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# Rhizosphere fungi actively assimilating plant-derived carbon in a grassland soil

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## ABSTRACT

Despite the advantages of the next generation sequencing (NGS) techniques, one of their caveats is that they do not differentiate between microbes that are actively participating in carbon cycling in the rhizosphere and microbes performing other functions in the soils. Here we combined DNA-SIP with NGS to investigate which rhizosphere fungi actively assimilate plant-derived carbon. We provided <sup>13</sup>C<sub>2</sub> to plants in intact soil cores collected from a grassland and sampled the rhizosphere in a time series to follow the fate of carbon in the rhizosphere mycobiome. We detected a difference between active rhizosphere fungi using plant-derived carbon and the total mycobiota: 58% of fungal species were using fresh rhizodeposits, and an additional 22% of fungal species received carbon several weeks later while 20% were not involved in cycling of freshly photosynthesized carbon. We show that members of Ascomycota, Mucoromycota, and basidiomycete yeasts were first users of freshly photosynthesized carbon, while fungi not using recently fixed carbon consisted mainly of mycelial (non-yeast) Basidiomycota. We conclude that a majority of fungi inhabiting the rhizosphere in this grassland ecosystem are actively using plant derived carbon either directly or via food-web interactions.

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## 1. Introduction

The introduction of high-throughput sequencing techniques has led to the creation of numerous inventories of fungal communities (coined as mycobiota), often based on sequencing of marker regions notably the internal transcribed spacer (ITS) region (Schoch et al., 2012; Lindahl et al., 2013). Sequence based information on the total fungal species composition in soils (Tedersoo et al., 2014) has been related to environmental conditions and has increased our understanding of fungal community assembly in various environments (e.g. Hartmann et al., 2015; Thomson et al., 2015). However, not all the sequences detected in a given soil sample are

from fungi that are active at the time of sampling (Emerson et al., 2017). Furthermore, a proportion of the sequences detected via DNA-based methods can be categorized as 'relic DNA' (Carini et al., 2016). This cell-free 'relic' DNA alongside DNA present within dead micro-organisms may act as sources of genetic material and bioavailable nutrients, whereas dormant structures such as spores act as a reservoir of individuals that may respond to a change in the environmental conditions (Carini et al., 2016; Emerson et al., 2017). Use of labeling techniques, such as the naturally occurring carbon isotope <sup>13</sup>C have allowed the tracking of carbon from plants to microbes and further to soil animals feeding on these microbes (Ostle et al., 2003; Leake et al., 2006; Drigo et al., 2010; Hannula et al., 2012) permitting a glimpse of the carbon transfer in almost real time. Other options to study microbial activity are the quantitative SIP with labeled H<sub>2</sub>O (Hungate et al., 2015) and use of RNA/DNA ratio (Hurt et al., 2001) based analysis that both give an estimate on the activity of the community.

Plant identity and, therefore vegetation composition, has been indicated as a major factor influencing the assembly of soil

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microbes (Peay et al., 2013) and consequently ecosystem function (Scherber et al., 2010; Cardinale et al., 2011). Plant roots release a wide range of compounds, collectively named rhizodeposits, into the rhizosphere (Haichar et al., 2014). Around 20% of the photosynthesized carbon is transferred to the rhizosphere as root-exudates (Lynch and Whipps, 1990; Kuzyakov and Domanski, 2000; Canarini et al., 2019) and the root-derived energy resources are a major input into the belowground food web (de Ruiter et al., 1995).

Traditionally, fungi have been placed in the soil food web in the detritus channel and as a mycorrhizal channel starting from living plant roots (Moore et al., 1996; de Boer et al., 2006). Bacteria are assumed to be better competitors than fungi for 'labile' carbon substrates released in the rhizosphere (Moore et al., 1996). This has led to a division into bacterial and fungal channels based on resource quality (de Vries et al., 2006; Malik et al., 2016). However, recent evidence has challenged this subdivision and studies have shown that fungi receive more carbon from the plants in the rhizosphere than assumed earlier (Buée et al., 2009; Hannula et al., 2012, 2017; Morriën, 2016). This lack of apparent separation between the fungal and bacterial energy channels is further fueled by the notion that fungi are functionally a very diverse group of organisms belonging to many trait-based categories (Treseder and Lennon, 2015).

Some species of saprotrophic fungi are characterized by rapid growth rates, prolific spore production and ability to use only simple carbon compounds, appearing thus as perfect rhizosphere inhabitants (Newsham et al., 1995; Broeckling et al., 2008; De Graaff et al., 2010). Fungal species from the order Mucorales, as well as filamentous ascomycetes (such as aspergilli and penicilli) and basidiomycete and ascomycete yeasts (Botha, 2011; Mestre et al., 2011) form together a group that utilise simple sugars efficiently and are able to grow quickly, thus competing effectively with bacteria (de Boer et al., 2005). In terrestrial ecosystems, input of plant derived carbon resources is not only from rhizodeposits but also from lignocellulose-rich residues of above and belowground plant biomass. This implies that both recalcitrant and labile carbon compounds are present in the vegetation during the growing season and it is unknown if there is a clear separation of fungal species that grow on these resources.

It has been shown that fungal interactions and community diversity can promote plant diversity and productivity (van der Heijden et al., 1998; Wagg et al., 2014; Peay et al., 2016) but can also have potential negative effects through actions of plant pathogenic fungi (Raaijmakers et al., 2009). Thus, the functionality of the mycobiota in the rhizosphere will affect both individual plants and plant community function (Hannula et al., 2017; Morriën et al., 2017). Furthermore, root-associated fungi can form an important link in the flow of energy and nutrients from plants to predatory soil organisms (Wardle et al., 2004). Together, these plant-microbe-consumer effects create complex multi-trophic interactions in the rhizosphere (Wardle et al., 2004; Bais et al., 2006; Philippot et al., 2013; Panke-Buisse et al., 2014). The contribution of fungi to rhizosphere processes is not limited to the plant-fungal interaction, since fungi also have the potential to modulate the food web interactions through competition with each other and bacteria and through parasitism of other fungi, insects and nematodes (Kerry, 1988; Boddy, 2016) thus forming a complex network of belowground multi-trophic interactions.

In the current study, we followed the fate of recently photosynthesized carbon in the rhizosphere of intact plant-covered soil cores collected from restored grassland ecosystems, using a  $^{13}\text{C}$  stable isotope labeling approach. These cores originate from a single grassland (Nieuw Reemst, NR), and were collected as part of a larger study investigating the effects of grassland restoration on

soil biota (Hannula et al., 2017; Morriën et al., 2017). Here, we were specifically interested in a more detailed estimation of fungal groups actively assimilating plant-derived carbon in the grassland ecosystem. We used a  $^{13}\text{C}$  labeling approach to separate the fungi that actively incorporate recently photosynthesized, plant-derived carbon from the inactive fungi or fungi fulfilling other ecological roles such as decomposition of soil organic matter. We used intact soil cores and followed presence and activity of fungal species using a time series of sample collection. We tested the hypothesis that: (1) AMF, endophytes as well as fungi known to have rapid growth rates and high competitive abilities in the rhizosphere would be part of the mycobiota actively using plant derived carbon and hence detected to have  $^{13}\text{C}$  immediately after labeling event while; (2) at the later sampling stages active fungal community structure shifts towards slower growing fungi and more fungi using secondary carbon sources such as insect and nematode parasites.

## 2. Material and methods

### 2.1. Experimental set-up and sampling

In July 2012, intact soil cores of 12 cm diameter and 20 cm deep with native grass-dominated vegetation were collected from a restored grassland ecosystem that has been taken out of agricultural production 21 y prior to sampling. The field (Nieuw Reemst, 52°2'33"N, 5°46'29"E) is located in the Veluwe region, in the center of the Netherlands. The dominant grass species in all the cores were *Holcus lanatus* and *Agrostis capillaris*, both common grassland species in the region. The soil type is glacial sandy soil (holtpodzol), soil pH was 4.66, total nitrogen content 1.36 g kg<sup>-1</sup> and total phosphorus content (Olsen P) 0.23 g kg<sup>-1</sup>. More details on sampling and the field location can be found in Hannula et al. (2017) and in Morriën et al. (2017). Cores used here are a subset of cores used in our previous studies, namely field site 'NR' (Hannula et al., 2017; Morriën et al., 2017). Soil cores were collected 1 week before labeling to allow the microbial communities to stabilize after coring and transportation. At three replicate spots in the field, four cores per spot were collected. The soil moisture at the time of labeling was 24% (w/w) and the average total plant biomass per core was 32 g (DW). Three cores from each spot (making a total of nine cores) were labeled with 99.99 atom-%  $^{13}\text{CO}_2$  (Cambridge Isotope Laboratories, Andover, MA, USA). Prior to the start of labeling the plants were allowed to assimilate carbon until the  $\text{CO}_2$  concentration fell below 300 ppm which took about 3 h. During this period the photosynthesis rate of the plants was monitored. When the  $\text{CO}_2$  concentration of 300 ppm and hence depletion of  $\text{CO}_2$  was reached  $^{13}\text{CO}_2$  was injected into the chamber using a gas-tight pumping system until the  $\text{CO}_2$  concentration reached 450 ppm. During the labeling period additional  $^{13}\text{CO}_2$  was injected when the concentration fell below 350 ppm. In total about 4.5 L of  $^{13}\text{CO}_2$  was injected into the chamber. The plants were labeled during 8 h in the light, after which the  $^{13}\text{CO}_2$  was partially removed by opening the chamber. The cores were left in the cabinet for the next 12 h (including a dark period) when no extra  $^{13}\text{CO}_2$  was added. The duration of labeling and amount of label added was optimized earlier using the same system (Drigo et al., 2007; Hannula et al., 2012). Three cores (one per spot) were placed in a similar chamber and kept under the same light and temperature conditions, but with a  $^{12}\text{CO}_2$  atmosphere, thus representing the control treatment. The  $\text{CO}_2$  concentrations in the chambers were monitored throughout the experiment. For more details on the labeling see (Hannula et al., 2017).

At 24 h after start of the labeling all the cores were removed from the chambers and 3 cores from the  $^{13}\text{CO}_2$  treatment and 1 control core from  $^{12}\text{CO}_2$  treatment were destructively harvested.

The remaining cores were kept in the same growth chambers under ambient  $^{12}\text{CO}_2$  conditions before destructive harvesting after 6 or 13 d after end of labeling (called 1 week and 2 weeks after start of labeling). The total number of samples was thus 12. Samples for molecular analysis were collected from the rhizosphere soil (i.e. soil adhering to roots on removal of plants from the soil core) from the upper, root filled part of the cores by brushing the roots, homogenized my mixing, collected to an Eppendorf tube and frozen at  $-80^\circ\text{C}$  prior to DNA-extraction (Hannula et al., 2017). Our previously published data (Morriën et al., 2017) relating to soil animal, plant and phospholipid fatty acids (PLFA) composition in this specific site are presented here as background information in Supplementary Fig. 1.

## 2.2. Molecular analysis

DNA was extracted from approximately 0.25 g of rhizosphere soil using MoBio PowerSoil Kit according to the manufacturer's instructions. The quantity of DNA was inspected using Quant-iT™ PicoGreen® dsDNA Assay Kit (Thermo Life sciences) using  $\lambda$ -DNA as standard and quality was inspected on an agarose gel stained with ethidium bromide and visualized under UV-light.  $^{13}\text{C}$ -enriched DNA was separated from  $^{12}\text{C}$  DNA by density-gradient centrifugation and analysed as described in Neufeld et al. (2007). In short, 2  $\mu\text{g}$  of DNA was centrifuged in a micro- ultracentrifuge (Sorvall Discovery M120 SE with S120-VT vertical rotor) for 70 h at 60 000 RPM (250 000 g) in CsCl with starting density of  $1.725\text{ g ml}^{-1}$ . A fraction collector (CMA 470; Harvard Apparatus) was used to collect 18 fractions (each ca. 100  $\mu\text{l}$ ) at a constant flowrate of  $350\text{ }\mu\text{l min}^{-1}$ . The exact densities of the fractions were determined using an AR200 refractometer (Reichert, Germany) and purified using ethanol precipitation with PEG. All the (18) fractions from one sample from each run were subjected to real-time PCR using the Rotor-Gene SYBR® Green PCR Kit (Qiagen) on a Rotor-Gene 3000 (Gorbet Research, Sydney, Australia) with primers ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') and ITS9 (5'- GAA CGC AGC RAA IIG YGA-3') (Ihrmark et al., 2012) to verify the presence of DNA in the fractions in desired densities (for a fragment to be considered to contain mainly  $^{13}\text{C}$  labeled fungi, the average density was  $1.74\text{ g ml}^{-1}$  and for the selected  $^{12}\text{C}$  fractions  $1.70\text{ g ml}^{-1}$ , Supplementary Fig. 2). Based on this, the fractionated DNA was combined into two subsets based on the presence of nucleic acids in the indicated densities, the first one containing fractions with mainly  $^{13}\text{C}$ -enriched DNA and latter fractions containing unlabeled  $^{12}\text{C}$  DNA. The density of the fragments used, the assignment to subsets and their cycling threshold (ct) values based on fungal qPCR are shown in Supplementary Fig. 2. The terms  $^{13}\text{C}$  fraction and  $^{12}\text{C}$  fraction based on their densities are used throughout the manuscript even though we note that GC-content of individual OTUs will have an effect on its location on the spectrum. The absence of observable fungal DNA in densities around  $1.74\text{ g ml}^{-1}$  in  $^{12}\text{C}$  labeled control samples was confirmed with PCR and these samples were subjected to sequencing as were the samples labeled with  $^{13}\text{C}$ .

The fungal ITS2 region was amplified from the twelve  $^{13}\text{C}$  pooled fractions' and twelve  $^{12}\text{C}$  pooled fractions' using the same primers as above. The PCR conditions used are described in more detail in Thomson et al. (2015). The PCR products were purified using QIAquick PCR purification Kit (Qiagen) with added sodium acetate (pH 5) according to the manufacturer's protocol. The length of the resulting fragment was inspected using gel electrophoresis and quantified using Quant-iT™ PicoGreen® dsDNA Assay Kit (Thermo Life sciences). Finally, the amplicons were pooled at equimolar concentrations and (pyro)sequenced by Macrogen (Korea).

## 2.3. Bioinformatic analysis

Sequences were analysed using a Snakemake workflow (Koster and Rahmann, 2012) that follows the SOP for 454 data in mothur version 1.33.2 (Schloss et al., 2009). Per sample standard flowgram format (SFF) files were created using the sffile command allowing no mismatches to the barcodes. Flowgrams were trimmed to be at least 470 and maximum 700 flows. Flowgrams were corrected using the shhh.flows command (Quince et al., 2011) and trimmed to be at maximum 700 bp. Afterwards the results of the different sff files were combined for further analysis. ITS2 regions were extracted using ITSx 1.0.10 (Bengtsson-Palme et al., 2013). Chimeric sequences were identified and removed by aligning the sequencing to the UCHIME release of UNITE of November 18, 2018 (Köljalg et al., 2013) using USEARCH 7.0.1090 (Edgar et al., 2011). Sequences were clustered into OTUs with ESPRIT-Tree with default settings and 97% cut-off (Cai and Sun, 2011). Clustering results were converted into mothur format and classified using the classify.seqs command in mothur against the UNITE database release for mothur of November 18, 2018 (Köljalg et al., 2013). Taxonomic classification and OTU clustering data are combined into the BIOM format (McDonald et al., 2012).

## 2.4. OTU definition

The fungal OTUs were assigned into functional group when possible (Hannula et al., 2017). This approach is similar to that taken earlier by Tedersoo et al. (2014) and refined by Nguyen et al. (2016). The functional groups defined here were AMF, coprotrophic fungi, endophytes (including dark septate endophytes), potential plant pathogens, aspergilli and penicilli, nematophagous fungi, saprotrophic fungi, wood pathogens and decomposers, yeasts, entomopathogens, animal pathogens and others (lichens, ectomycorrhizal fungi and mycoparasites). Both data on presence-absence of the OTUs and OTUs percentage of total reads were used in the analysis. For each analysis the data type used is specified in the text.

The relative labeling of each OTU was calculated using formula (modified from Kramer et al. (2016)). We did not subtract the presence in  $^{12}\text{C}$  labeled control samples as was suggested by Kramer et al. (2016) as the  $^{13}\text{C}$  fraction of the control samples had very little reads that led to almost solely zero values. If an OTU was not detected in one of the fractions of a sample, but confirmed to be present in the same fraction of another sample, the relative abundance of the OTU in the sample it was present in was used. We did not use taxon specific correction for GC content as there is no correction available for fungi.

$$\text{Labeling of OTUs} = \frac{\text{relative abundance of OTU in } ^{13}\text{C fraction}}{\text{relative abundance of OTU in } ^{12}\text{C fraction}} \quad (1)$$

## 2.5. Statistical analysis

The difference in the amount of reads per sample was standardized by using relative abundance of total OTUs in the same sample. Absolute values were used only for the diversity and rarefaction calculations. No effect of sequencing depth on number of OTUs detected was found (i.e. no correlation between number of sequences obtained and number of species observed was detected). The effect of sampling time and fraction ( $^{13}\text{C}$  or  $^{12}\text{C}$ ) on fungal community composition at the level of phylum, class, order and OTU was estimated using ANOVA combined with Tukey's pairwise comparisons as the post-hoc test. When the distribution of data was not in accordance with assumptions of ANOVA (i.e. due to

complete absence of a group in one or more treatments), Kruskal-Wallis test was used in combination with Mann-Whitney as a post-hoc test. The average labeling of OTUs assigned to each phylum was compared to the average labeling across all fungi using ANOVA and a pairwise *t*-test. Similarly, the average labeling of OTUs belonging to classes were compared to the average labeling of their respective phyla.

The OTUs explaining most differences between time points were analysed using SIMPER implemented in program PAST (Hammer et al., 2001) and their statistical significance tested using ANOVA. We evaluated the difference in community structure of fungi in relation to isotope incorporation and time with two-way PERMANOVA with Bray-Curtis as a distance measure. Estimates of alpha-diversity were calculated using PAST and both Simpson and Shannon-H indexes were calculated using the non-transformed raw data and resulting values were subjected to ANOVA to compare treatment effects. Rarefaction curves of the observed richness were calculated in PAST using 1000-fold resampling without replacement.

### 3. Results

After removing the  $^{13}\text{C}$  fraction samples of  $^{12}\text{C}$  labeled controls due to their low sequencing depth (<100 reads obtained), the remaining samples had on average 15 838 sequences per sample with a range of 7926–25982 reads. A total of 1669 OTUs were detected in the soil. Approximately 28% of the OTUs could not be assigned to a phylum and are referred to as unknown fungi. The most abundant phylum making up 51.3% of the OTUs was Ascomycota. Basidiomycota made up 12.9% of the OTUs and Mucoromycota, (including Glomeromycotina) comprised 5.6% of OTUs. The largest ascomycetal classes were Sordariomycetes (18% of all OTUs) and Dothideomycetes (9%), the largest basidiomycete class was Agaricomycetes (9%). Largest subphylum of Mucoromycota were Glomeromycotina (making up 4% of total OTUs). The OTUs were further divided into 16 functional groups based on their functional guild and growth morphology as indicated in the Material and Methods. OTUs which could only be assigned to phylum level were classified as ‘unclassified Ascomycota’, ‘unclassified Basidiomycota’ or ‘unclassified Chytridiomycota’. The most common functional classification after ‘unknown function’ and ‘unknown ascomycete’ was ‘saprotroph’ with 182 OTUs assigned to

this group.

The  $\alpha$ -diversity was not different between the sampling times ( $F = 0.56$ ,  $p = 0.59$ ), but the  $^{12}\text{C}$  DNA fractions had significantly more taxa present ( $F = 6.12$ ,  $p = 0.04$ ) than the  $^{13}\text{C}$  fractions (Fig. 1). We further evaluated the difference in community structure of fungi in relation to isotope incorporation and time with two-way PERMANOVA on Bray-Curtis distances. The fungal community structure was not significantly different between the  $^{12}\text{C}$  and  $^{13}\text{C}$  DNA-fractions ( $F = 0.74$ ,  $p = 0.34$ ) or the sampling times ( $F = 0.85$ ,  $p = 0.38$ ).

At the first sampling time point, 1 d after labeling, 42% of all OTUs detected were found only in the  $^{12}\text{C}$  fraction. Of these OTUs restricted to the  $^{12}\text{C}$  fraction in the first sampling moment, 275 (68%) were still in the  $^{12}\text{C}$  fraction one week later, and 218 OTUs (20.43% of total OTUs) were consistently found in the  $^{12}\text{C}$  fraction of the soils but never found to have incorporated  $^{13}\text{C}$ . The rhizosphere fungi not using root-derived  $^{13}\text{C}$  were assigned to Ascomycota (110), Basidiomycota (30), and Mucoromycota (15). Of the 15 Mucoromycota OTUs, the majority (11) were classified as Glomeromycotina. From the remaining 63 OTUs, 62 OTUs could not be identified at the phylum level and one was assigned as Rozellomycota. Some OTUs (22; 2.06% of total OTUs) were consistently found in  $^{13}\text{C}$  labeled fractions at all time points (and in the  $^{12}\text{C}$  fraction of the control) but never detected in  $^{12}\text{C}$  labeled fraction of the  $^{13}\text{C}$  labeled samples. Of these, 10 were assigned to Ascomycota, one to Mucoromycota, one to Basidiomycota, and one to Rozellomycota each and the remaining 9 were unclassified.

The average abundance of all OTUs decreased albeit not significantly with time since labeling while the average labeling of OTUs (calculated using formula eq (1)) was highest 1 week after labeling (Fig. 2). The average abundances of OTUs assigned to phyla and classes remained constant over time while their average labeling varied (Figs. 2 and 3, Table 1). At the phylum level, members of the phylum Ascomycota ( $t = 49.76$ ,  $p < 0.05$ ) were the most labeled 1 d after labeling with  $^{13}\text{C}$ , while Basidiomycota ( $t = 45.32$ ,  $p < 0.05$ ), and unclassified fungi ( $t = 4.85$ ,  $p < 0.05$ ), were on average less labeled with  $^{13}\text{C}$  compared to the average  $^{13}\text{C}$  labeling of OTUs across phyla (Fig. 2). One week after  $^{13}\text{C}$  labeling, members of phylum Chytridiomycota ( $t = 3.82$ ,  $p < 0.05$ ) were on average the most labeled fungi, even though their contribution to the total amount of labeled fungal OTUs was low due to the small number of non-abundant OTUs (Fig. 2).

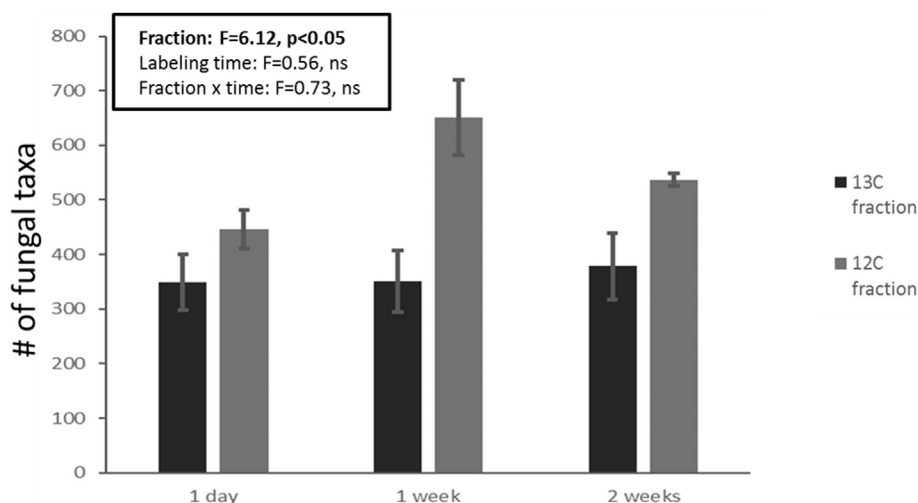
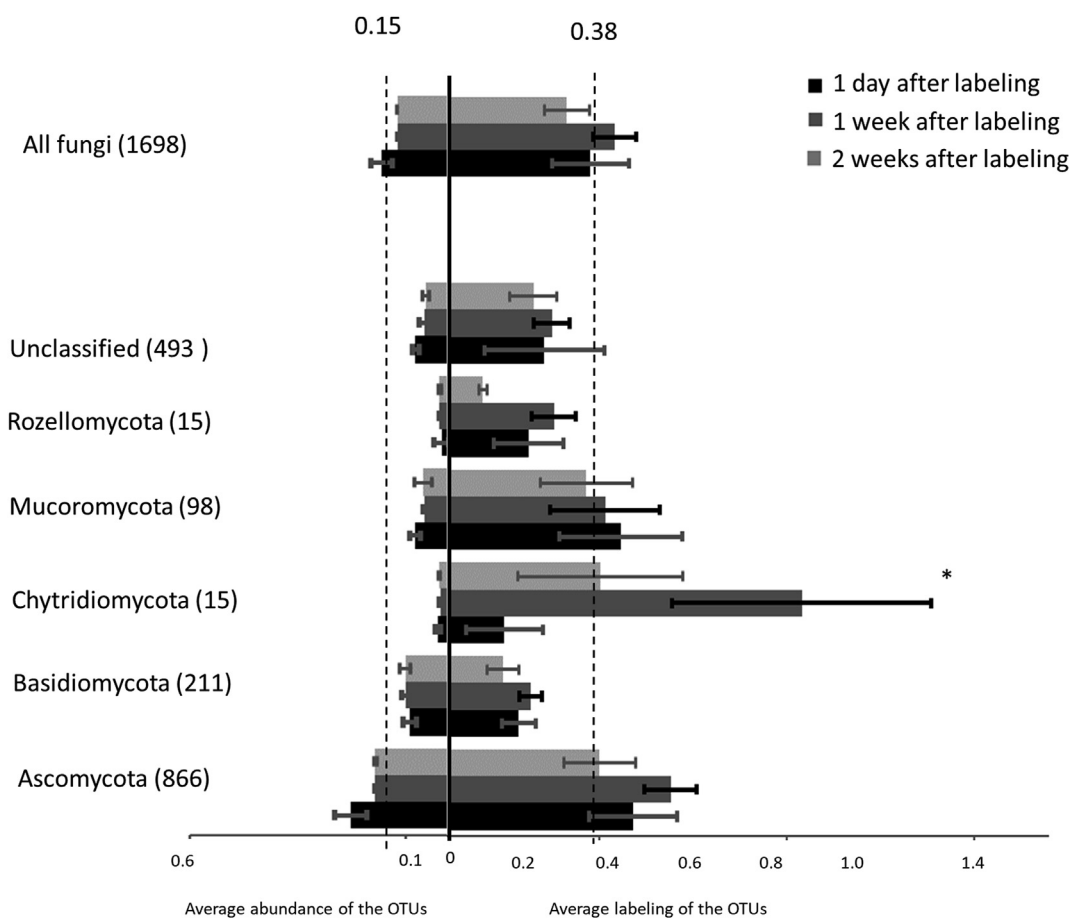


Fig. 1. The number of fungal taxa detected in intact grassland soil cores in  $^{13}\text{C}$  (black) and  $^{12}\text{C}$  (light grey) fractions 1 d, 1 week and 2 weeks after labeling with  $^{13}\text{CO}_2$ . Bars represent averages with standard errors.



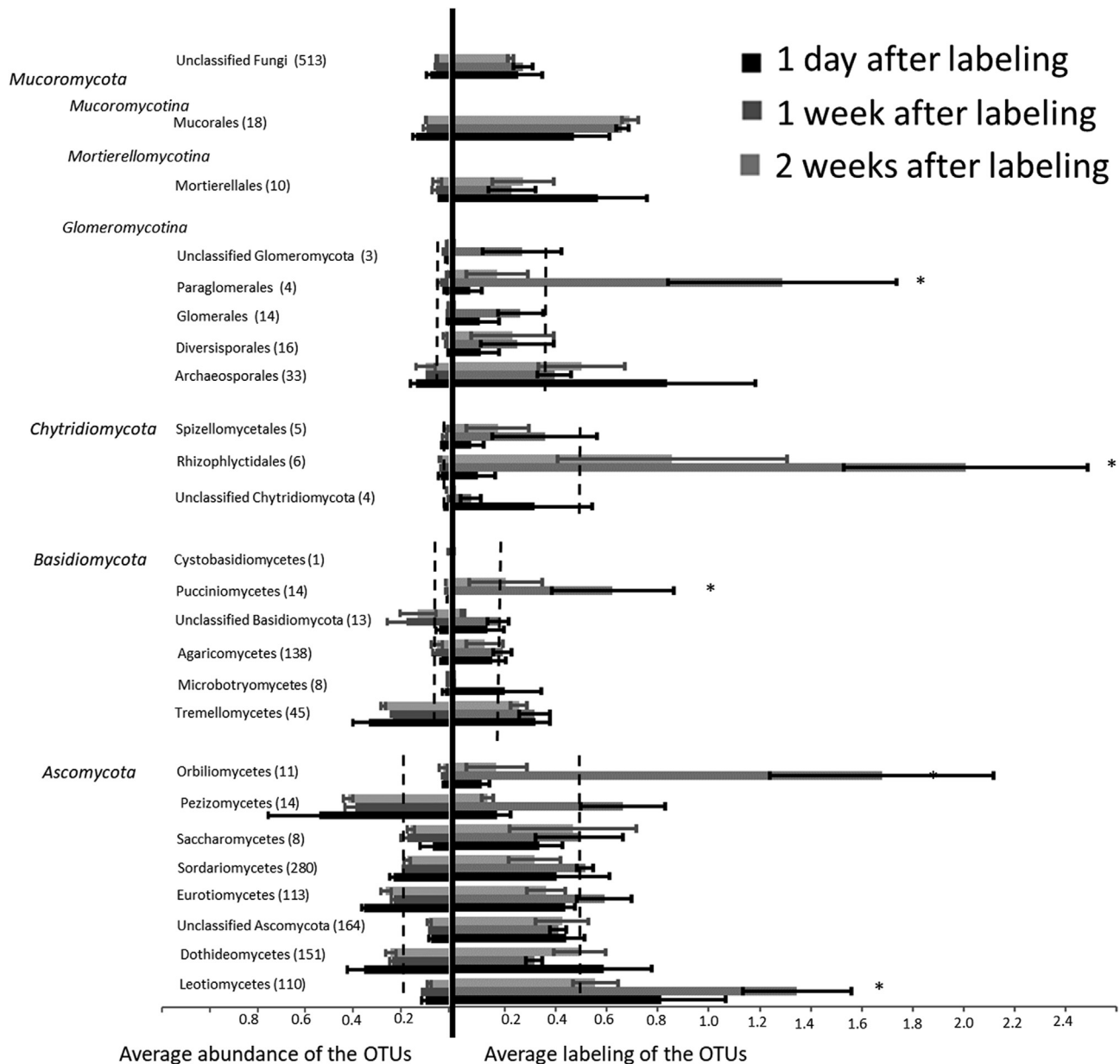
**Fig. 2.** Average labeling (right side) calculated using formula eq (1) and average abundance (left side) of OTUs belonging to major fungal phyla in time after labeling. Black bars represent averages in OTUs 1 day after labeling, dark grey bars OTUs 1 week after labeling and light grey bars OTUs 2 weeks after labeling. Bars represent averages with standard errors. The dotted lines are average OTU abundance (on the left) and average OTU labeling (on the right) of all OTUs. Stars indicate phyla that are significantly affected by time since labeling.

There was also large variation in average  $^{13}\text{C}$  labeling of classes within phyla, both within and between harvest dates (Fig. 3, Table 1). Taxa most enriched with  $^{13}\text{C}$  one day after labeling compared to the average labeling of that phylum were ascomycete classes Leotiomyces ( $F = 20.69$ ,  $p < 0.01$ ) and Dothideomycetes ( $F = 8.92$ ,  $p < 0.05$ ), and orders Archaeosporales ( $F = 12.25$ ,  $p < 0.05$ ) and Mortierellales ( $F = 10.20$ ,  $p < 0.05$ ) from Mucoromycota. Notably, Archaeosporales were substantially more labeled with  $^{13}\text{C}$  than other orders of AMF ( $F = 12.25$ ,  $p < 0.05$ ). The class Tremellomycetes was the most labeled of basidiomycete classes ( $F = 17.88$ ,  $p < 0.05$ ). Low amount of labeling at the first sampling time was detected for Orbiliomycetes, Pezizomycetes, and unclassified Glomeromycota (Fig. 3, Table 2). One week after labeling the average abundances of these taxa had not changed as compared to the first day but the intensity of labeling had increased or decreased (Fig. 3). Of the most  $^{13}\text{C}$  labeled taxa at the first sampling timepoint (1 d), Leotiomyces and Archaeosporales were the only taxa that remained significantly more labeled than average 1 week later. The taxa that became labeled between 1 d and 1 week sampling were Orbiliomycetes (previously among unlabeled taxa;  $F = 7.27$ ,  $p < 0.05$ ), Pucciniomycetes (and especially Platygloeales) ( $F = 21.34$ ,  $p < 0.05$ ), Paraglomerales ( $F = 6.48$ ,  $p < 0.05$ ) and Mucorales ( $F = 11.46$ ,  $p < 0.05$ ). Two weeks after the labeling there were no groups containing  $^{13}\text{C}$  that were previously found only in  $^{12}\text{C}$  fractions, which makes that time point comparable to the situation 1 week after labeling albeit with on average less  $^{13}\text{C}$  in all groups

(Fig. 3).

At the level of orders, differences in labeling of OTUs between time points became increasingly apparent for orders belonging to Ascomycota and Basidiomycota (Supplementary Fig. 3). One day after labeling GS37, Thelebolales, Myrmecridiales and Capnodiales in phylum Ascomycota were the OTUs most enriched in the  $^{13}\text{C}$  fraction. The OTUs classified as GS37 and Thelebolales were on average 6-fold more abundant in the  $^{13}\text{C}$  fraction than average ascomycete OTUs. The OTUs belonging to the orders Tremellales and Sebaciniales were the most  $^{13}\text{C}$ -labeled from the phylum Basidiomycota one day after labeling. Basidiomycete orders Russulales, Polyporales and Auriculariales consisting mostly of ectomycorrhizal fungi and wood degrading saprotrophs were not labeled at all one day after labeling. One week after labeling, Helotiales and Orbiliales were the ascomycete orders found in the  $^{13}\text{C}$  fraction which had not been present in the  $^{13}\text{C}$  fraction at 1 d; and Trichosporonales and Platygloeales were the most  $^{13}\text{C}$  labeled basidiomycete orders.

When OTUs were subdivided into functional groups, we observed that OTUs classified as endophytes were on average the most  $^{13}\text{C}$  labeled group at the first two time points (Fig. 4). Other functional groups that were labeled with  $^{13}\text{C}$  more than average at the first sampling moment were yeasts, unknown Ascomycota, and aspergilli and penicilli (molds) (Fig. 4). At the second time point, 1 week after labeling, nematophagous fungi and coprotrophic fungi, together with endophytes, were the groups most abundant in the



**Fig. 3.** Average labeling (right side) and average abundance (left side) of OTUs belonging to major fungal classes in time after labeling. Black bars represent averages in OTUs 1 day after labeling, dark grey bars OTUs 1 week after labeling and light grey bars OTUs 2 weeks after labeling. Bars represent averages with standard errors. The dotted lines are average OTU abundance (on the left) and average OTU labeling (on the right) of all OTUs belonging to the same phylum. Stars indicate classes that are significantly affected by time since labeling.

$^{13}\text{C}$  fractions. In addition, a large group of saprotrophic fungi were found more commonly in the  $^{13}\text{C}$  labeled fraction. After 2 weeks since labeling with  $^{13}\text{C}$ , situations looked similar to the one a week earlier except that the average amount of  $^{13}\text{C}$  was lower in all the groups. Yeast (both ascomycete and basidiomycete) OTUs were among the groups that quickly responded to plant-derived carbon, as they were above average labeled in the beginning, but did not use carbon during prolonged incubation. Groups such as ectomycorrhizal fungi and animal pathogens were neither abundant nor incorporating plant-derived carbon in these cores.

When considered in detail, it became apparent that the individual OTUs that were most labeled with  $^{13}\text{C}$  right after labeling belonged to the phylum Ascomycota (Table 2). Most (13 out of 20) of the OTUs receiving most of the carbon from the plant after 1 d were no longer among the most  $^{13}\text{C}$  labeled OTUs by the next

sampling time one week later. Furthermore, out of the 20 OTUs that were highly labeled with  $^{13}\text{C}$  1 week after sampling, only two were labeled immediately, but the rest acquired plant-derived carbon later on (Table 2).

#### 4. Discussion

We used  $^{13}\text{C}$  labeling of typical grassland plants in intact soil cores to demonstrate that in this ecosystem over half of the members of rhizosphere mycobiota are actively involved in the turnover of freshly photosynthesized carbon released to the rhizosphere as root exudates. Furthermore, time series of sampling after the labeling gave a glimpse into the different positions of fungal groups in the rhizosphere food web. The use of intact soil cores provided a realistic view of the fungal community under field

**Table 1**

ANOVA on  $^{13}\text{C}$  labeling of the OTUs in fungal phyla compared to average fungal labeling and in fungal classes compared to average labeling of the phyla they belong to after 1 d and 1 and 2 weeks after labeling. Significant values are marked in bold.

	Average labeling $^{13}\text{C}/^{12}\text{C}$ (1 day)		Average labeling $^{13}\text{C}/^{12}\text{C}$ (1 and 2 weeks)	
	F	p	F	p
Ascomycota	4.525	0.101	0.176	0.689
Leotiomyces	<b>20.690</b>	<b>0.010</b>	<b>23.900</b>	<b>0.003</b>
Dothideomycetes	<b>8.922</b>	<b>0.040</b>	0.983	0.360
Unclassified Ascomycota	4.457	0.102	1.073	0.340
Eurotiomycetes	0.059	0.820	0.504	0.504
Sordariomycetes	0.623	0.474	<b>6.974</b>	<b>0.038</b>
Saccharomycetes	4.160	0.111	2.109	0.197
Pezizomycetes	<b>21.700</b>	<b>0.010</b>	<b>15.260</b>	<b>0.008</b>
Orbiliomycetes	<b>22.010</b>	<b>0.009</b>	5.437	0.059
Basidiomycota	1.538	0.283	3.526	0.110
Tremellomycetes	<b>17.880</b>	<b>0.013</b>	4.480	0.079
Microbotryomycetes	6.125	0.069	Nd	
Agaricomycetes	5.620	0.077	4.789	0.071
Pucciniomycetes	Nd		<b>21.340</b>	<b>0.004</b>
Unclassified Basidiomycota	6.532	0.063	<b>6.035</b>	<b>0.049</b>
Cystobasidiomycetes	Nd		Nd	
Chytridiomycota	7.200	0.055	0.430	0.536
Unclassified Chytridiomycota	0.053	0.995	1.940	0.213
Rhizophlyctidales	0.346	0.588	<b>4.432</b>	<b>0.032</b>
Spizellomycetales	1.571	0.278	0.617	0.462
Mucoromycota: Glomeromycotina	0.314	0.605	0.239	0.642
Archaeosporales	<b>12.254</b>	<b>0.021</b>	<b>7.077</b>	<b>0.029</b>
Diversisporales	3.494	0.135	0.080	0.785
Glomerales	0.468	0.532	3.642	0.093
Paraglomerales	5.604	0.077	<b>6.481</b>	<b>0.034</b>
Unclassified Glomeromycota	7.239	0.055	0.312	0.592
Mucoromycota: other	0.243	0.648	1.614	0.251
Mortierellales	<b>10.201</b>	<b>0.035</b>	0.732	0.441
Mucorales	0.760	0.432	<b>11.460</b>	<b>0.015</b>
Unclassified	0.968	0.381	0.326	0.589

conditions, as no disturbances known to affect fungi, such as homogenization of soils and planting seeds, were used. Also, we showed that functional assignment of fungal species into functional guilds (Nguyen et al., 2016; Kvaschenko et al., 2017) can be done relatively accurately based on sequence data as we saw relevant functional guilds positioned in accordance with the measured flow of carbon through the soil food web (Morriën et al., 2017).

In traditional food-web models (Moore et al., 1996) fungi are situated in the second trophic level, together with bacteria, being the consumers of root exudates and litter from plants. We were able to place just over half (58%) of the members of the rhizosphere fungal community in the strict category of root exudate consumers (i.e. they were immediately after labeling incorporating plant-derived carbon in their DNA). We showed that phyla of fungi often associated with fast growth in soils (namely classes belonging to Ascomycota and Mucoromycota (Veresoglou et al., 2018)) were the first rhizosphere fungi to receive labeled carbon from plants. As expected, the differences in growth and response to root exudates was not conserved at the level of phylum. For example, Tremellales (basidiomycete yeasts) and Sebaciales, an order known to be closely associated with plants (Weiß et al., 2016), were actively accumulating recently labeled plant-derived carbon, while other members of the phylum Basidiomycota were less actively involved in rhizosphere carbon related processes (Fig. 3). Similarly, for Ascomycota, the Leotiomyces were over-represented in the fraction actively assimilating plant-derived carbon while

Orbiliomycetes appeared to be mostly using other resources than recently fixed plant-derived carbon for growth. Leotiomyces is a class of fungi containing many plant pathogenic, endophytic and rhizosphere species, as well as species known as mycoparasites and litter saprotrophs (Zhang and Wang, 2015). This multitude of functions might explain why Leotiomyces were found in  $^{13}\text{C}$  fraction immediately after labeling and became even more enriched in  $^{13}\text{C}$  1 week after labeling; the labeled community after prolonged incubation did not consist of the same species that were labeled after 1 d.

Yeasts and some fast-growing ascomycetes (e.g. aspergilli and penicilli), in particular, seem to play an important role in the soil food web using labile carbon sources exuded by the roots. They are likely to compete with bacteria, thereby occupying a comparable ecological niche (Botha, 2011; Treseder and Lennon, 2015). These species have rapid growth rates and prolific spore production, and are able to use simple carbon compounds and to incorporate the carbon quickly into their DNA making them good competitors in the rhizosphere environment (Newsham et al., 1995; Broeckling et al., 2008; De Graaff et al., 2010; Aguilar-Trigueros et al., 2015). The slower growing, less competitive fungi (for example several members of phylum Basidiomycota) often invest more resources in exploration of new patches (Veresoglou et al., 2018) and in production of extracellular enzymes to degrade more complex biopolymers (Aguilar-Trigueros et al., 2015). Our data suggest that, in this system, these slower growing fungi (K-strategists) received



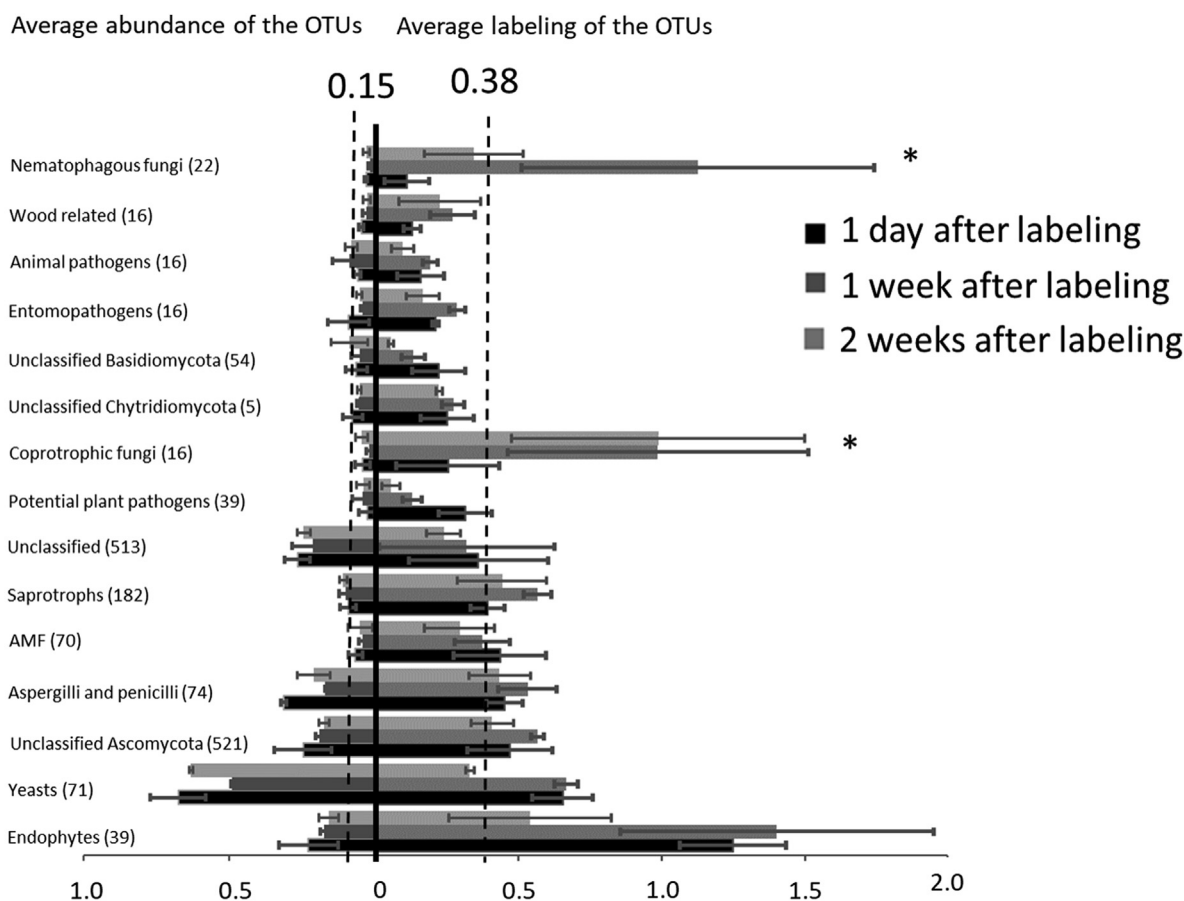
**Table 2**  
The OTUs with highest relative labeling and their relative abundance at 1 d and 1 week after labeling.

	#OTU ID	Phylum	Order	Species	Function	1 day		1 week	
						average labeling $^{13}\text{C}/^{12}\text{C}$	Abundance	average labeling $^{13}\text{C}/^{12}\text{C}$	Abundance
<b>1 day</b>	Otu05129	Unclassified	Unclassified	Unknown	Unclassified	49.51	0.008	1.55	0.003
	Otu04252	Ascomycota	Helotiales	Helotiales sp.	Unclassified	24.43	0.006	6.23	0.001
	Otu04459	Ascomycota	Dothideomycetes insertae sedis	<i>Oidiodendron</i> sp.	Mold	16.78	0.001	1.25	0.001
	Otu05124	Mucoromycota	Archaeosporales	Archaeosporaceae sp.	AMF	13.43	0.001	<1	0.001
	Otu05052	Ascomycota	Helotiales	Helotiales sp.	Unclassified	12.05	0.003	2.87	0.001
	Otu04663	Ascomycota	GS37	GS37	Unclassified	10.42	0.003	<1	0.001
	Otu04234	Ascomycota	Helotiales	<i>Phialocephala</i> sp.	Endophyte	9.98	0.003	<1	0.003
	Otu05101	Ascomycota	Unclassified	Unknown	Unclassified	9.74	0.005	<1	0.013
	Otu04679	Ascomycota	Sorariomycetes	Myrmecridiales sp.	Unclassified	8.39	0.002	1.61	0.000
	Otu04766	Ascomycota	Sordariales	Sordariales sp.	Unclassified	8.39	0.001	0.00	0.001
	Otu04489	Basidiomycota	Agaricales	<i>Entoloma sericeum</i>	Saprotrophic fungus	7.91	0.003	<1	0.001
	Otu04389	Ascomycota	Eurotiales	<i>Penicillium adametzii</i>	Mold	7.74	0.003	<1	0.001
	Otu05043	Ascomycota	Capnodiales	<i>Devriesia</i> sp.	Unclassified	6.56	0.018	<1	0.004
	Otu03331	Ascomycota	Sordariomycetes insertae sedis	<i>Myrmecridium</i> sp.	Saprotroph	6.10	0.001	0.00	0.000
	Otu04801	Ascomycota	Unclassified	Unknown	Unclassified	6.10	0.001	<1	0.001
	Otu05277	Ascomycota	Chaetothyriales	Chaetothyriales sp	Unclassified	5.93	0.003	7.51	0.001
	Otu03230	Ascomycota	Sordariomycetes insertae sedis	<i>Colletotrichum</i> sp.	Endophyte	5.87	0.001	0.00	0.000
	Otu04688	Ascomycota	Chaetothyriales	<i>Exophiala opportunistica</i>	Animal pathogen	5.66	0.003	<1	0.001
	Otu04938	Ascomycota	Pleosporales	Pleosporales sp.	Unclassified	5.59	0.005	0.00	0.000
	Otu05252	Unclassified	Unclassified	Unknown	Unclassified	5.59	0.002	1.22	0.004
<b>1 week</b>	Otu04776	Ascomycota	Helotiales	Vibrissaceae sp.	Saprotrophic fungus	<1	0.001	35.43	0.001
	Otu04469	Ascomycota	Hypocreales	<i>Acremonium</i> sp.	Saprotrophic fungus	<1	0.001	18.19	0.001
	Otu03938	Ascomycota	Orbiliiales	Orbiliaceae sp.	Ascomycota	0.00	0.000	17.64	0.001
	Otu04747	Ascomycota	Helotiales	Vibrissaceae sp.	Endophyte	0.00	0.000	17.57	0.001
	Otu03570	Unclassified	Unclassified	Unknown	Unclassified	0.00	0.000	17.30	0.000
	Otu04347	Ascomycota	Hypocreales	<i>Hypocrea virens</i>	Endophyte	<1	0.001	17.30	0.001
	Otu04909	Ascomycota	Sordariales	<i>Podospora curvicolla</i>	Coprotrophic fungus	4.70	0.002	14.83	0.002
	Otu04487	Unclassified	Unclassified	Unknown	Unclassified	0.00	0.000	12.33	0.001
	Otu04112	Ascomycota	Leotiomycetes	Leotiomycetes sp.	Unclassified	<1	0.002	12.00	0.002
	Otu04295	Unclassified	Unclassified	Unknown	Unclassified	0.00	0.000	11.54	0.001
	Otu04228	Ascomycota	Helotiales	Helotiales sp.	Unclassified	0.00	0.000	11.07	0.001
	Otu05084	Chytridiomycota	Rhizophydiales	Rhizophydiales sp.	Chytridiomycota	0.00	0.002	9.67	0.001
	Otu05146	Ascomycota	Chaetothyriales	<i>Exophiala salmonis</i>	Animal pathogen	0.00	0.000	8.65	0.000
	Otu05151	Unclassified	Unclassified	Unknown	Unclassified	0.00	0.000	8.65	0.000
	Otu04917	Ascomycota	Pezizales	Ascobolaceae sp	Coprotrophic fungus	0.00	0.000	7.51	0.000
	Otu04036	Ascomycota	Hypocreales	Hypocreales sp.	Unclassified	4.70	0.002	6.86	0.001
	Otu04957	Ascomycota	Leotiomycetes	Leotiomycetes sp.	Unclassified	<1	0.000	6.50	0.002
	Otu04195	Ascomycota	Unclassified	Unknown	Unclassified	0.00	0.000	6.21	0.001

root-derived carbon later, or not at all.

Archaeosporales were the most active AMF order in the rhizosphere using recently photosynthesized carbon. Quite surprisingly, Paraglomerales, Glomerales and Diversisporales were detected in the rhizosphere with DNA based methods, but were apparently not actively assimilating carbon from the root despite their lifestyle as obligate mutualists. Furthermore, we detected an enrichment of  $^{13}\text{C}$  in Paraglomerales only 1 week after labeling, at a time point that roots had almost no  $^{13}\text{C}$  left (Supplementary Fig. 1). Hempel et al.

(2007) investigated differences in AMF-taxa between roots and extraradical hyphae in soils and showed that Paraglomerales were absent in the roots but forming extensive extraradical hyphae, which may explain why labeling in their DNA occurred 1 week later than in the DNA of Archeosporales (Fig. 3). The discrepancy between our results and those of earlier studies may be attributed to the higher copy numbers in the intraradical hyphae compared to extraradical hyphae. Also, AMF allocation into intraradical vs. extraradical fractions can vary among taxonomic groups (Hart et al.,



**Fig. 4.** Average labeling (right side) and average abundance (left side) of OTUs belonging to fungal guilds in time after labeling. Black bars represent averages in OTUs 1 d after labeling, dark grey bars OTUs 1 week after labeling and light grey bars OTUs 2 weeks after labeling. Bars represent averages with standard errors. The dotted lines are average OTU abundance (on the left) and average OTU labeling (on the right) of all OTUs. Stars indicate guilds that are significantly affected by time since labeling.

2001). Furthermore, AMF spores, and thus inactive structures, can contribute the majority of AMF extraradical DNA (Gamper et al., 2008), which could explain the relatively large proportion of AMF taxa not receiving carbon from the plant in our study.

Besides the majority of the fungi assigned to the second trophic level of the soil food web, we detected fungi making up 20% of the rhizosphere mycobiota receiving labeled carbon after it was no longer found to be enriched in plant roots (Suppl. Fig. 1), making them potential consumers of other carbon sources than plant roots and exudates. Placing fungi only at the second trophic level may not thus give a complete picture of their ecology. We attribute this to the large diversity in fungal lifestyles and to their ability to parasitize other fungi and animals (Boddy, 2016). There is evidence that parasites can make up a large fraction of the soil protist community (Mahé et al., 2017) and this may also be the case for fungi. In aquatic systems, Chytridiomycota play key roles as parasites (Kagami et al., 2007), and also here their later labeling would indicate that they play a similar role in the soils. Furthermore, it is known that, for example, nematode communities can be controlled by fungi (Mankau, 1980). We showed that one week after labeling, the class Orbiliomycetes, which was underrepresented in the  $^{13}\text{C}$  fraction after one day, was among the most heavily labeled taxa. At the same time, we found that especially root feeding and bacterivorous nematodes were strongly labeled (Suppl. Fig. 1). This is in line with its known position as a secondary or tertiary carbon user and higher trophic position in the soil food web using nematodes as food/prey (Pfiester, 1997). These fungi using secondary carbon should be included in soil food-web models and taken into account

when studying functionality of rhizosphere mycobiota. On the other hand, Platyglloeales (in the class Pucciniomycetes) was the order of Basidiomycetes most enriched with  $^{13}\text{C}$  1 week after labeling (Fig. 3). This order contains mainly rusts, which would indicate that part of the aboveground leaf-derived carbon has ended up in the rhizosphere by 1 week after labeling.

We estimate that approximately 80% of fungal taxa detected using DNA based methods, obtained recently produced root carbon in the rhizosphere, of which 60% are using freshly assimilated carbon and a further 20% secondary carbon sources. This high activity in the rhizosphere is likely to be due to the selection by plants of the rhizosphere microbiome and the temporal dynamics included in this study that add insight in the complexity of food-web interactions (Hiltner, 1904; Hartmann et al., 2009; Raaijmakers et al., 2009). Many fungal species produce prolific numbers of spores (Carlile et al., 2001), which might explain the proportion of them being inactive when DNA is targeted. Furthermore, there might be relic DNA left in the soil from previously active organisms that we detect in the non-active pool of fungi (Carini et al., 2016). Scientists have tried to circumvent this by using RNA to study only the active fraction of the soil microbiome, however, using RNA does not ensure the capture of the active part of the community (Blazewicz et al., 2013). Another option is that these rhizosphere fungi are active but using other carbon sources than freshly photosynthesized carbon, such as dead plant material (Zhang et al., 2016), and are detected in this study in the  $^{12}\text{C}$  pool despite being active. Here we did not replicate the  $^{12}\text{C}$  control cores and urge future studies to use more replicates both for samples and

controls (see approach in Hungate et al., 2015) and study more soils simultaneously in order to gain better understanding of the variation in carbon transfer to rhizosphere fungal communities. We further recommend use of intact soil cores and/or *in situ* measurements in the field instead of mixing the soils and using pot experiments.

In conclusion, we showed that a majority of fungi present in the rhizosphere are actively assimilating labile pools of root-derived carbon being either in direct contact with plants taking up recently photosynthesized carbon or acquire the carbon through soil food-web interactions. We showed differences between rhizosphere fungi using root-derived carbon and all rhizosphere fungi, and conclude that certain taxa are more likely to be involved in the plant-related rhizosphere processes, whereas others fulfil other ecological functions, such as being pathogenic or parasitic to insects, or decomposing soil organic matter and dead roots, or are simply dormant. Our results support the hypothesis that fungi characterized by rapid growth rates and competitive abilities in rhizosphere (r-strategists), and endophytes, are components of the active rhizosphere mycobiota assimilating labile root carbon. Ascomycete and basidiomycete yeasts, aspergilli and penicilli, endophytes and 'sugar fungi' initially used rhizodeposits, most likely competing with bacteria, while at the later sampling stages we saw a shift in community structure to slower growing saprotrophic fungi (K-strategists) and fungi using secondary carbon sources. We further show that the ability of fungi to use rhizodeposits is not conserved at phylum level.

#### Availability of data and materials

All sequences are deposited in ENA under accession number PRJEB15250.

#### Authors' contributions

SEH, EM, WvdP, and WdB designed and came up with the experiment. SEH and EM conducted the experiment and SEH performed data analysis and wrote the first draft of the manuscript. SEH, EM, WvdP, and WdB commented and contributed all to subsequent version of the manuscript.

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#### Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.funeco.2020.100988>.

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