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

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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

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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.