Received Date : 13-Oct-2015
Revised Date : 08-Jun-2016
Accepted Date : 22-Jun-2016
Article type : Original Article

Original Articles

Title:

Life in leaf litter: novel insights into community dynamics of bacteria and fungi during litter decomposition

Running title:

Microbial communities during litter decomposition

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Conflict of interest: The authors declare there to be no conflict of interest.

Abstract:

Microorganisms play a crucial role in the biological decomposition of plant litter in terrestrial ecosystems. Due to the permanently changing litter quality during decomposition, studies of both fungi and bacteria at a fine taxonomic resolution are required during the whole process. Here we investigated microbial community

succession in decomposing leaf litter of temperate beech forest using pyrotag sequencing of the bacterial 16S and the fungal internal transcribed spacer (ITS) rRNA genes. Our results reveal that both communities underwent rapid changes. Proteobacteria, Actinobacteria and Bacteroidetes dominated over the entire study period, but their taxonomic composition and abundances changed markedly among sampling dates. The fungal community also changed dynamically as decomposition progressed, with ascomycete fungi being increasingly replaced by basidiomycetes. We found a consistent and highly significant correlation between bacterial and fungal richness ($R = 0.76$, $P < 0.001$) and community structure ($R_{Mantel} = 0.85$, $P < 0.001$), providing evidence of coupled dynamics in the fungal and bacterial communities. A network analysis highlighted non-random co-occurrences among bacterial and fungal taxa as well as a shift in the cross-kingdom co-occurrence pattern of their communities from the early to the later stages of decomposition. During this process, macronutrients, micronutrients, C:N ratio and pH were significantly correlated with the fungal and bacterial communities, while bacterial richness positively correlated with three hydrolytic enzymes important for C, N and P acquisition. Overall, we provide evidence that the complex litter decay is the result of a dynamic cross-kingdom functional succession.

Introduction

The biological decomposition of plant litter and the subsequent release of nutrients to soils play an essential role in global carbon and nutrient cycling (Sayer 2006; Manzoni *et al.* 2008; Talbot & Treseder 2011). Decomposition is a dynamic process, during which the substrate's composition changes permanently (Berg 2000). The different compounds are not decomposed at the same rate, leading to a rapid decrease in readily available nutrients and therefore a relative increase in more recalcitrant compounds (Berg & McClaugherty 2003). Litter decomposition processes are triggered and mediated to a large extent by microorganisms that colonize the litter material during the degradation process (Romaní *et al.* 2006; van der Heijden *et al.* 2008; Cline & Zak 2015). Microbes play an essential role in all biogeochemical cycling and in doing so, they act as pools of both C and N (Prosser *et al.* 2007). Many studies have demonstrated that fungi are highly important for the degradation process due to their ability to secrete enzymes that catalyze the turnover of complex macromolecules such as cellulose, hemi-cellulose and lignin (Aneja *et al.* 2006; Talbot *et al.* 2013; Kuramae *et al.* 2013; Purahong *et al.* 2014b; Peršoh 2015). Fungal community assembly history is also known as a priority effect was found to influence the colonization success of later-arriving fungal species and leaf litter decomposition rate (Cline & Zak 2015). A recent diversity analysis of oak leaf litter revealed that its loss of

mass is coupled with changes in the fungal community during the decomposition process (Voříšková & Baldrian 2013). Fungal diversity was lowest at the beginning of the degradation process, reached a peak after 4 – 8 months, and leveled off at later decomposition stages (Voříšková & Baldrian 2013). However, the precise factors driving such succession are not fully understood.

The role of bacteria in the degradation of litter is still under debate. Although some authors consider that bacteria probably facilitate the fungal degraders by providing electrons or essential micronutrients and are thus indispensable for litter degradation (Frey-Klett *et al.* 2011), other authors postulate that bacteria simply colonize the soil-litter interface where they benefit from readily available substances formed during the degradation of complex macromolecules by fungal exoenzymes (Boer *et al.* 2005; Romaní *et al.* 2006). Some recent studies using molecular methods have suggested that diverse bacteria and fungi co-occur and may interact during the course of litter decomposition (Purahong *et al.* 2015; Urbanová *et al.* 2015). However, detailed studies on such cross-kingdom interactions and on their dynamics in relation to litter degradation are missing.

In the present study, we (i) investigated the successional changes in the communities of both bacteria and fungi during the decomposition of European beech (*Fagus sylvatica*) leaf litter over 473 days using a molecular meta-barcoding approach, (ii) tested the correlation of the physicochemical properties of leaf litter on successional changes of bacterial and fungal communities, (iii) unraveled the relationships between bacterial and fungal communities by exploring their co-occurrence patterns, and (iv) tried to relate such richness patterns to changes in potential enzymatic activities. We postulate that the bacteria–fungi interactions become increasingly important during leaf litter degradation as this process occurs in parallel with a relative enrichment in complex macromolecules of lower degradability. Thus we expected the microbial co-occurrence network pattern to become increasingly complex over time. Furthermore, we hypothesized that fungi with the potential to produce enzymes involved in the degradation of macromolecules, such as lignin or cellulose, are crucial components of the co-occurrence network of bacteria and fungi. Finally, in light of recent work by Hoppe et al. (2015) we expected to reveal correlative relationships between the potential enzymatic activities related to C, N and P turnover, and the variations in bacterial and fungal richness along the decomposition process.

Materials and Methods

Study site

The study was conducted at the Hainich-Dün Biodiversity Exploratory (about 1,300 km^2; 51°16'N 10°47'E) in Central Germany (Fischer *et al.* 2010). According to the World Reference Base of Soil Resources the main soil

type is stagnosol on a limestone bedrock (Fischer *et al.* 2010). The soil pH is weakly acidic (5.1 ± 1.1; mean ± SD) with a litter layer of 2 cm – 5 cm (Purahong *et al.* 2015). The annual mean temperature and precipitation ranges from 6.5 ºC – 8 ºC and 500 mm – 800 mm respectively. All information pertaining to the experimental area has been described in detail in Fischer et al (2010). We assigned three replicate study plots (2 m × 8 m) located on flat land within the experimental site (HEW12) characterized as an unmanaged deciduous forest reserve of 10 000 m^2 dominated by European beech with an uneven age distribution (tree age up to >100 years, cover tree layer (trees > 10 m) = 85%) (Purahong *et al.* 2015).

Litterbag design and sampling

We collected freshly fallen leaves of European beech from the study site in October 2009. The leaves were air dried to constant weight at room temperature. Ten grams of air-dried leaves were placed into nylon litterbags (25 cm × 25 cm, mesh size 2 mm). At the end of the litter fall period (13 November 2009) 15 litterbags were placed horizontally in the upper litter horizon of each replicate study plot. Nine additional litterbags were retained to determine the initial dry mass (oven-dried at 105 °C ≥ 24 hr until constant weight), nutrient element concentrations, and lignin content of the litter. The initial chemical composition of the litter material is given in Table S1, Supporting Information. Litterbags were retrieved on five sampling dates: in 2010 on February 10th (89 days), May 12th (180 days), August 24th (284 days), November 10th (362 days), and in 2011 on March 1st (473 days). On each sampling date, three randomly chosen litterbags per plot (nine litterbags in total) were removed, each placed into a separate clean plastic bag to reduce the loss of small fragments, and transported in an ice-box (0 °C) to the laboratory within 4 h. In the laboratory, litterbags were processed immediately. First, the three replicate litterbags retrieved from the same plot were pooled in a composite sample and their wet weight was determined. This resulted in three composite sample replicates at each sampling time. Each composite sample was then ground to pass a two mm sieve, homogenized, divided into two parts: one original homogenized and one freeze-dried subsample, which were both stored at -20 °C for further analysis.

Physicochemical analysis of leaf litter

The water content and pH (in 0.01 M $CaCl_2$) of the leaf litter samples were measured. Water content was used as a proxy for annual variation in precipitation. Total C and N concentrations were determined by dry combustion at 1000 °C with an Elementar Vario EL III elemental analyzer (Elementar Analysensysteme GmbH, Hanau, Germany) according to DIN/ISO 10694. Nutrient ions (Mg, K, P, Ca, Fe, Cu, V, Mn, Co) were determined with the same device using inductively coupled plasma (ICP) optical emission spectrometry (ICP-OES) and mass

spectrometry (ICP-MS) according to manufacturers' specifications. Total lignin was calculated by summing Klason lignin (acid insoluble lignin) and acid soluble lignin (Raiskila *et al.* 2007). Klason lignin content was determined gravimetrically as the dry mass of solids after sequential hydrolysis with sulfuric acid (72 % w/w); in a second step, acid soluble lignin was measured UV-photometrically in 4 % H_2SO_4 (Effland 1977; Liers *et al.* 2011). All physicochemical analyses were conducted in triplicate on the same subsample.

Enzyme assays

A total of eight potential enzyme activities were measured from original homogenized leaf litter samples (Sinsabaugh *et al.* 2003; German *et al.* 2011; Purahong *et al.* 2014b). These include five hydrolytic enzymes important for the acquisition of polymeric carbon (β-glucosidase, cellobiohydrolase, and xylosidase), nitrogen (N-acetylglucosaminidase), and phosphorus (acid phosphatase); and three oxidative enzymes related to the chemical modification of lignin (laccase, general peroxidase, and manganese peroxidase - MnP).

Microbial DNA extraction and sequence library preparation

DNA was extracted from 100 mg of each homogenized and freeze-dried leaf litter sample using the ZR Soil Microbe DNA MiniPrep kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's protocol. The presence and quantity of genomic DNA was checked using a NanoDrop ND-1000 spectrophotometer (ThermoFisher Scientific, Dreieich, Germany). DNA extracts were then stored at −20 °C for further analysis.

Bacterial and fungal amplicon libraries were obtained for pyrosequencing using custom fusion primers. We used the primer pair BAC 341F (5′-CCTACGGGAGGCAGCAG-3′) and BAC 907R (5′-CCGTCAATTCMTTTGAGTTT-3′) to amplify the V3–V5 region of the bacterial 16S rRNA gene (Muyzer *et al.* 1995; Muyzer & Smalla 1998), and the primer pair ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′, Gardes & Bruns 1993) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′, White *et al.* 1990) to amplify the fungal internal transcribed spacer (ITS) rRNA region. The custom primers were constructed with the barcodes and sequencing primers attached at the BAC 341F and ITS4 primers for unidirectional sequencing (see Wubet *et al.* 2012; Lentendu *et al.* 2014 and Supporting Information). PCR conditions are shown in the Supporting Information. Amplification products were visualized with eGels (Life Technologies, Grand Island, New York). Products (three replicate reactions per sample) were then pooled in equimolar amounts and each pool was cleaned with Diffinity RapidTip (Diffinity Genomics, West Henrietta, New York), and size selected using Agencourt AMPure XP (BeckmanCoulter, Indianapolis, Indiana, USA) following Roche 454 protocols (454 Life Sciences, Branford, Connecticut, USA). Size-selected pools were then quantified and 150 ng of DNA were

hybridized to Dynabeads M-270 (Life Technologies, Oslo, Norway) to create single stranded DNA. Single stranded DNA was diluted and used in emPCR, which were performed and subsequently enriched for amplicon sequencing following the established manufacturer's protocols (454 Life Sciences) at the Research and Testing Laboratory (Lubbock, TX, USA).

Bioinformatics analysis

The raw demultiplexed reads were first quality trimmed using MOTHUR 1.33.3 (Schloss et al. 2009). Reads satisfying the following criteria were considered for further analyses: holding one of the expected barcodes with maximum 1 mismatch; the forward primer with a maximum of 4 mismatches; a minimum length of 350 nt; a minimum average quality of 30 or 25 Phred score; containing homopolymers with a maximum length of 8 nt; without ambiguous nucleotides for bacteria and with a maximum of 8 ambiguous nucleotides for fungi. The quality filtered reads were shortened to their first 350 bases and potential chimeras were removed using UCHIME 4.2.40 (Edgar *et al.* 2011) as implemented in MOTHUR. The read counts were normalized to the smallest read number per sample (2,368 and 2,471 reads for bacteria and fungi, respectively). Unique sequences were sorted according to decreasing abundance and were clustered into OTUs using CD-HIT-EST 4.5.4 (Fu *et al.* 2012) at a threshold of 97 % pairwise similarity. Bacterial 16S OTU representative sequences were assigned against the Silva SSU reference sequence database (version 115) using the MOTHUR implementation of the Wang *et al* (2007) classifier. Fungal ITS OTU representative sequences were first classified against the dynamic version of the UNITE fungal ITS sequence database (version 6, released on 15.01.2014; Kõljalg *et al.* 2013). The sequences with only Fungi identification were further classified against the full version of the UNITE database in order to improve their taxonomic annotation. Rare OTUs (singletons to tripletons), which could potentially have originated from sequencing errors (Kunin *et al.* 2010) were removed from the dataset. We used a Mantel test based on Bray-Curtis distance measure with 999 permutations to assess the correlation between the whole matrix and a matrix excluding the rare OTUs for both bacterial and fungal data sets (Hammer *et al.* 2001; Hoppe *et al.* 2015). The results indicated that the removal of rare OTUs from the bacterial and fungal communities had no effect (bacterial dataset: R_{Mantel} = 0.995, P = 0.001; fungal dataset: R_{Mantel} = 1.000, P = 0.001).

Statistical analyses

Bacterial and fungal OTU richness values and Shannon diversity were calculated using the 'diversity' function in the software PAST 3.0 (Hammer *et al.* 2001). In this work we used observed bacterial and fungal OTU

richness (simply referred to as OTU richness) as the measure for microbial diversity. To assess the coverage of the sequencing depth, individual rarefaction analysis was performed for each sample using the function 'diversity' in PAST (Fig. S1, Supporting Information).

Non-metric multidimensional scaling (NMDS) analysis was used to visualize the impact of bacterial and fungal community succession over time on the relative distribution of the samples on the ordination plot. We used the relative abundance OTU matrices of the fungal and bacterial communities and Bray-Curtis dissimilarity distances to calculate the NMDS ordination plot. Leaf litter quality parameters and physicochemical properties were fitted to the NMDS ordination plots of bacterial and fungal communities using the 'envfit' function in the vegan package, and Goodness-of-fit statistics (R^2) were calculated with P values based on 999 permutations (Oksanen 2013). All significant environmental variables ($P < 0.05$) were plotted in the respective NMDS ordination plots using PAST. Dynamics of bacterial and fungal abundances over time were assessed by the Friedman test using SPSS as the data sets vary in time. With this test, time is used as a between-subject factor because it cannot be randomized. Correlations between bacterial and fungal communities were analyzed using Mantel tests based on the Bray-Curtis dissimilarity distance matrix in PAST. The relative abundances of eubacterial and fungal phyla over time were visualized using Treemap 3.7.0 (Macrofocus, Switzerland) (Purahong *et al.* 2014a). Correlations between bacterial and fungal richness as well as between enzyme activities and fungal and bacterial richness were assessed using the Pearson product-moment correlation in PAST. All datasets were log-transformed and tested for normality using the Shapiro–Wilk W test. Correlations between specific dominant bacterial and fungal OTUs (those that account for at least 1 % of the relative abundance at one or more sampling times) and leaf litter quality parameters and physicochemical properties were assessed using Spearman's rank correlations.

C-score and checkerboard pair analysis of the fungal and bacterial communities was done using EcoSim's default settings (Gotelli & Entsminger 2004) to check for the non-random occurrence of the microbial communities. We used the extended local similarity analysis (eLSA) to calculate the local similarity (LS) scores between fungal, bacterial, and fungal and bacterial OTUs using 1000 permutations (Xia *et al.* 2011) to assess significant co-occurrence network patterns of the fungal and bacterial communities during the litter decomposition. The significant local similarity correlations ($P < 0.01$) were visualized and sub-network patterns were extracted using Cytoscape 3.0.2. Network properties were calculated using Network Analyser as implemented in Cytoscape (Shannon *et al.* 2003). These correlation networks include nodes that consisted of

fungal and bacterial OTUs as a proxy for 'species' and the local similarity scores among the OTU's referred as edges connecting nodes (Steele *et al.* 2011).

Results

Bioinformatics processing of the sequence datasets

A total of 86,609 quality filtered bacterial 16S and 85,979 fungal ITS reads were obtained after removal of chimeric sequences and were clustered into 3,346 bacterial and 771 fungal OTUs. By removing the low abundance OTUs (i.e. singletons to tripletons), the final analyses datasets contained 908 bacterial and 225 fungal OTUs.

Bacterial succession

The bacterial OTUs were assigned to thirteen phyla and 186 genera (complete taxonomy provided in Table S2, Supporting Information). Members of the Proteobacteria (55.8 %), Actinobacteria (24.9 %) and Bacteroidetes (17.8 %) were the dominant phyla among the entire bacterial community accounting for 98.5 % of all bacterial OTUs. From the 42 most abundant OTUs (> 1 % of the relative abundance in at least one of the sampling times), 34 displayed a significant shift during the litter decomposition (Fig. 1a and 2, Fig. S2a, Supporting Information). The NMDS ordination plot also revealed distinct bacterial communities in leaf litter at different sampling times (Fig. 1a). As decomposition progressed, the average Bray-Curtis dissimilarity of bacterial communities compared to the initial community increased from 51.35 % (after 89 days) to 79.44 % (after 473 days) (Table S3, Supporting Information). We found the lowest bacterial richness at the early stages of leaf litter decomposition (0 – 180 days, observed OTUs = 216 – 236) as compared to later stages (362 – 473 days, observed OTUs = 357 – 404) (Fig. S3a, Supporting Information). Shannon diversity also showed a similar trend (Fig. S3b, Supporting Information).

The leaf litter was initially (day 0) highly dominated by two bacterial OTUs: OTU 002 identified as *Frigoribacterium* sp. (8.9 %) and OTU 004 identified as *Sphingomonas* sp. (8.4 %; Figs 1a and 2, Table S2, Supporting Information). These two OTUs remained dominant after 89 and 180 days of decay with relative abundances ranging from 5.2 % to 12.8 %, but their relative abundances gradually decreased to 1.4 % and 3.5 % after 473 days. We found the majority of the early-stage dominant bacterial OTUs to decrease in abundance or become absent in the later stages (Figs 1a and 2). At the early decomposition stage (89 and 180 days), the bacterial communities were also co-dominated by ten additional OTUs (Figs 1a and 2). The two bacterial OTUs from this early decomposition stage (*Nitrobacter* sp. and *Kineosporia* sp.) maintained their high abundances at

all sampling dates until the end of the experiment (180 – 473 days). In contrast, *Streptomyces* and *Bradyrhizobium* co-dominated only during the later decomposition stages of leaf litter (284 – 473 days) (Figs 1a and 2).

Fungal succession

Overall, 225 fungal OTUs belonging to three phyla and 64 genera were detected. Members of the phylum Ascomycota (69.9 %) and Basidiomycota (30.06 %) dominated the fungal community (Table S4, Supporting Information). The polyphyletic phylum Zygomycota was only represented by 0.04 % of all the sequence recovered in this study and *Mortierella* was the only genus identified (*Mortierella globulifera* and *Mortierella* sp.). As observed for bacterial OTUs, the majority of the most abundant fungal OTUs (24 out of 39) significantly shifted over time (Figs 1b and 3, Fig. S2b, Supporting Information). The NMDS ordination also shows a clear and distinct pattern of fungal communities in leaf litter at different sampling times (Fig. 1b). The average Bray-Curtis dissimilarity of fungal communities compared to the initial community increased as decomposition progressed from 58.97 % (after 89 days) to 96.52 % (after 473 days) (Table S5, Supporting Information). Although members of the two phyla always coexisted, a clear shift from Ascomycota to Basidiomycota was observed in the later stages of litter decomposition. Fungal richness and diversity was lowest during the early stages of the leaf litter (0 - 89 days, observed OTUs = 52 – 63; 0 day, Shannon diversity = 1.31), and increased over time to reach a peak after 473 days (observed OTUs = 98) or 180 - 284 days (Shannon diversity = 3.05) (Figs S3c and d, Supporting Information).

The patterns of the fungal community successions were from ascomycetes (*Mycosphaerella* sp.) and aquatic hyphomycetes (*Gyoerffyella* sp.1) (0 day) via biopolymer decomposing ascomycetes (Xylariales OTU 3, *Cylindrosympodium* sp.1, Leotiomycetes OTU 12) (89 – 180 days) and basidiomycetous molds (*Ceratobasidium*) to basidiomyceteous litter-decomposers (*Mycena* spp., *Clitocybe* spp. *and Lepiota* spp.) (284 – 473 days).

Leaf litter quality and microbial community structure and function

Leaf litter physicochemical parameters during decomposition corresponded to changes in bacterial and fungal communities (Figs 1a and b). Specifically, the C:N ratio, macronutrients (C, N, K, Mg and Ca), micronutrients (Cu, Co and Mn), leaf litter water content and pH were significantly correlated with bacterial and fungal

communities ($P < 0.05$; Supporting Information; Fig. S4, Supporting Information). When comparing the response pattern of microorganisms to changes in the physicochemical factors of the leaf litter, we found very similar correlation patterns in the NMDS plots for bacteria and fungi (Figs 1a and b).

We found both positive and negative relationships between microbial richness and enzyme activities measured as potential predictors of ecosystem functions (Fig. 4). Bacterial richness was positively correlated with activities of β-glucosidase ($R = 0.53$, $P < 0.05$), N-acetylglucosaminidase ($R = 0.53$, $P < 0.05$) and acid phosphatase ($R = 0.80$, $P < 0.001$). In contrast, fungal richness was negatively correlated with laccase ($R = -0.57$, $P < 0.05$) and general peroxidase ($R = -0.52$, $P < 0.05$). The appearance of *Mycena* spp., *Clitocybe* spp. and *Lepiota* spp. in the later stages of litter decomposition was coupled with a marked increase of manganese peroxidase enzyme activity and a great reduction of lignin (Figs 1b and 3; Figs S5 and S6; Table S4, Supporting Information).

Concordance between bacterial and fungal communities and network patterns

Bacterial OTU richness was significantly correlated with fungal OTU richness (Fig. 4a; $R = 0.76$, $P < 0.001$). We found a consistently significant concordance between the bacterial and fungal community compositions ($R_{\text{Mantel}} = 0.85$, $P < 0.001$). Both fungal and bacteria communities showed a non-random distribution pattern (bacteria: C-score = 9.38, $P < 0.0001$, checkerboard = 117565.00, $P < 0.0001$; fungi: C-score = 8.41, $P < 0.0001$, checkerboard = 7326.00, $P < 0.0001$). Further, co-occurrence network pattern analysis revealed distinct network patterns of microbial communities at the early (0 – 180 days) and later (284 – 473 days) stages of leaf litter decomposition (Figs 5a and b). Compared with the early stage specific network, the later stage had higher number of modules (4 *vs*. 3), much higher weighted degree (57 *vs*. 29) and lower neighborhood overlap (0.28 *vs*. 0.55) (Figs 5a and b; see detailed information on the structure of the networks in Table S6, Supporting Information). Sub-networks of fungal and bacterial communities connected with three of the important fungal genera frequently detected both at the early (*Gyoerffyella*) and later (*Mycena* and *Clitocybe*) stages of decomposition, show a strong dominance of the bacterial communities (79 % - 82 % of the overall fungal and bacterial OTUs). There were 57 – 112 bacterial OTUs from different phyla (Proteobacteria > Bacteroidetes > Actinobacteria) co-occurring and potentially interacting with members of the respective fungal genera (Tables S7 – S9, Supporting Information). Notably, all these sub-networks included potential nitrogen fixing bacterial taxa.

Discussion

Microbial community dynamics during leaf litter degradation

After 473 days, almost 40 % of the total leaf litter mass had been lost; by day 284 the C:N and Lignin:N ratios had also leveled off (Purahong *et al.* 2015). Substantial amounts of lignin were decomposed between days 284 to 473 (~20 % – 40 %), demonstrating that leaf litter decomposition reached the later or second phase of decomposition stage mentioned in Berg (2000). In relation with this advanced level of decomposition, we found a significant shift in the structure of both bacterial and fungal communities from the early to later sampling dates, as all community profiles from the different sampling times taken at intervals of approximately 3 months were markedly distinct from each other.

Members of *Frigoribacterium* and *Sphingomonas,* that are known to produce proteolytic or cellulolytic enzymes (Aislabie *et al.* 2006), dominated the early colonizer bacterial communities. This pattern is consistent with the culture-based study of bacterial succession on black alder leaf litter (Dilly & Irmler 1998). *Frigoribacterium* and *Sphingomonas* as well as many of the dominant bacterial genera initially present in the leaves (*Pseudomonas*, *Rhizobium*, *Massilia*, *Mythylobacterium*, *Kineosporia*, *Flavobacterium*, *Variovorax*) were reported as phyllosphere bacteria, including both leaf endophytes and epiphytes as well as those saprobic bacteria commonly isolated from plant debris (Bodenhausen *et al.* 2013; López-Guerrero *et al.* 2013). Some of these genera persisted at relatively high abundances during the early stages of leaf litter decomposition (*Pseudomonas*, *Massilia*) or even at later stages (*Frigoribacterium*, *Sphingomonas*, *Rhizobium*, *Kineosporia*, *Flavobacterium*), which is consistent with a number of studies that have reported on the abilities of these genera to use some complex substrates or to fix nitrogen (Aislabie *et al.* 2006; Bernhard 2010; López-Guerrero *et al.* 2013). It is also interesting that at the later stage of litter decomposition we found filamentous bacteria (*Streptomyces*) known to have a potential for transforming humus and lignin (Trigo & Ball 1994). Interestingly, we found diverse microbes able to fix nitrogen including *Frankia*, *Rhizobium* and *Bradyrhizobium* (Bernhard 2010; Seipke *et al.* 2012; Sellstedt & Richau 2013). We also found high relative abundance of nitrifiers at most sampling dates, indicating the leaf litter environment to be an oligotrophic habitat in which autotrophic microbes could also survive. The coincident finding of these two groups might reflect the high spatial heterogeneity of the leaf litter habitat: while the nitrogen fixing organisms might be dwelling inside the leaf material, where conditions are reductive and carbon is released by exoenzymatic degradation of complex substrates, the nitrifiers may be settled more at the leaf surface as they require oxic conditions for nitrification of ammonium

(Kowalchuk & Stephen 2001). Both microbial groups might at the end contribute to improve N resource availability, which otherwise would tend to decrease during the decomposition process.

We also found clear successional patterns of fungal communities, with an initial decomposer community being dominated by ascomycetous fungi typical of the phyllosphere (Unterseher *et al.* 2013). This fungal community was replaced by other ascomycetes known to be biopolymer decomposers, specialized in substrates such as cellulose, hemicelluloses, pectin and/or chitin. At the later decomposition stage, fungal species (chiefly members of the phylum Basidiomycota) able to decompose recalcitrant substrates such as lignin and humic substances became dominant. It is interesting to note that, apart from the phyllosphere fungi, the initial leaves were highly dominated by aquatic hyphomycetes affiliated with the genus *Gyoerffyella* (Ascomycota). *Gyoerffyella* was originally isolated from healthy ectomycorrhizal *Picea abies* roots (Czeczuga & Orłowska 1997; Selosse *et al.* 2008), thus fulfilling the definition of an endophytic microbe (Purahong & Hyde 2010; Dean *et al.* 2014). In the present study *Gyoerffyella* was highly abundant in leaf litter from 0 until 180 days: the organism may thus be already present in the leaves before litter fall, and through time it changes its metabolism from being that of an endophyte during leaf growth, to one of saprophytism during litter decomposition. This is consistent with studies showing that hyphomycetes are generally able to produce cellulases and a range of other enzymes (including laccase) to decompose the cell wall polysaccharides and detoxify plant compounds (Abdel-Raheem & Ali 2004; Junghanns *et al.* 2005; Krauss *et al.* 2011). The leaves of European beech were also initially co-dominated by fungi of the genus *Mycosphaerella,* which have been described as being able to adapt to various life strategies including endophyte, saprotroph and plant pathogen (Crous 2009; Unterseher *et al.* 2013; Voříšková & Baldrian 2013). According to its relative abundance, *Mycosphaerella* in our study might function as an early decomposer, which is consistent with the findings of another study of fungal community dynamics in oak leaf litter (Voříšková & Baldrian 2013). During succession, members of Leotiomycetes, Xylariales, *Cylindrosympodium*, and *Apodus* replaced the phyllosphere fungal communities. Most of these taxa are known as specialized biopolymer decomposers. Leotiomycetes have been reported as the main cellulase producers in decomposing European beech leaf litter based on a meta-proteomics study (Schneider *et al.* 2012). The members of Xylariales consist of both cellulose and lignocellulose decomposers that can cause soft-rot type II (Martínez *et al.* 2005; Nghi *et al.* 2012).

The dominant later stage basidiomycetous lignin decomposers include members of the genera *Mycena, Clitocybe, and Lepiota,* which produce both laccase and manganese peroxidases (Steffen *et al.* 2000; Erden *et al.* 2009; Kellner *et al.* 2014). A peroxidase gene expression study on European beech leaf litter from the same

study site confirmed that class II peroxidase reference sequences were assigned to a putative manganese peroxidase sequence of *Mycena* sp. with 98 % amino acid identity (Kellner *et al.* 2014). A transcriptional expression study of pure cultures of *Clitocybe nebularis* exhibited, among members of all three super-families, transcripts of secretory peroxidases (manganese peroxidase belonging to the ligninolytic class II peroxidases, dye-decolorizing peroxidases, unspecific or aromatic peroxygenases) (Kellner *et al.* 2014). Members of the genus *Lepiota* are saprotrophic and normally grow in the lower litter layers of the forest floor where they decompose both cellulose and lignin (Erden *et al.* 2009).

Bacteria – fungi interactions: The case of nitrogen and phosphorus

Bacteria may facilitate the leaf litter decomposition processes by interacting with or mediating other members of the decomposer communities, especially fungi (Romaní *et al.* 2006; Frey-Klett *et al.* 2011). Our experiments supported this prediction given the correlations between richness and community structures of both bacteria and fungi. In our study nitrogen accumulation in leaf litter was observed over the whole decomposition experiment. In both the initial and later phases of leaf litter decomposition, the lignin:N as well as the C:N ratios did not reach the critical value (the end point of the immobilization phase) for N mineralization. The presence of microbes that feed on leaf litter C but obtain nitrogen from the environment for their growth and reproduction, results in a net increase in litter N and a decrease of lignin:N or C:N ratios (Berg & Staaf 1981). Our results suggest that some nitrogen fixing bacteria may have contributed to the accumulation of N in leaf litter. Atmospheric N_2 can be captured by heterotrophic nitrogen fixing bacteria and be used for their multiplication thus leading to a net increase of N in leaf litter (Kowalchuk & Stephen 2001). The high abundance of nitrogen fixing bacteria (diazotrophs) may reflect a constant nitrogen addition that supports other decomposers, especially those fungi which have been reported to form a commensal interaction with nitrogen fixing bacteria in a spatially and chemically similar substrate, N-limited dead wood (Hoppe *et al.* 2014). Indeed, in our study we also found that the potential lignocellulose decomposers such as *Clitocybe* spp. *and Mycena* spp. co-occurred with many bacterial OTUs identified as bacterial genera able to fix nitrogen (e.g., *Bradyrhizobium*, *Mesorhizobium*, *Pseudomonas* and *Rhizobium*) (Tables S8 and S9, Supporting Information). The nitrifying bacteria that were detected might contribute to the N cycle through N resource mobilization by the co-occurring diazotrophs and make them available to lignolytic fungi (Table S8, Supporting Information). Interestingly, our results also revealed that bacterial richness significantly correlated with acid phosphatase activity. Many bacterial OTUs known as phosphate solubilizers (i.e. *Burkholderia, Flavobacterium, Pseudomonas* and *Rhizobium*) co-occurred with the most dominant fungal OTUs in the early stages of decomposing litter

(*Gyoerffyella* spp.) as well as the potential lignocellulose decomposers (*Clitocybe* spp. *and Mycena* spp.) (Rodríguez & Fraga 1999). Thus besides N, the bacterial communities might also provide P to the fungal decomposers.

Fungi – fungi interactions: The case of laccases and peroxidases

Fungi have been shown to be primary decomposers of litter and are the main group of soil microorganisms actively secreting hydrolytic and oxidative enzymes (Keiblinger *et al.* 2012; Schneider *et al.* 2012; Baldrian 2014). Previous results based on microbial biomass clearly showed that fungi dominated during the entire decomposition experiment (Purahong *et al.* 2014c). We found a negative correlation of fungal richness and the two oxidative enzyme activities (laccase and general peroxidase), which may reflect fungal-fungal interactions. Specifically, in diverse communities, fungi are known to invest more energy for antagonistic interactions (i.e. production of secondary metabolites, Boddy *et al.* 1989; Boddy 2000) than for growth and the production of decomposition enzymes (Fukami *et al.* 2010; Dickie *et al.* 2012). Biosynthesis of extracellular enzymes is highly nutrient demanding and energetically expensive for fungi, as these biocatalysts have to be secreted in substantial amounts, which means substantial losses of C and N (Whalen & Sampedro 2010). An alternative explanation could be that the fungi producing high quantities of these oxidative enzymes were also the most competitive, and thus could exclude other fungi from their territories (Tsujiyama & Minami 2005).

Microbial community structure and leaf litter quality

Our work supports that bacterial and fungal communities in leaf litter are partially structured by environmental filters over time. This is consistent with the results from soil bacterial and fungal communities in a long-term chronosequence (Welc *et al.* 2012; Freedman & Zak 2015). Leaf litter decomposition is a dynamic process, during which the nutrient composition changes permanently at different points in time (Berg 2000). Microbes generally have rapid growth and short generation times, thus under favorable conditions some specific microbes can rapidly turn to dominate the community (Prosser *et al.* 2007). Our results show clearly that the abundances of some specific bacterial and fungal OTUs positively or negatively correlated with leaf litter physicochemical factors including nutrients, pH and water content (Fig. S4, Supporting Information). This scenario may explain the changes of microbial richness and community patterns over different temporal scales (Prosser *et al.* 2007). We have also identified major abiotic factors of beech leaf litter that are correlated with the structures of bacterial and fungal communities. It is interesting that the abiotic factors driving bacterial and fungal communities were identical, and that the directions of the correlations were similar. In particular, the C:N ratio

of the leaf litter is significant factor as it relates both to the nutrient status of the leaf litter and to its stage of decomposition (Purahong et al. 2014c, 2015). Furthermore, microbial macronutrients such as C, N, K, Mg and Ca, which are required for microbial growth and reproduction (Prescott et al. 1999), and micronutrients such as Co, Cu and Mn, which have important roles as structural components of enzymes involved in catalysis of reactions (Aggarwal 2007), are significantly correlated with bacterial and fungal community structure. Additionally, water content and pH were significant factors confirming earlier studies (McGuire et al. 2011; Matulich et al. 2015; Purahong et al. 2015).

Conclusions and outlooks

In the present study, we combined a litterbag experiment with a high-resolution molecular culture-independent meta-barcoding approach in order to investigate bacterial and fungal community successions during beech litter decomposition in a Central European beech dominated forest. Keeping possible biases inherent to molecular techniques in mind (Tedersoo *et al.* 2010; Voříšková & Baldrian 2013), our work provides insights into bacterial and fungal community dynamics, co-occurrence network patterns and their roles in leaf litter decomposition. Apart from identifying litter decomposition traits related to bacterial and fungal community successions, we revealed a cross-kingdom fungal and bacterial co-existence relationship and its implication for potential enzyme secretion and related ecosystem services.

Overall, parallel to decomposition progress, we identified the succession of fungal communities from an ascomycete to a basidiomycete dominated community, composed of taxa capable of degradation of lignocellulose compounds, which confirms the crucial role of fungi in litter decomposition. Positive linear correlations between bacterial richness and three hydrolytic enzymes important for C, N and P acquisition suggest that bacteria make a significant contribution to carbon, nitrogen and phosphorus dynamics in leaf litter decomposition. Furthermore, the dominance of bacterial taxa in the cross kingdom microbial community co-occurrence networks, both at the early and later stage of litter decomposition indicate that bacteria may play a more important role in leaf litter decomposition processes than previously thought (Boer *et al.* 2005; Romaní *et al.* 2006; Keiblinger *et al.* 2012; Schneider *et al.* 2012). However, the direct role of bacteria in litter decomposition cannot be drawn from our study as at least a part of their detected diversity may reflect parasites, mutualists or commensals associated to the fungi. In general our results revealed that litter decomposition is a dynamic process controlled by dynamically shifting and interacting cross kingdom microbial communities specialized to the different stages of the litter decomposition processes.

Acknowledgments

Our work was funded, in part, by contributing projects to the DFG Priority Program 1374 on Infrastructure-Biodiversity Exploratories (BU 941/17-1, KR 3587/1-1, KR 3587/3-2, HO 1961/4-1, and HO 1961/5-2). We thank the managers of the three exploratories, Swen Renner, Sonja Gockel, Kerstin Wiesner, and Martin Gorke, for their work in maintaining the plot and project infrastructure; Simone Pfeiffer and Christiane Fischer for providing support through the central office; Michael Owonibi for managing the central database; and Markus Fischer, Eduard Linsenmair, Dominik Hessenmöller, Jens Nieschulze, Daniel Prati, Ingo Schöning, ErnstDetlef Schulze, Wolfgang W. Weisser, and the late Elisabeth Kalko for their role in setting up the Biodiversity Exploratories project. We thank Jessica L. M. Gutknecht (UMN-Twin Cities) for the collaboration to generate the enzyme data. The authors would also like to thank the anonymous reviewers for their valuable and constructive comments to improve the quality of the paper.

Figure legends

Fig. 1 Bacterial and fungal community succession in European beech leaf litter over 473 days. a) NMDS ordination of all bacterial OTUs detected in different sampling dates (left panel) and heat-map of 42 dominant bacterial OTUs which account for at least 1 % of the relative abundance at one or more sampling times (right panel). b) NMDS ordination of all fungal OTUs detected in different sampling dates (left panel) and heat-map of 39 dominant fungal OTUs which account for at least 1 % of the relative abundance at one or more sampling times (right panel). In NMDS ordinations, the numbers 0 – 5 represent the sampling time; 0 = 0 day, 1 = 89 days, 2 = 180 days, 3 = 284 days, 4 = 362 days, 5 = 473 days. All significant leaf litter quality parameters and physicochemical properties ($P < 0.05$) were plotted in the respective NMDS ordination plots. Colors in the heat map represent the relative abundances in each sampling time ranging from: less than 1 % = blue; 1 % - 2 % = yellow; 3 % - 4 % = pink; 5 % or more = red. A significant effect of time on bacterial and fungal OTU abundances based on Friedman test ($P < 0.05$) is indicated by an asterisk.

Fig. 2 The relative abundances of eubacterial phyla over time generated using Treemap 3.7.0 (Macrofocus, Switzerland). Pebble treemap in circular layout with piecharts, hierarchically sorted by phylum and OTU. Colors indicate the time points. Size of each pie corresponds to a bacterial OTU relative abundance in the total community at all time points combined. Dominant bacterial OTUs which account for at least 1 % of the relative abundance at one or more sampling times are shown in color. The only OTUs labeled are those large enough and with at least genus known. TP = time point: TP0 = 0 day, TP1 = 89 days, TP2 = 180 days, TP3 = 284 days, TP4 = 362 days, TP5 = 473 days.

Fig. 3 The relative abundances of fungal phyla over time generated using Treemap 3.7.0 (Macrofocus, Switzerland). Pebble treemap in circular layout with piecharts, hierarchically sorted by phylum (the third grey circle contains other Fungi) and OTU. Colors indicate the time points. Size of each pie corresponds to a fungal OTU relative abundance in the total community at all time points combined. Dominant fungal OTUs which account for at least 1 % of the relative abundance at one or more sampling times are shown in color. The only OTUs labeled are those large enough and with at least genus known. TP = time point: TP0 = 0 day, TP1 = 89 days, TP2 = 180 days, TP3 = 284 days, TP4 = 362 days, TP5 = 473 days.

Fig. 4 Correlations between bacterial and fungal richness (a) and between enzyme activities and microbial richness (b - f) in leaf litter.

Fig. 5 Co-occurrence networks of bacterial and fungi in leaf litter at early (0 – 180 days) (a) and later (284 – 473 days) (b) stages of decomposition. Bacterial taxonomy: Acido = Acidobacteria, Alpha = Alphaproteobacteria, Armat = Armatimonadetes, Betap = Betaproteobacteria, Deltap = Deltaproteobacteria, Gamma = Gammaproteobacteria, Actin = Actinobacteria, Bacte = Bacteroidetes, Chlor = Chloroflexi, Fibro =

Fibrobacteres, Firmi = Firmicutes, NA = not assigned. Fungal taxonomy: Ascom = Ascomycota, Basid = Basidiomycota and Zygom = Zygomycota.

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Data accessibility

Raw pyrosequencing reads and metadata were submitted to the European Nucleotide Archive under study accession numbers PRJEB9175 and PRJEB9300. Bacterial and fungal biom OTU tables, OTU representative sequences, the bioinformatic scripts and data on leaf litter physicochemical properties are available at Dryad: doi:10.5061/dryad.q1q10.

Contributions

D. Krüger, W.P., F.B., M.H., T.W., D. Kapturska and M.P. conceived and designed the experiments. W.P, M.P. and D. Kapturska performed the experiments. T.W., G.L. and W.P. analyzed the data. D.Krüger, T.W., G.L., F.B., M.H., and W.P. contributed reagents/materials/analysis tools. W.P., M.S., T.W., G.L., F.B., M.P., D. Krüger and M.H. wrote the paper. D. Krüger and M.H. were PI of one project phase, D. Krüger and F.B. of another.

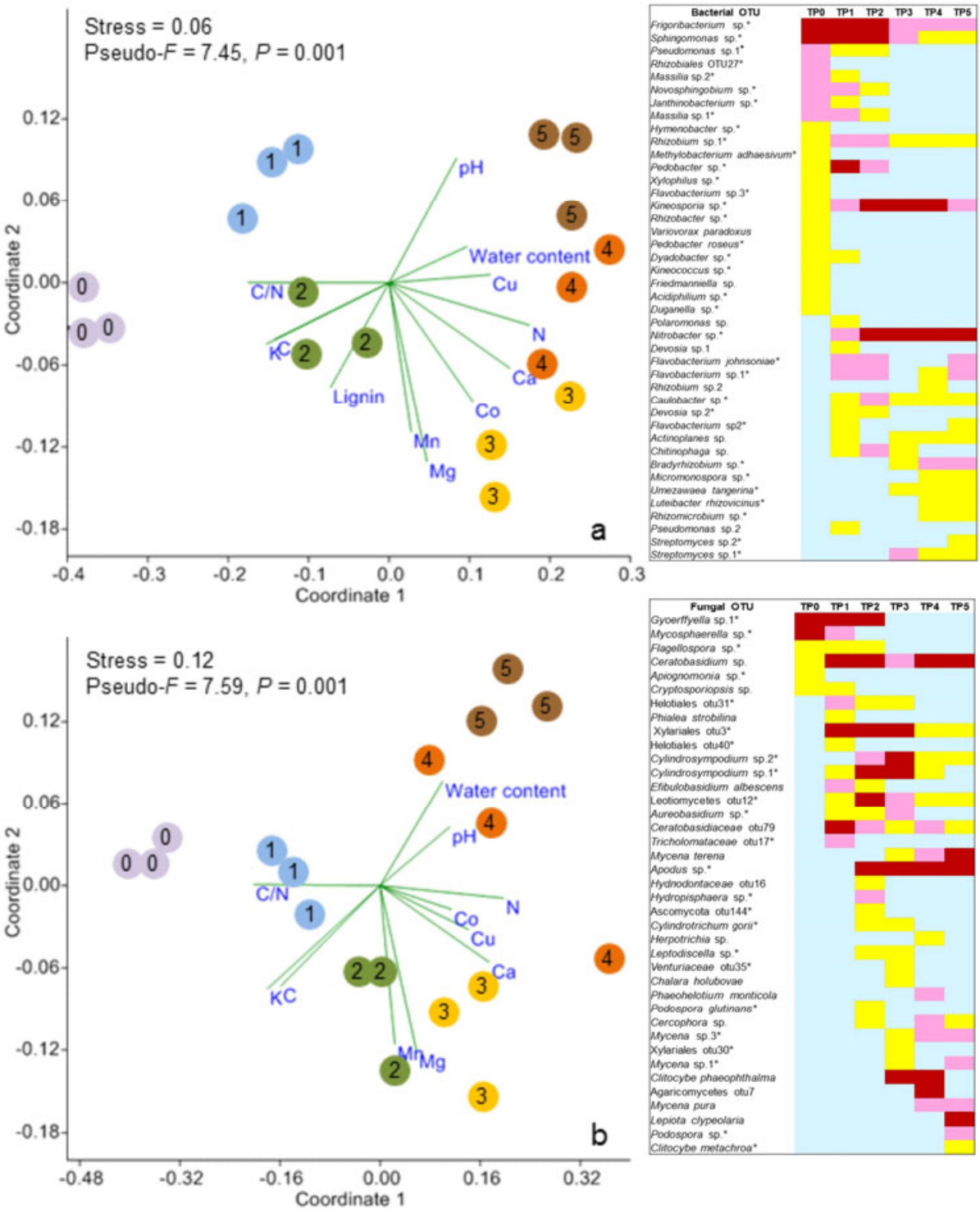
Conflict of Interest: The authors declare no conflict of interest.

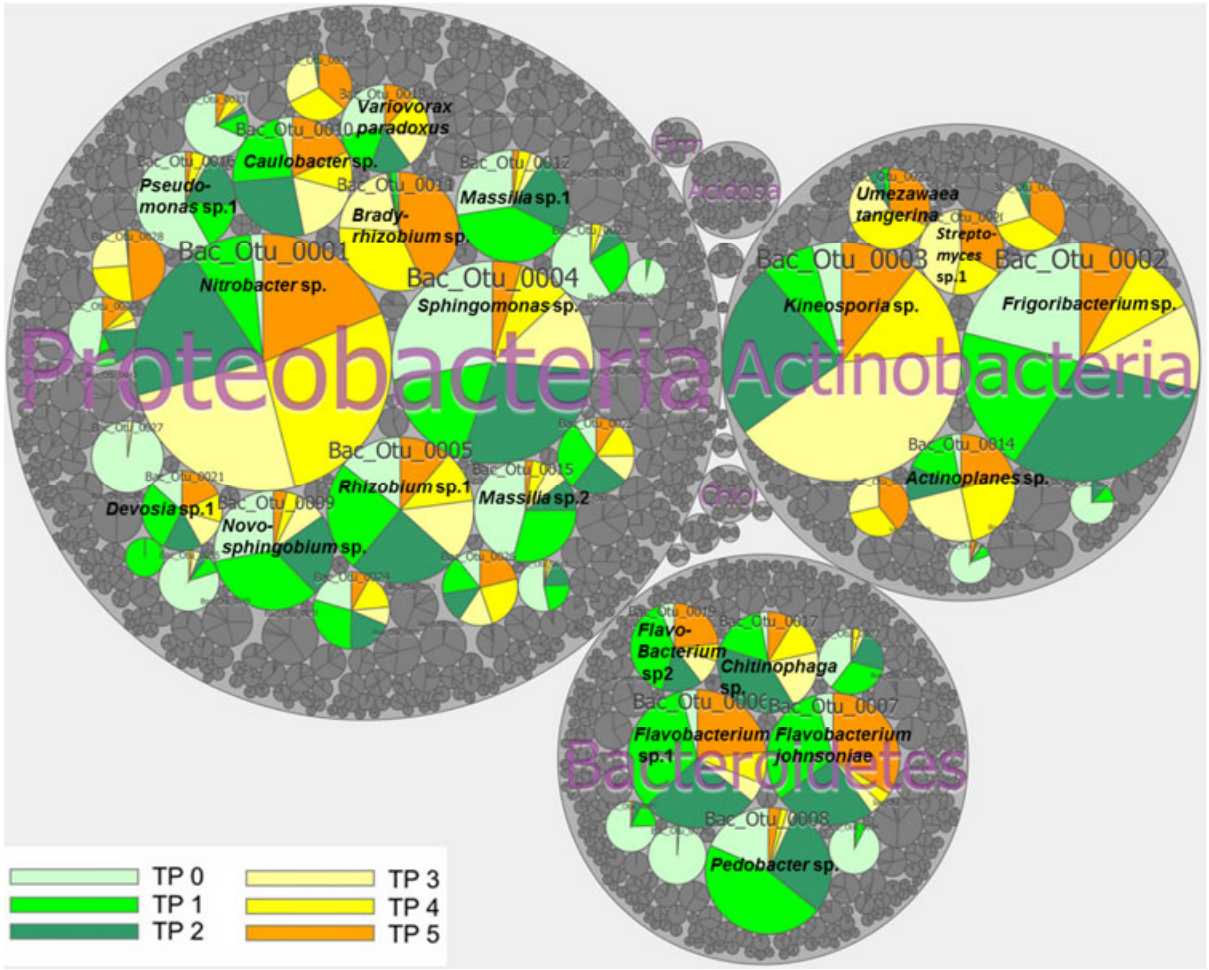
Supporting Information

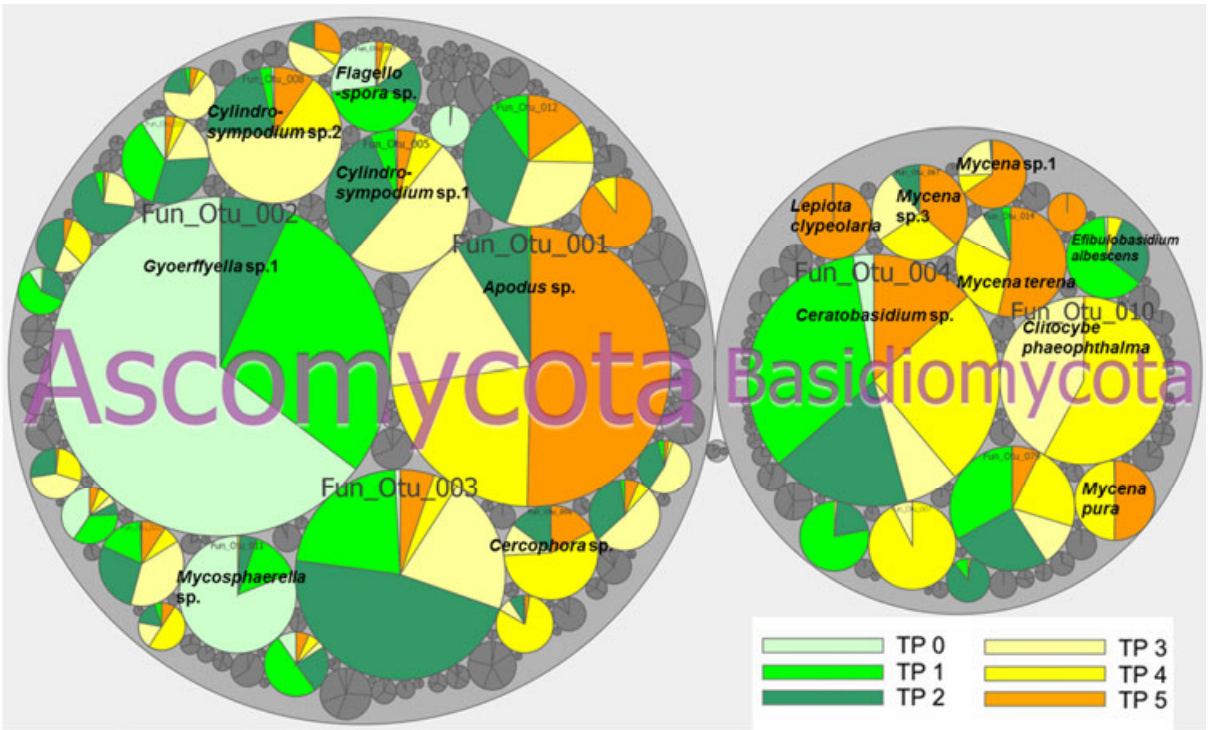
Additional supporting information may be found in the online version of this article

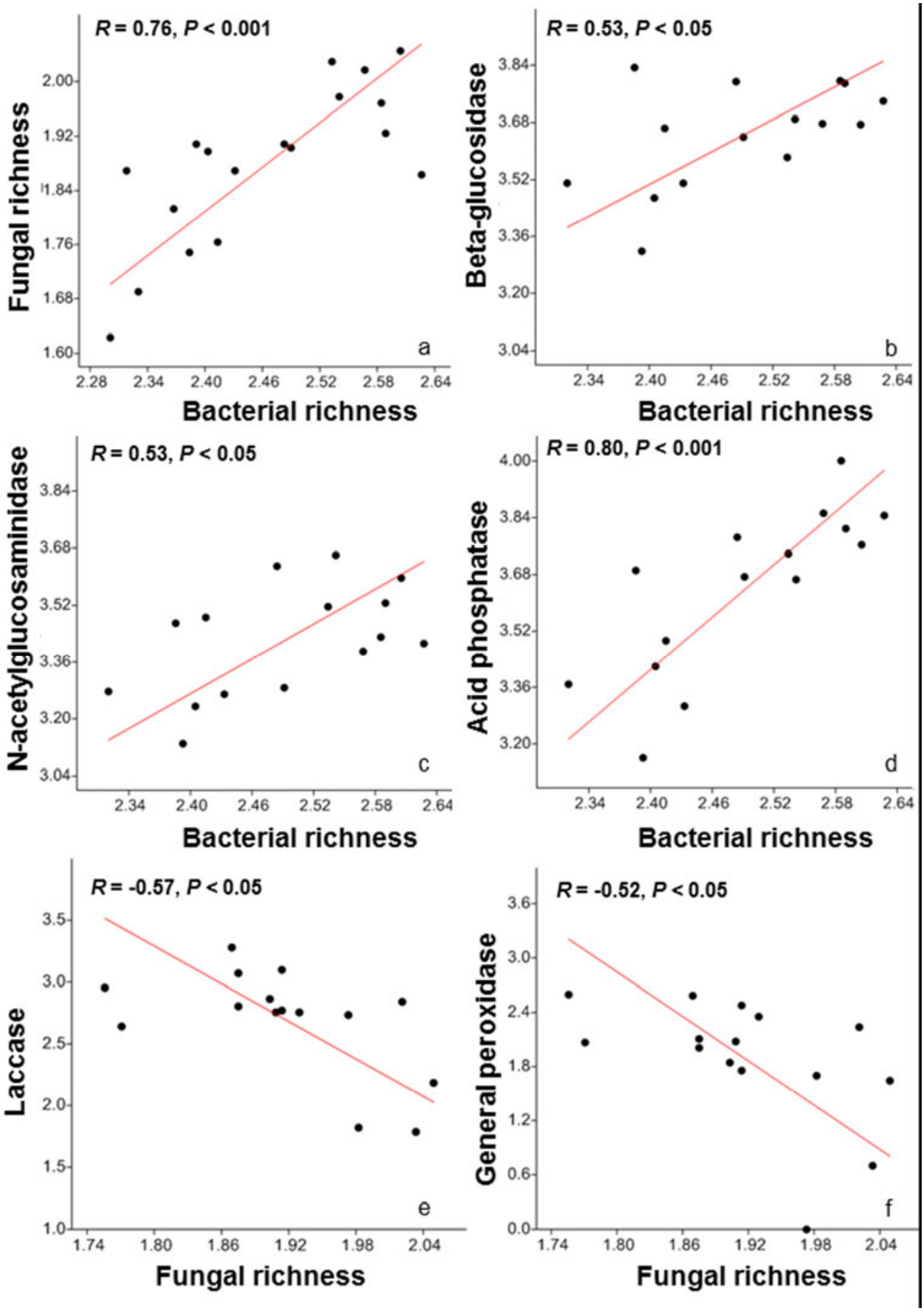
- **PCR conditions**
- **Decomposition of European beech leaf litter**
- **Bacterial community dynamics**
- **Fungal community dynamics**
- **Factors significantly corresponding with microbial community successions**
- **Fig. S1** Individual rarefaction analysis for each sample
- **Fig. S2** Heat-map of relative abundances of dominant bacterial and fungal OTUs (mean and standard error, n = 3).

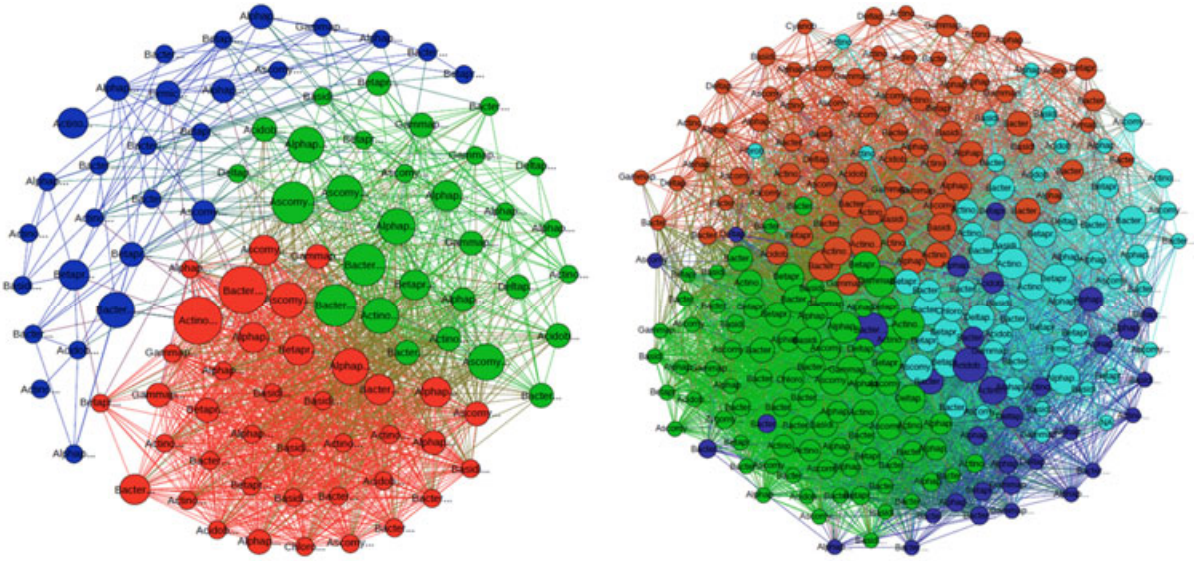
- **Fig. S3** Observed bacterial and fungal OTU richness and Shannon diversity in leaf litter across different sampling dates.
- **Fig. S4** Spearman's rank correlations between specific bacterial and fungal OTUs (account for at least 1 % of the relative abundance at one or more sampling times) and leaf litter quality parameters and physicochemical properties.
- **Fig. S5** Mean manganese peroxidase activities (mU/g litter dry mass) in beech leaf litter at different sampling times
- **Fig. S6** Mean amount of total lignin remaining during decomposition
- **Table S1** Initial chemical composition of dried beech leaf litter
- **Table S2** Taxonomically resolved bacterial community dynamics (with relative abundances) at different sampling dates
- **Table S3** Dissimilarities of bacterial communities in beech leaf litter at different sampling dates as determined by Bray–Curtis distance measures
- **Table S4** Taxonomically resolved Fungal community dynamics (with relative abundances) at different sampling dates
- **Table S5** Dissimilarities of fungal communities in beech leaf litter at different sampling dates as determined by Bray–Curtis distance measures
- **Table S6** Detailed information on the structure of bacterial and fungal networks
- **Table S7** Bacterial and fungal OTUs co-occurring with *Gyoerffyella* spp.
- **Table S8** Bacterial and fungal OTUs co-occurring with *Clitocybe* spp.
- **Table S9** Bacterial and fungal OTUs co-occurring with *Mycena* spp.











a Early stage

b Later stage

Network	Module	Colour	Contribution	Total OTU number	Bacterial OTUs	Fungal OTUs	Bacterial : Fungal ratio	Weighted degree	Number of triangles
Early stage	A	Green	31%	30	25	5	5.0	28.47	304.33
Early stage	B	Red	40%	39	30	9	3.3	44.23	801.51
Early stage	C	Blue	29%	28	25	3	8.3	7.75	11.04
Early stage	**A, B, C**	**-**	**100%**	**97**	**80**	**17**	**4.7**	**28.82**	**419.57**
Later stage	D	Green	33%	84	65	19	3.4	72.79	1431.62
Later stage	E	Red	30%	75	60	15	4.0	42.33	469.37
Later stage	F	Purple	13%	34	32	2	16.0	50.74	697.06
Later stage	G	Sky blue	24%	61	49	12	4.1	56.33	800.72
Later stage	**D, E, F, G**	**-**	**100%**	**254**	**206**	**48**	**4.3**	**56.89**	**897.65**