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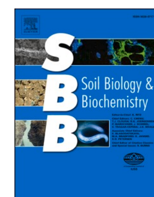

Microbial utilization of simple and complex carbon compounds in a temperate forest soil

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ABSTRACT

Forest soil processes carried out by microorganisms are critical for the global carbon (C) cycle and climate. Characterizing the utilization of differently recalcitrant C sources is an important step towards understanding the ecosystem-level function of microorganisms in temperate forest soils. Here, using stable-isotope probing (SIP), we tracked C incorporation into bacterial and fungal biomass by quantifying ^{13}C incorporation into phospholipid fatty acids (PLFA-SIP), its respiration (i.e., content in the produced CO_2) and C accumulation by individual microbial taxa (DNA-SIP), following the addition of ^{13}C-labelled substrates of different recalcitrance (citric acid, glucose, chitin, cellulose, hemicellulose, and plant biomass) in microcosms. The highest ^{13}C respiration was observed after the addition of the low-molecular-mass substrates citric acid and glucose, while the highest ^{13}C incorporation into microbial biomass was observed during growth on chitin. Communities of fungi and bacteria that incorporated ^{13}C of various origins into their biomass differed from the original soil communities, as well as between treatments. The most distinct microbial community was observed in microcosms containing ^{13}C-chitin, indicating its utilization by both fungi and bacteria. Bacterial taxa were more often versatile, incorporating C of various origins, while there was a higher share of fungi that were specialists. Together, our results show that low-molecular-mass compounds that belong to typical root exudates are more readily respired, while the C from biopolymers studied was relatively more incorporated into microbial biomass. Various C sources are targeted by distinct microbial communities, although their composition partly overlaps due to the existence of generalist bacteria and fungi that are capable of utilizing various C sources.

1. Introduction

Terrestrial ecosystems represent an important carbon (C) pool, as well as a sink that is able to sequester a substantial percentage of the total CO_2 emitted to the atmosphere by humans (Soong et al., 2020; Friedlingstein et al., 2020). Forests cover 31% of the global land area and store an estimated stock of 861 Pg C, nearly half of which is contained in soil (Keenan et al., 2015; Pan et al., 2011). The understanding of processes that lead to C storage in forest soils is essential to estimate the role of soils in the global C budget, as well as their potential responses to global change (Chen et al., 2018).

The flow of C in forest ecosystems is mainly driven by the activity of primary producers that fix atmospheric CO_2 and supply it into soil in two contrasting forms: as recalcitrant (known as difficult and/or slow to degrade) biopolymers and as simple (nonpolymeric, small molecular mass) organic compounds. Plant litter and deadwood, representing the former, are mostly composed of a complex combination of recalcitrant polymers, including cellulose, hemicelluloses and lignin. Simple compounds are supplied to soils through rhizodeposition, either through root exudation or by direct transfer to root symbiotic fungi or bacteria (Nguyen, 2003). Root exudates of forest trees are composed of low-molecular-mass compounds, including monosaccharides, amino acids and carboxylic acids (Sasse et al., 2017; Shen et al., 2020; Yu, 2018) and may account for up to 20% of the assimilated C by plants (Shen et al., 2020). The transformation of this simple labile C strongly influences the C and nitrogen (N) dynamics and has been noted to account not only for a significant proportion of soil respiration but also for the formation of stable soil organic matter (Meier et al., 2017; Zhou et al., 2021; Schmidt et al., 2011; Sokol et al., 2019). In addition to C from plant origin, the biomass of microorganisms also represents an

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abundant pool in forest soils with relatively fast turnover (Brabcová et al., 2018; Bukovská et al., 2021). Dead microbial biomass is rich in N-acetylglucosamine-containing biopolymers: chitin in fungi and peptidoglycan in bacteria. Due to the large amount of biomass produced in forest soils yearly by fungi—both ectomycorrhizal and saprotrophic (Ekblad et al., 2013)—the transformation of fungal biopolymers such as chitin is a key component in C cycling (Hendricks et al., 2016; Starke et al., 2020).

Fungi and bacteria are essential players in the recycling and turnover of soil organic matter (Schimel and Schaeffer, 2012; Trivedi et al., 2018) and can thus influence the feedback between climate and C storage. Understanding the roles of soil microbes in C cycling is thus essential for predicting the soil response to future climatic conditions (Graham et al., 2016). Saprotrophic filamentous fungi appear to be best suited to the decomposition of biopolymers due to their ability to produce a variety of extracellular enzymes and filamentous growth that allows them to colonize bulky substrates efficiently (De Boer et al., 2005; Starke et al., 2021); however, several bacteria also participate in the transformation and mineralization of organic matter (Glassman et al., 2018; López-Mondéjar et al., 2019). Recent studies show that certain bacteria are also involved in the degradation of complex (polymeric) compounds of plant origin (López-Mondéjar et al., 2020; Štursová et al., 2012; Wilhelm et al., 2019) or of fungal origin (Brabcová et al., 2016, 2018).

However, the specific roles and quantitative contributions of distinct guilds of microbiota in the decomposition processes and the flow of C in soils is an ongoing matter of debate (Kramer et al., 2016; López-Mondéjar et al., 2018; Rousk and Frey, 2015). There are contradictory results that indicate the association of specific microbial taxa with certain biopolymers (Algora Gallardo et al., 2021; Bhatnagar et al., 2018; Brabcová et al., 2018), while others demonstrate the ability of many taxa to utilize various resources (López-Mondéjar et al., 2018). Moreover, several bacteria appear to depend on the utilization of simple organic compounds (Lladó et al., 2019), while decomposition abilities also vary among fungi, being limited in most yeasts and several micromycetes (moulds) (Baldrian et al., 2011; Mašínová et al., 2018).

Tracking the microbial use of C sources of varying recalcitrance, and the assignment of functional roles to microorganisms represents an essential step for understanding C cycling in ecosystems (Morrissey et al., 2017; Pepe-Ranney et al., 2016; Wang et al., 2021).

Moreover, defining the relative contribution of fungi and bacteria to the use of C sources is an important prerequisite for the modelling of C flow and soil C sequestration potential, since it is assumed that fungal biomass contributes more to C stabilization than the biomass of bacteria (Busse et al., 2009; Clemmensen et al., 2013).

The aim of this study was to track the utilization of C from compounds of different complexity in a temperate forest soil and to track its flow into the microbial decomposer food web using stable isotope probing (SIP). To accomplish this, we prepared soil microcosms with the addition of ^{13}C-labelled compounds of different origins and followed the respiration, biomass production and ^{13}C accumulation by individual microbial taxa classified into ecological guilds. The complexity of the C compounds used ranged from simple organic acids and monosaccharides entering soil as root exudates (glucose and citric acid) through biopolymers of plant origin (cellulose, hemicellulose) and fungal origin (chitin) to complex plant biomass. In addition to the classification of the above compounds in terms of their priority use by fungi or bacteria, we also aimed to identify major microbial taxa specifically using each substrate. Due to the higher expression of fungal enzymes targeting plant biomass in forest soils (Starke et al., 2021; Žifčáková et al., 2017) and the enrichment of bacteria on dead fungal biomass (Brabcová et al., 2016, 2018), we hypothesize that C from plant biopolymers is more incorporated into fungal biomass, while C from chitin accumulates preferentially in bacterial biomass. Because bacteria can be distinguished into guilds of decomposers with slow growth and fast-growing opportunists utilizing easy C (Lladó et al., 2019; Lladó and Baldrian, 2017), we hypothesize that the former bacteria (e.g., the members of the phylum *Acidobacteria*) are more able to accumulate C from biopolymers and plant biomass, while bacterial opportunists prioritize C from the low-molecular-mass compounds. In fungi, we hypothesize that unicellular yeasts with low decomposition abilities (Mašínová et al., 2017) and moulds (Algora Gallardo et al., 2021) are the primary utilizers of simple C compounds.

2. Materials and methods

2.1. Soil collection and experimental setup

The study used soil collected in a temperate forest in the Training Forest Enterprise Masaryk Forest Křtiny of Mendel University in Brno. The Křtiny Forest has a total area of 103 km^2 of mixed temperate forest and is located north of Brno, Czech Republic. The area has an altitude range of 210–575 m; the mean annual temperature is 7.5 °C and the mean annual precipitation is 610 mm (Martinović et al., 2021). Soil was collected on September 26th, 2016, in a forest dominated by *Quercus petraea* agg. (49.26148°N 16.64807°E, 388 m a.s.l.). The soil was a cambisol with developed litter, organic and mineral layers. The organic-mineral horizon of soil (Ah) was approximately 3 cm thick, with a pH of 5.12 and contained 4.35% C and 0.321% N. This organic-mineral horizon (further referred as "soil") was collected at four close locations, passed through a 2-mm sterile sieve, and pooled to create one composite sample. Soil was kept at 4 °C for three weeks and preincubated at 12 °C for one week prior to the experimental setup.

Microcosms were set up in 100-ml flasks containing 5 g of soil and 0.08 g of one of the six ^{13}C-labelled substrates, including ^{13}C-glucose (99 atom% ^{13}C; Cambridge Isotope Laboratories, MA, USA), ^{13}C-citric acid (99 atom% ^{13}C; Saint Louis, MI, USA), ^{13}C-chitin from *Aspergillus* (97 atom% ^{13}C), ^{13}C-cellulose from *Zea mays* (97 atom% ^{13}C), ^{13}C-hemicellulose from *Zea mays* (97 atom% ^{13}C), and ^{13}C-plant biomass from ground maize leaves (97 atom% ^{13}C; all from Isolife, Wageningen, Netherlands); all substrates were either finely milled or powdered. The substrate was mixed with the whole soil volume of each sample, and each microcosm was moistened by the addition of 2 mL of sterile distilled water to ensure proper substrate distribution. The soil water content reached 50%, a value regularly observed during the moist periods of the vegetation season at the study site. Twelve microcosms per treatment as well as controls without substrate addition were set up. Beakers with 5 ml of 1 M NaOH solution were placed inside each flask to capture CO_2. Microcosms were incubated in the dark at 12 °C, the mean summer temperature of the study site (Štursová et al., 2020), and four replicates per treatment were destructively harvested immediately after being set up and after 7 and 21 days following the substrate addition. Microcosm materials were frozen immediately at −80 °C, freeze-dried, and stored frozen at −40 °C.

2.2. Measurement of carbon isotopic composition of CO_2 and PLFA

The CO_2 content, ^{12}C/^{13}C ratio and PLFA content were measured as described previously (López-Mondéjar et al., 2018). CO_2 absorbed in the NaOH was quantified by titration with 0.1 M HCl immediately after the microcosm was opened for processing. Furthermore, $SrCl_2$ was added to precipitate $SrCO_3$. The $SrCO_3$ pellet was washed three times with deionized water to completely remove NaCl and other soluble impurities. After washing, $SrCO_3$ was dried at 60 °C and used for analyses of C isotopic composition. The ^{13}C abundance in the CO_2 released from the $SrCO_3$ samples using phosphoric acid in a helium atmosphere was analysed using a GasBench II equipped with a cold trap and coupled with a Delta V Advantage isotope ratio mass spectrometer (ThermoFischer Scientific, Waltham MA, USA). From the CO_2 content and ^{12}C/^{13}C ratio, ^{13}C–CO_2 production (the mineralization of the added substrate) was calculated.

The samples (approx. 2 g each) were extracted by a mixture of chloroform–methanol–phosphate buffer (1:2:0.8), according to Bligh

and Dyer (1959) and Šnajdr et al. (2008). Immediately before the extraction, each sample was spiked with the mixture of internal standards, containing 30 μg 1,2-dinonadecanoyl (C19:0)-sn-glycero-3-phosphocholine (Avanti, Polar Lipids, Birmingham AL, AL USA) and 30 μg heneicosanoic acid (C21:0, Sigma-Aldrich, St. Louis MO, USA), dissolved in chloroform. Samples were incubated at room temperature overnight upon mild horizontal shaking (100 rpm, amplitude 2 cm). The samples were then centrifuged at 750×*g* for 10 min, decanted and added with 10 ml citrate buffer each, and centrifuged again at 750×*g* for 10 min to attain phase separation. The lower (chloroform) fraction was collected and filtered through hydrophobic syringe filters (0.45 μm Chromafil Xtra PTFE, Macherey-Nagel, Düren, Germany), transferred to 50 ml glass vials and evaporated to dryness at 50 °C under stream of nitrogen gas. Further, these total lipid extracts were fractionated using LiChrolut® Si (25–40 μm) 200 mg columns as described by Welc et al. (2012) to obtain neutral, glyco- and phospholipid fractions. The latter (phospholipid) fraction eluted with methanol was evaporated to dryness and transmethylated using the rapid trimethylchlorosilane-methanol derivarization approach (Konvalinková et al., 2017; Welc et al., 2012). The abundance and isotopic (^{13}C) enrichment of the individual phospholipid fatty acid (PLFA) methylesters (dissolved in 300 μl hexane and filtered through the hydrophobic filters as above) were analysed by gas chromatography (Trace 1310 gas chromatograph equipped with a DB-5 column, 60 m × 0.25 mm, 2 μm coating, instrument by ThermoFisher Scientific, Bremen, Germany and the column by Restek, Centre County PA, USA), using 5 μl injection volume, 1:50 split injection and temperature gradient from 60 °C to 240 °C as reported previously (Konvalinková et al., 2017; López-Mondéjar et al., 2018). The chromatograph was online connected to an isotope ratio mass spectrometer Delta V Advantage via Isolink (ThermoFisher Scientific). Different fatty acids were identified according to comparison of compound retention times with several commercial and in house fatty acid standards. Abundance of the different compounds in the original samples was calculated using the C19:0 abundances in each sample (this effectively integrated all losses during extraction, derivatization and split injection). The assignment of different signature PLFA followed Frostegård et al. (1993), Joergensen (2022), López-Mondéjar et al. (2018), Welc et al. (2012) and references therein. Specifically, the amount of 18:2ω6,9 fatty acid in the samples was used as a proxy of fungal biomass (PLFAF), whereas the sum of the amounts of fatty acids i14:0, i15:0, a15:0, 16:1ω7t, 16:1ω9, 16:1ω7, 10Me-16:0, i17:0, a17:0, cy17:0, 17:0, 10Me-17:0, 10Me-18:0 and cy19:0 served as a proxy of bacterial biomass (PLFAB). The content of all PLFA molecules (PLFAT) was used as a proxy of total microbial biomass in all treatments except the addition of plant biomass, where PLFAs were also present in the added substrate. The fungal/bacterial biomass ratio (F/B) was calculated as PLFAF/PLFAB. The ^{13}C abundance in the individual PLFAs was analysed as described previously (López-Mondéjar et al., 2018) using a trace 1310 gas chromatograph equipped with a DB-5 column (60 m × 0.25 mm) coupled to the mass spectrometer (see above) via IsoLink. The ratio of ^{13}C incorporation into microbial biomass (PLFA) and ^{13}C respiration was expressed in mg ^{13}C incorporated into PLFA/mg ^{13}C respired (data provided in Supplementary Table S1).

2.3. DNA extraction and ^{13}C DNA separation

DNA was extracted from 0.5 g aliquots of freeze-dried microcosm material using the Fast DNA Spin Kit for Soil (MP Biomedicals, Solon, OH). DNA yield and purity were checked on Qubit. SIP fractionations were carried out from triplicate independent microcosms. Labelled DNA was separated by isopycnic centrifugation using a caesium trifluoroacetate solution (CsTFA, GE Healtcare, Piscataway, NJ) with a starting buoyant density (BD) of 1.6 g mL^{-1} as previously described (Štursová et al., 2012). Briefly, the CsTFA solution was combined with 2.3 μg DNA and subjected to ultracentrifugation for 48 h at 141400×*g* using 5.1-ml tubes in an NVT 100 rotor and L-100XP Optima Ultracentrifuge (Beckam Coulter, Brea, CA, USA). Each centrifugation run included duplicates of a blank control (no DNA) for BD determination, three replicates of microcosm samples and corresponding controls without added substrates. Gradients were fractionated into 250 μl fractions, and the BD of each fraction was determined gravimetrically using fractions from the blank gradients. Sample DNA was precipitated from fractions with 1 mL of 2-propanol overnight at −20 °C and then centrifuged at 10,000×*g*. The pellets were cleaned twice with 0.5 mL 2-propanol, vacuum dried, and resuspended in water.

The distribution of microbial DNA in the fractions was determined with qPCR using 1108F/1132R primers for bacteria (Amann et al., 1995; Wilmotte et al., 1993) and FF390/FR1 primers for fungi (Prévost-Bouré et al., 2011). For each fraction, qPCR was performed in duplicate reactions as previously described (Baldrian et al., 2012). The labelled ^{13}C-DNA and unlabelled ^{12}C-DNA were determined by plotting the normalized DNA concentrations vs. the fraction number. Fractions representing the ^{13}C-DNA and the ^{12}C-DNA in each sample were pooled to form representative samples for each microcosm; fractions with unclear assignment were omitted. The typical buoyant density of bacterial ^{13}C-DNA fraction was >1.585 g mL^{-1}, and ^{12}C-DNA fraction <1.580 g mL^{-1}. For fungi the buoyant density of the ^{13}C-DNA fraction was >1.580 g mL^{-1} and <1.575 g mL^{-1} for the ^{12}C-DNA fraction. For the control microcosms, fractions corresponding to those identified as ^{13}C and ^{12}C in the same run were separately pooled as well in the same manner as in our previous experiments (López-Mondéjar et al., 2018; Štursová et al., 2012).

2.4. Microbial community analysis and statistics

The bacterial primers 515F and 806R (Caporaso et al., 2011) barcoded on both ends were used to amplify 16S rRNA gene, and the fungi-specific primers gITS7 and ITS4 (Ihrmark et al., 2012) barcoded on both ends were used to amplify the fungal ITS2 region in three PCR reactions per sample. Each 25 μl PCR contained 5 μL of 5x buffer for Q5 High-Fidelity DNA polymerase (New England Biolabs, Inc.), 5 μL of 5x Q5 HighGC Enhancer (New England Biolabs, Inc.), 0.5 μL of 10 mM PCR Nucleotide mix (Bioline), 1.5 μL of 10 mg ml^{-1} BSA (GeneON), 0.25 μL of the Q5 High-Fidelity DNA polymerase (New England Biolabs, Inc.), 1 μL of each 10 μM forward and reverse primer (Sigma-Aldrich), 9.75 μL of H 2 O, and 1 μL of the template DNA. The PCR conditions for fungal ITS2 region were: initial denaturation for 5 min at 94 °C; 30 cycles of 30 s at 94 °C, 30 s at 56 °C, 30 s at 72 °C; followed by an extension at 72 °C for 7 min. The PCR conditions for bacterial 16S rRNA were: initial denaturation 4 min at 94 °C; 25 cycles of 45 s at 94 °C, 60 s at 50 °C, 75 s at 72 °C; followed by an extension at 72 °C for 10 min. All PCR reactions were performed on a Mastercycler personal thermocycler (Eppendorf, Hamburg, Germany).

PCR amplicons were pooled by samples, purified, and mixed in equimolar quantities. Sequencing libraries were prepared using a TruSeq PCR-free kit and sequenced in-house on Illumina MiSeq (2 × 250 base paired-end reads). Both ^{13}C-DNA and ^{12}C-DNA was sequenced from all the samples including the no-substrate control, as well as the total DNA from the no-substrate control prior to separation of fractions (further referred to as "control"). For clarity, we will refer to the sequenced ^{13}C-DNA pooled fractions ("heavy" fraction) as "^{13}C-community", and ^{12}C-DNA fractions ("light" fraction) as "^{12}C community".

The amplicon sequencing data were processed using the pipeline SEED 2.01 (Větrovský et al., 2018) as described in (Žifčáková et al., 2016). Briefly, paired-end reads were merged using fastq-join (Aronesty, 2013) and the quality filtering was performed with the mean quality score 30 cut-off. The ITS2 region was extracted using ITSx v1.0.11 (Bengtsson-Palme et al., 2013) and whole amplicons of the bacterial 16S rRNA gene or the extracted ITS2 regions of fungal amplicons were cleaned from chimaeras and clustered into operational taxonomic units (OTUs) at a 97% similarity level using UPARSE implemented in USEARCH (Edgar, 2013). Consensus sequences were constructed for

each OTU, and the closest hits at the genus or species level were identified using BLASTn against RDP (Cole et al., 2014) for bacteria or UNITE 8.1 (Nilsson et al., 2019) for fungi. Non-bacterial and non-fungal sequences were discarded. The assignment of fungi to putative ecological categories was based on genus-level identification (Põlme et al., 2021). Sequence data were deposited in the SRA under project number PRJNA755183.

Two requirements were defined for assuming an OTU to be ^{13}C-accumulating (i.e., ^{13}-C-enriched): (1) the OTU showed higher relative abundance in ^{13}C community than in ^{12}C community and in all ^{13}C-microcosms containing the same substrate and (2) the OTU also showed a higher ^{13}C-DNA/^{12}C-DNA abundance ratio in all ^{13}C-microcosms containing the same substrate than in control microcosms. The first criteria selects for OTUs with heavy DNA that incorporated ^{13}C from the substrate; and the second criteria allowed us to distinguish that the heavy DNA is related with ^{13}C incorporation and not due to naturally heavy DNA (e.g. GC-rich DNA). Only the OTUs that fulfilled both criteria were considered enriched. This approach allowed us to distinguish the share of ^{13}C-accumulating OTUs (enriched or labelled) from the rest of OTUs (considered as unlabelled). These criteria were previously used in our earlier study (López-Mondéjar et al., 2018), which showed that the microbial community composition in the "light" fraction is not impacted by the substrate addition. Despite the often-cited and unavoidable limitations of SIP-based experiments (Leung et al., 2015; Neufeld et al., 2007), the use of these strict criteria for identifying the ^{13}C-enriched taxa in our experiment minimizes false positives.

Community and statistical analyses were performed using R (R core team, version 3.6.3. www.r-project.org) with *phyloseq* (McMurdie and Holmes, 2013) and *vegan* (Oksanen et al., 2019) packages in R Studio (version 1.2.5033, www.rstudio.com). Differences in CO_2 production and PLFA content among treatments were tested using analysis of variance (ANOVA), after checking the data for ANOVA assumptions; for the Day 21 time point with different substrates as a fixed effect. ANOVA was followed by Tukey's or Dunn's post hoc tests. Significant differences between treatments and the control were tested using Dunnett's test. Bray-Curtis was used as a metric of similarity, and NMDS (nonmetric multidimensional scaling) was used to visualize the differences between the ^{13}C communities in substrate-supplied microcosms. The Adonis function from the *vegan* package (permutational multivariate analysis of variance) on Bray-Curtis distances was used to test for the significance of the observed differences for each time point. Differences at $p < 0.05$ were considered statistically significant.

3. Results

3.1. Response of microbial community to substrate addition

Microbial activity increased significantly in substrate-supplemented microcosms compared to that of the control microcosms, with the exception of cellulose, as reflected by higher CO_2 production (Fig. 1a). The $^{13}CO_2$ production was higher than that in the control upon the addition of all ^{13}C substrates, being the highest after the addition of citric acid and glucose and relatively modest after cellulose addition. Interestingly, while the respiration of most substrates levelled off after Day 7, the opposite was true for chitin (Fig. 1b).

The incorporation of ^{13}C into microbial biomass was fastest in hemicellulose- and glucose-supplemented microcosms, but after 3 weeks, most ^{13}C was incorporated into the biomass of the chitin treatments, while little ^{13}C-labelled PLFAT was observed after cellulose and citric acid addition (Fig. 1c). The ^{13}C–F/B ratio, i.e., the ratio of fungi-specific PLFAs to bacteria-specific PLFAs, was significantly higher than that in the control in all treatments except for chitin addition, indicating the disproportionate accumulation of C by fungi than by bacteria. The highest enrichment of fungi was observed after the addition of citric acid and cellulose. (Fig. 1d). Total PLFAs, as well as PLFAF, PLFAB and F/B ratio are shown in Supplementary Fig. 1.

The ratio of ^{13}C-carbon incorporated into PLFAs to ^{13}C lost by respiration differed among substrates and was high in the microcosms with chitin, hemicellulose and cellulose. In microcosms supplemented with glucose, the ratio was 2x lower and in microcosms with citric acid, 4x lower compared to chitin and cellulose, indicating that the C in the former two low-molecular-mass substrates was more intensively mineralized and less intensively used for biomass formation than C in the biopolymers in this soil (Fig. 2).

3.2. Microbial accumulation from C compounds of varying complexity

Both fungal and bacterial communities incorporating C from different sources were significantly different from each other ($p < 0.01$), while the change over time was not significant. Bacterial communities showed higher Bray-Curtis similarity across treatments, yet separation by treatments was still evident (Fig. 3).

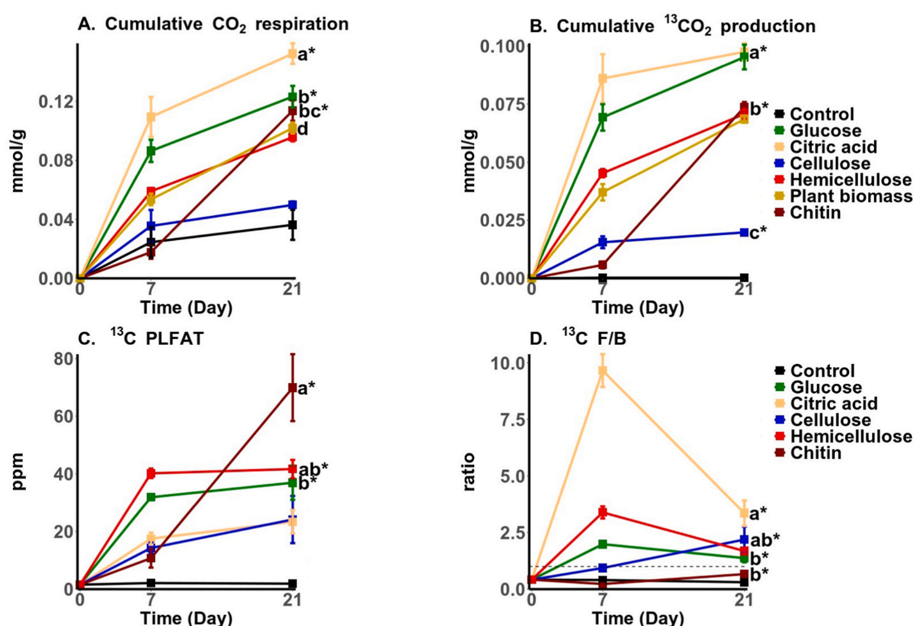

Fig. 1. Respiration and microbial biomass following the addition of ^{13}C-labelled substrates into oak forest soil. A) Cumulative CO_2 respiration and B) cumulative $^{13}CO_2$ respiration in microcosms supplemented with ^{13}C substrates. C) Total ^{13}C-containing microbial biomass (^{13}C PLFAT) and D) the ratio of fungi-specific ^{13}C-PLFA to bacteria-specific ^{13}C-PLFA. The dashed line represents ratio of 1. The data represent means and standard errors. Different letters indicate significant differences ($p < 0.05$) on Day 21 (Tukey's HSD test), and asterisks indicate significant differences from the control (Dunnett's test). Unit mmol/g represents mmol CO_2 per g soil.

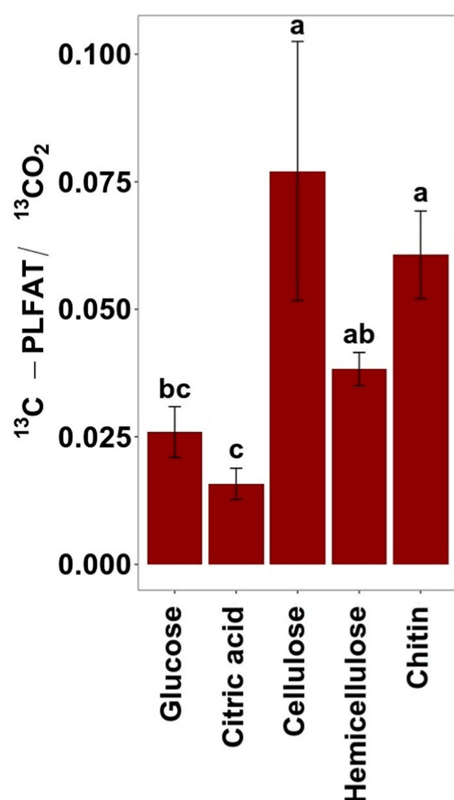

Fig. 2. The ratio of ^{13}C incorporation into microbial biomass (PLFA) and its respiration following the addition of ^{13}C-labelled substrates into forest soil after 21 days of incubation. The data represent means and standard errors. Different letters indicate significant differences ($p < 0.05$) on Day 21 (Dunn's test).

Soil fungal communities were dominated by Ascomycota and Basidiomycota with a small share of Mucoromycota. Ascomycota also dominated in most of the ^{13}C communities, typically representing 60–70% of the total ^{13}C community. The only exception was the ^{13}C community on chitin, where Ascomycota were lower and Mucoromycota dominated. In individual substrates, the dominance of a few fungal genera was often recorded, such as *Mortierella* and *Pseudocosmospora* on chitin, *Lycoperdon* on cellulose and maize, and *Penicillium* and *Apiotrichum* on citric acid (Fig. 4). Most fungi in the original soil were ectomycorrhizal root symbionts, saprotrophs, and moulds. Yeast sequences represented only 3% in the control community but as many as 20–25% in the ^{13}C- communities on citric acid, glucose, and maize leaves. The share of moulds was high on chitin and citric acid and very low on cellulose. Animal pathogens were found in ^{13}C community on chitin, and plant pathogenic fungi increased on hemicellulose (Fig. 4).

Actinobacteria and Proteobacteria dominated the bacterial community in the original soil. The same was recorded in the ^{13}C communities, although the share of Proteobacteria was typically higher and that of Actinobacteria was typically lower. The members of the genus *Pseudomonas* were particularly enriched in glucose and citric acid and *Kitasatospora* was high on chitin. Importantly, the relative abundance of Bacteroidetes (*Mucilaginibacter* and *Flavobacterium*) was higher in complex substrates (cellulose, hemicellulose, maize leaf, and chitin) (Fig. 4). For the detailed comparison of the ^{12}C and ^{13}C communities in both the control and the treatments see Supplementary Fig. 2.

In total, 220 bacterial OTUs and 59 fungal OTUs fulfilled the criteria to be considered accumulators of C from ^{13}C-labelled substrates. Of these, 49% fungi and 53% bacteria were enriched on only one of the six substrates, while 2 fungal and 10 bacterial OTUs accumulated C from all substrates (Supplementary Tables S2 and S3). Enriched OTUs belonged to 38 fungal and 117 bacterial genera. It appears that bacterial genera are more versatile since 15 of 117 were found to accumulate C from five or six substrates, while in fungi, it was only 2 of 38 genera (*Lycoperdon* and *Pseudogymnoascus*; Fig. 5). In fungi, five genera were highly abundant in the control soil but not enriched on any substrate—four of them were ectomycorrhizal fungi—*Clavulina*, *Elaphomyces*, *Russula*, and *Sebacina*; also in bacteria, members of several dominant genera were not significantly enriched on any of the substrates (Fig. 5).

Approximately 62% of fungi and 43% of bacteria accumulated C from glucose or citric acid (or both), while 34% of fungi and 26% of bacteria used C from chitin. Altogether, approximately 60% of fungi and 80% of bacteria were found to be enriched in plant biomass (maize leaf) or its components (cellulose or hemicellulose) (Fig. 5, Supplementary Tables S2 and S3). C accumulation reflected the ecology of fungi. No enrichment of C from any substrate was observed for ectomycorrhizal fungi, which represented the most abundant taxa in the original soil (Supplementary Table S3). Yeast sequences were more frequently recorded on the least-recalcitrant substrates: glucose, citric acid, and hemicellulose, and moulds were most frequently observed on chitin, cellulose, and glucose. Animal pathogens were frequent among chitin-enriched taxa (Supplementary Table S3). In bacteria, Bacteroidetes and Verrucomicrobia were frequent among OTUs utilizing biopolymers (cellulose, hemicellulose, and chitin) and low on simple C substrates. Planctomycetes and Chloroflexi were exclusively enriched in citric acid. The share of Proteobacteria was higher among OTUs utilizing glucose and citric acids than on biopolymers (Supplementary Table S2).

4. Discussion

Our results show that both fungi and bacteria were able to utilize

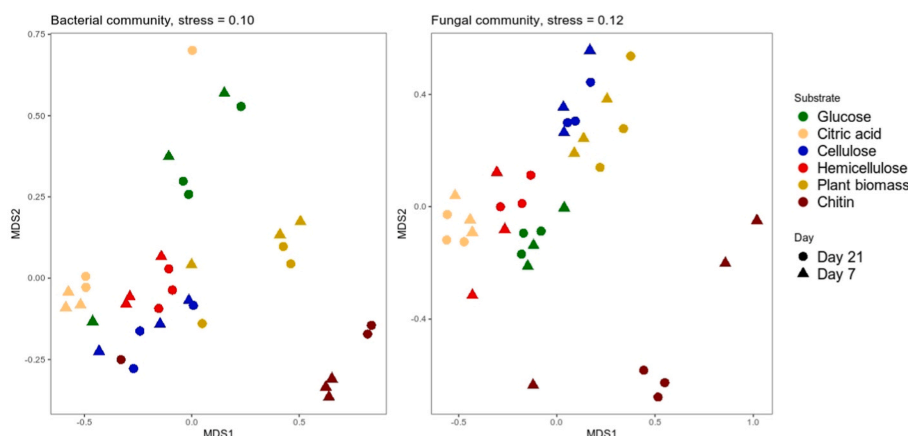

Fig. 3. Nonmetric multidimensional scaling (NMDS) analysis of soil fungal and bacterial communities accumulating carbon from different sources.

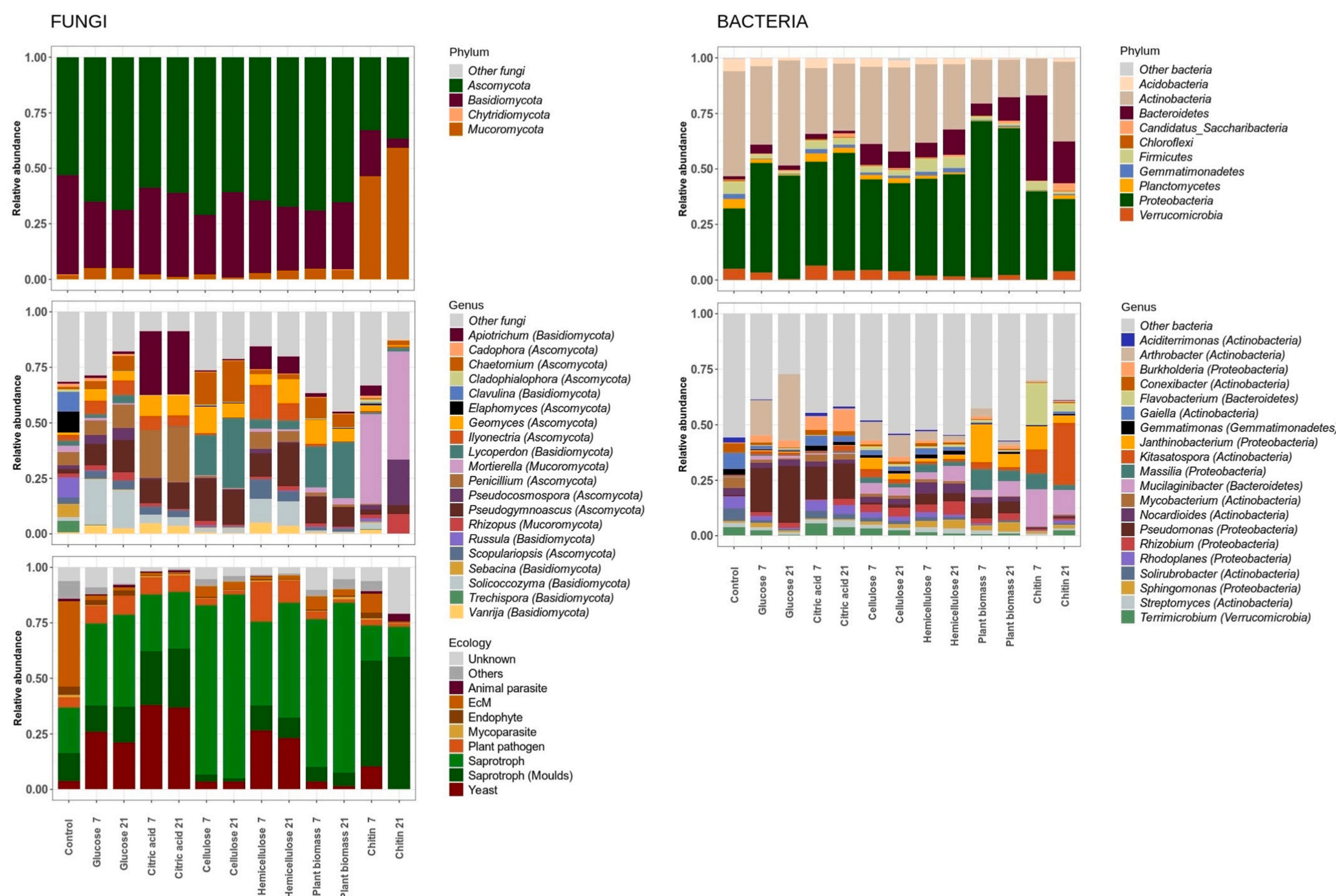

Fig. 4. Composition of microbial communities accumulating ^{13}C from substrates added to oak forest soil. Relative abundance of fungal and bacterial phyla, genera, and ecological guilds of fungi. The twenty most-abundant fungal and bacterial genera across all treatments are displayed in the genus panels. Control represents the total community.

diverse carbon-containing compounds in the forest soil. These findings support previous evidence about the important role of bacteria as significant players in the degradation of complex compounds in soil (Pepe-Ranney et al., 2016; Wilhelm et al., 2019) and confirm that both fungal and bacterial communities participate in the utilization of labile (rapidly degraded) and recalcitrant substrates (Bhatnagar et al., 2018; Kramer et al., 2016; López-Mondéjar et al., 2020; Rousk and Frey, 2015). It is, however, important to note that the replication in this study is of technical sort and represents only one area in the studied forest.

The compounds are utilized both as sources of energy through aerobic respiration, observable as CO_2 production, and as a source of C for biomass production. We show that the ratio of C used for biomass production/respiration is low in the case of glucose and especially citric acid, indicating their potential importance as energy sources in this experiment. Analysis of ^{13}C incorporation into PLFAs indicates that fungi accumulate more C from all substrates except chitin compared with bacteria. Similar results have been previously reported, showing that ^{13}C from plant origin is more rapidly and to a greater extent incorporated by fungi in comparison to bacteria (Koechli et al., 2019; Štursová et al., 2012). Fungi also dominated the ^{13}C incorporation from citric acid in relation to bacteria, in accordance with previous studies showing that numerous forest soil fungi are able to grow on citric acid and other simple C-containing substrates (Baldrian et al., 2011). In the case of chitin, the ^{13}C F/B ratio was similar to that in the control without substrate addition (Fig. 1d), indicating a similar rate of ^{13}C incorporation from chitin by bacteria and fungi. While bacteria quantitatively dominate fungi during fungal biomass degradation (Brabcová et al., 2018), they are able to utilize diverse components of this biomass, not exclusively chitin (Algora et al., 2022; López-Mondéjar et al., 2020; Starke et al., 2020).

Even though both fungal and bacterial decomposers were able to degrade all substrates, less specialization was observed for bacteria (Fig. 5). More than 50% of the ^{13}C-enriched bacterial taxa belonged to the typically slow-growing (oligotrophs) Acidobacteria, Actinobacteria, Alphaproteobacteria, or Verrucomicrobia (Supplementary Table S2) (Fierer et al., 2007). Actinobacterial taxa such as Micrococcales and Streptomycetales are known to degrade polysaccharides of plant origin in forest soils (Morrissey et al., 2016; Wilhelm et al., 2017, 2021), and the vast majority of these highly abundant bacteria (Lladó et al., 2018) were enriched when supplied with plant biomass or hemicellulose (e.g. *Kitasatospora*, *Cellulomonas*, *Leifsonia*). Interestingly, while bacteria from those phyla that are regarded as slow growers seemed to utilize all substrates well, there was no indication of fast-growing bacteria with a low ability to degrade biopolymers as previously assumed, e.g., Betaproteobacteria (Lladó et al., 2019; Lladó and Baldrian, 2017). In general, proteobacterial genera such as *Pseudomonas* and *Burkholderia* were slightly more frequent among the taxa that accumulated C from simple C compounds compared to biopolymers, although both genera of common fast-growing opportunistic bacteria in soil (Goldfarb et al., 2011; Lladó et al., 2019; Lladó and Baldrian, 2017; Peix et al., 2009), were enriched in several substrates (Fig. 5, Supplementary Table S2). In addition, taxa belonging to Burkholderiaceae and Verrucomicrobia have been recently reported as early colonists of the rhizosphere in comparison to other bacterial groups and may have adapted to compete for and assimilate root exudates (Dang et al., 2021; Nuccio et al., 2020), indicating their preference for plant exudates such as the glucose and citric acid tested

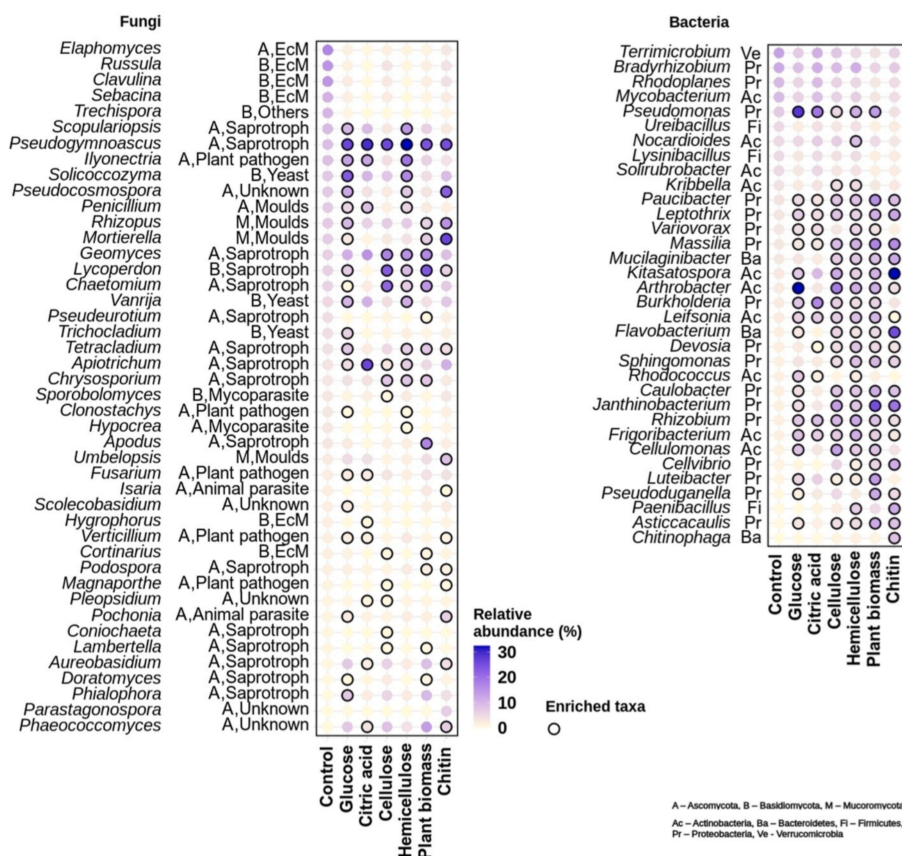

Fig. 5. Maximum relative abundance of fungal and bacterial genera following the addition of ^{13}C-labelled substrates to forest soil. The fill colour represents the maximum relative abundance in each treatment, either on day 7 or 21. The enrichment in the ^{13}C community is indicated with a black outline. The ten most abundant genera from all treatments were included, as well as any other enriched genera. Control represents the total community. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

here. In our experiment, both groups showed higher abundance on these substrates. Bacteroidetes taxa reported in our study, such as *Mucilaginibacter*, *Flavobacterium* or *Chitinophaga*, were previously identified as important decomposer taxa in forest ecosystems (Brabcová et al., 2016; Eichorst and Kuske, 2012; López-Mondéjar et al., 2016; Mieszkin et al., 2021), presenting a complex enzymatic repertoire for chitin, cellulose, and hemicellulose degradation and expresses this potential in situ (Lladó et al., 2016, 2019). Consistently, these taxa were more frequent and more abundant in the communities accumulating C from these complex biopolymers (Fig. 4; Supplementary Table S2).

In fungi, guild-specific substrate preferences were observed. Yeasts were, consistent with expectations, most frequently recorded as enriched on the three most rapidly utilized substrates: glucose, citric acid, and hemicellulose. Due to their unicellular nature, yeasts are often compared to bacteria in terms of their limited ability to degrade complex carbon sources (De Boer et al., 2005; Mašínová et al., 2017). The relatively high abundance and enrichment of some yeasts in the hemicellulose treatment (e.g., *Vanrija*, *Soilicoccozyma*) (Fig. 5) can be explained by their ability to degrade hemicellulose components (Mašínová et al., 2017; Mestre et al., 2011; Yurkov, 2018). Moreover, hemicellulose microcosms were rich in efficient fungal decomposers (Fig. 4) that have enzymatic potential for hemicellulose decomposition (Baldrian et al., 2011; Eichlerová et al., 2015; Talbot et al., 2013), leaving an opportunity for yeasts to feed as commensalists. Although certain yeast species are known to efficiently degrade cellulose (Mašínová et al., 2018) or incorporate cellulose-derived C (Štursová et al., 2012), no such taxa were observed here except for the members of the genus *Apiotrichum*.

Previous studies have shown that moulds are involved in the degradation of fungal biomass (Brabcová et al., 2016, 2018), and many of them express chitinases (Baldrian et al., 2011). In agreement with that, moulds dominated the fungal community accumulating C from chitin (Fig. 4, Supplementary Table S3). *Mortierella*, the most abundant mould genus in this work, comprises common chitinolytic fungi (Gortari and Hours, 2008; Swiontek Brzezinska et al., 2014) that have also been associated with the decomposing biomass of fungi (Brabcová et al., 2018) or soil-inhabiting mites (Werner et al., 2018). Moulds also readily accumulated C from simple compounds, while they were almost absent from cellulose, which is in agreement with their observed growth preferences (Algora Gallardo et al., 2021). In addition to moulds, fungal animal parasites also accumulated C from chitin (Supplementary Table S3, Fig. 4) since this polymer forms the exoskeletons of arthropods.

5. Conclusions

Our results show that simple C substrates that represent compounds that enter soil as root exudates—glucose and citric acid—are more often used for energy generation and relatively less incorporated into microbial biomass, indicating distinct use of these C sources in forest soils. Various C sources are targeted by distinct groups of microbial taxa, although their composition partly overlaps. Despite some level of specialization and substrate preference, many fungal and especially bacterial taxa are able to use C from different sources for their growth. Microbial taxa considered opportunistic, including yeasts, moulds, and Proteobacteria, were enriched in communities accumulating C from the most readily decomposing substrates—glucose, cellulose, and hemicelluloses—while Bacteroidetes were more frequent among C accumulators from biopolymers. Our results show the importance of bacteria and moulds in chitin utilization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

the work reported in this paper.

Data availability

Sequence data were deposited in the SRA under project number PRJNA755183.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2022.108786.

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