer the incubation is the lower variable the microbial C-glucose consumption. Moreover, priming effects in biopores at 3 soil depths and bulk soil at 75–105 cm (Fig. 5a) were associated with SOM decomposition (real priming effect) (Blagodatskaya and Kuzyakov, 2008).

The triggered absolute turnover of pre-existing SOM was higher in biopores than in root-affected soil or bulk soil (Fig. 5). As previously suggested (Blagodatskaya and Kuzyakov, 2008; Blagodatskaya et al., 2009; Pausch et al., 2013b), exudate input in the rhizosphere creates important but highly dynamic hotspots, resulting in priming effects. The lifetime of such hotspots, however, is short (Pausch and Kuzyakov, 2011) due to the rapid decomposition of exudates. Consequently, microbial activities quickly return to baseline, as seen in the similarity of enzyme activities between rhizosphere and bulk soil at 0–30 cm (Fig. 2). In contrast, biopores are stable and long-lived microhabitats for microorganisms, even exceeding the lifetime of *Lumbricus terrestris* itself (Tiunov and Scheu, 1999; Stromberger et al., 2012). The persistence of biopore walls creates stable microhabitats that favor the maintenance of vast and active microbial communities (Tiunov and Scheu, 1999), and positively contributed to the priming effect observed in our soil profile. Moreover, such pore walls are casted with excreted mucus (Brown et al., 2000), which is made up of low molecular weight carbohydrates, amino acids, glycosides and glycoproteins (Scheu, 1991; Lavelle et al., 1995; Pan et al., 2010). Such mucus compounds are released from earthworm's body surfaces acting as a "paradox" (Brown et al., 2000) which stimulates dormant microbial communities for SOM mineralization over a period of years (Brown et al., 2000). Similarly, Bityutskii et al. (2012) proposed earthworm mucus as an important primer in the soil profile, but suggested that this effect can be delayed up to 30–90 days. Additionally, higher priming effect in biopores than bulk soil was partly explained by a higher catalytic efficiency in the former than the latter. As mentioned in previous section, higher catalytic efficiency is associated with a faster dispersion of enzyme-substrate complex than its formation, i.e. more SOM is decomposed. Combining with higher microbial biomass in biopores, glucose amendment induced priming of pre-existed SOM. However, priming effect is more attributed to glucose addition in bulk soil than biopores as normalizing this value with added glucose volume. In brief, the elevated priming effect in biopores compared to rhizosphere and bulk soil results from the stability of biopore walls and favorable properties of excreta by earthworms.

5. Conclusions

The positive effects of earthworms on nutrient mobilization (N and P) have been broadly known for decades, but there has been no consensus concerning the role of earthworms in soil organic matter decomposition (priming effects) due to easily-degradable C inputs. Total organic C and total N decreased more with depth in bulk soil than in biopores, suggesting that earthworms reduced the C input gap between top- and subsoil. The negative correlation between

enzyme activities and C:N ratios in bulk soil was unexpected, but can be explained by the abundance of ancient, slow-cycling C in the subsoil, compared to young-fast cycling C in the topsoil. Together with nutrient limitation in the bulk subsoil, this suggests that enzyme activities were increased in the subsoil to help microorganisms access limited nutrients. Higher priming effect in biopores than bulk soil and rhizosphere was determined by the stability of biopore walls, C and N enrichment and higher catalytic efficiency of hydrolytic enzymes. Overall, earthworms boost SOM turnover by stimulating microbial activities, especially in the subsoil.

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Appendix ASupplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2017.06.021.

Appendix 1

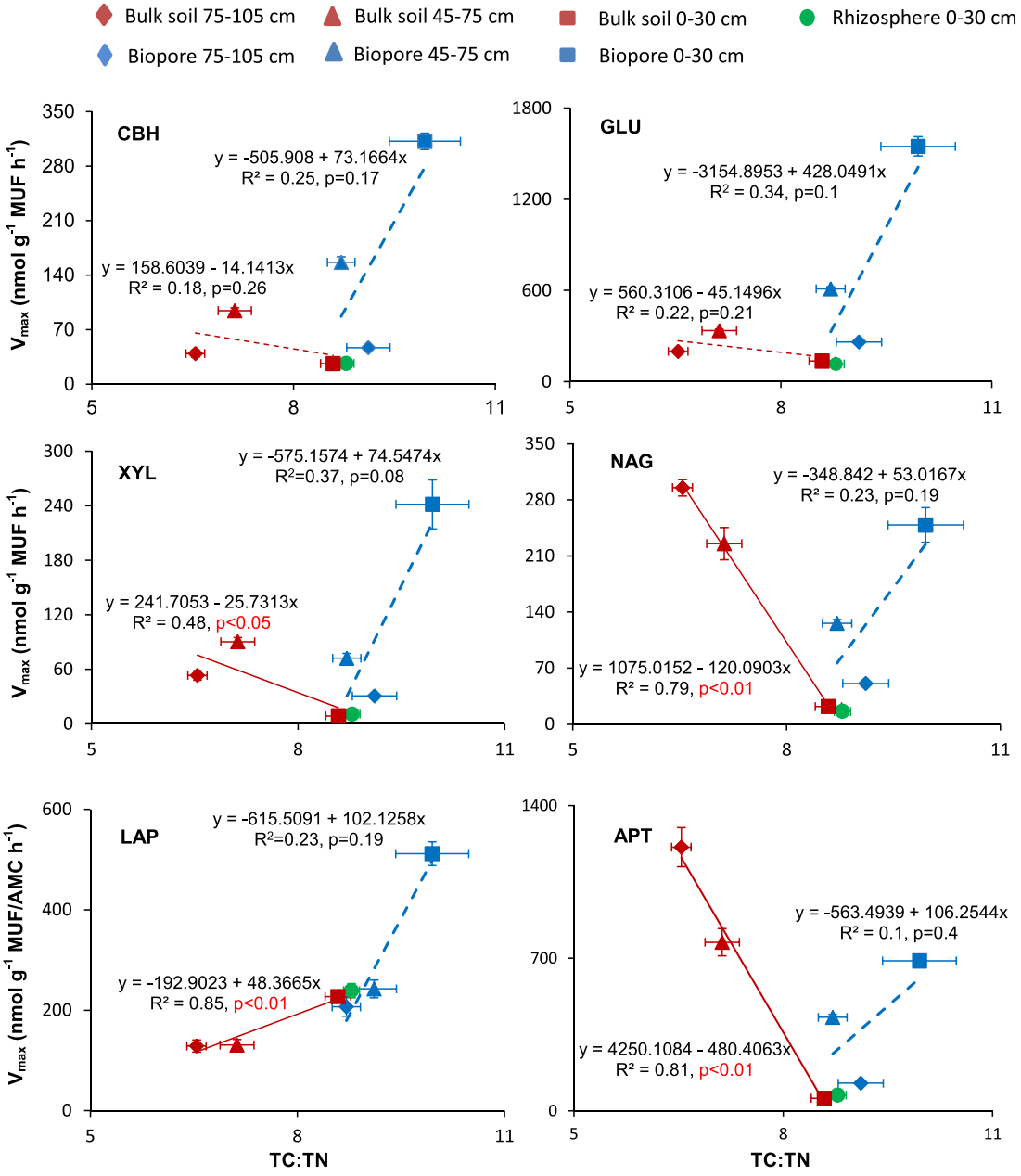

Fig. S1. Correlation between C:N ratio and enzyme activities with soil depth. The regression lines are based on all replicates, but only means are presented. Non-significant relation is indicated with dashed line.

Appendix 2

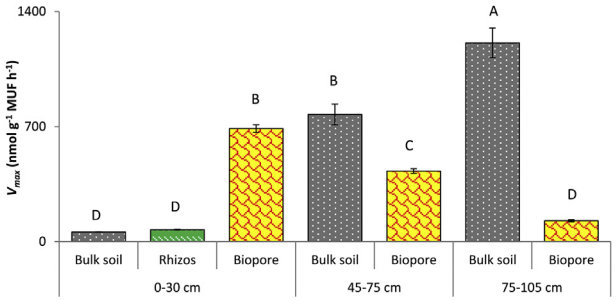

Fig. S2. Phosphatase activity in biopores is higher than in bulk soil and rhizosphere in the topsoil, but lower in the subsoil.

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