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Earthworm burrows: Kinetics and spatial distribution of enzymes of C-, N- and P- cycles

Duyen T.T. Hoang [a, d, *, 1], Bahar S. Razavi [b, 1], Yakov Kuzyakov [a, b], Evgenia Blagodatskaya [a, c]

[a] Department of Soil Science of Temperate Ecosystems, University of Göttingen, Göttingen, Germany
[b] Department of Agricultural Soil Science, University of Göttingen, Göttingen, Germany
[c] Institute of Physicochemical and Biological Problems in Soil Science, Pushchino, Russia
[d] Department of Soil Science, Forestry University, Hanoi, Viet Nam

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ABSTRACT

Earthworms boost microbial activities and consequently create hotspots in soil. Although the presence of earthworms is thought to change the soil enzyme system, the distribution of enzyme activities inside worm burrows is still unknown. For the first time, we analyzed enzyme kinetics and visualized enzyme distribution inside and outside worm burrows (biopores) by *in situ* soil zymography. Kinetic parameters (V_{max} and K_m) of 6 enzymes – β-glucosidase (GLU), cellobiohydrolase (CBH), xylanase (XYL), chitinase (NAG), leucine aminopeptidase (LAP) and acid phosphatase (APT) – were determined in pores formed by *Lumbricus terrestris* L. In earthworm burrows, the spatial distributions of GLU, NAG and APT become observable in zymogram images. Zymography showed a heterogeneous distribution of hotspots in the rhizosphere and worm burrows. The hotspot areas were 2.4–14 times larger in the burrows versus reference soil (soil without earthworms). The significantly higher V_{max} values for GLU, CBH, XYL, NAG and APT in burrows confirmed that earthworms stimulated enzyme activities. For CBH, XYL and NAG, the 2- to 3-fold higher K_m values in burrows indicated different enzyme systems with lower substrate affinity compared to reference soil. The positive effects of earthworms on V_{max} were cancelled by the K_m increase for CBH, XYL and NAG at a substrate concentration below 20 μmol g^{-1} soil. The change of enzyme systems reflected a shift in dominant microbial populations toward species with lower affinity to holocelluloses and to N-acetylglucosamine, and with higher affinity to proteins as compared to the reference soil. We conclude that earthworm burrows are microbial hotspots with much higher and denser distribution of enzyme activities than reference soil.

1. Introduction

Soil microbial functioning is frequently assessed in terms of enzyme activities because all biochemical transformations in soil are facilitated by enzymes (Burns, 1981). Most enzymes are assumed to originate from microorganisms, but plant roots and soil animals can contribute to enzyme abundance either directly, by enzyme production, or indirectly, by releasing organic substrates that stimulate microorganisms producing enzymes (Gianfreda and Rao, 2014).

Earthworms, which are the most important soil-dwelling animals, play the role of "engineers" by mixing soil materials, aggregating soil particles and digesting plant litter (Jones et al., 1994; Lavelle et al., 1997). The pore system formed by earthworms is termed the drilosphere and is among the most important microbial hotspots in soil (Kuzyakov and Blagodatskaya, 2015). The high microbial activity in the drilosphere is explained by the input of labile organic materials within the well-aerated and stable structure of worm burrows. High microbial activities, in turn, accelerate the transformation and redistribution of carbon (C) and nutrients such as nitrogen (N) and phosphorus (P).

Earthworms may accelerate the decomposition (C loss) and, conversely, promote C storage or protection from decomposition (C accumulation) in stable aggregates (Brown et al., 2000). Therefore, the net effect of earthworms on the C-cycle remains controversial. Earthworm activity has been shown to both enrich soluble organic C in the drilosphere (Parkin and Berry, 1999) and lead to the loss of dissolved and particulate forms of soil C (Bohlen et al., 2004). Sensitive nerves in the pharyngeal region enable earthworms to selectively feed on specific compounds such as proteins and soluble

* Corresponding author. Department of Soil Science of Temperate Ecosystems, University of Göttingen, Büsgenweg 2, 37077 Göttingen, Germany.
E-mail addresses: dhoangt1@gwdg.de, duyenhoang42@gmail.com (D.T.T. Hoang).
[1] The first two authors contributed equally to this study.

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carbohydrates (Judas, 1992; Benckiser, 1997). Thus, earthworm casts are usually enriched with polysaccharides (Marinissen et al., 1996), providing available substrate for cellulolytic enzymes (β-glucosidase, cellobiohydrolase) to produce glucose and for the hydrolysis of hemicelluloses (xylanase) to xylose (Bayer et al., 2006).

Both C- and N- cycling can be simultaneously accelerated by hydrolytic enzymes such as N-acetyl glucosaminidase and proteases (Binet and Trehen, 1992; Bohlen and Edwards, 1995; Amador and Görres, 2005). NAG releases N-containing amino sugars from chitin, which is one of the dominant forms of organic N in soils (Olander and Vitousek, 2000). Chitin is a naturally abundant mucopolysaccharide accounting for 5–8% of total N content in soil (Kumar, 2000), derived mainly from fungal cell walls and arthropod exoskeletons. The surface of earthworm burrows can be strongly enriched with chitin due to the colonization of biopores by fungi and arthropods (Don et al., 2008). Fungal mycelium passing through earthworm guts may further increase the concentration of chitin along biopores. This, in turn, can increase NAG activity, which catalyzes chitin degradation by cleaving a bond between the C1 and C4 atoms of two consecutive N-acetyl glucosamine residues of chitin (Flach et al., 1992). Beside environmental N sources, earthworms themselves contribute to the organic N in soil by secreting mucus (Brown and Doube, 2004). Mucus consists of proteins and polypeptides which are decomposed by proteases and peptidase, e.g., leucine-amino-peptidase (LAP) (Matsui et al., 2006).

Accelerated turnover of microbial C and N in biopores can induce the competition for P, which is a main limiting nutrient for microbial growth. In soil, phytate is the most abundant and recalcitrant form of organic P (Richardson et al., 2001). It is hydrolyzed by phosphatase enzymes to form available P for microbial and plant growth. Acid phosphatase in soil is produced by both plants and microorganisms (fungi, bacteria) (Turner et al., 2002; Lee et al., 2008). Phosphatase activity is increased when P-solubilizing bacteria colonize biopores (Wan and Wong, 2004). Furthermore, since the digestive tract of earthworms secretes phosphatase, phosphatase activity is predicted to increase after soil has passed through the gut (Vinotha et al., 2000).

The contribution of earthworms to the C-, N- and P-cycle could be detected through their interactions with soil microorganisms. Enzyme activities in burrow walls are a crucial indicator reflecting the mechanism behind the role of earthworms in plant litter decomposition. At the same time, extracellular enzymes – as macromolecules – are susceptible to adsorption by soil particles (Chenu and Stotzky, 2002), which challenges the quality of enzyme analysis (Nannipieri et al., 2012). Determination of enzyme activities by fluorogenically labeled substrates is frequently applied in soil studies. Only very few studies, however, have compared enzyme kinetics in burrow walls with that in reference soil. It is still unclear whether earthworms affect only enzyme activities (i.e., the rate of catalytic reactions) or whether they also alter intrinsic enzyme properties (e.g., enzyme affinity to substrate). Moreover, there are no studies on the spatial distribution of enzyme activity inside burrow linings. Zymography visualization techniques have successfully combined biochemical assays with two-dimensional *in situ* measurements. The zymography technique (Gross and Lapiere, 1962) has seen application in scientific fields as diverse as medicine, biochemistry and agriculture. This approach non-destructively visualizes the conversion of the substrate into an altered reaction product (Vandooren et al., 2013). It yields spatially resolved quantitative and qualitative information about hydrolase activities in a sample (Vandooren et al., 2013). Zymography has previously been adapted to visualize the spatial and temporal dynamics of enzyme activities in soil with living and dead roots (Spohn et al., 2013, 2014). Such a visualization inside earthworm habitats remains a challenge. Our study was therefore designed to i) determine the effects of earthworms on C-, N- and P- cycles by measuring enzyme kinetic parameters in worm burrows and reference soil; ii) visualize enzyme distribution inside and outside earthworm burrows. Earthworms were reported to affect soil enzyme activities by (1) enriching organic matter in their burrows, (2) enhancing microbial biomass, and (3) processing organo-mineral soil by gut enzymes (Judas, 1992; Kristufek et al., 1992; Jégou et al., 2000; Tiunov and Scheu, 1999, 2002; Don et al., 2008). Thus, we hypothesized that i) enzyme activities are higher inside worm burrows than in reference soil, but that the change in enzyme kinetics according to substrate concentration is enzyme specific, ii) the drilosphere microhabitat is enriched with available substrates, resulting in a higher percentage of hotspots than in reference soil without earthworms. Considering that earthworm engineering activity is strongly dependent on their interactions with growing roots (Ross and Cairns, 1982), we placed earthworms into unsieved soil containing living roots.

To this end, we incubated soil-filled rhizoboxes with *Lumbricus terrestris* L. and maize plants for two weeks to obtain burrow systems. The enzyme kinetics of six hydrolytic enzymes (β-glucosidase, cellobiohydrolase, xylanase, chitinase, leucineaminopeptidase, phosphatase) was analyzed inside the burrows and in reference soil (with plants but without earthworms). Non-destructive zymography was applied along the lining of earthworm burrows, on the front panel of earthworm boxes, and in reference boxes to visualize the distribution of hotspots.

2. Materials and methods

2.1. Experimental setup

Lumbricus terrestris L. was collected manually with *in-situ* soil in the botanical garden of Göttingen University and placed in a black pot at room temperature for one week to adapt the earthworms to the new environmental conditions. Water was added at a rate of 0.3 g water g^{-1} soil dry weight. After this pre-incubation, earthworms were removed from *in-situ* soil to sandy loam Haplic Luvisol, which was collected from the Ap-horizon (0–30 cm depth) of an arable field in Göttingen. Earthworms thrive under moist, but well-aerated conditions (Lavelle et al., 2004). Thus, in order to create an optimal environment, the soil was hand-sorted rather than sieved to remove roots and detritus. The soil properties were as follows: bulk density 1.1 g cm^{-3}, total carbon (TC) 28 g C kg^{-1} soil, total nitrogen (TN) 2 g N kg^{-1} soil, sand 49.5%, silt 42% and clay 8.6%. A transparent plastic box (15 × 20 × 15 cm) was used for the experiment; a removable front panel enabled opening without affecting the earthworm habitat or root distribution. Before filling the boxes with soil, a layer of gravel (1–2 cm diameter) was laid on the bottom for drainage, to prevent water saturation. Three mature earthworms (5–10 cm long) were placed in each box.

Maize seeds (*Zea mays* L.), 72 h after germination, were simultaneously planted in the soil, 0.5 cm away from the front panel. Growing maize roots regulate air and moisture in the soil, enhancing the conditions for earthworms. The experiment comprised two treatments: boxes with maize and *Lumbricus terrestris* L.; and reference boxes with maize but without earthworms. The boxes were kept in a climate chamber at a controlled temperature of 18 ± 1 °C and a daily light cycle of 16 h, with light intensity set at 300 μmol m^{-2} s^{-1}. Aluminum foil was used to cover the boxes to protect them from the light and prevent algal growth. During the growth period, the soil water content was maintained at 60% of field capacity (Spurgeon and Hopkin, 1999). After two weeks of incubation, many burrows had been formed and the maize roots reached the bottom of the box.

2.2. Enzyme assays

In order to assess the effect of earthworms on soil enzyme activities, we analyzed the kinetics of 6 enzymes, employing fluorimetric microplates based on 4-methylum-belliferone (MUF) and 7-amino-4-methylcoumarin (AMC) according to Razavi et al. (2015). The six selected enzymes reflect the C-cycle group, including cellulolytic enzymes: 1) β-glucosidase (GLU) measured with MUF-β-D-glucopyranoside (MUF-G), 2) cellobiohydrolase (CBH) measured with MUF-β-D-cellobioside (MUF-C), and xylanase (XYL) measured with MUF-β-D-xylopyranoside; the N-cycle group: 4) chitinase (NAG) measured with MUF-N-acetyl-β-D-glucosaminide (MUF-N); 5) leucine-amino-peptidase (LAP) measured with L-Leucine-7-amido-4-methylcoumarin hydrochloride (AMC-L), and the P-cycle group: 6) acid phosphatase (APT) quantified with MUF-phosphate (MUF-P). We determined enzyme activities at a range of substrate concentrations (0, 10, 20, 30, 40, 50, 100, 200 μmol g^{-1} soil). Saturation concentrations of fluorogenic substrates were determined in preliminary experiments.

In each earthworm box, we carefully sampled soil material along burrow walls (≥5 burrows); these materials were homogeneously mixed and a part of them (each 0.5 g soil) was spent for a preliminary test to define an appropriate substrate concentration. The rest of soil samples were used for the real experiments. The same procedure was applied to the reference boxes, i.e., we sampled soil materials randomly in each reference box and homogeneously mixed. Totally we had 3 replicate boxes of earthworm and other 3 replicate boxes of reference soil. This yielded a total of 3 replicates for worm burrow and 3 replicates for reference soil for enzyme assays. Biopore samples were taken from earthworm burrows that were not used for zymography in order to compare with reference samples collected from reference boxes at positions far from plant roots. Suspensions of 0.5 g soil (dry weight equivalent) with 50 mL sterilized water (Stemmer et al., 1998; Koch et al., 2007) were prepared. 50 μL of soil suspension was added to 50 μL buffer (pH: 6.5) and 100 μL of each substrate solution in a 96-well microplate (Puregrade, Germany). The activity of each enzyme was measured at three time points: 30, 60 and 120 min. The fluorescence was measured using a Victor 1420-050 multi-label counter (PerkinElmer, USA) at an excitation wavelength of 355 nm and an emission wavelength of 460 nm. Enzyme activities were calculated as released MUF or AMC in nmol per g dry soil per hour (nmol g^{-1} h^{-1}) (Awad et al., 2012).

2.3. Enzyme kinetics and statistical analysis

The Michaelis-Menten Equation (1) was applied to calculate the K_m and V_{max} for each enzyme:

$$V = \frac{V_{\max}[S]}{K_m + [S]} \quad (1)$$

where V is the reaction velocity (nmol g^{-1} h^{-1}), V_{max} is the maximum reaction velocity at saturated substrate concentration, and K_m is an affinity constant for each enzyme, equal to the substrate concentration at which the reaction rate is half of the maximum rate (½V_{max}). V_{max} and K_m values were determined using non-linear curve fitting (OriginPro 8.5 software).

Potential differences of means between the two treatments (reference and biopores) were tested with one-way ANOVA using STATISTICA 64, where $p < 0.05$ of Turkey's HSD test indicated significance. Homogeneity of variance and normality of the values were tested by Levene's test and Shapiro-Wilk's W test. Error bars indicate the standard error of the means.

2.4. Zymography of earthworm burrows

Zymography was performed by incubating a substrate-infused membrane on the soil surface of the front panel and on the inner burrow walls. For this purpose, the front panel of the box was opened, exposing the pore systems and roots. Three substrates (Sigma-Aldrich, Germany) were used, corresponding to the enzymes GLU, NAG and APT. These enzymes were selected because they are produced not only by microorganisms in the burrow surface, but are also found in the earthworm gut (Tracy, 1951; Brown et al., 2000; Wan and Wong, 2004). Thus, we expected to see strong differences in enzyme activities in soil with and without earthworms.

The substrates were dissolved separately in universal buffer to a concentration of 12 mM. The amounts of substrate were chosen based on preliminary tests. Polyamide membrane filters (diameter 20 cm, pore size 0.45 μm – Tao Yuan, China) were cut into pieces of the required size and soaked in the prepared substrate solutions. Enzyme activities were detected by the appearance of fluorescent reaction products on the membrane (Dong et al., 2007); these were captured by digital camera (Spohn and Kuzyakov, 2014). The procedure of Spohn and Kuzyakov (2014) was modified to make the technique applicable for studies of worm burrows by placing membranes in direct contact with the soil surface, reducing the necessary incubation time. Furthermore, direct attachment does not require adjusting the incubation time for each enzyme, i.e. all enzymes were incubated for the same amount of time. Quenching effects of soil particles were also tested by a 60-min application of membranes saturated with a series of MUF and AMC concentrations to the soil surface. No significant quenching effect on fluorescence intensity was detected.

Substrate-soaked small pieces of membrane (matching the shape and size of the burrow area) were separately placed inside burrows, which were then covered by a layer of soft plastic stuffing. Finally, flint glass beads (1 mm) were placed on top to ensure the proper membrane attachment to the burrow wall. At the same time, a large membrane with dimensions matching the box side (15 × 20 × 15 cm) was attached to the whole exposed soil surface. After 1 h incubation, the membranes were carefully removed, placed in a dark room, and exposed to UV light of 360 nm wavelength, which excites the fluorescent molecules (Spohn et al., 2013).

To quantify the zymogram images, we calibrated against standards that related the enzyme activities to the gray-value projected onto the zymograms. The calibration function for each enzyme was obtained by zymography of 3 cm^2 membranes soaked in solutions of MUF or AMC at concentrations of 0.01, 0.2, 0.5, 1, 2, 4, 6 and 10 mM. The amount of MUF or AMC per area basis was calculated from the volume of solution taken up by the membrane and membrane size. The calibration membranes were imaged under UV light and analyzed in the same way as the samples.

2.5. Image analysis

Zymography images were taken with a digital camera (SX10IS, Canon). Image processing and analysis were done in the Matlab environment according to Razavi et al. (2016). The zymograms were transformed to 16-bit grayscale image as matrices. Then, all zymograms were first referenced based on the grayvalue of a reference object embedded in all the zymograms. We used the common image-processing approach of selecting the grayvalue obtained from the blank sides of the image as the referencing point. After referencing the zymograms, we calculated an average background grayvalue through the zymograms of calibration lines at a concentration of zero and subtracted this value from all the zymograms. Note that we applied the same membrane type to both

the zymograms of the biopores and to the calibration baseline. To illustrate the results, we depicted the values of the grayscale image in color (Spohn and Kuzyakov, 2014). The hotspot length and visible area were calculated using Euclidean distances (Moradi et al., 2011). The edges were detected in places where the second derivative of the intensity crossed the zero axes, which highlighted areas with rapid change in pixel intensity values (Moradi et al., 2011). Hotspots were segmented from surrounding area by their contrast color intensity in digital images. Based on referencing of images and of calibration line, color intensity all pixels with the color intensity exceeding average value (i.e., >0.7) were assigned to the hotspots (represented by red color) for enzyme activities (Appendix. 1). To confirm the boundaries, one-way analysis of variance (ANOVA) was applied to assess the significant differences between independent variables (mean values of four 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) (Appendix. 1). Thus, ANOVA, followed by Turkey HSD test at a probability level of $p < 0.05$, confirmed the categories of enzyme activity. Homogeneity of variance and normality of the values were tested by the Levene's test and Shapiro Wilk's W test. The pixel-wise grayvalue in the zymography was converted to enzyme activity using the calibration function obtained for each enzyme.

We used Matlab environment to obtain xy coordinates of each hotspot and then used spatial point pattern analysis (Diggle, 1983; Arnold et al., 1997) to determine the effect of earthworms on hotspot distribution. Spatial point pattern analysis and Quadrat methods were used to determine whether the distribution of hotspots in space was aggregated or dispersed (Arnold et al., 1997; Diggle, 1983). Quadrat methods equally partition an area of study into sub-regions, or quadrats, and count the number of points within each quadrat. For randomly dispersed points, the variance of the number of points per quadrat is approximately the same as the average number of points per quadrat. The ratio of variance/mean is defined as the dispersion index and is less than 1 for dispersed spatial distribution, greater than 1 for more clustered distribution patterns.

3. Results

3.1. Enzyme activities and enzyme kinetics

After two weeks, *Lumbricus terrestris* L. were found alive and the presence of hatchlings and cocoons indicated that they had acclimatized well to soil conditions. In both reference and burrow soils, the substrate-dependent enzyme activity corresponded well to Michaelis-Menten kinetics (Fig. 1). For most of tested enzymes, the differences in activity between worm burrows and reference soil were less pronounced at low amounts of substrate, but strongly increased under substrate saturation (Fig. 1). Thus, at substrate concentrations below the threshold value of 20 μmol g^{-1} soil of CBH, XYL and NAG, the overlapping of the curves (Fig. 1) showed the absence of significant differences in enzyme activities between burrow and reference soil. Above the threshold concentrations, these activities increased faster in worm burrows than in reference soil. In contrast, for LAP the differences in reaction rates between treatments were much stronger at low (<20 μmol g^{-1} soil) than at high substrate concentrations (Fig. 1).

Earthworms showed the positive influence on all tested enzymes in comparison with reference soil. The maximum rate (V_{max}) of enzyme-mediated reactions in worm burrows was higher than that of reference soil by the factor of 1.1–2.5 (Fig. 2). The differences were significant ($p < 0.05$) for five out of the six enzymes, excepting LAP.

Significant differences in K_m values between reference soil and worm burrows were detected for three enzymes: CBH, XYL and NAG. Their K_m values were around two-to threefold higher in worm burrows. The other tested enzymes showed there were no significant differences of K_m values between worm burrows and reference soil, with the exception of LAP (K_m in burrow was lower than in reference).

3.2. Zymography of earthworm burrows

3.2.1. Zymography outside worm burrows

The earthworm burrows were distributed across the soil volume and were well exposed on the front panel (soil surface) of the earthworm boxes (three red-marked areas A, B and C on Fig. 3a). The distribution of enzyme activities outside the burrows was visible as red, yellow and green colors, respectively representing high to low enzyme activities, while dark blue corresponded to very low activity (Fig. 3b). In reference boxes, the hotspots with strong color intensity were mainly associated with roots, while enzyme activity at a distance from the roots was much weaker (coldspot) (Appendix. 2).

The spatial distribution of hotspots was calculated based on the dispersion index, which was seen higher than 1 unit for reference box, earthworm box and burrow edge (Appendix. 3). The dispersion index of reference box was approximately 1.5 and 1.125 times higher than earthworm box and burrow edge, respectively. The significant difference ($p < 0.05$) of the dispersion index was found between reference box and earthworm box or earthworm burrow. Although earthworm box and earthworm burrow did not show the significant difference of the dispersion index, these two treatments followed different trend and direction – increase in aggregation in the earthworm burrow in relation to the earthworm box. In significant results were expected as earthworm box contains earthworm burrow.

3.2.2. Zymography inside worm burrows

As a consequence of enzyme specificity, the activities of 3 tested enzymes (APT, NAG, GLU) were different inside worm burrows (Fig. 4). The contribution of hotspot area to the total soil surface of reference soil was very small, varying between 0.1 and 0.2% for the three enzymes. This area in earthworm boxes increased up to 1%. In contrast, the percentage of hotspot area inside worm burrows was much larger than that of the soil surface of earthworm and reference boxes, comprising 1.8%, 1.2% and 0.5% for GLU, NAG and APT, respectively (Fig. 5). The differences between reference soil and burrow wall were particularly more striking for the C- and N-facilitated enzymes than those of the P cycle (Fig. 5). The largest differences in hotspot area between worm burrow and reference boxes were observed for GLU (13.8 fold), while the smallest burrow effect was detected for APT (2.4 fold). The proportion of hotspot areas inside exceeded that outside worm burrows and in reference soil by a factor of 1.8 and 13.8 times, respectively, for GLU, but only by a factor of 1.2 and 2.4 for APT, and 1 and 9.8 for NAG (Fig. 5).

4. Discussion

4.1. Enzyme activities

Enzyme activities were measured to quantify the effect of earthworms on soil enzyme systems. Within burrow walls, significant increases in activity (1.5–2.6 times, relative to the reference soil) of three extracellular enzymes (GLU, CBH, XYL) were in line with several previous findings (Tiunov and Scheu, 1999; Aira et al., 2006; Don et al., 2008; Ernst et al., 2009) (Fig. 2). Higher enzyme activities, and consequently accelerated decomposition of plant residues in worm burrows, are explained by the physiology and

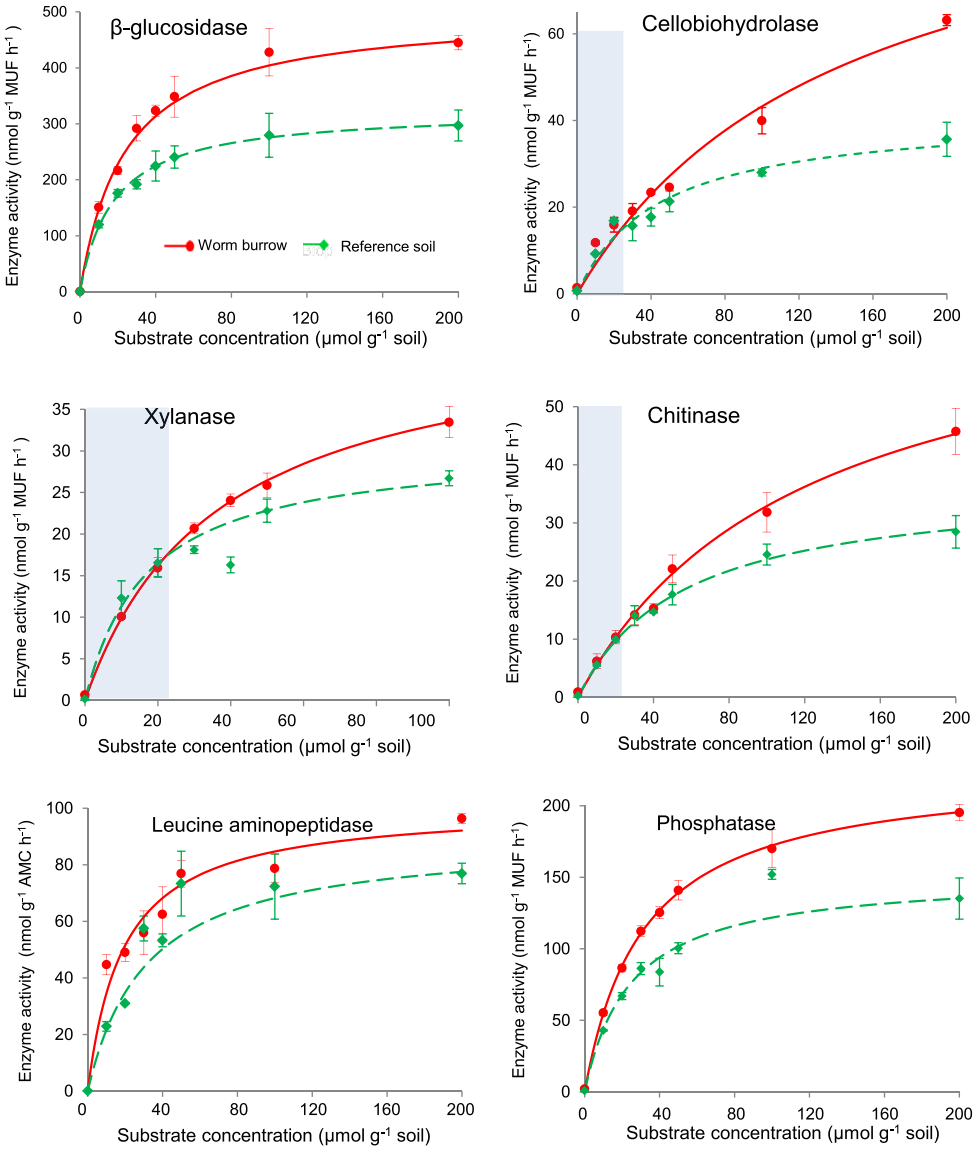


Fig. 1. Enzyme kinetics of 6 enzymes (β-glucosidase, cellobiohydrolase, xylanase, chitinase, leucine aminopeptidase and phosphatase) are shown as symbols and model simulations (Equation (1)) as curves. The sections emphasized by color shading show the concentration range at which no earthworm burrow effects occurred. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

metabolic activity of earthworms. Firstly, earthworms create a mixture of mineral and organic materials inside their guts (Brown et al., 2000). This mixing stimulates physical and biochemical degradation of plant litter inside the gut, releasing polysaccharides, proteins and amino acids with casts, mucus and urine (Tiunov and Scheu, 1999; Brown et al., 2000; Jégou et al., 2000). Such a modified organic matter pool becomes more labile, and is most abundant in the innermost surface of the burrows (Don et al., 2008). In addition, the mucus secreted by earthworms provides moisture and organic resources that activate microorganisms (Brown et al., 2000). The microbial community in burrow walls includes many litter-associated cellulolytic microorganisms, e.g. *Cellulomonas, Cytophaga* or fungi (such as *Trichoderma* spp.) in fresh casts. They are responsible for breaking-down both cellulose and hemicellulose (Tiunov and Scheu, 1999). Evidently, the high activity of cellulolytic and hemicellulose-decomposing enzymes in worm burrows (Fig. 2) reflected the availability of these substrates, resulting from earthworm feeding on plant residues. The higher activities of cellulolytic versus hemicellulose-degrading enzymes (XYL) were caused by differences in chemical properties. Although cellulose and hemicellulose are major components of plant tissues, the latter is more structurally recalcitrant.

Doubled NAG activity was detected in burrow walls compared with reference soil, but no substantial differences were observed for LAP (Fig. 2). Theoretically, the activity of both NAG and LAP is associated with N-acetyl-glucosamine hydrolysis (Flach et al., 1992). In soil, N-acetyl-glucosamine is produced by fungal (chitin) and bacterial (peptidoglycan) cells (Tracy, 1951). To cleave N-acetyl-glucosamine, the chitinolytic and saccharolytic soil microorganisms produce NAG and LAP to exploit this substrate as a source of C and N. The activity of N-cycle enzymes therefore increases the availability of organic N and C for microorganisms (Stone et al., 2012). The doubled NAG activity in the presence of earthworms (Fig. 2) was still less than the 4.0- and 2.5-fold increases observed by Shan et al. (2013). The higher NAG activity in burrow walls resulted from its production by *Lumbricus terrestris* L. during litter

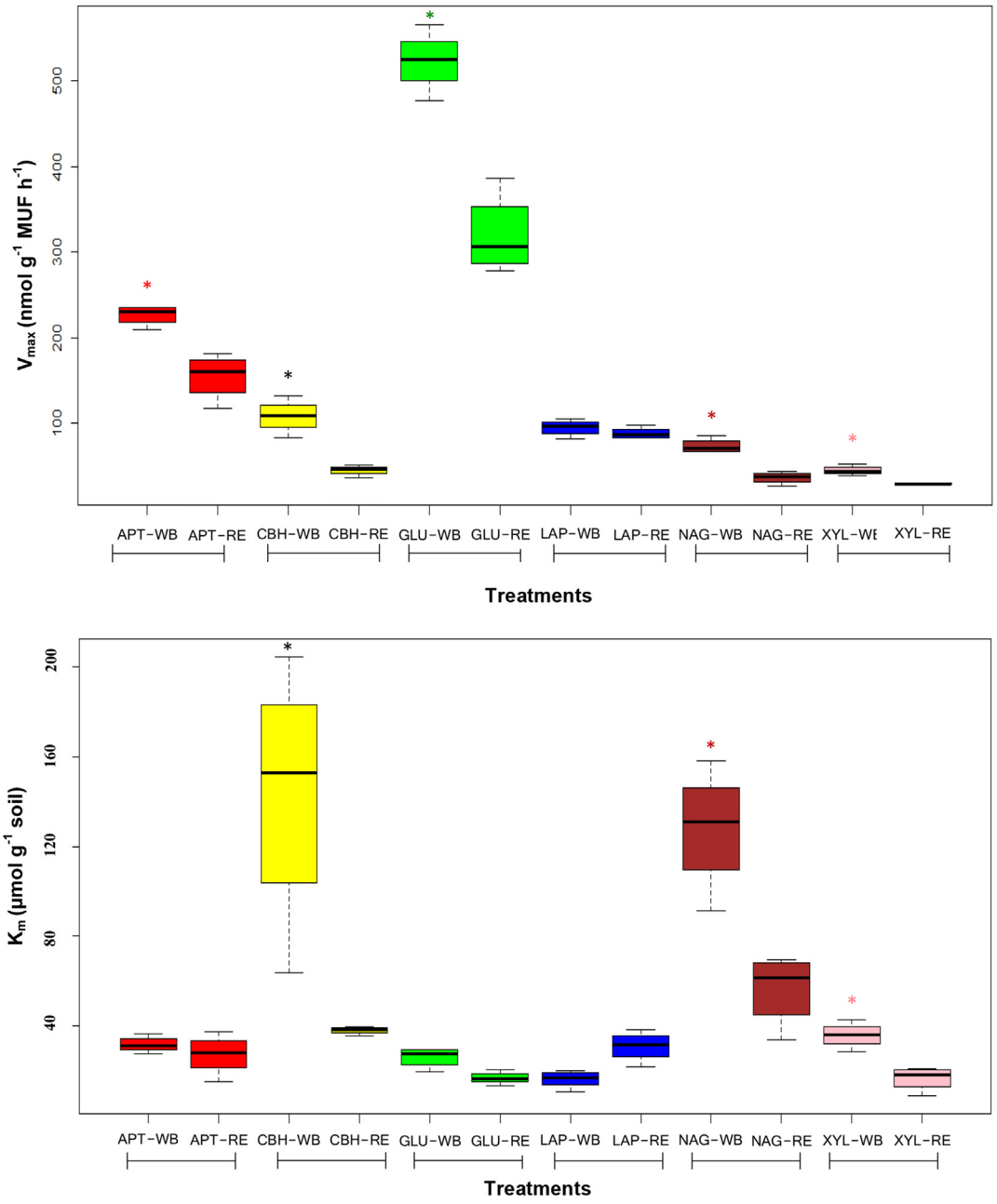


Fig. 2. Boxplot of V_{max} and K_m for the six enzymes tested. Boxes with similar colours present results for the same enzymes but different treatments (worm burrow (WB) and reference (RE) soil). The boxplots show the lower, median, and upper quartiles, with whiskers extending to the most extreme data point. Asterisks (*) indicate significant differences between reference and burrow samples for each enzyme at $p < 0.05$ as determined by Tukey's HSD test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

digestion (Tracy, 1951; Shan et al., 2013). Moreover, *Lumbricus terrestris* L. feeds on fungi and releases them within casts onto burrow surfaces (Tiunov and Dobrovolskaya, 2002; Jégou et al., 2000). The enrichment of fungal hyphae in their intestines and inside worm burrows accelerates production of NAG. N is also one of the most crucial nutrients for cell development in the earthworm's body, but it is a limited resource for both earthworms and microbes (Bohlen and Edwards, 1995). Earthworms might incorporate a large high amount of N in their tissues and therefore leave less N remaining in their casts for immobilization in microbial biomass (Ernst et al., 2009). This may lead to competition for N between microorganisms and earthworms, thus stimulating fungi (abundant in earthworm casts and burrow walls) to excrete more NAG to extract N. Despite also having a function in N-cycle processes, LAP showed a different trend to NAG. No difference in LAP activity between worm burrow and reference soil showed that earthworms likely did not influence LAP activities after two-week incubation.

A fifty percent higher activity of APT in burrows versus reference soil is in line with the profound differences observed by Lee (1985), Don et al. (2008) and Ernst et al. (2009). These findings can be explained by the presence of APT in earthworm feces and gut, as previously demonstrated (Vinotha et al., 2000). Moreover, the higher APT activity associated with walls compared to reference soils also reflects the higher abundance of P-solubilizing bacteria when earthworms are present (Wan and Wong, 2004).

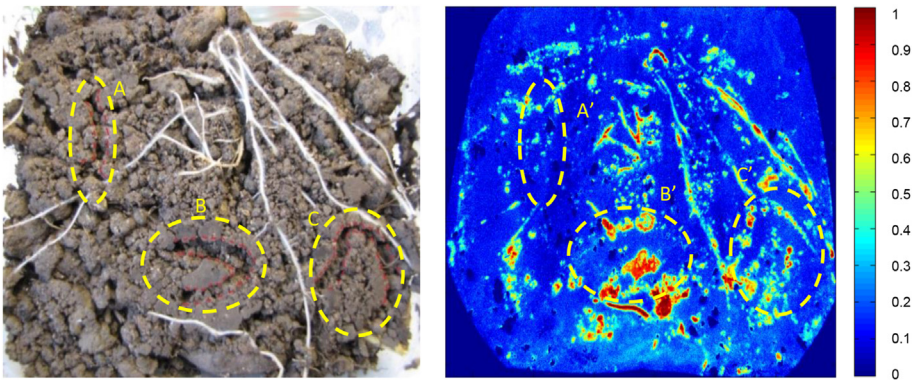


Fig. 3. An example of the earthworm boxes and corresponding zymography (β-Glucosidase) for the soil surface. a) Earthworm burrow exposed on the soil surface at yellow dashed-line positions A, B and C. The red dashed-line positions inside the yellow dashed-line are the borders of burrows b) Zymography image: A′, B′ and C′ were corresponding positions of burrows on the membrane. Note the higher color intensity in the area surrounding burrows but not inside the burrows. (Zymography images inside the opened burrows are shown in Fig. 4). Side color map is proportional to the calibration line of MUF substrate. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

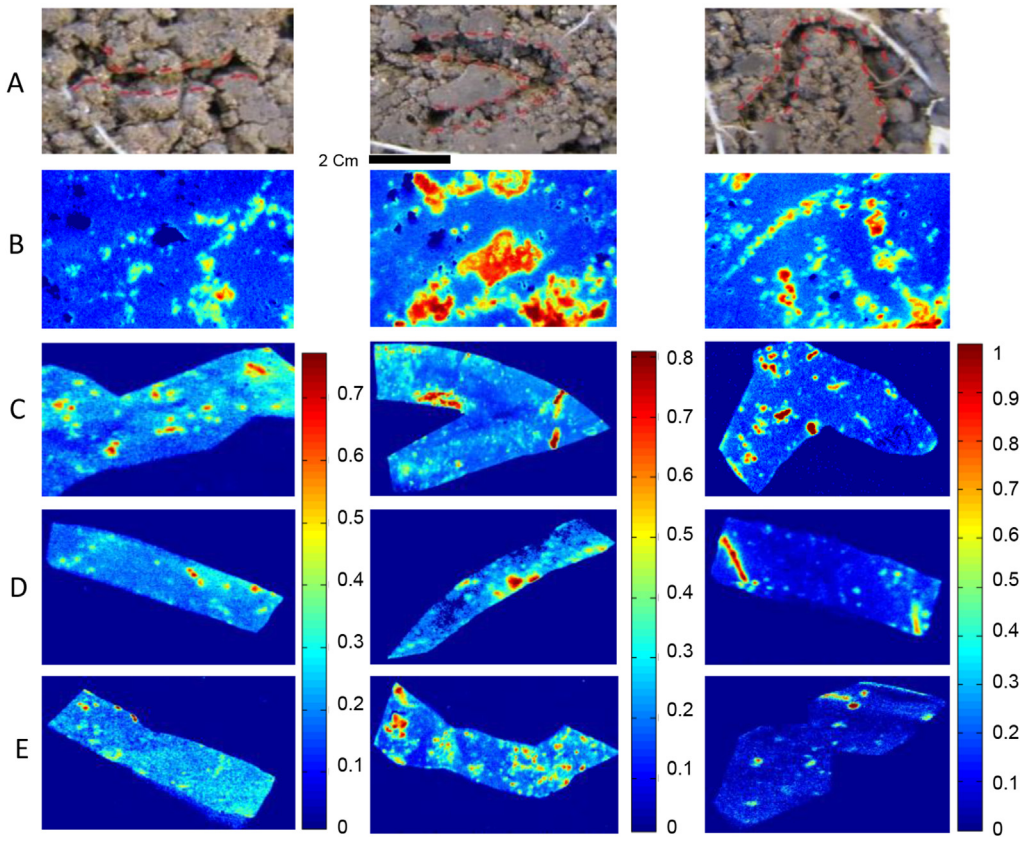


Fig. 4. Zymography images inside the opened worm burrows: phosphatase, chitinase and β-glucosidase activities in order from left to right side. Picture A shows a photo example of the burrows in the first replicate, B is an enlarged part of the zymography image from Fig. 3, showing the distribution of hotspots along burrow borders; C, D and E show 3 replicates of zymography application inside burrows within 3 earthworm boxes.

4.2. Enzyme kinetics

The two-to threefold increase in K_m for XYL, CBH and NAG in burrows versus reference soil indicated the presence of different enzyme systems with lower affinity to substrate. Lower substrate affinity (K_m increase) indicated a decrease in overall enzyme function under substrate limitation (Stone et al., 2012; German et al., 2012). Earthworms affect enzyme activity both directly and indirectly by altering microbial community structure and dynamics (Aira et al., 2006). Differences in K_m reveal differences in the functional traits of microorganisms in hotspots of various origins (Kuzyakov and Blagodatskaya, 2015). Thus, the K_m increase likely indicated a shift in the functional structure of the microbial community toward the domination by fast-growing but less efficient populations with lower substrate affinities (Fontaine and Barot, 2005; Blagodatskaya et al., 2009; Blagodatskaya and Kuzyakov, 2013). The insignificant differences in K_m of GLU and APT between burrows and reference indicated that earthworms did not affect the corresponding enzyme systems

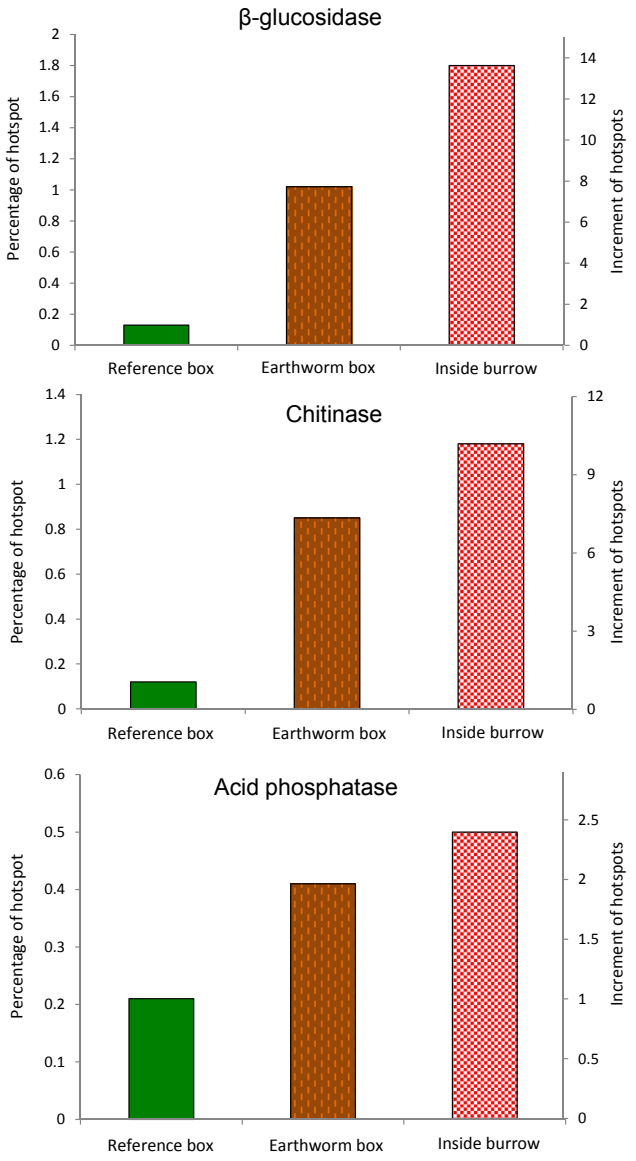


Fig. 5. Comparison of the percentage of hotspot area in reference soil, earthworm boxes and inside burrows (left axis) and the increment of hotspots (right axis).

(Fig. 2). The two enzymes demonstrated the highest activity rates in our assay (GLU and APT) constitute a major group of widespread enzyme activities occurring in all domains of life. Given that the K_m of GLU and APT was unaffected by worms, we assume that these enzyme systems are relatively well conserved across microbial species. This assumption is apparently not valid for different soils because K_m values of GLU and APT differed significantly across a land-use gradient (Tischer et al., 2015). The variations in K_m, however, are much smaller for soil enzymes than for enzymes extracted from pure microbial cultures (Tischer et al., 2015). We conclude, therefore, that GLU and APT isoenzymes of the same or similar families (Asif Shah et al., 2011) were expressed in reference soil and in worm burrows within same soil type. The K_m of LAP in worm burrows was 23% lower (not significant) than reference soil (Fig. 2), which indicated a higher affinity of enzyme systems to protein and polypeptides (Fontaine et al., 2004). Overall, we observed a significant increase in K_m in worm burrows compared to the reference for XYL, CBH and NAG (Fig. 2).

At low substrate concentration (<20 μmol g^{-1} soil), we detected a similar reaction rate for NAG, CBH and XYL in burrows and reference soil (Fig. 1). These results can be explained by a much greater increase in K_m (substrate affinity decreased) which is in the denominator of the Michaelis-Menten equation (1) than in V_{max} canceled the differences in XYL, CBH and NAG activities at substrate concentrations below 20 μmol g^{-1} soil in both treatments (Fig. 1). Thus, the decomposition of microbial and plant residues in worm burrows was accelerated only at substrate levels exceeding that threshold. In particular, because K_m and V_{max} vary independently due to the change of substrate concentration, both parameters of the Michaelis-Menten kinetics need to be considered to assess the influence of earthworms on soil microorganisms.

4.3. Visualization of hotspots outside and inside worm burrows

Most of the hotspots demonstrated by strong color intensity in earthworm soil were associated with worm burrows and roots (Fig. 3). These enzyme activity hotspots (Figs. 3 and 4) result from i) earthworms' action and mucus release, promoting the proliferation of micro-organisms; or ii) root development, accelerating microbial activity in the rhizosphere. In this study, we did not distinguish the area of hotspots of rhizosphere origin from those created by earthworms. In some cases, earthworm burrows may become occupied by roots too, but we selected burrows without roots to avoid mixed effects. A future challenge will be to investigate these interactions in order to assess the mutual formation of hotspots of different origin.

Despite decades of study about the effects of earthworms on enzyme activities (Burns, 1981; Ross and Cairns, 1982; Matsui et al., 2006; Dong et al., 2007), none have focused on the spatial distribution and kinetic properties of enzymes. More recently, zymography techniques have been applied to soil-root interactions, but not to earthworm activity. Our study is the first successful application of zymography to worm burrows and soil processed by earthworms. The percentage of hotspot area was up to 1.8 times higher inside the burrow than in the surrounding soil. This demonstrated that active microorganisms preferentially inhabit areas inside and at the edge of burrow walls (Figs. 3 and 4). However, the pattern of spatial distribution of hotspots demonstrated relative increase in aggregation in the earthworm burrow in comparison with the earthworm boxes (Figs. 5 and 6). These results mean earthworms reused their burrows many times (Capowiez et al., 2001) so their enzymatic effect were more evident there. The mucus secreted with casts motivated microorganisms to decompose organic matter because of the labile C richness (Brown

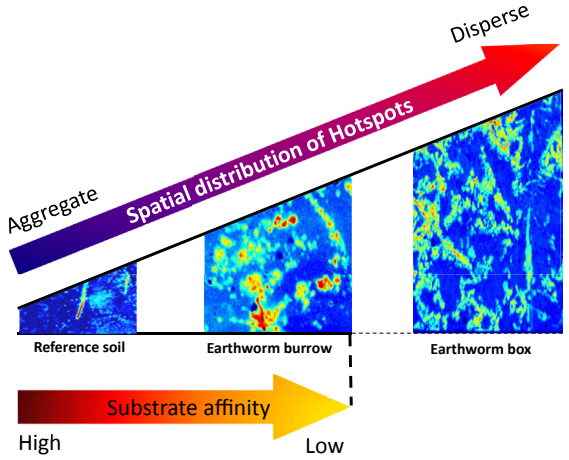
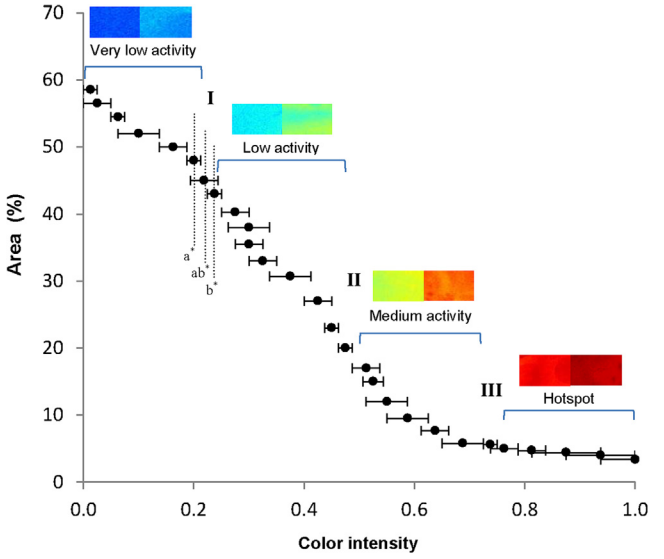


Fig. 6. Substrate affinity was lower in burrows compare to control soil for Cellobiohydrolase, Chitinase, Xylanase. Spatial distribution of hotspots was more spread in burrow compare to control box which were more aggregated (around root).

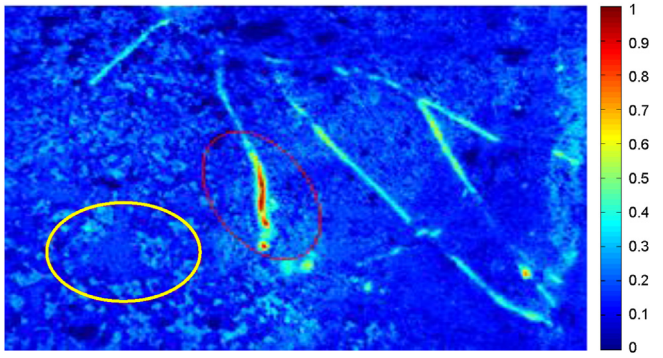
et al., 2000). This finding is explained by the continuous movement of earthworm in the box, which spread hotspots on soil surface, while in reference box hotspots focus along plant roots.

5. Conclusions

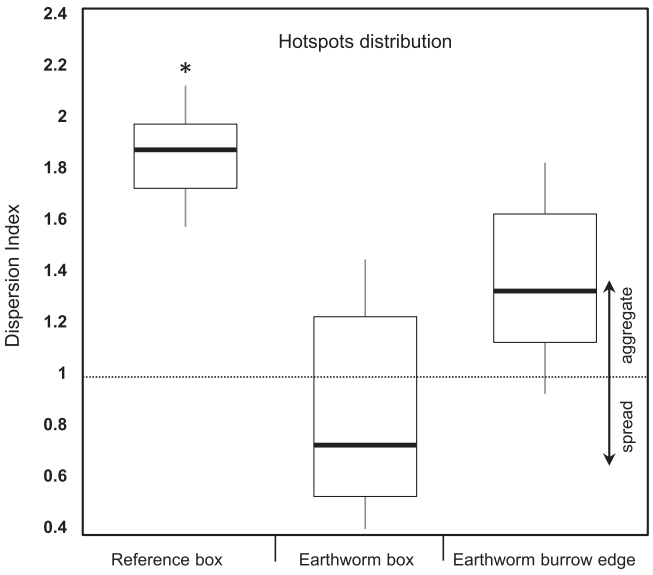
Hotspots were twice as concentrated close to earthworm burrows as in surrounding soil. The prevalence of hotspots inside burrows proves that earthworms accelerate microbial enzyme activities. Above the substrate threshold of 20 μmol g^{-1} soil, the activities of CBH, XYL and NAG in burrows were up to 2 to 3 times higher than in reference soil. Not only activity but also the enzyme systems differed, as revealed by the two-fold increase of K_m for CBH, XYL and NAG and by the 23% decrease for LAP. These different enzyme systems point to a shift in dominant microbial populations to burrow-related species with lower affinity to holo-cellulose and to N-acetylglucosamine and with higher affinity to proteins. The combined application of zymography and enzyme kinetic assays enabled relating the distribution of enzyme activity to enzyme kinetic properties in soil modified by earthworms.



Appendix.1. An example of detecting the boundaries of four categories of enzyme activities. The percentage of the area of MUF concentration in the total image is considered as a function of color intensity. Data points depict means calculated from four adjacent pixels. Asterisks indicate significant differences between the mean values of four adjacent pixels.



Appendix.2. Zymography (β-glucosidase activity) of the reference soil surface. The red circle positions show the distribution of hotspots along fine roots. Yellow circles illustrate areas of low enzyme activity (coldspots) in reference soil.



Appendix.3. Box plot representations of data from spatial point pattern analyses in reference box, earthworm box and earthworm burrow edges (>1 aggregate, and <1 spread). *Asterisk* indicates significant differences between the reference box, earthworm box and earthworm burrow edge.

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