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ORIGINAL PAPER

Biopore history determines the microbial community composition in subsoil hotspots

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Abstract Biopores are hotspots of nutrient mobilisation and shortcuts for carbon (C) into subsoils. C processing relies on microbial community composition, which remains unexplored in subsoil biopores. Phospholipid fatty acids (PLFAs; markers for living microbial groups) and amino sugars (microbial necromass markers) were extracted from two subsoil depths (45–75 cm ; 75–105 cm) and three biopore types: (I) drilosphere of *Lumbricus terrestris* L., (II) 2-year-old root biopores and (III) 1.5-year-old root biopores plus six 6 months of *L. terrestris* activities. Biopore C contents were at least 2.5 times higher than in bulk soil, causing 26–35 times higher Σ PLFAs g^{-1} soil. The highest Σ PLFAs were found in both earthworm biopore types; thus, the highest soil organic matter and nutrient turnover were assumed. Σ PLFAs was 33% lower in root pores than in earthworm pores. The treatment affected the microbial community composition more strongly than soil depth, hinting to similar C quality in biopores: Gram-positives including actinobacteria were more abundant in root pores than in earthworm pores, probably due to lower C bioavailability in the former. Both earthworm pore types featured fresh litter input, promoting growth of Gram-negatives and fungi. Earthworms in root pores shifted the composition of the microbial community heavily and turned root pores into earthworm pores within 6 months. Only recent communities were affected and they reflect a strong heterogeneity of microbial activity and functions in subsoil hotspots, whereas biopore-specific necromass accumulation from different microbial groups was absent.

Keywords Amino sugars · Biomarkers · Carbon sequestration · Carbon turnover · Detritusphere · Drilosphere · Phospholipid fatty acids

Introduction

After decades of disregard, the subsoils have only recently regained interest within soil science, despite the fact that they store approximately half of the terrestrial carbon (C) and contain pools of nutrients such as magnesium, calcium and phosphorus significant to plant nutrition (Kell 2012; Kuhlmann and Baumgärtel 1991; Rumpel and Kögel-Knabner 2011; Salome et al. 2010). Aside from dissolved C transport, large amounts of C are transported into the subsoil by earthworms and roots, i.e. in biopores (Don et al. 2008; Kautz 2015). Rooting plants and burrowing earthworms leave not just voids behind through which plants reach the deeper soil faster to explore soil resources (Ehlers et al. 1983; Han et al. 2015) but they additionally induce hotspots of increased microbial activity (Kautz et al. 2013; Nakamoto 2000). Apart from C transport, they have further functions such as soil organic matter (SOM) turnover or possibly C sequestration depending on their genesis.

Biopores make up about 1–10% of the total soil volume (Ehlers et al. 1983; Kuzyakov and Blagodatskaya 2015) and are only persistent in subsoils, i.e. below the ploughed horizon, or in topsoils which are not frequently tilled (Ehlers et al.

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1983). Large, vertical biopores reaching into the subsoils are in particular created by crops with allorhizic root systems like common chicory (*Cichorium intybus* L.) (Ehlers et al. 1983; Perkons et al. 2014). Roots deposit large amounts of C into their surroundings, which partly remain after root death, creating the rhizo-detritusphere. Alternatively, anecic earthworms, such as *Lumbricus terrestris* L., create earthworm biopores. They feed on plant residues near the soil surface and deposit residues, mucus and casts in their burrows, creating the drilosphere (Bouché 1975; Jégou et al. 1998). Root detritus and earthworm activities enrich the inner walls of biopores with C, N and P, which induces microbial growth, enzyme activities and, therefore, greater C and N turnover compared to the surrounding bulk soil (Graff 1967; Hoang et al. 2016; Jégou et al. 2001; Parkin and Berry 1999). This leads to nutrient release from SOM and from the solid phases, enhancing soil fertility (Jégou et al. 2001; van Groenigen et al. 2014; Volkmar 1996).

C input into subsoils is usually much lower than into topsoils (Hafner and Kuzyakov 2016; Rumpel and Kögel-Knabner 2011). It is more and more questioned if subsoil C turnover is governed by the very same mechanisms as topsoil C turnover—since environmental and soil conditions are rather different in subsoils (Salome et al. 2010; Sanaullah et al. 2011; von Luetzow et al. 2006). Even though biopores are thought to be the main locations of C turnover in the subsoil, little is known about these hotspots (Kuzyakov and Blagodatskaya 2015). For climate change mitigation, it is desirable to sequester C in subsoils through biopores, e.g. by deep rooting plants or deep burrowing earthworms (Kell 2012). Prior to this, the role and relevance of biopores for C turnover and sequestration, particularly in the subsoil, need to be clarified. The importance of the microbial community composition for C turnover is frequently mentioned in the literature, but its investigation has received surprisingly little attention (Fierer et al. 2003; Schmidt et al. 2011; Struecker and Joergensen 2015). The links between the microbial community composition and C turnover are not straightforward, and microbial activity may be more important than diversity (Nannipieri et al. 2003). Nevertheless, the microbial community composition influences the enzyme activities (Waldrop et al. 2000). So, functional microbial groups, such as fungi or Gram-positives, and their residues are key to assess the relevance of subsoil biopores for short- and long-term C turnover. We characterised both by the following biomarkers:

1. Phospholipids are parts of microbial cell membranes and quickly decomposed after cell death, thus accounting for the living microorganisms (Frostegård and Bååth 1996; Zelles 1999). Microbial group-specific fatty acids in the phospholipids (PLFAs) allow broad characterisation of the microbial community with some limitations (Zelles 1999), and the total PLFA content (∑ PLFAs) is a proxy of the living microbial biomass (Frostegård et al. 1991).
2. Amino sugars make up microbial cell walls and are more persistent to decomposition as their polymers need to be broken down first and the resulting amino sugars are likely stabilised in soil (Amelung 2001, 2003; Glaser et al. 2004; Lauer et al. 2011; Miltner et al. 2012). Thus, they reflect mainly microbial necromass (Glaser et al. 2004; Glaser and Gross 2005; Parsons 1981). Prokaryotic bacterial cell walls consist of peptidoglycan, a polymer of *N*-acetylglucosamine (GlcN) and *N*-acetylmuramic acid (MurAc), while fungi produce chitin, an (*N*-acetyl) glucosamine polymer, and galactosamine (GalN) (Amelung 2001; Engelking et al. 2007; Glaser et al. 2004). The ratios of amino sugars to MurAc are used to qualitatively assess long-term changes in the microbial community composition (Glaser et al. 2004).

This work aims at better describing subsoil hotspots and their heterogeneity in situ by characterising functional microbial groups. We hypothesised that different biopore types featured deviating abiotic (e.g. water fluctuations, pH) and biotic factors (e.g. C content and C quality) causing a strongly different microbial community composition. However, our study mainly focussed on biotic controls to link C dynamics with microbial community composition. We assumed that the frequent C input by earthworms would lead to microbial communities adapted to abundant fresh C, i.e. mainly enrichment of Gram-negatives (Bird et al. 2011; Gunina et al. 2014; Treonis et al. 2004), while the one-time C input by roots would promote communities of more complex SOM degraders, i.e. mainly Gram-positives including actinobacteria (Kramer and Gleixner 2008). Furthermore, we hypothesised that the necromass pattern would reflect the recent community pattern. Biopore wall coatings were sampled from root pores, earthworm pores and their combination in the subsoil and were analysed for broad taxonomic groups of microorganisms (PLFAs) and microbial residue composition (amino sugars).

Material and methods

Study site and sampling design

The study site was the Campus Klein-Altendorf experimental research station near Bonn, Germany. The site is characterised by a maritime climate with temperate humid conditions (9.6 °C mean annual temperature, 625 mm annual precipitation). The soil type is a Haplic Luvisol (Hypereutric, Siltic) developed from loess, resulting in a loamy soil with a high silt content (IUSS Working Group WRB 2008). C contents of the bulk soil were 0.41 ± 0.02% and 0.35 ± 0.05% for the 45–75 and the 75–105 cm layers, respectively. The soil was comprehensively described by Vetterlein et al. (2013). These layers were chosen according to the ploughing depth and our

definition of the subsoil, i.e. the soil below the ploughed (Ap) horizon. In this field, the ploughing depth was 30 cm and we added another 15 cm to safely exclude any effects related to ploughing.

The preparation for the experiment started in 2009: common chicory (*Cichorium intybus* L., var. Puna) was grown for three consecutive years (2009–2012), creating many roots in the subsoil. In 2012, the topsoil down to 45 cm depth was removed and different biopore types were induced by the experimental setup: old root pores, earthworm-incubated root pores and native earthworm pores (Fig. 1).

1. *Old root pores:* after excavating the topsoil in 2012, transparent plastic films were put onto the soil surface prepared in 45 cm depth. The locations of only fresh and live chicory roots ≥5 mm were manually mapped on plastic films by a permanent marker. Large nails were also pushed into the soil—marking the positions of the plastic films, which were then taken off the surface. The topsoil was filled back and the plots were kept fallow, i.e. weeds were manually removed, until the sampling in autumn 2014 to allow the decay of the chicory roots. For the sampling, the topsoil was removed again and the plastic films were put back onto the soil surface in 45 cm depth and aligned to match the locations of the nails. This allowed relocation of the now decayed roots. Since the last C input was the plant roots and the last input predominantly drives the microbial community differentiation, the simplified term *root pores* is used herein. Although these pores did not always contain visible root tissue anymore, their environment can be described as detritusphere as this pore type showed enrichment in suberin and lignin (data not shown).
2. *Earthworm-incubated root pores*: in spring 2014 after 1.5 years of fallow and root decomposition, per replicate more than 25 pores, which previously contained chicory roots, were incubated with tagged earthworms (*Lumbricus terrestris* L.). For the tagging, red elastomer tags were injected into the earthworm bodies (Butt and Lowe 2007). The incubation was performed by placing tubes (8 mm diameter) containing the tagged earthworms onto the pores' opening at 45 cm depth, adding the topsoil again, and then removing the tube, thus creating a void. Earthworms were fed for 6 months with clovergrass put on the soil surface until sampling of the biopores in autumn 2014. The number of earthworms incubated was chosen to be comparable to the native earthworm abundance. The pore history is well known: only 1.5-year-old root channels of chicory were incubated with earthworms of one species and fed with known food sources. Only pores were selected from which the tagged earthworms were expelled in 45 cm depth. Thus, this pore type's full description is 'root biopores incubated with earthworms for six months', or in short, *earthworm-incubated pores*.
3. *Native earthworm pores* were treated similarly to the incubated earthworm pores, i.e. the sites were kept fallow from 2012, grass-clover litter was regularly added to the soil surface for 6 months from spring 2014 and they were expelled in autumn 2014. For this, a horizontal soil surface was prepared in 45 cm depth and covered with plant litter for three full days. Pores with visible earthworm middens were considered colonised with native earthworms. We assumed this pore type to be representative of the average native earthworm population in this field. Despite the large majority of earthworms identified being *L. terrestris*, colonisation by different earthworm species cannot be fully excluded.
4. *Bulk soil* samples, i.e. soil not containing any biopores, were taken from the sides of the profile wall.

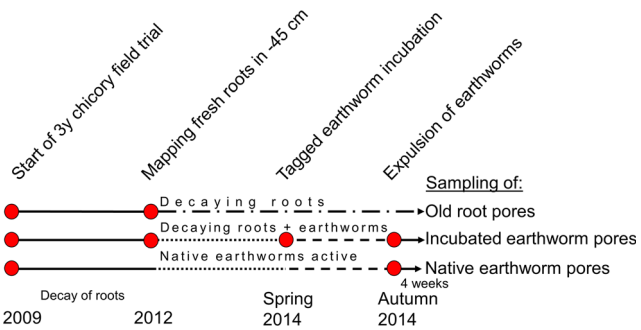

Fig. 1 Timeline of the experiment. Chicory was grown for three consecutive years (2009–2012), followed by 2 years during which the three biopore types differentiated

In September 2014, a trench was dug next to the plots with an excavator to facilitate sampling. The location of each pore opening on the soil surface in 45 cm was labelled with a tiny flag, and the soil around the pore was manually removed down to 75 cm. Each pore was opened vertically with a knife, and samples were taken by carefully shaving off the inner wall coating using microspatulas (Andriuzzi et al. 2013). Only pores with a minimum diameter of 4 mm were selected. Thirty-two samples were taken: four replicates were taken from each of the four treatments (three biopore types; bulk soil) and from two subsoil depths (45–75 cm; 75–105 cm). Sample material for each treatment combination was pooled from about 25 pores. All samples were stored at 5 °C until PLFA extraction within 3 weeks. Sample material not required for the PLFA analysis was then dried at 60 °C for 48 h to determine the soil moisture and amino sugar contents.

Phospholipid fatty acid analysis

Phospholipids were extracted by a method modified after Frostegård et al. (1991). All chemicals were of at least p.a. grade and obtained from Sigma-Aldrich, Munich, Germany. Prior to extraction, 25 μg of the first internal standard (IS 1) phosphatidyl cholinedinonadecanoic acid (Larodan, Sweden) was added to each sample, and additionally for the neutral lipid fraction, 25 μg of dodecanoic acid triglyceride (1 μg μl^{-1}; Sigma-Aldrich, Munich, Germany). About 3.5 g fresh pore wall material and 6 g of bulk soil were extracted twice with a solution of methanol, chloroform and citrate/KOH buffer (pH 4, *v*/*v*/*v* = 1:2:0.8) (Bligh and Dyer 1959). Following purification of phospholipids by solid phase extraction (SPE), derivatisation to fatty acid methyl esters (FAMEs) was by hydrolysation by NaOH in MeOH for 10 min at 100 °C and subsequent methylation by BF_3 in MeOH (~1.25 M) at 80 °C for 15 min. Samples were transferred to autosampler vials after adding 15 μg of the second internal standard (IS 2; 1 μg μl^{-1}) tridecanoic acid methyl ester and measured by the GC-MS system (GC5890 with MS 5971A, Agilent, Waldbronn, Germany) equipped with a 45 m DB5-MS column (5%-Phenyl)-methylpolysiloxane, 0.25 mm I.D., 0.25 μm film thickness; Agilent, Waldbronn, Germany). Stock solutions containing external standards of 27 fatty acids and IS 1 with contents of 1, 4.5, 9, 18 and 24 μg were derivatised and measured together with the samples. The relation between the integrated peak area of each FAME and the peak area of the IS 2 was calculated. Calibration lines were determined by a linear regression from the external standard substances at five different concentrations. The quantifications of each FAME considered the losses during the sample preparation, which were corrected for by the recovery of the IS 1. The GC parameters were as follows: the injection was splitless, the inlet temperature was set to 270 °C and the detector temperature to 280 °C. Column head pressure was kept constant at 0.79 bar. The initial oven temperature was 80 °C, held for 1.5 min, then increased at 10 °C min^{-1} to 167 °C and further at 0.7 °C min^{-1} to 196 °C, and finally at 10 °C min^{-1} to 300 °C and held for 8 min. The MS parameters were scan mode, *m*/*z* 50–550 and 1.5 cycles per second.

Single fatty acids are assigned to broad microbial groups, but the relationship between the groups and the fatty acids may not be 100% accurate, e.g. because the classification of marker fatty acids to taxa comes from pure culture studies (Zelles 1999). Thus, redundancies and mismatches, e.g. due to changing environmental conditions, might occur and only cultivatable taxa are used for the classification (Frostegård et al. 2011). Briefly, branched PLFAs represent Gram-positive, while monounsaturated PLFAs mostly represent Gram-negative bacteria. Actinobacteria produce 10-methyl-branched PLFAs, whereas polyunsaturated PLFAs represent eukaryotes and the PLFA 18:2ω6,9 fungi (Drenovsky et al. 2004; Fierer et al. 2003; Frostegård and Bååth 1996; Harwood and Russell 1984; Zelles 1997).

Neutral lipid analysis

During the PLFA purification, the neutral lipid fraction was collected from the SPE columns using 5 ml of chloroform and subsequently derivatised like the PLFA samples. The PLFA 16:1ω5 represents arbuscular mycorrhiza fungi (AMF) but it may also be derived from Gram-negative bacteria. Therefore, Olsson (1999) suggested that the ratio of the storage lipid NLFA 16:1ω5 and the phospholipid PLFA 16:1ω5 is a more sensitive indicator for AMF. A ratio of PLFA/NLFA <1 indicates Gram-negative origin of the PLFA 16:1ω5, while PLFA/NLFA >1 is indicative for AMF.

Total bacterial biomass and fungal/bacterial biomass ratio

The total bacterial biomass was calculated as the sum of all bacterial PLFAs. The ratio of PLFA 18:2ω6,9 to bacterial PLFAs represents the fungal/bacterial biomass ratio in soils (Frostegård and Bååth 1996).

Amino sugar analysis

Amino sugars were extracted by a method modified after Zhang and Amelung (1996). All chemicals of at least p.a. grade were obtained from Sigma-Aldrich, Munich, Germany. About 450 mg of each dried and ground soil sample, containing ~0.3 mg N, were subjected to hydrolysis by 6 M HCl under N_2 atmosphere for 8 h at 105 °C, filtration through glass fibre filters (Whatman GF6, GE Healthcare, Pittsburgh, PA, USA) and drying by a rotary evaporator to remove the remaining acid. One hundred micrograms of methylglucamine (MeGlcN) were added as the IS 1 after neutralisation. For the removal of iron and salts, the pH was adjusted to 6.6–6.8 by KOH and samples were centrifuged at 2000×*g* for 15 min. The supernatant was taken and lyophilized. Amino sugars were extracted from this by anhydrous methanol. Derivatisation to aldononitrile acetates was by the derivatisation reagent 32 mg ml^{-1} hydroxylamine hydrochloride and 40 mg ml^{-1} 4-(dimethylamino) pyridine in pyridine-methanol (4:1 *v*/*v*) for 30 min at 75–80 °C. Samples were then reheated for 30 min after adding 1 ml of acetic anhydride. Excess derivatisation agents were removed by three washing steps after addition of 2 ml dichloromethane, first by 6 M HCl and subsequently twice by 1 ml of deionised water. The organic phase was then dried under N_2 and dissolved in 185 μl of ethyl acetate-hexane (1:1), and 15 μg of the IS 2 tridecanoic acid methyl ester (1 μg μl^{-1}) in ethyl acetate-hexane (1:1) were added. Compounds were separated gas chromatographically on a 30 m OPTIMA® 17 column (phenylmethyl polysiloxane, 50% phenyl, 0.25 mm I.D.,

0.50 μm film thickness; Macherey-Nagel, Dueren, Germany) followed by flame ionisation detection (GC-FID system Agilent GC7820A, Waldbronn, Germany). The split ratio was set to 1:10, injector temperature was 250 °C, the detector temperature was 300 °C and the column flow was kept constant at 1.1 ml min^{-1}. The oven temperature programme was set as follows: initial temperature was 120 °C, held isothermal for 1 min, then increased at 5 °C min^{-1} to 250 °C, held for 2 min and increased at 10 °C min^{-1} to the final temperature 280 °C, which was held for 10 min. Peak identification was performed by analysing retention times of single amino sugar standards. Stock solutions of external standards of the amino sugars GlcN, GalN, MurAc and MeGlcN containing amounts of 25, 50, 125, 250 and 500 μg were derivatised and measured together with the samples. The relation between the peak area of each amino sugar and the peak area of the IS 2 was calculated. By a linear regression of five external standards' peak areas and their concentrations, analytes were quantified. The recovery rate was determined based on the peak area of the IS 1 and applied to the quantifications of the amino sugars.

C and N contents and $\delta^{13}C$ determination

For the analysis of C and N contents and $\delta^{13}C$ values, 40–50 mg of dried and ground sample were filled into 12 mm tin capsules (IVA, Meerbusch, Germany). The samples were measured on the FLASH 2000 CHNS/O Elemental Analyser (Thermo Fisher Scientific, Cambridge, UK) coupled by a ConFlo III interface to the Delta V Advantage isotope ratio mass spectrometer (both Thermo Fisher Scientific, Bremen, Germany). $\delta^{13}C$ may act as a proxy for SOM quality, as during SOM decomposition, $\delta^{13}C$ increases as ^{12}C is preferentially lost (Werth and Kuzyakov 2010).

Statistical analyses

Outliers between field replicates were identified using Nalimov's test (Lozán and Kausch 1998). No more than one replicate was removed by this outlier test. In case only three values were available, no outlier test was carried out. Through factor analysis, microbial groups of similar statistical behaviour according to factor loadings (>0.7) and algebraic sign were determined based on the normalised dataset. Thus, ubiquitous and plant-derived PLFAs were excluded from the statistical analysis. One-way analyses of variance (ANOVA) were carried out for each depth, and significances were calculated by Tukey's Honest Significant Difference test on the $\alpha < 0.05$ level. Levene's test was used to test for homogeneous variances. Normality of the residues was visually checked in Q-Q plots. No bulk soil data were included in the ANOVA as the assumptions were not met due to missing normal distribution of residues and missing data, so only trends regarding bulk soil were reported. All data were given as percent of Σ PLFAs, except for the two summative parameters Σ PLFAs per gram of soil and Σ PLFAs per gram of soil organic carbon (SOC). PLFA contents were normalised to SOC to express the microbial colonisation of the organic matter. Pairwise *t* tests for dependent samples were used to determine differences between soil depths within each pore type. Error bars in all charts were calculated as standard errors of means (SEM). The contributions of the factors pore type, depth and their interactions to the total variance were calculated by dividing the factor's type III sum of squares by the total sum of type III sum of squares.

Constrained redundancy analysis (RDA) was performed on the relative abundances of the PLFA dataset showing statistically relevant behaviour in the factor analysis, and the three explanatory environmental variables TOC, TON and $\delta^{13}C$. Response scores are reported herein as weighted average scores and type I scaling plots are shown. The RDA was performed in Addinsoft XLSTAT 2015 (Addinsoft SARL, Paris, France).

For the analysis of similarities (ANOSIM), the PLFA dataset showing statistically relevant behaviour was taken. ANOSIM tests if datasets are significantly different in their species composition, i.e. the PLFA fingerprints as markers for microbial groups. A Bray-Curtis similarity matrix was calculated, which was then used to calculate the non-parametric ANOSIM. *p* values reported herein are Bonferroni-corrected sequential *p* values. For this analysis, PAST 3.08 was used (Hammer et al. 2001).

For the amino sugar data set, non-parametric Kruskal-Wallis ANOVAs were calculated for each depth due to missing homoscedasticity, followed by post hoc comparisons of mean ranks of all pairs of groups (Kruskal-Wallis test). Error bars reported are SEM. Wilcoxon matched pair tests for dependent samples were used to determine differences between soil depths within each pore type. Unless otherwise specified, all statistical analyses were performed in Statsoft Statistica version 12.5 (StatSoft Inc., Tulsa, OK, USA).

Results

Σ PLFAs per gram of soil was similar between the two soil depths, indicating constant microbial biomass irrespective of depth (Table 1; Online Resource 1). This was also true for Σ PLFAs per gram of SOC (Table 1; Fig. 2). There was a close correlation between Σ PLFAs and the SOC content ($R^2 = 0.85$, $p < 0.001$). Earthworm-influenced pores, i.e. root pores after 6 months of earthworm presence (2.89 ± 0.18 mg g^{-1} SOC) and native earthworm pores (3.14 ± 0.31 mg g^{-1} SOC), showed ~33% higher PLFA amounts in 45–75 cm than root pores (2.05 ± 0.09 mg g^{-1} SOC) and ~8.5 times higher PLFA amounts than bulk soil (0.36 ± 0.08 mg g^{-1} SOC) (Fig. 2). Significant differences were mainly found between biopore

Table 1 Summary of the PLFA data, including complimentary data

	Root pores		EW-incubated pores		Earthworm pores		Bulk soil	
	45–75 cm	75–105 cm	45–75 cm	75–105 cm	45–75 cm	75–105 cm	45–75 cm	75–105 cm
Total organic carbon (%)	0.81 ± 0.03b	0.93 ± 0.06A	1.16 ± 0.04c	1.07 ± 0.04A*	1.17 ± 0.05c	1.05 ± 0.04A	0.41 ± 0.02a	0.35 ± 0.05B*
Total organic nitrogen (%)	0.09 ± 0.00b	0.10 ± 0.01A	0.12 ± 0.00c	0.11 ± 0.01A	0.11 ± 0.00c	0.10 ± 0.01A	0.06 ± 0.00a	0.05 ± 0.00B*
C/N	8.6 ± 0.2a	9.7 ± 0.5A	9.6 ± 0.1b	9.9 ± 0.4A	10.3 ± 0.2c	10.3 ± 0.5A	7.2 ± 0.1d	7.7 ± 0.9A
$\delta^{13}C$ (‰)	−25.66 ± 0.46ab	−23.87 ± 1.17A	-26.47 ± 0.21a	−25.55 ± 0.51A	−25.30 ± 0.25ab	−23.76 ± 0.66A	−25.00 ± 0.05b	−23.51 ± 0.75A
Σ PLFAs (μg g^{-1} soil)	16.65 ± 1.04a	23.02 ± 3.30A	33.51 ± 2.13b	25.48 ± 9.11A	36.61 ± 3.43b	26.42 ± 3.85A	1.09 ± 0.46[b]	0.70 ± 0.12[b]
Σ PLFAs (mg g^{-1} SOC)	2.05 ± 0.09a	2.20 ± 0.02A	2.89 ± 0.18b	3.24 ± 0.59A	3.14 ± 0.31b	2.57 ± 0.45A	0.36 ± 0.08[b]	0.197 ± 0.04[b]
Σ bacterial PLFAs (mg g^{-1} SOC)	1.09 ± 0.05a	1.35 ± 0.11A	1.45 ± 0.10b	1.69 ± 0.32A	1.56 ± 0.14b	1.31 ± 0.24A	0.12 ± 0.06c	0.03 ± 0.01B
Fungal/bacterial biomass (%)	3.6 ± 0.3a	2.3 ± 0.5A	8.2 ± 0.9b	4.4 ± 0.1A	4.9 ± 1.7ab	4.1 ± 1.2A	0.0 ± 0.0c	[a]
(NLFA/PLFA) 16:1ω5	6.56 ± 0.75a	5.03 ± 0.85A	3.30 ± 0.32b	2.85 ± 0.69A	3.23 ± 0.34b	3.10 ± 0.53A	[a]	[a]

Mean values (±SEM) are given. Different letters indicate statistically significant differences. Lowercase letters indicate parameters from 45 - 75 cm, while uppercase letters indicate parameters from 75-105 cm. Differences between soil depths were significant on $^{*}p < 0.05$—given next to the lower depth letters

[a] Too many missing data

[b] Not tested, assumptions not met

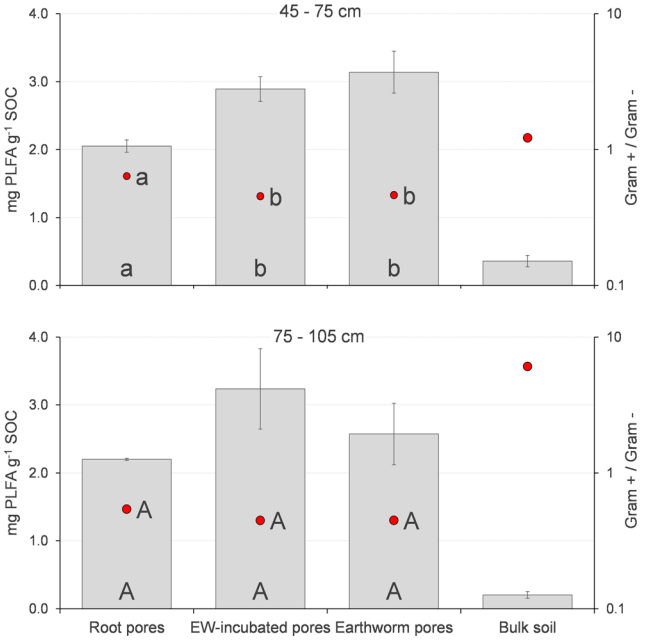

Fig. 2 The distribution of Σ PLFAs per unit SOC in each biopore type (root pores, earthworm-incubated pores, native earthworm pores) and the bulk soil in two subsoil depths (four samples × four treatments × two depths) and ratios of Gram-negative to Gram-positive PLFAs (*top*: 45–75 cm, *bottom*: 75–105 cm). *Bars* show Σ PLFAs (*left vertical axis*), while *red circles* show the ratios of Σ Gram-positive/Σ Gram-negative PLFAs (*right vertical axis*, note the logarithmic scale). Mean values (±SEM) are given. *Letters* indicate significant differences between pore types in each depth ($p < 0.05$). Differences between soil depths were not significant. Σ PLFAs per gram of SOC in the three pore types (root pores, incubated pores, native earthworm pores) was in 45–75 cm 7.5 times, and in 75–105 cm 13.5 times higher than in bulk soil. Pores with earthworms showed higher Σ PLFAs per gram of SOC than root pores and bulk soil

types in 45–75 cm, indicating that pore genesis gets less relevant with depth when gradients to bulk soil increase. However, the Σ PLFAs per gram of SOC increased with depth in root pores and the earthworm-incubated pores (2.2 ± 0.02 mg g^{-1} SOC and 3.24 ± 0.59 mg g^{-1} SOC). Biopores in both soil depths had on average 7.5–13.5 times higher Σ PLFAs per gram of SOC than bulk soil (Fig. 2).

Microbial community composition

Grouping of PLFAs to functional microbial groups was achieved by combining factor analysis of the PLFA contents and literature data (Apostel et al. 2013; Gunina et al. 2014). Mean values of total bacterial biomass followed the pattern of Σ PLFAs not showing any differences between pore types in 75–105 cm (Table 1). Two distinct groups of Gram-positive bacteria (based on a15:0; i15:0 and i17:0) and one group of actinobacteria (10Me16:0 and 10Me18:0) were identified (Fig. 3). Both Gram-positive groups were predominantly found in root pores, i.e. microhabitats characterised by low amounts of available C. Based on the signature fatty acids

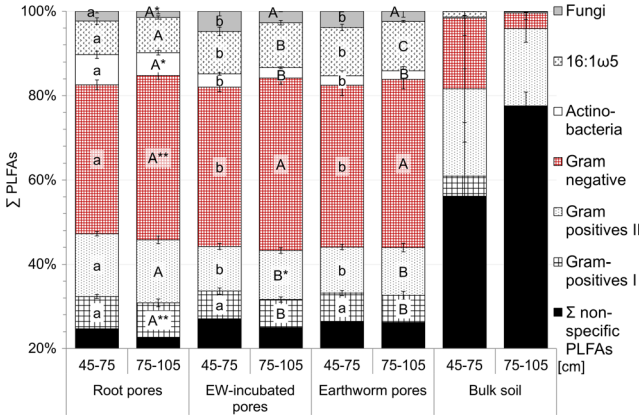

Fig. 3 Microbial communities in the three biopore types and bulk soil from two subsoil depths (four samples × four treatments × two depths): note the truncated *y-axis*. Mean values of percentage of Σ PLFAs (±SEM) are given. *Letters* indicate significant differences between pore types in each depth ($p < 0.05$). *Lowercase and uppercase letters* indicate the 45–75 and 75–105 cm depths, respectively. Differences between soil depths were significant on $*p < 0.05$ and $**p < 0.01$—given next to the *lower depth letters*. Gram-positives I and Gram-positives II are both Gram-positive groups, which showed different behaviour in the factor analysis. Root pores featured enrichment of Gram-positives and actinobacteria, whereas both earthworm pore types showed enrichment of Gram-negatives and saprotrophic fungi. Note the very high contribution of non-specific PLFAs to bulk soil samples

16:1ω7c, 18:1ω7c and cy17:0, one group of Gram-negative bacteria was identified (Fig. 3). In contrast to the Gram-positives, this group was enriched in 45–75 cm in both earthworm pores types compared to root pores and bulk soil (Fig. 3). All three pore types featured a trend towards higher Gram-negative abundance in 75–105 cm compared to 45–75 cm. Earthworm-influenced pores contained higher amounts of saprotrophic fungi (18:2ω6,9) compared to root pores (Fig. 3). For all biopores, fungal abundances decreased with soil depth, which was also represented by the corresponding fungal/bacterial biomass ratios (Table 1). The biomarker PLFA 16:1ω5 had generally lower contents in root pores compared to the other biopores (Fig. 3). However, ratios of NLFA/PLFA 16:1ω5 indicated the highest arbuscular mycorrhiza fungi contribution in root pores (~5.0–6.5, Table 1), and lower AMF contribution to the 16:1ω5 fatty acid in earthworm-influenced pores (~3). Higher Gram-negative abundance in earthworm-influenced pores explained the higher 16:1ω5 contents there.

The analysis of similarity (Table 2) showed that the microbial community fingerprints of both earthworm biopores were not different from each other, but different to the root pore fingerprint. Bulk soil showed no deviation in community composition from the three pore types. The community differentiation was also discernible in the constrained redundancy analysis (Fig. 4), which explained 48% of the inertia. A strong scattering of the bulk soil community data in the redundancy analysis indicates that bulk soil communities were affected by various biotic and abiotic factors and obviously in some cases also by macroscopically non-visible biopores (Fig. 4). It also clearly showed that the depth affected the PLFA composition in bulk soil much more strongly than in the biopores. The depth effect was almost eliminated from the biopores. Both earthworm pore types were overlapping, indicating a high degree of similarity. The variability of each biopore type was smaller than the bulk soil's variability. The three biopores combined variability was also smaller than the bulk soil's. Comparing the constrained RDA with an unconstrained principal components analysis (Online Resource 4), the grouping improved considerably. The *x*-axis of the RDA, defined by C and N contents, clearly separated biopores from bulk soil. The *y*-axis defined by $\delta^{13}C$, a proxy for SOM quality, separated the earthworm pores from the root pores.

Variance partitioning showed that most variance (>60%) of all microbial groups, except Gram-negative and fungi, was explained by the pore type and not by soil depth (Fig. 5).

Amino sugars

Total mean amino sugar contents among all treatments were for the 45–75 cm depth 1179 ± 183 μg g^{-1} soil, and for 75–105 cm 1673 ± 214 μg g^{-1} soil, i.e. an increase of 42% with depth (Table 3; Online Resource 2). Both amino sugar ratios GalN/MurAc and GlcN/MurAc showed no different patterns among biopores (Fig. 6a, b) and gave smaller fungal/bacterial necromass ratios with depth for earthworm and root pores and bulk soil. Muramic acid contents were similar among the biopore types (Table 3). Highest bacterial contribution to the necromass was in bulk soil and earthworm-incubated pores, whereas root pores and earthworm pores showed the highest fungal contribution to the necromass (Fig. 6a, b).

Discussion

The decisive factors for the microbial community composition were the biopore history and the biopore properties (Fig. 5). We assumed that the heterogeneous C inputs of varying frequency (root detritus vs. digested shoot biomass of clovergrass) in the pore types have mainly driven the community development (Fig. 3) and that abiotic soil factors like the texture (Sleutel et al. 2012), pH (Rousk et al. 2010) and moisture (Chen et al. 2007) have likely contributed to this community differentiation. Earthworms and roots strongly increased the C contents in the biopores, which led to 26–35 times higher PLFA abundances than in bulk soil (Table 1). This corresponds to a larger living microbial biomass and higher activity (Hoang et al. 2016), which are often linked to increased SOM decomposition rates and C turnover. In contrast, very low PLFA contents in bulk soil indicate lower C turnover but higher mean residence times (Don et al. 2008).

Table 2 ANOSIM results

		45–75 cm				75–105 cm			
		Root pores	EW-incubated pores	Earthworm pores	Bulk soil	Root pores	EW-incubated pores	Earthworm pores	Bulk soil
45–75 cm	Root pores								
	EW-incubated pores	*							
	Earthworm pores	*							
	Bulk soil								
75–105 cm	Root pores		*	*	*				
	EW-incubated pores	*					*		
	Earthworm pores	*					*		
	Bulk soil	*	*	*	*		*	*	*

Values reported are Bonferroni-corrected sequential p values based on 9999 permutations on the Bray-Curtis similarity matrix of the PLFA fingerprint. Differences between pores were significant on $*p < 0.05$. Both earthworm types were similar. Both were, however, different from root pores

The root pore community was unique to the communities of both earthworm pore types (ANOSIM, Table 2), which was also supported by their lower PLFA contents (Fig. 2). Both earthworm pore type fingerprints were not different from each other, suggesting that earthworm activity was the strongest factor for the microbial community composition in the pore walls (Table 2). Especially the earthworm gut taxa influence the microbial composition rather strongly in the casts (Brown 1995; Sampedro and Whalen 2007). Six months of earthworm activities have been long enough to turn a root pore into an earthworm pore regarding the microbial community composition. This is also supported by the overlapping of the native earthworm and the earthworm-incubated pores in the RDA plot (Fig. 4). In this experiment, this pore type was specifically designed to assess the effect of short-term earthworm activity in old root pores.

The PLFA fingerprints remained constant with depth (Table 2), indicating that depth is a minor factor for the microbial community composition in continuous pores due to root detritus and earthworm activities throughout the biopores. The contrast between pores and bulk soil increased with soil depth, as pore PLFA contents remained constant but bulk soil contents decreased with depth. This emphasises the importance of such hotspots, especially in the deeper subsoil. Very comparable findings for the bulk soil and native drilosphere using 16S rRNA gene fingerprinting were reported for the same chicory-planted soil (Uksa et al. 2014). Such mutual validation underlines the power of the PLFA analysis, even though it comes with some pitfalls and uncertainties (Frostegård et al. 2011). The bulk soil PLFA fingerprint was not statistically different from the biopores. This might be explained by the high variability of the bulk soil, especially

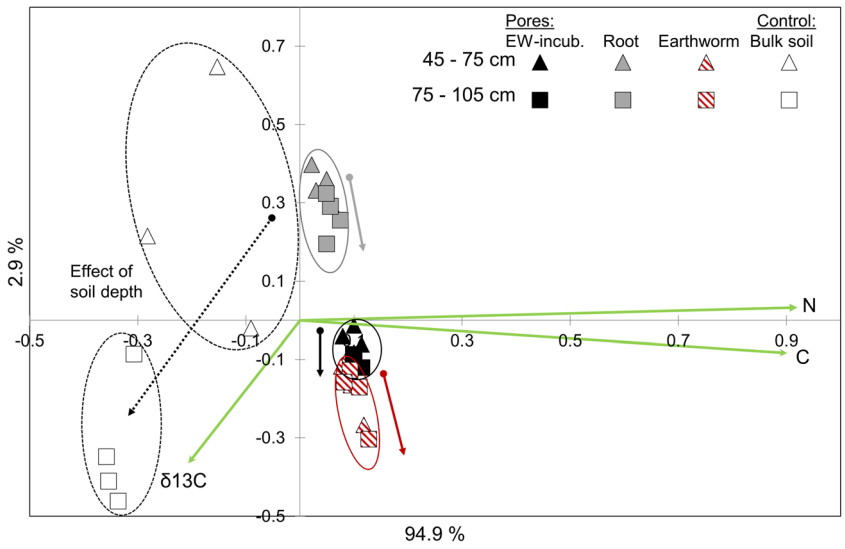

Fig. 4 Constrained redundancy analysis on the PLFA fingerprints from Fig. 3. Response scores were calculated as weighted average scores. The overall RDA was significant based on 9999 permutations. A type I scaling (distance) plot is shown. *Green vectors* illustrate the explanatory variables C, N and $\delta^{13}C$. *Arrows* illustrate the depth effects within a pore type. Forty-eight percent of the inertia was explained by the soil parameters C and N content, and $\delta^{13}C$. Fifty-two percent was explained by other factors (unconstrained) (color figure online)

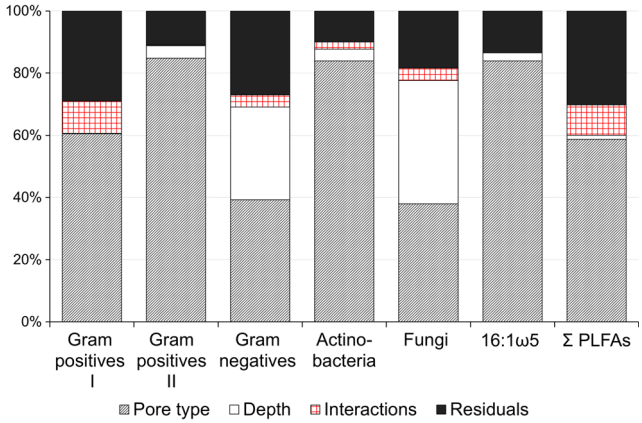

Fig. 5 Contribution of the factors soil depth, pore type and their interactions to the total variance of microbial community composition. Bulk soil data was not included, i.e. in total 24 samples were analysed. The contribution of the factors and their interactions to the total variance was calculated by dividing the factor's type III sum of squares by the total sum of type III sum of squares. Most variance (40–85%) of microbial groups is explained by the pore type. Gram-negatives and fungi are also influenced by soil depth

in 45–75 cm. This high variability might also be caused by small, non-visible biopores in the bulk soil subsample, which may not have been 100% excluded, as compared to the lower subsoil layer. Additionally, due to the C inputs—which are pore-specific, but partly similar—certain microbial groups preferentially grew in pores. Thus, pores can be differentiated from each other, but they are not necessarily different from bulk soil regarding their community composition.

Irrespective of the reasons for this, biopores increase or decrease variability depending on the scale: they increase the overall ecological variability in soil (Ehlers et al. 1983; Stromberger et al. 2012), but among biopores, it is considerably lower and even lower within one biopore. Therefore, different types of biopores presumably increase habitat diversity (as a function of substrate quality, input frequency, moisture, texture, aggregation or pH)—even if individual pores are along their vertical axis less diverse. Vertical variability of one biopore is rather low, as the defining factors, e.g. C quality, oxygen availability (Gliński and Lipiec 1990) and moisture controlling their properties remain rather constant along the biopore. The variability between biopores and bulk soil increases with soil depth, as the bulk soil's variability decreases while the pore's properties remain constant (Zhou et al. 2002). This increased variability is linked to higher resilience, a classic ecosystem property—in this case, attributed to soils (Ponge 2015). As biopores can be reused, different subsequent crops may cause further increased variability in e.g. C quality. Likewise, earthworms facilitate the introduction of species from the soil surface into the subsoil.

Multivariate statistics considerably improved the grouping of the principal component analysis (Online resource 4) as soon as the explanatory variable $\delta^{13}C$ was included in the analysis (Fig. 4). $\delta^{13}C$, a proxy accounting for SOM quality and related to turnover and decomposability, separated the upper bulk soil from the lower bulk soil reflecting the increased C processing with depth. However, also the lower bulk soil was clearly separated from the pore habitats by the RDA along the $\delta^{13}C$ vector suggesting a difference in C quality between biopores and bulk soil in 75–105 cm (Dorodnikov et al. 2007; Gunina and Kuzyakov 2014). Thus, the differences in microbial community composition between bulk soil and biopores also depend on SOM decomposability. A clear separation was also visible between root and earthworm-influenced pores which was partially along the $\delta^{13}C$ vector, i.e. explained by SOC quality. However, as the second axis describes a much lower proportion of the variance, this effect

Table 3 Summary of the amino sugar data

	Root pores	EW-incubated pores	Earthworm pores	Bulk soil	
45–75 cm (μg g^{-1} soil)					
Glucosamine	727 ± 305A	729 ± 57A	1308 ± 296A	544 ± 133A	
Mannosamine	18 ± 2A	23 ± 4A	26 ± 2A	3 ± 1A	
Muramic acid	12 ± 5A	19 ± 2A	22 ± 8A	13 ± 2A	
Galactosamine	225 ± 43A	306 ± 34A	583 ± 167A	203 ± 32A	
Σ	921 ± 264A	1071 ± 86A	1933 ± 464A	762 ± 164A	1179 ± 183
75–105 cm (μg g^{-1} soil)					
Glucosamine	1600 ± 340b	1010 ± 77ab*	1230 ± 195ab	606 ± 56a	
Mannosamine	42 ± 13a	11 ± 1a	28 ± 9a	1 ± 0a	
Muramic acid	23 ± 7a	23 ± 4a	25 ± 5a	23 ± 5a	
Galactosamine	776 ± 177b	394 ± 83ab*	605 ± 98ab	209 ± 30a	
Σ	2425 ± 516c	1517 ± 79ab*	1875 ± 296b	838 ± 85a	1673 ± 214

Mean values (±SEM) are given. Different *letters* indicate statistically significant differences. *Lowercase letters* indicate amino sugar contents for 45–75 cm. *Uppercase letters* indicate 75–105 cm. Different letters indicate significance differences. *$p < 0.05$ differences between soil depths are significant

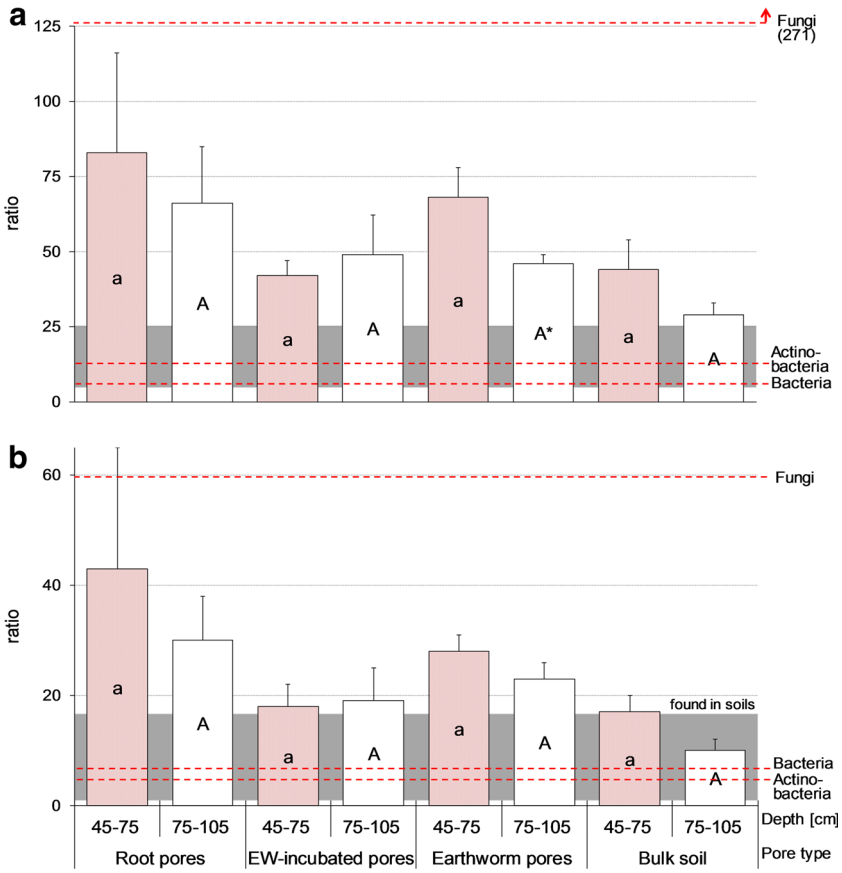

Fig. 6 Amino sugars ratios of (**a**) glucosamine to muramic acid and (**b**) galactosamine to muramic acid. Data from four samples × four treatments × two depths. Mean values (±SEM) are given. *Letters* indicate significant differences between pore types in each depth. Differences between soil depths were significant on $*p < 0.05$—given next to the *lower depth letters*. *Red* and *white bars* show the 45–75 and 75–105 cm depth, respectively. The *shaded areas* indicate the ratios found in a broad range of bulk soils, and the *dashed lines* indicate ratios of pure cultures of fungi, bacteria and actinobacteria (data taken from Glaser et al. (2004)). Both ratios represent fungal/bacterial necromass (45–75 cm, 75–105 cm). No pore effects were found (color figure online)

is much weaker than the difference between pores and bulk soil. To summarise, biopores featured 26–35 times higher PLFA abundances than bulk soil, and earthworm activities induced microbial communities unique to the root pores within 6 months.

Microbial community composition

Bacterial abundances

The bacterial group patterns are well explained by the organic matter input history and assumed quality. The root pores' most recent input of C was 2 years prior to sampling, so the more easily degradable C has been largely mineralised, having left behind less available compounds. The two groups of Gram-positive bacteria (I and II) and actinobacteria were enriched in the root pores and in the bulk soil compared to other microbial groups (Fig. 3). Two Gram-positive groups were distinguished as they showed statistically different behaviour from each other (Fig. 3), but more precise taxonomic description is not possible with PLFAs. In both habitats, older, more complex and more processed SOM is expected, of which Gram-positives and actinobacteria are frequently described to be decomposers of (Brant et al. 2006; Heuer et al. 1997; Kramer and Gleixner 2008; McCarthy and Williams 1992). The root pores in 75–105 cm were also significantly drier than the earthworm pores (data not shown). Soil moisture modulates the activity of bacteria, but it is not yet known how the microbial communities react to moisture fluctuations in biopores, e.g. through physiological adaptions to episodic macropore flow (Chen et al. 2007; Lundquist et al. 1999). In the root pores, biofilm-forming bacteria may have endured lower moisture conditions more successfully (Hueso et al. 2012; Vu et al. 2009). However, soil moisture may not strongly affect the soil C stock or its turnover (Aira et al. 2009; Guenet et al. 2012).

Earthworm activities, such as mucus secretion, selective ingestion of plant litter and microbial-rich aggregates, create very distinct habitats (Aira et al. 2009; Lal and Akinremi 1983; Sampedro and Whalen 2007; Stromberger et al. 2012; Tiunov and Dobrovolskaya 2002). Earthworms import fresh labile C into their burrows, which had the highest amount of microbial biomass with a clear predominance of Gram-negative bacteria (Fig. 3). Gram-negatives are thought to be decomposers of easily available organics (Bird et al. 2011; Griffiths et al. 1998; Gunina et al. 2014; Paterson et al. 2007; Treonis et al. 2004). Obviously, the C quality, represented by the $\delta^{13}C$ value, was linked to the abundances of Gram-

negative and Gram-positive bacteria. In the course of SOM decomposition, $\delta^{13}C$ increases as the lighter ^{12}C gets preferentially lost (Werth and Kuzyakov 2010), leading to higher abundances of Gram-positives—a pattern also discernible in the RDA plot (Fig. 4). The earthworms' mucus secretion, selective grazing on and selective survival of microorganisms in the presence of gut enzymes increase activities of microbes specialised on earthworm faeces. Sampedro et al. (2006) have shown that the prokaryote population in the earthworm gut was mainly Gram-negative. This is in line with the Gram-negative dominance in the earthworm pores (Fig. 3), as well as our analysis of fresh earthworm casts, which contained predominantly PLFAs representing Gram-negatives (Online Resource 3). Moreover, the resulting environmental conditions in the drilosphere, i.e. higher moisture due to mucus secretion, aggregation and more neutral pH in casts (Brown 1995; Parkin and Berry 1999; Tiunov and Scheu 1999), may also shape the community composition and activity. At higher soil moisture, higher growth rates may be sustained due to a greater diffusion of the limiting C resource (Zhou et al. 2002). While the pH effect on the ∑ PLFA content is often not significant (Rousk et al. 2010), small pH changes likely influence the communities and the abundances of single PLFAs (Bååth and Anderson 2003). Individual groups like fungi might cope better with a lower pH (Sleutel et al. 2012), while at a more neutral pH growth of bacteria might be promoted. However, we did not assume the pH to change throughout these pores as the earthworms were active in both soil depths and the pH of the bulk soil increased only weakly from 45–75 to 75–105 cm (Vetterlein et al. 2013). Earthworms also affect the texture of their burrow wall compared to the bulk soil (Lal and Akinremi 1983), but this effect is likely more pronounced in sandy soils (Zhang and Schrader 1993). The important role of the texture for microbial activity (Bach et al. 2010; Sleutel et al. 2012) may not play a large role in affecting the microbial community in our field site because of the low sand content of about 3.8%.

After 2 years of bare fallow and therefore absence of C inputs, the microbial abundance in the root pores was still eight times higher compared to bulk soil, with a trend towards increased bacterial biomass and significantly increased abundances of Gram-positive 1 and actinobacteria with depth. This may be explained by less decomposed root material in 75–105 cm compared to 45–75 cm, which is supported by an increase in the C content and C/N ratio from 45–75 cm to 75–105 cm (Table 1). The slow and continuous decomposition of roots may have led to the continuous release of bioavailable C over 2 years (Fontaine et al. 2003; Kuzyakov 2010; Kuzyakov and Blagodatskaya 2015), resulting in increased bacterial PLFAs with depth in both pore types that contained roots. Thus, positive effects of biopores on microbial nutrient cycling and consequently plant nutrition are expected for at least 2 years. These results are in good agreement with those of Sanaullah et al. (2016), who incubated root detritus and bulk subsoil for 3 years. They reported sequential growth of first Gram-negatives and fungi on fresh root detritus, while Gram-positives appeared only much later and were linked to turnover of more processed and native SOM.

Finally, it can be summarised that root pores without fresh C input for 2 years and potentially drier conditions were mainly colonised by general decomposers (Gram-positives, actinobacteria) whereas in earthworm pores featuring recent C inputs, additional moisture and near neutral pH, a higher Gram-negatives abundance was found in 45–75 cm, i.e. degraders of more easily available low molecular weight organic substances. This coincides with the general shift of Gram-negative dominance near the soil surface towards Gram-positive dominance in deeper soil layers as a function of C content, C quality, mean annual temperature and soil moisture (Blume et al. 2002; Franzmann et al. 1998; Kramer and Gleixner 2008; Stromberger et al. 2012).

Fungal abundances

Higher fungal PLFA contributions were found in both earthworm pore types than in root pores in 45–75 cm. This may be connected to a lack of plant residues since fungi are primary decomposers of structural plant material. In the 2-year-old root pores, visible cellulose fibres or lignocellulose structures were absent; thus, this late decomposition state accounts for the rather low importance of fungi in subsoil root pores (Sanaullah et al. 2016). Regarding earthworm pores, often no increases of fungi relative to bacteria are reported (Devliegher and Verstraete 1997; Stromberger et al. 2012; Tiunov and Scheu 1999).

PLFAs and amino sugar ratios were consistent as both methods returned decreased fungal contribution with depth for almost all treatments (Figs. 3 and 6). Consequently, this community shift was present not only in the living microflora but had already affected the accumulated microbial necromass. Such a decrease of fungal abundance with depth is common for bulk soils (Fierer et al. 2003; Moll et al. 2015) and mainly explained by a decrease of available C with depth. However, in biopores, where the C content is rather constant throughout the pores because earthworms distribute organic matter vertically (Table 1; Jégou et al. 1998, 2000), this explanation is not valid. Other mechanisms and soil properties which co-regulate the fungal biomass may need to be considered: apart from a change in SOM quality with depth, lower oxygen availability and the promotion of bacterial growth by an increased pH in the bulk soil (6.9 to 7.1) may help explain this pattern in this loess soil.

Comparing the microbial necromass data with the literature, smaller amino sugars to muramic acid ratios have been reported for agricultural soils (Amelung 2001; Engelking et al. 2007; Glaser et al. 2004), indicating that mainly fungal

residues make up the microbial necromass in these biopores. The amino sugar ratios GalN/MurAc and GlcN/MurAc were similar among the pore types (Fig. 6a, b), leading to the conclusion that, despite deviating communities, the amount of necromass accumulated during 2 years of pore wall genesis has not been sufficient to achieve a representative imprint of the PLFA pattern on the necromass. The ratio of MurAc/GlcN may be also skewed since after the depolymerisation of peptidoglycan a single, yet very strong, ether bond needs to be broken up to convert muramic acid to glucosamine. Such reactions are catalysed by high pH conditions, e.g. found in earthworm guts (Amelung 2001; Millar and Casida 1970; White et al. 1996).

The highest ratios of NLFA/PLFA16:1ω5 (values were approximately six) were found in root pores showing residual storage lipids by former mycorrhization. Lower ratios around three were found in pores with earthworm activity (45–75 cm), and thus, 16:1ω5 needs to be interpreted as a Gram-negative marker fatty acid there. We also found PLFA 16:1ω5 in fresh earthworm casts (Online Resource 3), and this further supports the generally high Gram-negative abundance in the earthworm pores (Fig. 3). To sum up, earthworm pores showed highest fungal biomass among treatments, while the ratio of fungal/bacterial necromass was not different between pores and got smaller with depth.

Implications for C turnover in subsoil biopores

The majority of studies on subsoil microbial communities have focussed on bulk soils, where C decreases with depth, the localisation of C inputs and biopores are not accounted for and the fluctuations of the environmental conditions are not as strong as near the surface. When the C content remains constant with depth, changing abiotic factors help explain the microbial community composition (Struecker and Joergensen 2015). As similar C contents with depth occur in the investigated biopores, it also likely that soil physical factors such as water fluctuations control the microbial community composition. The root pores in the deeper subsoil were significantly drier than the earthworm pores, and this is one of the likely explanations for the higher abundance of biofilm-forming Gram-positives under such circumstance. However, apart from the C content in the pores, the quality of the C input is likely a key factor governing abundances and activities of microbes (Fierer et al. 2003).

In bulk soil, more stable and less bioavailable compounds are usually found in deeper soil (Rumpel and Kögel-Knabner 2011), leading to stronger specialisation of microbial communities compared to the topsoil. It has been suggested that subsoil microbial communities were specialised to their environment and distinct from the topsoil communities (Fierer et al. 2003; Moll et al. 2015). This might not be true in the case of biopores as they feature high oxygen availability (Gliński and Lipiec 1990; Stewart et al. 1999), abundant C sources (Hafner and Kuzyakov 2016) and microbes and C distributed nearly homogeneously throughout the pores (Fig. 2, Table 1). Also, biopores have been likened to topsoil due to repeated fresh C inputs (Don et al. 2008). Compared to the bulk soil, microorganisms in biopores live in the 'land of plenty'. Although absolute C contents are not unusually high, they are three times higher than in bulk soil.

Earthworm pores

Earthworms influence larger soil volumes than the 2 mm around their burrows (Don et al. 2008; Jégou et al. 2000; Tiunov and Scheu 1999). However, horizontal diffusion is not an important process in earthworm pores (Don et al. 2008; Schrader et al. 2007), due to higher bulk densities and higher hydrophobicity compared to root pores (Lipiec et al. 2015). This hampers C export from the burrow into the bulk soil, creating distinct burrows of C accumulation. Also, this would explain the not decreasing C contents along the vertical extension of the pores, as C contents and bacterial biomass did not change significantly with depth.

It remains to be determined to which degree C stabilisation occurs in earthworm pores (Kögel-Knabner et al. 2008). Long-term stabilisation depends on physical disconnection, sorption on reactive mineral surfaces (Lee 1985; Schmidt et al. 2011) and absence of labile C sources promoting priming (Kuzyakov 2002). Large C inputs, increased moisture and good oxygen supply (Dziejowski et al. 1997; Gliński and Lipiec 1990; Görres et al. 1997) paired with frequent disturbance by the earthworms destabilise organic matter. The mixing of C inputs with the mineral phases during the gut passage may enhance stabilisation. However, no increased adsorption of C on iron oxides by earthworms was found so far (Don et al. 2008). The C sequestration may be favoured in earthworm-incubated pores with higher fungal abundance compared to native earthworm pores. It was hypothesised that this may be due to improved aggregate formation by hyphae (Rillig et al. 2015; Six et al. 2006), the decomposability of the melanised necromass (Clemmensen et al. 2015) or higher C use efficiency of fungal-dominated communities (Herrmann et al. 2014; Jastrow et al. 2007). Furthermore, extended hyphal networks may help sequester more C by exporting it to the bulk soil. Don et al. (2008) found no evidence of persistent C enrichment, short mean residence times of 3–8 years of earthworm-imported C and also high turnover rates. This does not necessarily contradict C sequestration: earthworm pores likely indirectly support C sequestration by stimulation of root growth through soil structure changes and improved nutrient and water supply in subsoils, which in turn increase belowground biomass (Brown 1995). Depending on the pore angle, relief, bulk density and moisture, roots growing in earthworm pores also may re-enter the bulk

subsoil after having benefitted from higher nutrient supplies in the pore, effectively increasing root biomass in deep soil layers (Athmann et al. 2013; Hirth et al. 2005). To conclude, repeated priming hampers C sequestration, boosts C turnover in the earthworm pores and they support C sequestration through large C accumulation and root growth promotion.

Root pores

To sequester high C amounts in the subsoils, deep rooting plants with abundant belowground biomass appear useful (Kell 2012; Lorenz and Lal 2007). Regarding C sequestration, first, the root pore walls may not be as hydrophobic as the earthworm pores (Lipiec et al. 2015), facilitating soluble C export into the bulk soil, where it can be stabilised on mineral surfaces. Second, lateral roots, root hairs and fungal hyphae are likely to leave the root pores and export C into the bulk soil. In contrast to the repeated labile C inputs in the earthworm biopores limiting the C stabilisation (Fontaine et al. 2007; Kuzyakov and Blagodatskaya 2015), in root pores, C input happens only once. As soon as the easily available C is respired, subsoil root C may be stabilised. C stabilisation was already apparent as a large microbial necromass accumulation relative to the bulk soil (75–105 cm; Table 3). For large C sequestration, new root pores are ideally created regularly and pores are cut off from the fresh C supply by harvesting.

Conclusions

At the heart of discussion on the roles of biopores lies the fundamental issue of promoting C turnover for nutrient supply or promoting C sequestration in unsaturated subsoils. In both cases, microorganisms are key actors and their community composition is one important factor regarding C turnover or microbial necromass production to be stabilised. Microbes in subsoil biopores live in the land of plenty compared to the bulk subsoil due to high C and oxygen supply, resulting in 26–35 times higher PLFA abundances in biopores. Soil depth affected the microbial community composition of the bulk soil much more strongly than of the biopores. The distribution of bacteria and fungi among pore types was an indicator for SOM quality in the pore walls. Decomposers of more complex organic matter (Gram-positives and actinobacteria) had higher abundances in the root pores, whereas the earthworm pores featured fungi and Gram-negatives. Earthworms had strong effects on microbial communities: the highest Σ PLFAs and highest amounts of rapidly metabolising Gram-negatives were found in both earthworm pore types, and, thus, the highest C and nutrient turnover are assumed. Introducing earthworms into decaying root pores influenced the microbial community heavily. The microbial community in these pores was rendered hardly distinguishable from native earthworm pores after 6 months of earthworm activity.

C turnover is inversely correlated with C sequestration. Therefore, low sequestration per unit of C input is expected in biopores unless C is stabilised in organo-mineral associations, exported to the bulk soil or occluded in aggregates. Earthworm pores support C sequestration through improving root growth in the subsoil. In the root pores, more of the remaining detritus C might be sequestered since no fresh C is repeatedly supplied from the surface. Overall, biopores strongly contribute to C input into subsoils. The functions of C and nutrient turnover, as well as C sequestration in subsoils, depend on the biopore history: earthworm biopores boost C turnover and plant nutrition in the subsoil, whereas root pores may be more responsible for C sequestration because of lacking priming. Biopores contribute to C sequestration directly by (1) large C inputs in the subsoil, (2) mixing with mineral phases for stabilisation and indirectly (3) by promoting deep root growth, i.e. increasing the total C input into subsoils.

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Compliance with ethical standards

Role of the funding source DFG KU 1184/29-1.

Conflict of interest The authors declare that they have no conflicts of interest.

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