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Six months of *L. terrestris* L. activity in root-formed biopores increases nutrient availability, microbial biomass and enzyme activity

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ABSTRACT

In arable fields, biopores are primarily formed by taproots, but may also be bored by earthworms. Irrespective of the pore origin, repeated use by anecic earthworms yields a wall coating that is rich in carbon, nutrients and microorganisms. However, this effect is halted by routine tillage, and it remains unclear how quickly earthworms are able to alter biopore properties in subsoil. We conducted an earthworm incubation field experiment in arable soil to test the capacity of *Lumbricus terrestris* to i. increase total nutrient contents including plant available P, ii. alter the microbial community and iii. increase enzyme activities in biopore walls over one vegetation period. Firstly, biopores that contained chicory roots were identified on a plot scale (4.2 × 1.5 m). After two years under fallow, roots were decomposed. We then inserted individual earthworms at 45 cm depth into a subset of these pores, afterwards refilling with topsoil. After six months, earthworms were removed and soil was opened at 45–75 cm and 75–105 cm soil depth layers. The inner pore wall (1 mm) of individual root biopores ('RBP') or root biopores modified by earthworms ('EBP') as well as the bulk soil were sampled in 6 depth intervals of 10 cm each and analyzed for total C, N, S content, plant available P, microbial biomass, phospholipid fatty acids (PLFA) and enzyme activity. Biochemical properties of bulk soil, RBP and EBP clearly differed after one vegetation period as indicated by principal component analysis. PLFA markers of fungi and protozoa were detected only in biopores. Compared with the bulk soil, total C, N, S were enriched in RBP by a factor of 2.0–3.1, plant available P by a factor of 8–10, and microbial biomass by a factor of 12–36. In EBP, all of these parameters were as in RBP or elevated even further (C, N, S: factor 1.0–1.4, plant available P: factor 1.3–1.5, microbial biomass: factor 1.5–2.0, PLFA markers of fungi: factor 2.6–4.4, PLFA markers of protozoa: factor 9.2–14.2). PLFA markers indicative of the ratio of Gram-positive to Gram-negative bacteria

(G+: G−) were 5–10 fold lower in RBP than in bulk soil, the microbial metabolic quotient (qCO_2) was 0.4–0.6 times as high. In EBP, these parameters were further reduced (ratio G+: G−: factor 0.7, qCO_2: factor 0.7–0.8). RBP were particularly characterized by high contents of 10-methyl branched fatty acid indicators of actinobacteria.

Activities of enzymes involved in the C-cycle (xylanase, cellobiohydrolase, ß-glucosidase) and N-cycle (chitinase, chitotriosidase, leucine aminopeptidase) were also elevated in RBP as compared to the bulk soil (factor 1.1–3.6) and further increased in EBP (factor 1.2–3.7). All these effects were more pronounced in the 45–75 cm soil layer. We conclude that, in only six months, *L. terrestris* in arable fields modified ordinarily nutrient-rich biopores into 'super-hotspots' of microbial biomass, enzyme activity and nutrient availabilities. Hence, even short-term promotion of earthworm populations by agricultural management practices can increase microbial biomass and enzyme activity in biopores and its coupling to nutrient mobilization in the subsoil.

1. Introduction

Tubular shaped, continuous vertical biopores in arable fields are typically created either by taprooted crops or, to a minor degree, by anecic earthworms (Kautz et al., 2014). These biopores have diameters of up to 12 mm (Edwards and Bohlen, 1996), can be several meters

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deep, and can persist for decades in the subsoil beneath the plow layer (Hagedorn and Bundt, 2002; Shipitalo et al., 2004). Crop roots have been reported to preferentially follow such pores especially in compacted soils (Logsdon and Linden, 1992; Passioura, 1991), allowing them to reach deeper soil horizons more rapidly, thereby facilitating water uptake during dry spells (Gaiser et al., 2012). Likewise, anecic earthworms repeatedly utilize biopores, covering the pore walls with material rich in organic matter and nutrients, particularly N and P. Thus, they have been characterized as hotspots of microbial activity (Kuzyakov and Blagodatskaya, 2015) and a potential source of nutrients in the subsoil (Kautz et al., 2013). Moreover, earthworm burrows markedly contribute to water infiltration (Edwards et al., 1988; Ehlers, 1975), which can be advantageous for draining heavy rainfalls.

In general, biopore generating earthworms can be promoted by reduced tillage intensity (Ehlers et al., 1983; Kuntz et al., 2013; Pelosi et al., 2014), because tillage operations remove their surface food supply and destroy the top portion of permanent burrows (Kladivko, 2001). Fodder crops such as grass clover also have beneficial effects on earthworm populations, especially when cultivated as perennials (Mäder et al., 2002; Riley et al., 2008). Recently, occasionally reduced tillage (ORT) was shown to also result in increased earthworm abundances (Moos et al., 2016). However, the positive effects of perennial fodder cropping or ORT are temporary, as increases in earthworm populations following ca. two vegetation periods of soil dissipate with the onset of tillage.

Anecic earthworms such as *Lumbricus terrestris* reach maturity after approximately one year under field conditions (Satchell, 1967). In the early growth stages, they are known to largely behave like endogeics (Lowe and Butt, 2005), i.e. they predominantly stay beneath the soil surface. As such, the time for *L. terrestris* to generate biopores and influence pore properties in the subsoil during cultivation of perennial crops or ORT may be on the scale of only a few months. Kautz et al. (2014) have shown that the number of anecic earthworms increased during cultivation of perennial fodder crops, but the worms hardly contributed to the formation of new biopores in the subsoil, primarily as a consequence of re-colonization of established pores. However, in another study X-ray computed tomography in combination with in situ endoscopy revealed considerable effects of short-term earthworm incubation in microcosms on physical pore properties such as pore diameter distribution, pore connectivity, and accessible pore surface area (Pagenkemper et al., 2015). The extent to which earthworms are able to alter biochemical properties and thus pore quality in the short-term remains uncertain. Graff (1967) distinguished 'young' burrows from 'old' burrows by the color of the pore wall and demonstrated that the former exhibited higher nutrient contents. Similarly, Athmann et al. (2014) found that only biopores showing visible signs of earthworm passage were significantly elevated in C and N contents in comparison to the bulk soil. For grassland subsoils, Don et al. (2008) reported that pores inhabited by earthworms exhibited higher nutrient contents and enzyme activities than abandoned earthworm burrows, but no such data are available for short-term effects in arable fields. In this study, we conducted an earthworm incubation field experiment in arable soil to test the capacity of *L. terrestris* to i. increase total nutrient contents including plant available P, ii. alter the microbial community and iii. increase enzyme activities in biopore walls over one vegetation period.

2. Material and methods

2.1. Site conditions and experimental design

The field trial was performed at 'Campus Klein Altendorf' experimental station in Rheinbach, Germany (50°37′N, 6°59′E) with a mean annual temperature of 9.4 °C and total annual precipitation of 603 mm. The soil is a Haplic Luvisol derived from loess with a clay content of 18% in the Ap and 29–32% in the Bt horizon. A detailed description of soil properties at the experimental site is given by Vetterlein et al. (2013).

The experiment had a completely randomized block design with four replications that had the following treatments: i. bulk soil, ii. root channels after two and a half years of decay (i.e., root biopores 'RBP') and iii. root channels after two years of decay followed by earthworm incubation for six months (i.e., earthworm modified biopores, 'EBP'). It is important to note that the age and origin of these pores was not known. It is likely that most were older biopores that had been visited by both roots and earthworms before chicory cultivation, while a smaller portion of biopores were newly generated by chicory taproots.

In detail, the treatments were established as follows: Four 6 × 10 m replicate field plots of a larger randomized field experiment with arable crops were selected for this study. Chicory 'Puna' (*Cichorium intybus* L.) was sown in spring 2009 with a sowing density of 385 seeds m^{-2} (5 kg ha^{-1}) and cultivated continuously for 3 years. On January 30th 2012, the trial was plowed to 30 cm depth. On September 10th 2012, the topsoil was removed down to 45 cm depth in subplots with surface areas of 4.2 × 1.5 m in each of the replicate field plots and stored adjacent to the study area. A depth of 45 cm was chosen to be sure to also remove the plow pan. The horizontal area in 45 cm depth was carefully planed and biopores were cleared from loose and smeared soil particles using a vacuum cleaner. The prepared surface was covered with transparent films and the locations of large biopores (diameter > 5 mm) containing roots were mapped. No visible signs of earthworm activity were detected in these root containing biopores. Afterwards, the topsoil was put back and the plots were left under fallow conditions for 19 months to allow for root decomposition and to remove all food sources, thereby discouraging earthworms native to the site from colonizing these biopores.

In April 2014, half of the subplots (2.1 × 1.5 m) were re-opened, and the topsoil was stored next to the experimental area as before. Again, a horizontal area in 45 cm depth was prepared for identification of biopores. In each field replicate, 25 biopores were identified that had previously contained roots, but were now found to be empty. Each selected pore was incubated with one dew worm (*L. terrestris*). The worms were adults obtained from Canadian wild harvesting (Superwurm e.K., Düren, Germany), and were kept for four weeks at 4–6 °C in buckets with nutrient rich soil and horse manure. Prior to incubation all worms were kept on filter tissues for 3 days to ensure complete defecation. Then they were labeled with visual implant elastomers (Butt and Lowe, 2007). Wooden sticks (diameter 8 mm, length 45 cm) were inserted into all incubated pores before the subplots were refilled with topsoil. Finally, removal of the wooden sticks created a connection between incubated pores and the soil surface. For the next six months all subplots were covered with mulching material from a grass clover field. Weeds that occasionally emerged were manually removed from the experimental area. After the incubation period the topsoil was removed from all subplots and incubated earthworms were removed via the octet method (Thielemann, 1986).

2.2. Sampling

In October 2014, an excavator was used to create a trench along the long side of the subplots as a base for collecting samples from the bulk soil and from pore walls of RBP and EBP. For RBP, no visible signs of earthworm activity were detected before or during sampling. EBP were only taken into account if an earthworm with a clearly recognizable implant elastomer was found after the incubation period. The recovery rate of labeled earthworms was 39% (39 of 100 pores), with 6–13 pores sampled in each plot. Within a layer of 10 cm (45–55 cm depth) single biopores were opened and approximately 1 mm of the inner wall material was sampled with microspatulas. The procedure was repeated for 6 depth intervals down to a maximum depth of 105 cm. Bulk soil was also collected from each treatment and depth layer with a spatula, keeping a distance to biopores of at least 5 cm. Samples from 45 to 75 cm and 75 to 105 cm depth layers were merged to gain sufficient

material for all analyses.

Due to the large volume of work, several persons were required to collect all of the samples. We were aware that a change in the sampling personnel might influence the results due to individual differences in the amount of material taken from the pore wall. To minimize such effects, the sampling personnel was trained prior to the field work and each field replicate was sampled by one person only.

2.3. Plant nutrients

Total C, N and S contents of pore wall and bulk soil samples were analyzed by dry combustion with an elemental analyzer. Soil pH was around 6.5 so we considered total C as soil organic content (C_{org}).

Calcium-acetate-lactate soluble P (CAL-P_i) was extracted according to Schüller (1969). Specific P pools were determined following the sequential extraction procedure of Hedley et al. (1982) as modified by Tiessen and Moir (1993); however, residual P was extracted by digestion in aqua regia. Concentrations of inorganic P (P_i) in each of the extracts were determined with a spectrophotometer by the molybdenum blue method (Murphy and Riley, 1962). Total P concentration in the extracts was determined by inductively coupled plasma optical-emission spectroscopy. Concentrations of organic P (P_o) were calculated as the difference of inorganic to total P. We classified P pools according to Negassa and Leinweber (2009), distinguishing highly available P (resin and $NaHCO_3$ extracts), moderately available P (NaOH extracts) and stable P (HCl_{dil}, HCl_{conc} and residual P in aqua regia) for interpreting our findings, but restrict to mentioning the chemical extractions as nomenclature.

2.4. Basal respiration, microbial biomass carbon (C_{mic}) and microbial metabolic quotient (qCO_2)

A fresh subsample (1 g oven-dry soil equivalent) was adjusted to 50% of the water holding capacity (WHC) and gravimetrically controlled. After two days preincubation at room temperature to avoid side effects of sieving (Blagodatskaya et al., 2011), samples were incubated at 20 °C (Creamer et al., 2014) in 12 mL septum-capped vials. Six other vials without soil were prepared as controls to correct for atmospheric carbon dioxide.

The basal respiration was measured at time 0 (t_0, i.e., right after adding the soil to the vials and flushing the vials with ambient air), then at 24 h intervals for three additional time points (t_1, t_2, t_3). Thereafter, 100 µL of a glucose solution (60 mg mL^{-1}) were amended to all the vials to determine the soil microbial biomass (µg C_{mic} g^{-1} soil dry mass) by the substrate induced respiration (SIR) method (Anderson and Domsch, 1978; Lin and Brookes, 1999; West and Sparling, 1986). The WHC reached 70% after aliquot amendment. Immediately after glucose addition, we used a manifold combined with a pump to standardize air inside vials with ambient air. All vials were then incubated for 2 h at 20 °C (Lin and Brookes, 1999). The measurements of basal respiration and substrate induced respiration were undertaken directly via gas chromatography. The CO_2 evolution (ppm) was calculated by subtracting the CO_2 concentrations of the blanks from those of the sample.

The basal respiration (µg C g^{-1} h^{-1}) was calculated by subtracting CO_2 concentrations in soil vials measured at t_1, t_2 and t_3 from CO_2 concentrations in blank vials and the initial CO_2 evolution of soil vials at t_0 (Creamer et al., 2014). Using the Ideal Gas Equation, CO_2 respiration was converted from ppm to µg C g^{-1} h^{-1} in accordance with headspace volume of the sealed flask containing the soil sample, incubation temperature and air pressure (Orchard and Cook, 1983).

The SIR was applied to calculate microbial biomass C (µg C g $soil^{-1}$) based on the equation by Anderson and Domsch (1978)

$$x = 40.4y + 0.37 \quad (1)$$

where y is the maximum initial rate of respiration (CO_2 evolution 2 h after adding glucose). Similarly, the microbial metabolic quotient (qCO_2) was calculated by dividing initial respiration (BR) by C_{mic} and expressed as µg CO_2-C mg^{-1} C_{mic} h^{-1} (Anderson and Domsch, 1990).

2.5. Phospholipid fatty acids (PLFA)

Phospholipids were extracted based on the protocol of Frostegård et al. (1991) with the modifications described by Apostel et al. (2013) using phosphatidylcholine-dinonadecanoic acid and tridecanoic acid methyl ester as internal standards (IS 1 and IS 2, respectively). Samples were measured on a coupled gas chromatography mass spectrometry (GC–MS) system employing a 45 m DB5-MS column (0.25 mm I.D., 0.25 µm film thickness). Stock solutions containing external standards of 27 fatty acids and IS 1, with total fatty acid contents of 1, 4.5, 9, 18 and 24 mg, were derivatised together with the samples. The detailed measurement procedure is described in supplement 1. Individual groups of microorganisms were determined based on previously published PLFA biomarker data (Zhang et al., 2015). Specifically, 18:2ω6,9 was used as a marker for fungal biomass (Frostegård and Bååth, 1996); 10Me16:0 and 10Me18:0 were used as markers for actinobacteria; a15:0, i15:0, i17:0, a17:0 were used as markers for Gram-positive bacteria, and 16:1ω5c, 16:1ω7c, 18:1ω7c, Cy17:0 were used as markers for Gram-negative bacteria. In addition, 20:4ω6 was used as a marker for protozoa (Fierer et al., 2003).

2.6. Enzyme activities

Enzymes were assayed according to the modified methodology of Razavi et al. (2015) for three out of four field replicates. Half a gram of fresh soil (dry weight equivalent) was dispersed in 50 mL sterilized water of which 50 µL soil suspension was pipetted to a 96-well microplate. Subsequently, 50 µL of buffer [MES $C_6H_{13}NO_4SNa_{0.5}$. (pH:6.5) buffer for 4-methylumbelliferone (MUF) substrate and TRIZMA $C_4H_{11}NO_3$·HCl, $C_4H_{11}NO$ (pH:7) buffer for 7-amino-4-methylcoumarin (AMC) was added. We measured activities of 3 enzymes regarding the C-cycle: 1) β-glucosidase (**EC** 3.2.1.21) measured with MUF-β-D-glucopyranoside (MUF-G), 2) cellobiohydrolase (**EC** 3.2.1.91) measured with MUF-β-D-cellobioside (MUF-C), and 3) xylanase (**EC** 3.2. 1.8) measured with MUF-β-D-xylopyranoside (MUF-X); and 3 enzymes regarding the N-cycle: 1) chitotriosidase (**EC** 3.2.1.14) measured with 4-methylumbelliferyl-β–DN, *N′,N″*-triacetylchitotrioside (MUF-Tr), 2) chitinase (**EC** 3.2.1.14) measured with MUF-*N*-acetyl-β-D-glucosaminide (MUF-N); 3) leucine-aminopeptidase (**EC** 3.4.11.1) measured with L-Leucine-7-amido-4-methylcoumarin hydrochloride (AMC-L).

The reaction solution was buffered at pH 6.5 whereas the optimal pH is 5.5 for xylanase (Schinner and von Mersi, 1990), 6.0 for cellobiohydrolase (Hong et al., 2003), 5.2 for chitotriosidase (Hollak et al., 1994), 5.5 for chitinase (Parham and Deng, 2000), 6.0 for β-Glucosidase (Eivazi and Tabatabai, 1988), and 7.5 for leucine aminopeptidase (Niemi and Vepsäläinen, 2005). For these assays that were not run at optimal pH, the results cannot be compared to other studies where the optimal buffered pH was used. Also, in these cases, the activities would be expected to be reduced compared to if it had been done at optimal pH. Thus, differences between treatments may have been underestimated.

The microplate was incubated with 100 µL/well of fluorescent substrate solution at the desired concentration range: 0, 10, 20, 30, 40, 50, 100, 200 nmol g^{-1} dry weight in a 96-well microplate. The concentration that resulted in saturation of fluorogenic substrate was determined based on preliminary experiments for which one field replicate was used. The assay of each enzyme at each substrate concentration was replicated three times in each plate, and each plate included a standard curve of the product (4-methylumbelliferone, MUF) or (7-amino-4-methylcoumarin, AMC), substrate controls (for each substrate concentration), and homogenate controls. Enzymatic activity (nmol product released h^{-1} g^{-1} dry soil) was calculated from the MUF

or AMC standard curve following Razavi et al. (2015). However, we did not run a control for autohydrolysis. We assumed autohydrolysis of the two substrates MUF and AMC to be ignorable because according to Rakels et al. (1993) substrate hydrolysis occurs for 0.5% of the total amount of substrate in 1 h and is thus negligible. The calibration solutions were prepared using soil suspension (50 µL) and MUF to obtain a series of concentrations 0–1.2 mM (Razavi et al., 2015). The time from substrate addition to the fluorescence measurement (30, 60 and 120 min) was the same for all enzymes and samples. Linear increase of fluorescence over time during the assay was checked and data obtained after 2 h used for further calculation (Razavi et al., 2015). The fluorescence was measured using a Multilabel Counter at an excitation wavelength of 355 nm and an emission wavelength of 460 nm. In order to calculate V_{max}, a calibration curve was prepared by adding MUF or AMC instead of substrates to the same amount of soil solution and buffers (MES or TRIZMA, respectively) following Freeman et al. (1995) and Razavi et al. (2015). Enzyme activities were calculated as released MUF or AMC in nmol g-1 h-1 (Marx et al., 2005). V_{max} values were determined by nonlinear curve fitting using the software OriginPro 8.5 (OriginLab, Massachusetts, USA).

2.7. Statistical analyses

Shapiro-Wilk tests were used to confirm normal distribution of the datasets. Means were compared by one-way ANOVA followed by Tukey-tests. For enzyme activities, only three field repetitions were considered, for all other parameters all four field repetitions were included in the analysis. For the 45–75 cm soil depth interval principal component analysis (PCA) was used for further data evaluation. All parameters analyzed were subjected to PCA, including individual PLFAs. Only components with Eigenvalues > 1 were considered. Principal components were not rotated. All calculations were performed using IBM SPSS version 22.

3. Results

3.1. Nutrient contents

In walls of pores with or without earthworm incubation total contents of C, N, P, S were generally higher than in the bulk soil (Table 1). This effect was particularly pronounced for C, N and S with at least two-fold increased contents in pore walls relative to the bulk soil in both depths (45–75 cm and 75–105 cm). Moreover, short-term earthworm incubation in EBP resulted in higher C contents than in RBP in the 45–75 cm soil depth layer.

Hedley fractionation of phosphorus revealed markedly higher contents of resin P_i, $NaHCO_3$ P_i, $NaHCO_3$ P_o, NaOH P_i, NaOH P_o, and HCL_{dil} P_i in pore walls than in the bulk soil, and the same was also observed for CAL-P_i. Furthermore, in EBP CAL-P_i and resin P_i were significantly enriched as compared to RBP in the 45–75 cm soil depth layer, and $NaHCO_3$ P_o in both soil depths.

In the bulk soil, increased soil depth resulted in lower contents of total N and S as well as NaOH P_o, while total P and HCl_{conc} P_i increased with depth. In RBP generally no effect of depth on nutrient contents was observed, but in EBP contents of total N and NaOH P_o and HCl_{conc} P_o were higher at 45–75 cm than at 75–105 cm.

3.2. Microbiological properties

Microbial biomass (C_{mic}) related to soil organic carbon was low in bulk soil regardless of soil depth (Fig. 1). In both biopore types, but especially in EBP, C_{mic} was considerably elevated, with the highest value in EBP at 45–75 cm soil depth. The microbial metabolic quotient (qCO_2) related to the microbial biomass was very high in bulk soil, especially in the 75–105 cm soil depth layer (Fig. 1), and decreased in both biopore types.

Table 1
Total nutrient contents and P fractions in bulk soil and the walls of different pore types. Different uppercase and lowercase letters indicate significant differences between depth levels and soil compartments respectively (one way ANOVA with Tukey-HSD, $p < 0.05$).

Soil depth	45–75 cm			75–105 cm		
Treatment	EBP[a]	RBP[b]	Bulk soil	EBP[a]	RBP[b]	Bulk soil
C (mg g^{-1})	11.6 a	8.10 b	4.10 c	10.7 a	9.30 a	3.50 b
N (mg g^{-1})	1.60 a A	1.40 a	0.70 b A	1.30 a B	1.30 a	0.60 b B
S (mg g^{-1})	0.15 a	0.14 a	0.07 b A	0.10 a	0.11 a	0.03 b B
P (mg kg^{-1})	917 a	800 a	454 b B	825 a	797 a	580 b A
CAL-P_i (mg kg^{-1})	242 a	163 b	15.8 c	194 a	153 a	19.8 b
Resin P_i (mg kg^{-1})	74.3 a	40.4 b	16.0 c	55.3 a	34.5 ab	14.8 b
$NaHCO_3$ P_i (mg kg^{-1})	35.1 a	33.1 a	15.9 b	27.9	31.8	18.9
$NaHCO_3$ P_o (mg kg^{-1})	23.9 a	7.19 b	n.d.	19.6 a	9.21 b	n.d.
$NaHCO_3$ P_t (mg $kg^{-1)}$	59.0 a	40.3 b	15.9 c	47.5 a	41.0 a	18.9 b
NaOH P_i (mg kg^{-1})	72.5 a	68.1 a	48.8 b	69.0	66.9	49.9
NaOH P_o (mg kg^{-1})	31.7 a A	24.7 ab	16.8 b A	20.4 a B	20.5 a	10.1 b B
NaOH P_t (mg kg^{-1})	104 a	92.7 a	65.6 v	65.6 b	87.4	59.9
HCl_{dil} P_i (mg kg^{-1})	194.4 a	207 a	135 b	214	231	240
HCl_{conc} P_i (mg kg^{-1})	171	186	144 B	187	159	166 A
HCl_{conc} P_o (mg kg^{-1})	40.6 A	30.2	35.9	10.7 b B	53.2 a	33.3 ab
HCl_{conc} P_t (mg kg^{-1})	211 ab	216 a	180 b	198	213	199
Residual P_t (mg kg^{-1})	31.5 ab	40.7 a	25.6 b	28.1	38.1	27.8

P_i: inorganic phosphorus, P_o: organic phosphorus, P_t: total phosphorus.
[a] EBP: Earthworm-modified biopores.
[b] RBP: Root biopores.

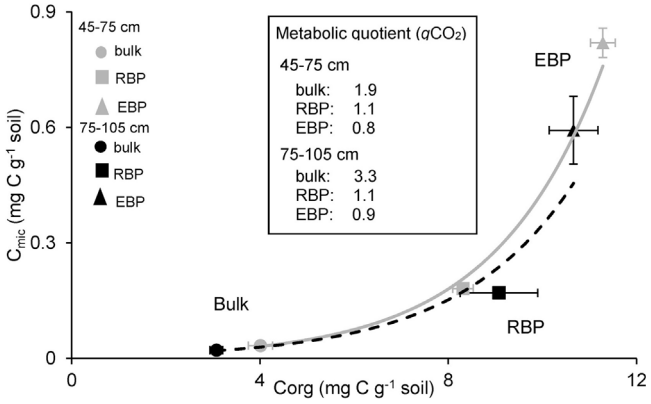

Fig. 1. Microbial biomass (C_{mic}) related to organic carbon content and microbial metabolic quotient (qCO_2). Error bars indicate standard deviation. RPB: root biopores, EBP: earthworm-modified biopores. C_{mic} is significantly higher in EBP than in RBP and bulk soil in both soil depth horizons and, only in EBP, significantly higher at 45–75 cm (ANOVA with Tukey-HSD, $p < 0.05$). qCO_2 is significantly lower in EBP and RBP as compared to the bulk soil and, only in bulk soil, significantly lower at 45–75 cm.

Similarly to C_{mic}, the total PLFA content (Fig. 2a) was much higher in both biopore types than in the bulk soil, increasing by a factor of 12–54 depending on biopore type and soil depth. Total PLFA was further increased by short-term earthworm incubation (factor 1.5–2.0 as compared to RBP; this difference was significant in the 45–75 cm soil depth layer). The ratio of Gram positive: Gram negative bacteria as determined with PLFA biomarkers (Fig. 2b) was much higher in bulk soil than in both biopore types (factor 3.4–16.3). In the bulk soil there was a significant effect of soil depth, with a much higher ratio in

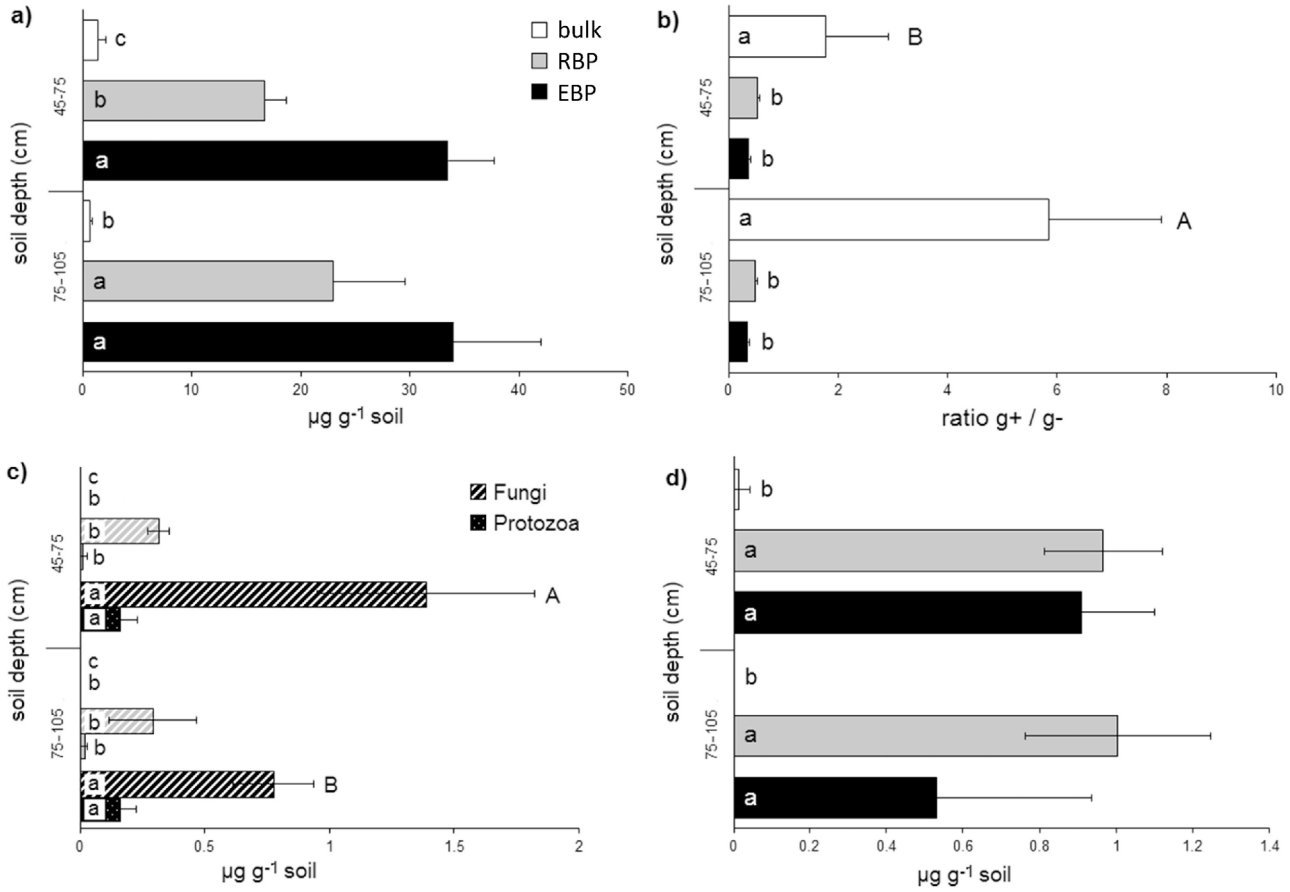

Fig. 2. a) Microbial biomass (total PLFA), b) ratio of biomarkers for gram-positive and gram-negative bacteria (a15:0, i15:0, i17:0, a17:0 and 16:1 ω 5c, 16:1ω7c, 18:1ω7c, Cy17:0), c) biomarkers for fungi (18:2ω6,9) and protozoa (20:4ω6) and d) biomarkers for actinobacteria (10Me16, 10Me18). Different uppercase and lowercase letters indicate significant differences between depth levels and soil compartments (ANOVA with Tukey-HSD, $p < 0.05$). Error bars indicate standard deviation.
RPB: root biopores, EBP: earthworm-modified biopores.

75–105 cm soil depth. Biomarkers for fungi and protozoa (Fig. 2c) were not found in bulk soil, and both parameters were significantly increased by short-term earthworm incubation as compared to RBP (factor 2.6–4.4 for fungi and 9.2–14.2 for protozoa). Biomarkers for actinobacteria (Fig. 2d) in bulk soil were present only at trace levels and only in the upper soil layer (45–75 cm). In the 45–75 cm soil depth layer there were almost equal amounts of actinobacteria in both biopore types, while at 75–105 cm, biomarkers for actinobacteria were about 1.8 times higher in RBP as compared to EBP. This increase was not significant.

Activities of enzymes related to the C-cycle (cellobiohydrolase, ß-glucosidase and xylanase) and of enzymes related to the N-cycle (chitinase, chitotriosidase, leucine aminopeptidase) were increased in EBP and partially also in RBP as compared to the bulk soil (Table 2). These effects were more pronounced in the 45–75 cm soil depth layer. While there was only one effect of soil depth in the bulk soil and depth effects in RBP were inconsistent, all enzyme activities except for chitinase were higher in the upper depth layer (45–75 cm) of EBP (Table 2).

3.3. Principal component analysis

Bulk soil, RBP and EBP were clearly distinguished by PC 1, explaining 68.3% of total variance (Fig. 3a). Additionally, RBP were separated from bulk soil and EBP by PC 2, which explained 14.6% of total variance. PC 1 loads high on C, N, CAL-P_i, microbial biomass, biomarkers for fungi and protozoa and enzyme activities such as xylanase

Table 2
Enzyme activities (Vmax) in bulk soil and the walls of different pore types. Different uppercase and lowercase letters indicate significant differences between depth levels and soil compartments respectively (one way ANOVA with Tukey-HSD, $p < 0.05$).

Soil depth	45–75 cm			75–105 cm		
Treatment	EBP[a]	RBP[b]	Bulk soil	EBP[a]	RBP[b]	Bulk soil
V_{max} Cellobiohydrolase (nmol g^{-1} MUF h^{-1})	89.0 a A	31.7 b B	16.1 c	70.7 a B	43.3 b A	12.1 c
V_{max} ß-Glucosidase (nmol g^{-1} MUF h^{-1})	1169 a A	511.1 b A	234.5 c	347.8 a B	295.3 ab B	256.8 b
V_{max} Xylanase (nmol g^{-1} MUF h^{-1})	66.6 a A	32.0 b	23.7 b	38.0 a B	27.1 b	23.1 b
V_{max} Chitotriosidase (nmol g^{-1} MUF h^{-1})	22.5 a A	7.58 b	6.11 b	15.4 a B	6.48 b	5.99 b
V_{max} Chitinase (nmol g^{-1} MUF h^{-1})	68.6 a	24.4 b	10.4 b	45.5 a	30.4 ab	13.3 b
V_{max} Leucine amino-peptidase (nmol g^{-1} AMC h^{-1})	540 a A	147 b	48.9 c B	354 a B	142 b	89.1 b A

[a] EBP: Earthworm-modified biopores.
[b] RBP: Root biopores.

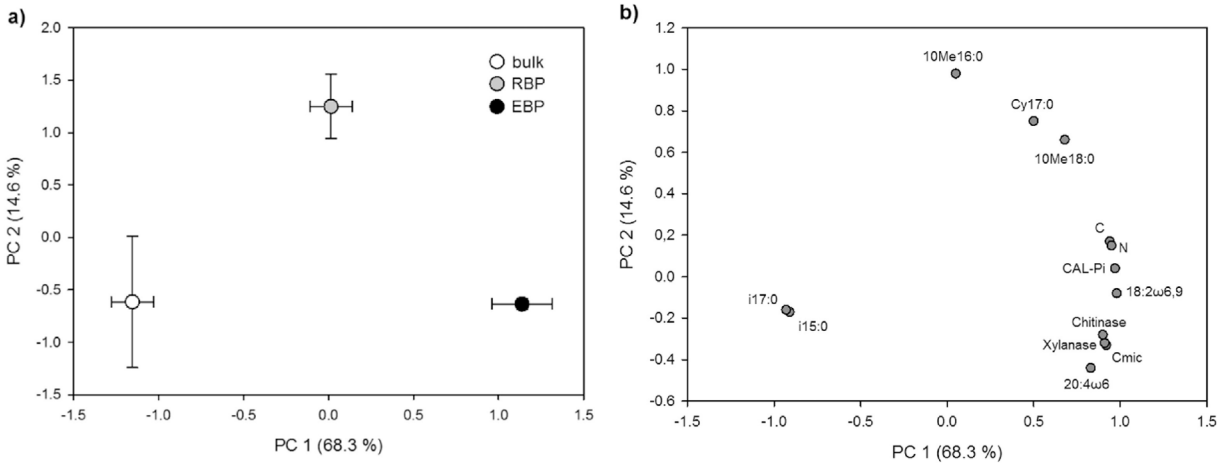

Fig. 3. a) Principal-component scores for bulk soil (open circles), root biopores (gray circles) and earthworm-modified biopores (black circles) in 45–75 cm soil depth and b) selected factor loadings of the first two principal components extracted from the dataset.

and chitinase (Fig. 3b). Negative loadings were recorded for iso-branched fatty acids. PC 2 loadings were particularly high for 10-methyl branched fatty acids.

4. Discussion

4.1. General properties of biopore walls

The increased C and N contents in pore walls vs bulk soil observed in our study are consistent with previous laboratory and field studies (e.g. Stromberger et al., 2012; Amador et al., 2003; Pankhurst et al., 2002; Tiunov and Scheu, 1999). These characteristics of biopores are obviously a result of organic material inputs by earthworms and plant roots. Accordingly, pore walls were previously found to be enriched in P, in particular in resin-P_i, $NaHCO_3$-P_i, NaOH-P_i and NaOH-P_o compared with the bulk soil (Barej et al., 2014). Also, increased microbial biomass (Stromberger et al., 2012; Tiunov et al., 2001; Tiunov and Scheu, 1999), bacterial counts (Parkin and Berry, 1999), PLFA contents (Pankhurst et al., 2002) and enzyme activities (Hoang et al., 2016; Jégou et al., 2001; Stehouwer et al., 1993; Uksa et al., 2015) in pore walls have repeatedly been reported.

4.2. Properties of root biopores

We differentiated between pores filled with roots prior to the experiment (RBP) and pores incubated with *L. terrestris* for one vegetation period (EBP). Despite RBP not being visited by earthworms for at least two years, their properties clearly differed from the bulk soil, with higher nutrient contents, microbial biomass and enzyme activity. In particular, this pore type was characterized by increased contents of the 10-methyl branched fatty acid biomarkers of actinobacteria (Figs. 2 and 3).

Actinobacteria are involved in late stages of plant residue degradation (Bernard et al., 2007; Goodfellow and Williams, 1983) and are adapted to survive at slower growth rates when resources are limited or consist of more complex organic matter (Bastian et al., 2009). Relative enrichment of these biomarkers in RBP supports the assumption that the resident microbes primarily respire old and recalcitrant plant residues.

4.3. Properties of earthworm-modified biopores

Short-term earthworm incubation resulted in import of carbon, increased enzyme activity and changes in the microbial community. *L. terrestris* removes plant residues from the soil surface and deposits organic matter on the burrow wall (Lavelle, 1988; Stromberger et al., 2012). Intake of primary organic matter into soil generally results in higher activity of enzymes related to the decomposition of polysaccharides from plant tissues (Bandick and Dick, 1999; Debosz et al., 1999; Kautz et al., 2004). Xylanase is mainly bound to particulate organic matter (Kandeler et al., 1999a,b) which is likely to be enriched in the drilosphere. Hence, it is plausible that presence of *L. terrestris* increases the activity of such enzymes in the burrow wall, as also reported by Don et al. (2008). Additionally, the composition of the microbial community in pore walls was evidently altered by the presence of *L. terrestris*: actinobacteria were reduced, as indicated by the relatively lower abundance of 10-methyl branched fatty acids, while fungal and protozoan fatty acids (i.e., 18:2ω6,9 and 20:4ω6, respectively) were relatively enriched in comparison with RBP. Both fungi and protozoa are assumed to form a substantial part of the earthworm diet (Curry and Schmidt, 2007; Edwards and Fletcher, 1988) and were previously determined to be enriched in burrow walls of *L. terrestris* (Tiunov et al., 2001). Moreover, 20:4ω6 was also found to be increased in drilosphere vs bulk soil samples (Stromberger et al., 2012). Thus, *L. terrestris* acts as an ecosystem engineer (Jones et al., 1994) by defecating and importing plant residues into the pore wall, thus creating a distinct habitat for microorganisms which in turn provide a suitable food source. The increased content of a PLFA-biomarker for fungi as a result of earthworm incubation is in line with higher activity of chitotriosidase and chitinase, possibly indicating a decomposition of chitin from fungal cell walls. These results indicate that fungal hyphae and spores may be transported downwards through biopores by earthworm activity. However, Tiunov and Scheu (1999) found a lower ratio of fungal:bacterial biomass in drilosphere samples than in the bulk soil, while Stromberger et al. (2012) reported no difference in this ratio in bulk soil and drilosphere as revealed by PLFA analysis. As explained by Stromberger et al. (2012), the reason for these contradictory results may be the complex equilibrium of fungi, bacteria, and fungivorous and bacterivorous fauna in drilosphere and bulk soil. This equilibrium is affected by earthworms via dispersal and activation, predation, habitat destruction, competition for organic matter, and production of fungicides and bactericides (Brown et al., 2004). Further research is required to determine the conditions under which earthworm activity promotes fungal or bacterial growth.

Apart from the diet, changes in PLFA contents in the burrow wall can also be related to processes in the digestive tract of *L. terrestris*. Sampedro and Whalen (2007) reported that the fatty acid pattern markedly changed in the gut of *L. terrestris* with enrichments of 16:1ω5 and 20:4ω6 in the gut content. These PLFA were also enriched in the walls of EBP.

Increased enzyme activity can also promote the mobilization of nutrients. The enrichment of total P and particularly of labile P forms in RBP vs. the bulk soil was similar to the results of Barej et al. (2014), who sampled biopores with a smaller lower diameter limit (> 2 mm) at the same site. This is plausible as smaller biopores are visited by earthworms less often than larger pores. Further significant enrichments of labile P as a result of earthworm activity suggest that walls of recently inhabited burrows can be hotspots of P acquisition by plants. This notion is supported by the finding of Kuczak et al. (2006) that earthworm casts are enriched in total P with higher proportions of P mainly in forms that are more readily extracted (resin, $NaHCO_3$, NaOH and HCl_{dil}). Moreover, Vos et al. (2014) noted that in a pot experiment the presence of *L. terrestris* increased plant growth and P uptake, but it remained questionable if this result could be applied to native, structured soils. Our results support the link between *L. terrestris* activity and P uptake by plants, as the shared burrows of *L. terrestris* and plant roots in natural conditions were significantly loaded with plant available P after earthworm incubation for a period of just six months.

4.4. Effects of soil depth

Microbial activity and availability of plant nutrients such as N and P have generally been reported to decline with soil depth (e.g., Fierer et al., 2003; Lynch and Brown, 2001). In the bulk soil from our field trial, significant decreases in the 75–105 cm depth layer were merely found for total N, total S and NaOH P_o, and significant increases were observed in the microbial metabolic quotient and the ratio of Gram positive: Gram negative bacteria. Interestingly, these depth effects were not observed in RBP, showing that these pores feature rather stable conditions throughout the soil profile and provide an attractive environment for plant roots even in greater soil depths. The effect of short-term incubation of *L. terrestris* on nutrient contents and microbial biomass was more pronounced at 45–75 than at 75–105 cm soil depth, resulting in many significant differences between both depth levels. Little is known about the in situ patterns of vertical movement of *L. terrestris*. One older study (Joyner and Harmon, 1961) indicates that earthworms tend to oscillate in rather stable day-and-night cycles between the soil surface around midnight and about 60 cm soil depth around noon. These observations coincide with our field experiment, where earthworm activity was obviously greater in the upper subsoil, although a clear impact on burrow properties was also detected in the deeper soil layers.

5. Conclusions

Subsoil biopores inhabited by roots for at least two years were enriched in microbial biomass and enzyme activity as well as N and plant available P compared to surrounding bulk soil. These hotspots turned into 'super-hotspots' with further inputs of C and nutrients, higher microbial and enzyme activities and altered microbial community composition as a consequence of colonization by *Lumbricus terrestris* – even during only one vegetation period. Both biopore properties and microbial performance in biopores are thus highly dynamic – and prone to effects of even short-term management practices that influence earthworm activity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.apsoil.2017.08.015.

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