How do microbial communities in top- and subsoil respond to root litter addition under field conditions?

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ABSTRACT

Contrasting microbial community composition and activity at different soil depths may affect root litter decomposition. These effects have up to now been investigated mainly in laboratory studies, which may not be able to take into account complex *in situ* conditions. Our study aimed to analyze the composition and activity of microbial communities after addition of ^{13}C labeled wheat root litter to a loamy soil under grassland at 30, 60 and 90 cm depths, during a three-year field experiment. We investigated the dynamics of bacterial and fungal abundances and community structures by DNA genotyping and pyrosequencing of 16S and 18S rDNAs. The genetic structures of bacterial and fungal communities were evaluated by automated ribosomal intergenetic spacer analysis. The functions of these communities were analysed by determination of extracellular enzyme activities and viable microbial communities involved in ^{13}C labeled organic matter decomposition studied by ^{13}C PLFAs.

The abundance of fungal and bacterial communities (16S and 18S rDNAs and PLFA) and the potential activities of enzymes involved in the C- and N-cycles were significantly higher at the top 30 cm compared with deeper soil throughout the experiment. Both were stimulated by fresh litter input. A trend to decreasing bacterial and fungal richness was noted after root litter addition at 30 cm, while richness of bacteria at 90 cm and those of fungi at 60 and 90 cm increased. Moreover, root litter addition caused a reduction of the Shannon Weaver Diversity index and a shift in microbial community structure at all three depths, which was more pronounced for bacteria at 30 and 60 cm and for fungi at 90 cm. The changes during litter degradation resulted in similar dynamics of most enzyme activities at all depths. Chitinase activity was enhanced after 29 months compared to initial conditions indicating the availability of high amounts of microbial detritus. The degrading microbial community as assessed by ^{13}C PLFA showed similar temporal dynamics at all three depths. Fungal contribution to this community decreased during later stages of litter degradation, while the contribution of Gram+ bacteria increased. We conclude that litter addition led to convergence of microbial communities of top- and subsoil through stimulation of copiotrophic populations. Soil microbial community structures are thus connected with the amount of fresh litter input. Enzyme activities and ^{13}C PLFA reflect to some extent the changes occurring during degradation, i.e. exhaustion of fresh plant material and accumulation of detritus.

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1. Introduction

Heterotrophic microbial community abundance and composition is closely related to plant growth, especially in rhizosphere and detritusphere. It is assumed that the highest microbial abundance,

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species richness and diversity is concentrated in the uppermost soil as a consequence of high litter input and rhizodeposition, whereas these parameters are declining with depth in accordance with substrate availability (Jumpponen et al., 2010; Oehl et al., 2005; Rosling et al., 2003; Zajicek et al., 1986). In addition, the composition of bacterial communities is stratified depending on root vicinity: whereas Gram negative bacteria (Gram−) are dominant in topsoil and the rhizosphere, Gram positive bacteria (Gram+) dominate in subsoil (Blume et al., 2002). This leads to a complete differentiation of the structure of bacterial communities in top- and subsoil (Eilers et al., 2012).

In subsoil, occurrence of carbon is scarce and dominated by stabilised organic matter compounds (Rumpel and Kögel-Knabner, 2011). Therefore, microbial biomass and activity are not homogenously distributed but concentrated near locations with C input, such as rhizosphere and pores (Bundt et al., 2001; Chabbi et al., 2009; Nunan et al., 2003; Kautz et al., 2013), leading to development of hotspots of microbial activity (Nunan et al., 2003; Kuzyakov and Blagodatskaya, 2015). The separation of fresh organic matter and degraders increases with depth and may be the major factor responsible for the slow turnover rate of soil organic matter in deep soil horizons (Holden and Fierer, 2005; Hafner et al., 2014).

Due to these particularities, microbial communities in subsoils may function differently and may have specific responses to land-use or climate changes compared with topsoil communities (Smith et al., 2015). For example, oxidative enzyme activities increase with soil depth (Uksa et al., 2015), while hydrolytic enzyme activities decrease, most probably because both types of enzymes bind to different physical fractions (hydrolytic enzymes to particulate organic matter and oxidative enzymes to clay minerals) (Kramer et al., 2012). Subsoil microbial communities are energy limited (Fontaine et al., 2007), and their community composition could shape enzyme activities, which could be an important control of organic matter decomposition (Wild et al., 2015), in a similar way as in topsoils. In response to contrasting microbial activities, the mechanisms of organic matter stabilization seem to affect different compounds in subsoil as compared to topsoil (Rumpel et al., 2012, 2015).

Global change and ecosystem disturbance may change belowground carbon allocations and water availability (Smith et al., 2016). To understand the changes occurring upon disturbance, it is important to investigate how the subsoil microbial populations respond to plant litter input under *in situ* conditions. The fate of fresh litter input due to disturbance or vegetation change and its effect on subsoil microbial communities and their activities is poorly understood. Nearly all studies on the response of subsoil microbial communities to substrate addition were based on laboratory incubations (e.g. Fierer et al., 2003; Fontaine et al., 2007; Naisse et al., 2015), leaving many open questions about the impact of contrasting conditions at different soil depths occurring *in situ*. In general, the former studies indicated that substrate addition to subsoil stimulates microbial activities depending on substrate quality and nutrient availability. Moreover, accessibility of organic matter (OM) may be a major determining factor for OM decomposition in subsoils (Salomé et al., 2010).

In a recent field incubation with ^{13}C labeled wheat litter aiming to investigate root degradation at three soil depths, we evidenced similar quantities of degraded litter after 36 months, despite contrasting decomposition dynamics (Sanaullah et al., 2011). Indeed, while root litter in topsoil followed exponential decay, at 60 and 90 cm, root litter decay began after a lag phase. Moreover, changes with time in soil organic matter (SOM) bulk chemical composition were horizon dependent (Baumann et al., 2013a). These observations suggest contrasting microbial functioning at different soil depths. However, up to now, it is unknown how microbial

communities at depths responded to wheat root addition and which strategy they used to overcome the initial lag phase: they may have changed their community structure or just their activities. Community structures could have converged at different soil depths directly after root litter addition and subsequently separated again, when easily decomposable material became exhausted.

To answer these questions, in the present study, samples from the incubation experiment were analysed for the abundance and activity of microbial communities by several biochemical and genetic tools. We used molecular tools such as soil DNA extraction, DNA fingerprinting with automated ribosomic intergenetic spacer analyses (ARISA) and pyrosequencing of 16S and 18S rDNA genes in order to study general microbial response to root litter addition (Ranjard et al., 2006). Moreover, we used compound specific isotope analyses of phospholipids (PLFA) to analyze the specific changes in the litter degrading community during decomposition at different soil depths (Frostegård et al., 2011). The response of microbial activity to root litter addition at different soil depths was studied by enzyme activities. The aims of this study were to investigate (1) if root litter addition caused increases in similar microbial groups at all soil depths, (2) if root litter additions stimulated similar enzyme activities at all soil depths but at different times (3) to determine if root litter addition causes similar shifts in microbial community structures at all three soil depths. We hypothesized that microbial community composition and functions before root litter addition are contrasting in top- and subsoil in agreement with former studies (Eilers et al., 2012; Ekelund et al., 2001) and that microbial community composition and functions of subsoil are strongly altered upon root litter addition.

2. Material and methods

2.1. Experimental set up

The field experiment was set up at Lusignan in the south-west of France ($46°25'12,91''$ N; $0°07'29,35''$ E) in 2006. The site had been under ley cropping systems since more than 50 years. Soil type at the site is Cambisol with a loamy texture. The details about the experimental setup are described by Sanaullah et al. (2011). Briefly, the soils were excavated with a corer from 30, 60 and 90 cm, mixed with ^{13}C labeled wheat roots and buried again at the same depth under grassland vegetation. To this end, soil-root mixtures were exposed in litterbags (10×10 cm, mesh size 100 μm) containing a mixture of 2 g ^{13}C labeled root material (dried at 40 °C, <1 cm pieces) and 100 g native loamy textured soil from each depth. Wheat root material was labeled continuously with ^{13}C ($\delta^{13}C = 1744 \pm 13‰$) by growing wheat hydroponically in a growth chamber under 2 atom% $^{13}C\text{-}CO_2$ atmosphere for 16 weeks. For analysis, three litterbags of each depth were excavated after 6, 12, 20, 29 and 36 months of incubation. The samples were taken back to the laboratory and stored at −20 °C before analyses.

2.2. Microbial biomass and structure

Microbial biomass is indicated as the amount of microbial DNA, which has been shown to be a robust, fast and easy way to quantify the microbial soil pool (e.g. Fornasier et al., 2014; Marstorp and Witter, 1999). For microbial DNA extraction we used 1 g of soil and the ISO-10063 procedure (Petric et al., 2011), which had been slightly modified by Plassart et al. (2012) to include a mechanical lysis step, using fastPrep®-24 instead of the recommended mini bead-beater cell disruptor. DNA concentrations of crude extracts were determined by electrophoresis in a 1% agarose gel using a calf thymus DNA standard curve (Dequiedt et al., 2011). After

quantification, DNA was purified using a MinElute gel extraction kit (Qiagen, Courtaboeuf, France).

Using extracted DNA, quantitative PCR was performed using primers 341F/515R to amplify 16S rDNA for bacteria and primers FF390/FR1 to amplify 18S rDNA for fungi (Vainio and Hantula, 2000). The real-time Q-PCR products were amplified on an ABI PRISM 7900HT (Applied Biosystems®, Courtaboeuf, France) with SYBRGreen® as detection system.

Automated ribosomal intergenic spacer analysis (ARISA) was carried out according to Ranjard et al. (2006). It was taken as an indicator of difference in microbial community structure at all three soil depths after 0, 6, 12, 20, 29 and 36 months. In brief, the ribosomal intergenic spacer regions of bacterial and fungal DNA were amplified by PCR with the primers S-D-Bact-1522-b-S-20/L-D-Bact-132-a-A-18 and ITS1F/3126T, respectively. The S-D-Bact-1522-b-S-20 and 3126T primers were labeled at their 5' end with the IRD800 fluorochrome for the detection of the resulting amplicons by a LiCor® DNA sequencer (ScienceTec, Les Ulis, France).

2.3. Pyrosequencing of 16S and 18S rRNA gene sequences

Bacterial and fungal compositions were determined before litter addition and after 6 months of incubation for the three soil depths (triplicates/depth) by 454 pyrosequencing of ribosomal genes. For bacteria, a 16S rRNA gene fragment with sequence variability and the appropriate size (about 450 bases) for 454 pyrosequencing was amplified by PCR using the primers F479 and R888. For fungi, an 18S rRNA gene fragment of about 350 bases was amplified using the primers FR1 and FF390. Primers and PCR conditions were as described previously (Tardy et al., 2015). The PCR products were purified using a MinElute gel extraction kit (Qiagen, Courtaboeuf, France) and quantified using the PicoGreen staining Kit (Molecular Probes, Paris, France). A second PCR of 9 cycles was then conducted under similar PCR conditions with purified PCR products and ten base pair multiplex identifiers added to the primers at 5' position to specifically identify each sample and avoid PCR biases. Finally, the PCR products were purified and quantified as previously described. Pyrosequencing was then carried out on a GS FLX Titanium (Roche 454 Sequencing System).

2.4. Bioinformatics analysis of 16S and 18S rRNA gene sequences

Bioinformatic analyses were done using the GnS-PIPE developed by the GenoSol platform (INRA, Dijon, France) and initially described by Terrat et al. (2012). First, all reads were sorted according to the chosen identifier sequences. The raw reads were then filtered and deleted based on their: (a) length, (b) number of ambiguities (Ns), and (c) primer sequence(s). PERL programs were then applied to obtain strict replication, alignment of reads using infernal alignments (Cole et al., 2009) and clustered at 95% sequence similarity into operational taxonomic units (OTU) that cluster rare reads with abundant ones, and do not count differences in homopolymer lengths. Another homemade filtering step was then applied to eliminate potential sources of errors (e.g., PCR chimeras, sequencing errors, OTU overestimation). In order to efficiently compare the datasets and avoid biased community comparisons, the samples reads were reduced by random selection closed to the lowest datasets (5000 and 12,000 reads for 16S and 18S rRNA gene sequences respectively). The retained high-quality reads were used for: (*i*) taxonomy-independent analyses, to determine several diversity and richness indices using the defined OTU composition, and (*ii*) taxonomy-based analysis using similarity approaches against dedicated reference databases from SILVA (http://www.arb-silva.de/). Silva (forest) is web based quality-controlled database of aligned ribosomal RNA (rRNA) gene

sequences from the Bacteria, Archaea and Eukaryota domains and supplementary online services (Quast et al., 2013).

Data have been used to calculate the Shannon-Weaver Index using the following formula:

$$H = -\sum[(pi) \times \ln(pi)]$$

where H is the Shannon-Weaver index and pi is OTUi/OTUtotal.

2.5. PLFA extraction and stable C isotope analyses

PLFAs were extracted from 5 g soil-root mixtures sampled after 6, 20, and 36 months to quantify the relative abundances of viable microbial groups within the soil profile. Briefly, PLFA fractions were obtained by one-phase chloroform-methanol-citrate buffer extraction and subsequent separation on silicic acid columns (Frostegård et al., 1991). The polar lipids were dried under N_2, followed by mild alkaline *trans*-esterification with BF3/Methanol (converting ester-linked fatty acids to the corresponding methyl esters (Morrison and Smith, 1964). At this step an internal quantification standard (19:0 Me) was added. After transesterification, PLFAs were taken up in dichloromethane and fatty acid methyl esters were separated by gas chromatography on a non-polar BPX-5 column. The initial column temperature in the GC was set at 100 °C for 1 min; temperature was increased to 325 °C within 76 min, and then hold for 2 min. Helium (ultrapure HeBIP, Air Products, France) was used as a carrier gas. Identification of PLFAs was based on comparison with methyl esters of the bacterial acid methyl esters mix BAME (Supelco, Sigma-Aldrich, USA). The concentration of individual PLFAs was expressed relative to that of C19:0 standard using the classical internal standard quantification approach. PLFA biomarkers were assigned to 3 different microbial groups: 14:0 and 16:1w9c for Gram−bacteria, i15:0, i16:0, i-17:0 and cy17:0 for Gram+ bacteria (Frostegård and Bååth, 1996) and (18:1w9c and 18:2w9c) for fungi (Zelles, 1997).

The contribution of microbial groups using root litter C was determined by analysing the isotopic composition of the extracted PLFAs by gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) using a Hewlett-Packard 5890 gas chromatograph coupled to an isotope ratio mass spectrometer (IRMS) (Isoprime, Micromass, UK) via a combustion interface (CuO, combustion reactor set to 850 °C). Separation of PLFAs was performed using the same column as described for quantitative analysis. Temperature specifications were set to an inlet temperature of 260 °C and the temperature program as described for quantitative analysis with an additional temperature ramp at the end of the program (20 °C min^{-1} to 350 °C, hold for 10 min). For accurate determination of highly ^{13}C enriched PLFAs, PLFA extracts were diluted 1:4 prior to analysis using the natural abundance standard BAME (diluted 1:10 in dichloromethane). The δ^{13}C values of the individual fatty acid methyl esters (FAMEs) were corrected for the addition of the methyl group added during esterification by simple mass balance:

$$\delta^{13}C_{FA} = \frac{[(nC_{FA} + 1)\delta^{13}C_{FAME} - \delta^{13}C_{MeOH}]}{nC_{FA}} \tag{1}$$

where, $\delta^{13}C_{FA}$ is the isotopic signature of the fatty acid (PLFA), nC_{FA} is the number of C atoms of the PLFA, $\delta^{13}C_{FAME}$ is the δ^{13}C value of the fatty acid methyl ester (PLFA after transesterifacation) and $\delta^{13}C_{MeOH}$ is the δ^{13}C value of the methanol used for transesterification.

2.6. Enzyme assays

In order to determine extracellular enzyme activities, we focused on three enzymes involved in the C cycle: xylanase, β-cellobiosidase and β-glucosidase, one involved in the nitrogen (N) cycle: leucine-aminopeptidase, and one enzyme involved in both cycles: chitinase. Extracellular enzyme activities were measured in 1 g of soil (Pritsch et al., 2004; Sowerby et al., 2005). In brief, four fluorogenic enzyme substrates based on 4-methylumbelliferone (MUF) were used for the assessment of enzyme activities: MUF-β-D-glucopyranoside (EC 3.2.1.21) for β-glucosidase, MUF-β-D-cellobioside (EC 3.2.1) for β-cellobiosidase, and MUF-β-D-xylopyranoside (EC 3.2.1) for xylanase. To study Leucine-aminopeptidase activity, L-Leucine-7-amino-4-methyl coumarin (AMC) substrate was used which is involved in the hydrolysis of peptidebonds. For the enzyme assay, 0.5 ml of soil:water extracts (1:10 w/v) were added to substrate solutions (containing either 200 μmol MUF or AMC) in microplates before incubation at room temperature (22 °C) for 1 h for enzyme releasing monomers (β-glucosidase, chitinase and leucine amino peptidase) and 3 h for enzymes releasing oligomers (β-Cellobiosidase and xylanase). Fluorescence was measured at an excitation wavelength of 355 nm and an emission wavelength of 460 nm (Victor[3] 1420-050 Multilabel Counter, PerkinElmer, USA). Enzyme activities were expressed as MUF or AMC release in μmol g^{-1} h^{-1}.

2.7. Statistical analyses

Normal data distribution was tested by the Kolmogorov-Smirnov test, the homogeneity of variances by the Levene test. After confirming that data were normally distributed, analyses of variance (ANOVA) with subsequent LSD tests were performed to analyze the effects of depth and time on microbial parameters using the statistical software R (R software 2008, version 2.13.1). The level of significance of statistical tests was $p < 0.05$ in this study.

For ARISA results, the data obtained from the 1D-Scan software were converted into band presence and intensity using the Pre-pRISA program (Ranjard et al., 2006) under R software version 2.11.1. For each sample 100 peaks and a resolution of 2 bp were used. The ARISA data matrices (taking into account the presence/absence and relative intensity of bands) were subjected to a Principal Component Analysis (PCA) on a covariance matrix. The significant discrimination of the genetic structures of the microbial communities revealed by PCA was confirmed by performing the Monte Carlo test with 1000 permutations. PCA and Monte Carlo tests were performed using the ADE-4 package (Chessel et al., 2004; Thioulouse et al., 1997) under R software version 2.11.1.

3. Results

3.1. Total microbial biomass as determined by DNA analyses

Throughout the experiment, topsoil at 30 cm showed much higher DNA concentrations than the other two depths (Fig. 1a). Before the start of the experiment, around 29 ng DNA were recorded at 30 cm compared to 0.4 and 3 ng DNA g^{-1} at 60 and 90 cm. Root litter addition did not influence the DNA concentrations at 30 cm. At 60 and 90 cm, DNA concentrations increased significantly up to 14.5 ng DNA g^{-1} during the experiment (Fig. 1a). While at 60 cm increasing DNA concentrations were recorded throughout the experimental period, a transitory increase of the DNA concentration was noted at 90 cm (Fig. 1a). After normalisation to total organic carbon, differences persisted but were much less pronounced, especially after root litter addition (Fig. 1b). Normalised to C, DNA contents were much higher at 30 cms at the beginning of

the experiment as compared to lower depths (34 ng mg^{-1} C at 30 cm versus 2 and 5 ng DNA mg^{-1} C at 60 and 90 cm). After root addition, the contents were reduced in all three depths varying between 3.2 and 0.1 ng mg^{-1} C. After 3 years, total DNA contents normalised to C were similar at 30 and 60 cm, while lower contents were recorded at 90 cm (Fig. 1b). In agreement with DNA concentrations and therefore microbial abundance, the carbon and nitrogen loss at the beginning of the experiment was also higher at the top 30 cm than at lower depths. After 6 months of field exposure, $60 \pm 5\%$ of root-derived C was remaining at 30 cm while at lower depths, this value ranged between 80 and 98% (Fig. 1c). However, at the end of incubation, similar amounts of root carbon, between 30 and 44% of initial, were remaining at all three depths (Fig. 1c, Sanaullah et al., 2011).

3.2. Dynamics of bacterial and fungal community structure described by ARISA

The structure of bacterial and fungal communities was assessed according to sampling dates for each of the three soil depths (Fig. 2). PCA analysis of ARISA profiles revealed a shift in the bacterial community composition 6 months after root litter addition regardless of soil depth. However, changes in the bacterial community structure were greater at 90 cm compared to the two other depths (Fig. 2). After this shift, patterns of the bacterial community dynamics differed according to the soil depth. At 30 cm, the bacterial community changes were resilient, with the community structure becoming similar to the initial state after 29 months. Contrastingly, at 60 and 90 cm resilience did not occur and community structures remained different from the initial state, but also stable until the end of the experimentation. It is interesting to note that after the initial shift observed after 6 months, amplitude of the bacterial community changes decreased with increasing soil depth.

For fungal communities the situation was quite different (Fig. 2): at 30 and 60 cm, patterns of community dynamics were similar with few changes in community structure occurring during the experiment. Contrastingly, shifts in the ARISA profiles were much greater at 90 cm than at lower depths. Fungal community structures were not resilient at any depth.

3.3. Taxonomic composition of fungal and bacterial communities before root litter addition and after 6 months of field exposure

Pyrosequencing was carried out for samples before root litter addition to soil and after 6 months, when the greatest effects on microbial community composition were observed following ARISA analyses (see above).

Before root litter addition, similar richness of bacterial communities was observed at 30 and 60 cm depth with more than 1000 molecular operational taxonomic unit (OTU) (Table 1). At 90 cm, bacterial richness was reduced by half. Fungal richness decreased with soil depth from 777 OUT at 30 cm to 149 OTU at 90 cm (Table 1). The fungal as well as bacterial communities showed a greater Shannon-Weaver diversity index and evenness at 30 and 60 cm depths as compared to those at 90 cm (Table 1).

Root litter addition changed microbial richness in contrasting direction at the different soil depths. While at 30 and 60 cm bacterial richness tended to decrease after root litter addition, an increase was noted for 90 cm (Table 1). For fungal richness we noted a decrease after root litter addition only at 30 cms, while increasing richness was noted at 60 and 90 cm. Moreover, root litter addition led to a decrease of the Shannon-Weaver diversity index as well as evenness at all three depths. Fungal communities showed greater change in these parameters as compared to bacterial ones. (Table 1).
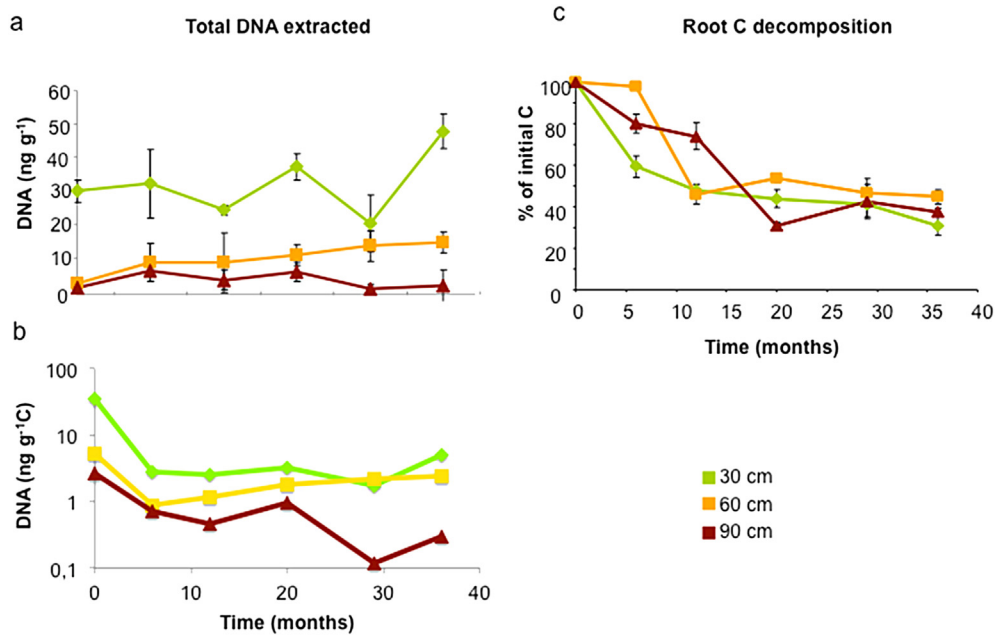
Fig. 1. Total soil DNA concentration (ng g^{-1}) (a) and DNA content (ng g^{-1} C) (b) and root C remaining (c) at different soil depths over time (data from Sanaullah et al., 2011). The data represented mean ± standard deviation (n = 3).
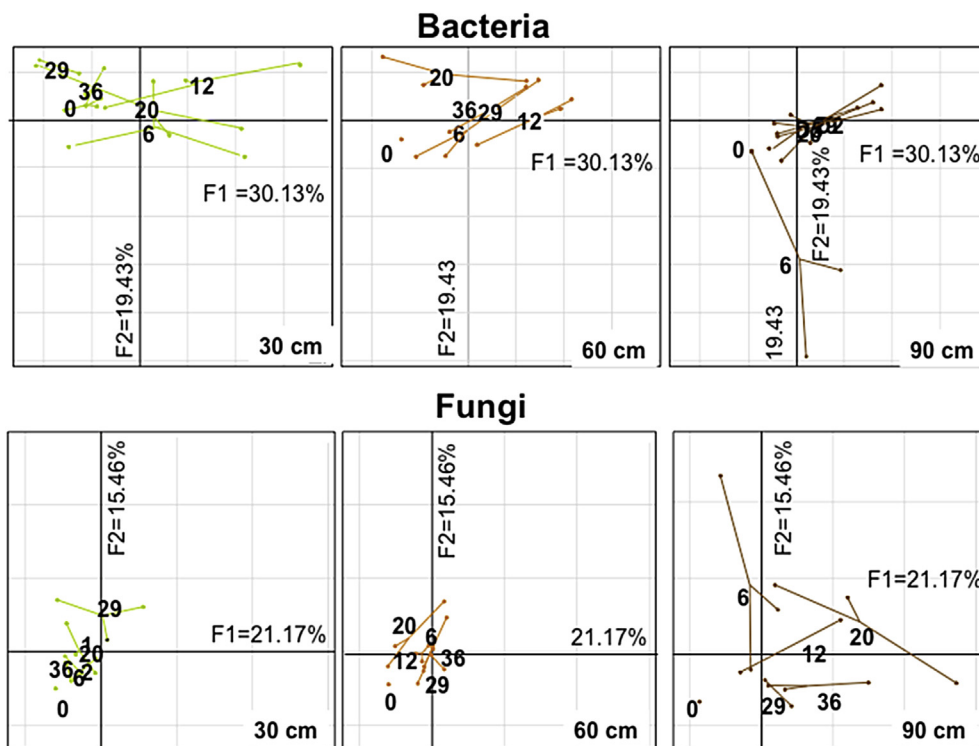

Fig. 2. PCA plan showing the distribution of bacterial and fungal community dynamics as determined by the first two factors (F1 and F2) at different soil depths (30, 60 and 90 cms) over time (0, 6, 12, 20, 29 and 36 months). Variables are microbial characteristics at the different sampling points with the first two factors as assessed by ARISA fingerprinting of extracted DNA. Lines within the PCA plan indicate variability of the community structures for three replicated field samples.

We also recorded differences in bacterial and fungal profiles at all three depths concerning relative intensity as well as the presence/absence of specific microbial groups (Fig. 3a and b). Before root addition, *Proteobacteria* were abundant at all three soil depths (Fig. 3a). At 30 cm, the community was dominated by bacteria belonging to *Actinobacteria* phyla, while *Acidobacteria, Bacteroidetes* and *Planctomycetes* were more abundant at 60 and 90 cm. 6 months after root litter addition, all soil depths showed a trend towards similar compositions. Most of the changes compared to the initial date were related to an increase in sequences affiliated to *Actinobacteria* and *Proteobacteria* populations, while *Acidobacteria* and *Firmicutes* decreased at all three depths.

Ascomycota and *Basidiomycota* were the main dominating fungal communities before root addition (Fig. 3b). The proportion of cloned rDNA sequences from *Ascomycota* was significantly higher at 90 cm as compared to 30 cm depth (Fig. 3b). 6 months after root litter addition, *Basidiomycota* decreased, while *Ascomycota* significantly increased.

3.4. Extracellular enzyme activities

Before the experiment, extracellular enzyme activities at 30 cm were much higher compared to those recorded for 60 and 90 cm (Fig. 4). Addition of root litter increased extracellular enzyme activities at all three depths. We recorded maximal activities after 6 months. Thereafter, enzyme activities were lower but in some cases remained still higher than those before litter addition. This was especially the case for chitinase, which showed after 29 months still significantly increased values at all three depths (Fig. 4).

Whereas leucine-aminopeptidase activity was evidenced at all dates at 30 cm, it could be detected in samples from 60 to 90 cm only during the first 12 months after root litter addition; thereafter this activity was absent at lower depths (Fig. 4).

3.5. PLFA content and root C incorporation into PLFA

In the whole soil profile, total PLFA concentrations ranged between 0.9 and 24 nmol g^{-1} before root litter addition and 7 and 39 nmol g^{-1} after root litter addition (Table 2). Total PLFA concentrations increased after root litter addition at all three depths. We recorded highest values after six months. Over all three depths, PLFA concentrations remained substantially higher than initial values even at the end of the experiment (Table 2). Fungal biomarkers were increased more than bacterial ones in all samples taken after root litter addition, as illustrated by the higher fungi/bacteria ratio. This ratio was higher for samples from 60 to 90 cms than those from topsoil (Table 2). ^{13}C analyses indicated that less than 0.5% of initial ^{13}C added was incorporated into PLFA. This proportion decreased during the experiment. We observed little differences in the allocation of the ^{13}C label to individual PLFA at the three different depths. At all three depths, most ^{13}C derived from added roots was incorporated in 16:0 PLFA, followed by incorporation into monosaturated or polysaturated 18C PLFAs, among which are two fungal markers (18:1w9c and 18:2w6). Incorporation of ^{13}C into fungal markers decreased from 6 to 36 months, while its incorporation into Gram+ bacterial markers (i-15:0 and i-16:0) increased (Fig. 5). Fungal contribution to total ^{13}C labeled PLFA was highest at 90 cm depth 6 months after root litter addition.

4. Discussion

4.1. Depth effects on microbial communities

Before root litter addition, total DNA extracted and potential enzyme activities were much higher at 30 cm than at lower depths (Figs. 1 and 4). These results are in agreement with other studies, showing quantitative differences of microbial biomass and their activity in top- and subsoil. The differences were related to contrasting physicochemical conditions (Holden and Fierer, 2005), organic C content (Blume et al., 2002) and the availability of fresh plant material, i.e. root residues and rhizodeposition (Müller et al., 2016). The low C concentration of subsoils (3.2—3.5 mg g^{-1} versus 8.7 mg g^{-1} for topsoil, Sanaullah et al., 2011) most probably does not provide enough energy to support large microbial communities (Fontaine et al., 2007).

Genetic fingerprinting using ARISA analyses of DNA extracted from soil, showed contrasting microbial community structures at all three depths. Pyrosequencing indicated that both bacterial and fungal richness decreased with soil depth. However, significant decrease of richness occurred for bacteria only at 90 cm (from 1391 OTU at 60 cm to 691 OTU at 90 cm), whereas it was observed from 60 cm downwards for fungi (from 777 OTU at 30 cm to 272 OTU at 60 cm) (Table 1). This may be explained by unfavorable conditions for microbial communities at greater depth, in particular lower oxygen content, which may favor bacteria rather than lignin degrading fungi (Chen et al., 2016). Microbial communities at greater soil depth were shown to be more adapted to the use of simple molecules as compared to topsoil communities, which are more adapted to the use of complex substrates (Salomé et al., 2010). This is supported by the observation of a lag phase during which the microbial communitiy compositions changed to be able to degrade root litter added in our field experiment (see below). Our data support previous results, which indicated that fungal contribution is strongly reduced in subsoil, not only because of oxygen limitations but also due to restricted availability of fresh plant material (Struecker and Joergensen, 2015).

Pyrosequencing indicated that the composition of microbial communities differed among the three horizons before root litter addition. Indeed, bacterial community composition at 60 and 90 cm indicated abundance of *Planctomycetes,* (Fig. 3a), which have been previously described as oligotrophic organisms (Pascault et al., 2013; Semenov, 1991). These oligotrophs are usually present in environments with low levels of nutrients (Koch, 2001), in accordance with conditions prevailing in subsoil (Fierer et al., 2003). The phylogenetic bacterial fingerprints at 30 cm depth before root addition (Fig. 3a) were dominated by bacteria belonging to *Actinobacteria* and *Firmicutes* phyla. These bacteria have copiotrophic properties, and can preferentially consume labile soil organic C

Table 1
Shannon index, evenness and operational taxonomic units of microbial populations at all three soil depths before incubation (0 months) and 6 months after root litter addition. Data are presented as mean ± standard deviation (n = 3).

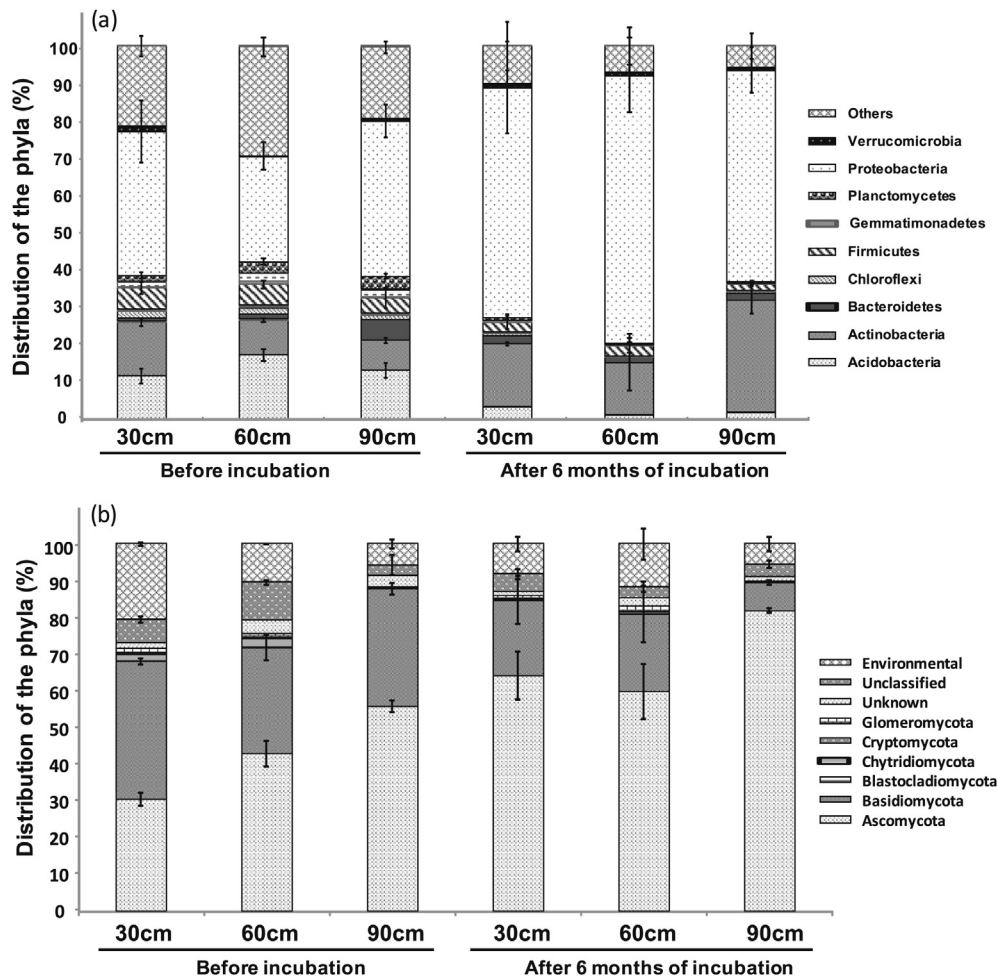|	Bacteria			Fungi		
	Shannon index	Evenness	OTU[a]	Shannon index	Evenness	OTU
30 cm						
0 months	5.62 ± 0.13	0.78 ± 0.02	1298 ± 111	4.22 ± 0.10	0.63 ± 0.00	777 ± 33
6 months	5.00 ± 0.48	0.72 ± 0.04	1088 ± 398	2.93 ± 0.40	0.50 ± 0.05	358 ± 94
60 cm						
0 months	5.70 ± 0.26	0.79 ± 0.01	1391 ± 345	3.64 ± 0.06	0.65 ± 0.01	272 ± 18
6 months	4.79 ± 0.61	0.69 ± 0.07	1060 ± 245	2.98 ± 0.49	0.50 ± 0.06	397 ± 126
90 cm						
0 months	5.20 ± 0.33	0.80 ± 0.01	691 ± 217	2.53 ± 0.36	0.50 ± 0.05	149 ± 45
6 months	4.38 ± 0.27	0.66 ± 0.04	742 ± 56	2.39 ± 0.06	0.43 ± 0.02	299 ± 33

[a] Operational taxonomic unit.

Fig. 3. Distribution of the phyla of (a) bacterial and (b) fungal sequences obtained by pyrosequencing of DNA extracted from at all three soil depths, before (0 month) and after 6 months of root litter incubation. For each sample, results are expressed as percentage of all classified sequences. Phylogenetic groups accounting for 1% or less of the classified sequences in all the samples are summarized in the artificial group "others". Data are presented as mean ± standard deviation (n = 3).

pools thus showing high growth rates when resource conditions are abundant (Fierer et al., 2007; Semenov, 1991). These phyla were less abundant at lower depths (Fig. 3a). High proportions of proteobacteria at all three soil depths indicated the presence of broad-range bacteria, which can grow under both C-rich or C-poor conditions (Semenov, 1991).

For fungi, the main difference between the three horizons before root addition was ascribed to greater contribution of *Ascomycota* at lower soil depth (Fig. 3b). The presence of *Ascomycota* and *Basidiomycota* represented the main saprophytic soil fungal decomposers classically identified in different soil niches (Vandenkoornhuyse et al., 2002). These fungal members may be indicative for the potential capability of the fungal community to degrade cellulose and ligno-cellulose (Osono and Takeda, 2006), in agreement with the slightly higher potential activities of β-cellobiosidase and β-glucosidase recorded in soil before root litter addition (Fig. 4). These results suggest that topsoil microbial communities are shaped by the abundance of fresh organic matter, which leads to strong differences of potential enzyme activities (Herold et al., 2014; Stone et al., 2014), as well as community structures of microorganisms in top- and subsoil horizons (Blume et al., 2002; Eilers et al., 2012). More species with more balanced contribution were present in 30 cm compared to lower depths (Fig. 3). Our results suggest that the microbial communities present at 30 cm depth were dominated by litter-degrading microbial

communities, whereas these contributed less to microbial communities at 60 and 90 cm. Therefore, we expected contrasting responses to root litter addition in top- and subsoil.

4.2. Effects of root litter addition on microbial community composition and activity

Root litter addition increased total extractable DNA at all three depths (Fig. 1), although it was always highest at 30 cm (Fig. 1, Table 2). Interestingly, the DNA content decreased after root litter addition and increased again slightly after 36 months, suggesting that new C after its addition is used in higher proportions for respiration rather than building new biomass. The PLFA content suggests that also the viable microbial communities increased greatly after root litter addition (2-fold at 30 cm; 8–10 fold at 60 and 90 cm) and remained higher than initial throughout the experiment. PLFA and DNA concentrations were found to be related at all three depths ($r^2 = 0.78$). Stimulation of microbial biomass after addition of fresh litter is a common phenomenon observed in laboratory studies for top-as well as subsoil horizons (e.g. Salomé et al., 2010). A decrease in microbial biomass during litter decomposition corresponds to the depletion of all readily available substrates and relative accumulation of recalcitrant ones during long-term incubation (Nannipieri et al., 1983). Especially in subsoil, initial microbial communities were most probably more specialized
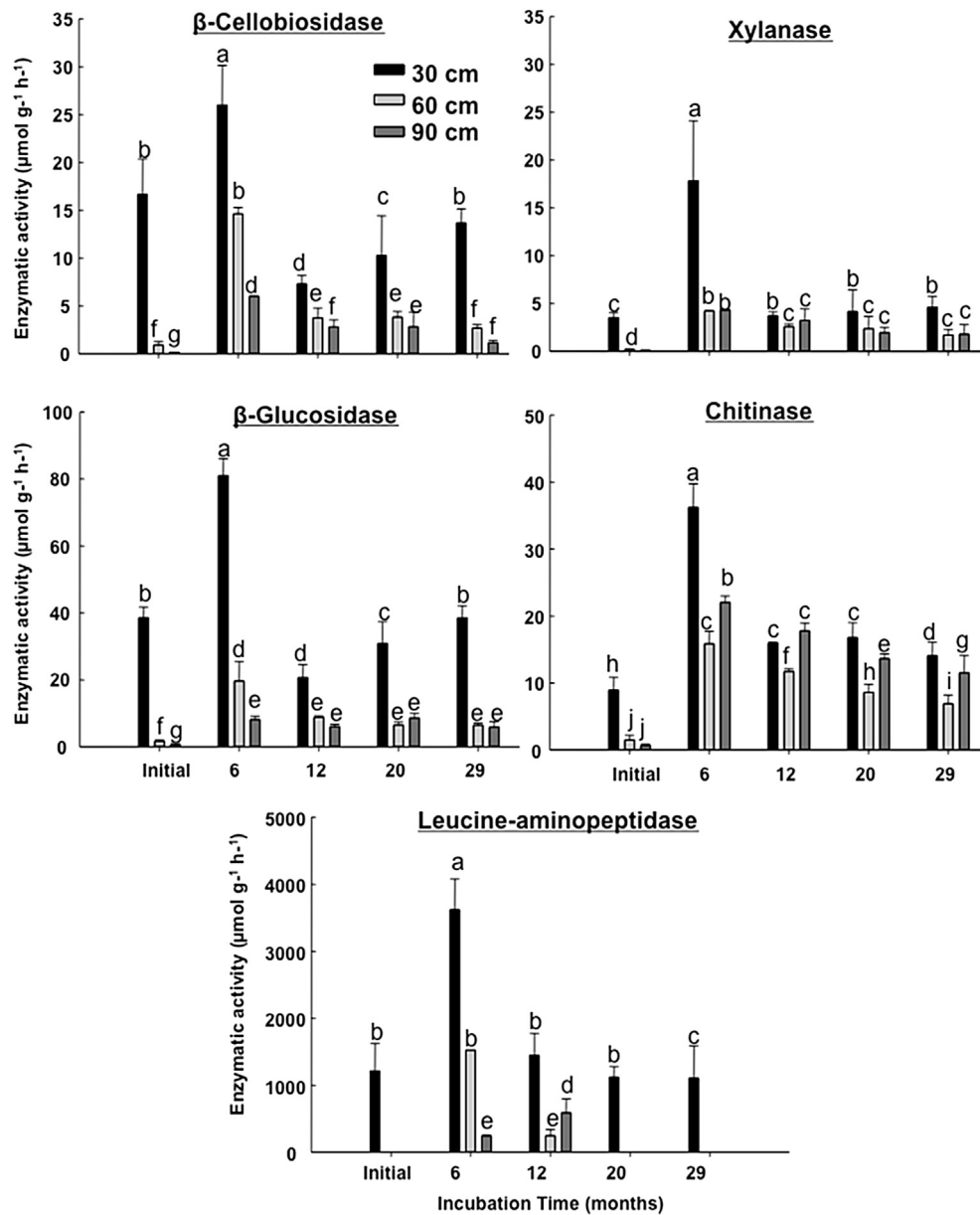
Fig. 4. Extra-cellular enzyme activities at different soil depths over time. Data are presented as means ± standard deviation (n = 3), different letters indicate significant differences (p < 0.05) of enzyme activities at a given depth over time (0, 6, 12, 20 and 29 months).

on degradation of SOM as compared to fresh litter. The addition of the latter increased the contribution of litter degrading phyla to the microbial communities, e.g. *Protobacteria, Actinobacteria* and *Ascomycota* capable of degrading cellulose and lignocellulose (Osono and Takeda, 2006), whereas a reduction of the relative abundance of *Firmicutes* and *Acidobacteria* was noted. [13]C labeled PLFA showed that in particular the contribution of fungi was stimulated 6 months after root litter addition (Fig. 5), in line with observations made by other authors (Bai et al., 2016). While root litter addition stimulated capiotropic microorganisms, those growing under nutrient limitations were reduced in such way that phyla characteristics of fungi and bacteria at 60 and 90 cm converged with those at 30 cm depth (Fig 3). Interestingly, this possibly resulted in lower phyla diversity, as can be noted by a decreased Shannon Weaver index and decreased evenness for bacterial as well as fungal communities at 30 and 60 cm to match those of microbial communities present at 90 cm depth (Table 1).

Such decrease of both indices after litter addition may be due to the stimulation of a subset of the total community represented by copiotrophic populations (Tardy et al., 2015).

Despite the inherent lower microbial richness and diversity of microbial phyla at 90 cm depth as compared to upper depths (Table 1), they were capable of root litter degradation (Fig. 1c). These data point to the fact that apparent taxonomical diversity described by the Shannon-Weaver Diversity Index is little related to potential functioning of soil microbial communities. This is in contrast to a former study showing that communities with a low Shannon-Weaver Diversity index have reduced capabilities to degrade litter (Baumann et al., 2013b). However, this study had been carried out in the laboratory under controlled conditions. The present study shows that in field soil despite apparently lower taxonomical diversity as indicated by the Shannon-Weaver Index and lower evenness at 90 cm depth, microbial communities had the potential to adapt to root litter degradation. This adaptation was

Table 2
Concentration of total PLFA (nmol g^{-1}), specific biomarkers for bacteria (Gram+ and Gram−) and fungi, as well as the ratio of fungal and bacterial biomarkers over time at all three depths. Data are presented as mean ± standard deviation (n = 3).

Soil depth	30 cm	60 cm	90 cm
0 months			
Total PLFA	23.8 ± 3.7	2.1 ± 0.8	0.9 ± 0.1
Gram−	2.4 ± 0.3	0.1 ± 0.1	0.1 ± 0.0
Gram+	6.4 ± 0.9	0.6 ± 0.3	0.2 ± 0.1
Fungi	1.8 ± 0.2	0.1 ± 0.0	0.1 ± 0.0
Fungi/bacteria	0.21 ± 0.01	0.24 ± 0.13	0.45 ± 0.19
6 months			
Total PLFA	38.9 ± 22.7	14.9 ± 4.9	12.0 ± 4.3
Gram−	3.4 ± 1.6	0.8 ± 0.4	0.8 ± 0.4
Gram+	5.5 ± 2.8	1.6 ± 0.7	1.1 ± 0.4
Fungi	7.2 ± 4.5	3.6 ± 1.2	3.7 ± 1.5
Fungi/bacteria	0.83 ± 0.27	1.50 ± 0.42	2.03 ± 0.36
20 months			
Total PLFA	26.3 ± 6.4	8.6 ± 1.2	7.8 ± 2.3
Gram−	2.2 ± 0.8	0.6 ± 0.2	0.6 ± 0.3
Gram+	4.5 ± 1.9	0.9 ± 0.5	1.0 ± 0.6
Fungi	3.3 ± 0.9	1.7 ± 0.2	1.3 ± 0.5
Fungi/bacteria	0.53 ± 0.18	1.31 ± 0.50	0.98 ± 0.57
36 months			
Total PLFA	32.61 ± 2.8	13.3 ± 2.7	9.2 ± 1.7
Gram−	2.9 ± 0.3	1.0 ± 0.2	0.7 ± 0.1
Gram+	7.6 ± 0.9	2.6 ± 0.6	1.8 ± 0.2
Fungi	3.1 ± 0.2	2.0 ± 0.4	1.3 ± 0.3
Fungi/bacteria	0.29 ± 0.01	0.54 ± 0.02	0.51 ± 0.08

evidenced by the decomposition dynamics in deep horizons, which showed similar degradation activity as in topsoil after a lag phase (Fig. 1c). During this phase, fungal communities showed the greatest changes, which were even more pronounced at greater soil depths (Table 2, Figs. 2 and 5). This is supported by the observation that fungal contribution to the degrading microbial communities increased most after the first 6 months as indicated by analyses of ^{13}C labeled PLFA (Fig 5). Our results are in agreement with recent work by Moll et al. (2015), who pointed out the central role of fungi for decomposition within the plant-soil system. Succession of microbial communities during plant litter decay is due to the replacement of rapidly growing r-strategists by more slowly growing K-strategists (e.g. Fierer et al., 2007; Bastian et al., 2009) because of the relative increase in the portion of recalcitrant

substrates (Baumann et al., 2009). This is in agreement with the increasing relative contribution of Gram+ bacteria and the decreasing contribution of fungi to the viable microbial community living on ^{13}C labeled organic matter (Fig. 5). It was interesting to note that the fungal contribution to ^{13}C PLFA during our experiment was related to the disappearance of fresh particulate organic matter (correlation with data shown by Sanaullah et al., 2011; r^2 = 0.64). This agrees with recent observations showing that fungi and Gram−bacteria are linked to the presence of fresh litter, while Gram+ bacteria are preferentially related to native and thus microbially processed SOM (Bai et al., 2016). The microbial communities present in later stages of litter degradation, i.e. from 20 months onwards may thus live on plant and microbial detritus left after fresh litter decomposition. Our results indicate that litter addition and its degradation stimulated similar communities at all three depths (Figs. 3 and 5), inducing strong differences of total microbial community structure changes at different soil depths detected by DNA fingerprinting (Fig. 2). Our data further show that a high Shannon Weaver diversity index and evenness of phyla are not required for litter degradation and its stabilization in soil.

Root litter addition increased the potential activity of all enzymes analysed at all depths (Fig. 4). The activity of chitinase remained higher than initial activity even three years after litter exposure (Fig. 4). Our data show that root litter addition to subsoil horizons stimulated enzymes activities (Allison and Vitousek, 2005). The enzyme pattern recorded at all three depths is in accordance with PLFA measurements as an indicator for viable microbial communities, showing highest values after 6 months and reduction thereafter in accordance with the exhaustion of particulate organic matter (Veres et al., 2015). It is interesting to note that the activities of chitinase recorded at all three depths were after 3 years still much higher than in soil before root litter addition in contrast to most other enzymes, which decreased to around initial values after 29 months. Chitinase is generally produced for the degradation of chitin, which is a cell wall component of fungi. Therefore, it may be related to the degradation of microbial detritus rather than fresh plant litter. The increased chitinase activity after 29 months is in agreement with the increasing contribution of Gram+ bacteria to the viable microbial community (Fig. 5). These bacteria are known for their ability to live on microbial detritus. Thus, enzyme activities may indicate availability of different types
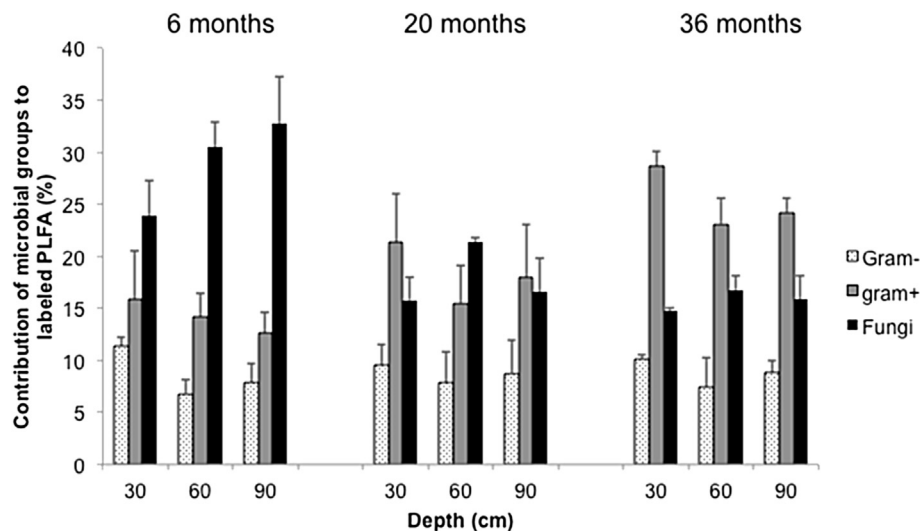

Fig. 5. Contribution (%) of specific PLFA to total ^{13}C labeled PLFA at all three depths at different times after root litter exposure (6 months, 20 months and 36 months). Data are presented as means ± standard deviation (n = 3).

of organic matter during litter degradation in soil (Veres et al., 2015) in accordance with microbial community changes. Higher relative increase of chitinase activity at 60 and 90 cm with regards to 30 cm after 29 months may indicate that microbial detritus was more important 3 years after root litter addition in deeper soil layers as compared to topsoil.

5. Conclusion

We analysed abundance and community composition of total as well as viable bacterial and fungal communities before and after addition of ^{13}C labeled root litter to topsoil and two subsoil depths. Succession of microbial communities occurred at 30, 60 and 90 cm during the three years of the *in situ* field experiment. Root litter addition led to convergence of microbial communities at all three soil depths through stimulation of a subset of the total community represented by copiotrophic populations. Fungal contribution to microbial communities was stimulated by fresh organic matter input, in particular in subsoil. Litter addition and its degradation were reflected by the dynamics of potential enzyme activities, indicating the changing availability of organic matter types at different times after the start of the experiment. Litter addition stimulated specific communities, which had similar characteristic and worked in similar way at all soil depths. Consequently, our study supports the general assumption that differences in microbial parameters between topsoil and subsoil are connected with the amount of fresh litter input.

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