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Who is who in litter decomposition? Metaproteomics reveals major microbial players and their biogeochemical functions

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Leaf-litter decomposition is a central process in carbon cycling; however, our knowledge about the microbial regulation of this process is still scarce. Metaproteomics allows us to link the abundance and activity of enzymes during nutrient cycling to their phylogenetic origin based on proteins, the ‘active building blocks’ in the system. Moreover, we employed metaproteomics to investigate the influence of environmental factors and nutrients on the decomposer structure and function during beech litter decomposition. Litter was collected at forest sites in Austria with different litter nutrient content. Proteins were analyzed by 1-D-SDS-PAGE followed by liquid-chromatography and tandem mass-spectrometry. Mass spectra were assigned to phylogenetic and functional groups by a newly developed bioinformatics workflow, assignments being validated by complementary approaches. We provide evidence that the litter nutrient content and the stoichiometry of C:N:P affect the decomposer community structure and activity. Fungi were found to be the main producers of extracellular hydrolytic enzymes, with no bacterial hydrolases being detected by our metaproteomics approach. Detailed investigation of microbial succession suggests that it is influenced by litter nutrient content. Microbial activity was stimulated at higher litter nutrient contents via a higher abundance and activity of extracellular enzymes.

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Introduction

Leaf-litter decomposition is the physical and chemical breakdown of dead plant material, a key ecosystem process that provides energy for microbial growth, releases nutrients for plant growth, influences ecosystem carbon (C) storage, and thus may have a long-term effect on the climate (Chapin *et al.*, 2002). Investigations of litter decomposition at different geographic locations have shed light on the factors that influence decomposition rates, for

example, that litter decomposition occurs above a certain threshold in mean annual temperature and depends on litter quality (Zhang *et al.*, 2008; Prescott, 2010). In spite of the vast amount of litter decomposition studies, little is known about specific functions contributed by the different microbial groups involved in litter decomposition, mainly due to the lack of suitable methods available for in-depth studies of ecosystem functioning.

Leaf litter consists mainly of cellulose, but also lignin, hemicellulose, pectin and proteins (Yadav and Malanson, 2007), requiring numerous enzymes for degradation. The presence and activity of extracellular enzymes link environmental nutrient availability to microbial nutrient demand (Sinsabaugh *et al.*, 2008), which in turn is determined by the elemental stoichiometry (that is, the C:N:P ratio) of the decomposer's biomass (Sterner and Elser,

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2002). Within the microbial biomass, phylogenetic groups such as fungi and bacteria exhibit different nutrient demands and thereby influence decomposition and the carbon use efficiency within the ecosystem (Sinsabaugh *et al.*, 2009; Keiblinger *et al.*, 2010). Studies on extracellular enzyme activities may reflect the constraints on microbial biomass stoichiometry and enzyme relationships to litter decomposition, that is, for hydrolytic enzymes ratios of C, N and P acquisition activities have been assessed and they converged on 1:1:1 (Sinsabaugh *et al.*, 2008). However, so far no attempt has been made to link enzyme activities to the respective protein abundances, their microbial origin, or the biogeochemistry of leaf litter.

In the last decade, metaproteomics has greatly advanced our understanding of microorganisms and their geochemical environment (reviewed in Newman and Banfield, 2002; Schneider and Riedel, 2010; Wilmes and Bond, 2009). In contrast to DNA and RNA, most proteins have an intrinsic metabolic function and can thus be used to relate specific microbial activities to defined organisms in multi-species communities. Therefore, the identification of the microbial proteins of a given habitat together with the analysis of their phylogenetic origin and their temporal distribution is expected to provide fundamentally new insights into the role of microbial diversity in biogeochemical processes. Recent large-scale characterisations of the entire protein complement of diverse microbial communities have proven useful to describe microbial functions in soil (Schulze *et al.*, 2005; Wang *et al.*, 2011), lake and ground water (Schulze *et al.*, 2005; Benndorf *et al.*, 2007, 2009), and leaf phyllosphere (Delmotte *et al.*, 2009). We have recently employed a proteomics approach to investigate a model system, consisting of the litter degrading fungus *Aspergillus nidulans* and the bacterium *Pectobacterium carotovorum* grown on sterilized leaf litter (Schneider *et al.*, 2010). In the current study, we expand our proteomics analyses to environmental litter samples.

The major goals of the present study were: (I) to link structure and function of microbial communities

involved in leaf-litter decomposition and (II) to investigate the impact of leaf-litter nutrient content (stoichiometry) and season on the abundance and activity of the leaf-litter decomposers. We analyzed the metaproteome from beech (*Fagus sylvatica* L.) litter collected in February and May 2009 at four Austrian sampling-sites that differed in their nutrient content. The newly developed bioinformatics pipeline 'PROteomics result Pruning & Homology group ANotation Engine' (PROPHANE, Schneider *et al.*, 2011) was employed to assign proteins to their phylogenetic and functional origin. Phospholipid fatty acid (PLFA) analyses, enzymatic measurements and qualitative assessment of fungal communities were used for data validation.

Materials and methods

Litter sampling sites

Beech leaf litter was collected at four forest sites in Austria: (I) Achenkirch (AK), Tyrol; (II) Klausenleopoldsdorf (KL), Lower Austria; (III) Ort (OR), Gmunden, Upper Austria; and (IV) Schottenwald (SW), Vienna that differed in their nutrient content. AK is located in the Achenal of the North Tyrolean limestone Alps. It is characterised by its location on calcareous bedrock that results in a high soil pH (Kitzler *et al.*, 2006b). OR is situated at an elevation of about 700 m above sea level and northeast orientation. The forest consists mostly of beech, but also of various conifers, and has an average slope angle of 34%. The soil is a brown loam on carbonatic rock. SW is situated in direct vicinity of Vienna (Kitzler *et al.*, 2006a) similar to KL that is located about 40 km south-west of Vienna (Kitzler *et al.*, 2006a). The predominant vegetation, stand age, exposition and elevation, as well as soil type and texture of the different sampling sites are described in Table 1.

Litter sampling

At the respective sampling sites, 500 g of beech leaf litter was sampled in February and May 2009 (exact

Table 1 Site and soil characteristics of the beech litter-sampling sites

	Achenkirch (Wanek <i>et al.</i> , 2010)	Klausenleopoldsdorf (Wanek <i>et al.</i> , 2010)	Ort	Schottenwald (Wanek <i>et al.</i> , 2010)
Location	47°35'N 11°39'E	48°07'N 16°03'E	47°51'N 13°42'E	48°14'N 16°15'E
Vegetation	Spruce-fir, beech	Beech	Mainly beech, conifers	Beech
Stand age (years)	127	62	90	142
Exposition	N	NNE	NE	SE
Elevation (m a.s.l.)	895	510	700	370
Soil type	Rendzic leptosols/ chromic cambisol	Dystric cambisol over sandstone	Cambisol	Dystric cambisol over sandstone
Soil texture	Loam	Loam-loamy clay	Loamy silt	Silty loam
Soil pH 0–7 cm	6.4	4.6	3.7	4.4

Abbreviation: a.s.l., above sea level.

Table 2 Different sampling sites and their mean air-temperature (T) and precipitation (Prec)

Site	Sampling _{Feb}	Sampling _{May}	T _{Feb} (°C)	T _{May} (°C)	Prec _{Feb} (mm)	Prec _{May} (mm)
Achenkirch	4th	18th	-1.66	5.88	55	65
Klausenleopoldsdorf	5th	27th	-0.11	9.64	37	46
Ort	27th	19th	0.88	11.0	51	57
Schottenwald	5th	27th	1.32	11.39	44	55

Abbreviation: Feb, February.

Samples were collected at the given dates in 2009. Climatic data were collected over a 3-month time period before litter sampling. Mean air temperature and precipitation were calculated by averaging the climatic data of this period.

sampling dates are listed in Table 2). In February, all sites were covered by snow, whereas in May all sites were free of snow. Three biological replicates were collected from three plots of 10 × 10 m within an area of 50 × 50 m. Only the L-horizon was sampled carefully, but excluding mineral soil material and O-horizon. Litter samples consisted predominantly of last autumn's foliage, which means that leaves were almost intact as beech litter at the investigated sites is completely decomposed during the course of 1 year. The litter was transported to the laboratory at ambient temperature, sorted, that is, leaves other than beech leaves were removed, cut into <0.5 cm² pieces using a kitchen knife and mixed thoroughly. Per sampling site, 10–50 g aliquots of cut leaf material from the three biological replicates per sampling site were stored at -20 °C until litter nutrient content had been analyzed, and at -80 °C until the metaproteome was analyzed.

Determination of environmental parameters and litter nutrient quality

Air temperature and precipitation at the respective sampling sites were monitored for 3 months before litter collection; mean air-temperature (T) and mean precipitation (Prec) were calculated by averaging these values.

Water content (WC) was determined gravimetrically by drying the litter at 105 °C for 24 h. Dry samples were ground with a mill (Retsch MM2000, Hanau, Germany) to a fine homogeneous powder. The total carbon (C) and nitrogen (N) contents of the litter were analyzed with an integrated oxidation and detection device (Leco CN2000, LECO corp. St Joseph, MI, USA). The ground samples were wet acidically oxidized (H₂SO₄ + HNO₃) in a microwave oven (CEM MARS Express), and elements (phosphorous (P), potassium (K), magnesium (Mg), manganese (Mn), calcium (Ca), iron (Fe)) were determined by inductively coupled plasma atomic emission spectrometry as described by Henschler (1988). Extractable nitrogen (N) in form of ammonium (NH₄-N) and nitrate (NO₃-N) and phosphorous (P) in form of phosphate (PO₄-P) were determined from freshly shred litter samples, extracted with 1 M KCl at a ratio of 1:20, by shaking on a reciprocal shaker for 60 min at 200 oscillations. Extracts were stored at -20 °C until analysis. Ammonium was

measured by the Berthelot reaction according to Schinner *et al.* (1996). Nitrate was determined with the vanadium chloride method as described by Hood-Nowotny *et al.* (2010). Phosphate was quantified photometrically based on the phosphomolybdate blue reaction (Schinner *et al.*, 1996) in a microplate reader (µQuant mQx200, Bio-Tek Instruments, Winooski, VT, USA).

PLFA analysis

Lipids were extracted from 1 g sub-samples using a modified Bligh and Dyer technique (Hackl *et al.*, 2005). Extracted PLFAs were analyzed with a HP 6890 Series GC-System and a 7683 series injector and auto sampler on a HP-5 capillary column and detected with a flame ionization detector. For identification of the fatty acid methyl esters, an external standard (bacterial acid methyl ester mix from SUPELCO) was used. For quantification of the peaks, methyl non-adeconoate fatty acid (19:0) was added. PLFA nomenclature is based upon Frostegard *et al.* (1993). The ratio fungal/bacterial PLFA was calculated by dividing the amount of 18:2ω6 through the amount of total bacterial PLFAs (Frostegard and Baath, 1996).

Cellulase and xylanase activity assay

Frozen cut leaf litter was ground in liquid nitrogen; 600 mg of the ground material were extracted with 4 ml extraction buffer (0.1 M sodium phosphate pH 6.0, 0.1% Triton X-100, 0.1% polyvinylpyrrolidone). After shaking at 4 °C for 1 h, extracts were centrifuged for 5 min at 14 000 g. The resulting supernatants were filter-sterilized by cellulose acetate filters and directly subjected to enzyme activity assays.

Cellulase and xylanase activity were determined as described earlier (Riedel *et al.*, 1998; König *et al.*, 2002). 250 µl filter-sterilized supernatant were mixed with 250 µl 1% (w/v) carboxymethyl cellulose (Sigma, Buchs, Switzerland) or 1.5% (w/v) xylan (from oat spelts, Boehringer Ingelheim, Lot Nr. 06995) in 0.1 M sodium phosphate buffer, pH 6.0, and incubated for 3.5 h (cellulase) or 2 h (xylanase) at 40 °C. The enzymatic release of reducing groups was determined with the dinitrosalicylic acid reagent (Wood and Bhat, 1988). One unit of enzyme

activity was defined as the amount of enzyme needed to release 1 μmol of glucose-equivalent reducing groups per minute.

Metaproteome analysis

From the cut and stored (-80°C) litter material, 5 g were ground in liquid nitrogen and mixed with extraction buffer containing 1% SDS, 50 mM Tris/KOH, pH 7.0 in a 1:5 ratio (w/v). Samples were sonicated for 2 min, followed by boiling for 20 min and shaking at 4°C for 1 h. To remove debris, extracts were centrifuged at 3000 g at 4°C followed by 5 min centrifugation at 14 000 g and 4°C . Supernatants were concentrated about 5-fold by vacuum-centrifugation (Eppendorf Vacuum Concentrator plus) at 30°C . Concentrated supernatants of 25 μl were subjected to 1D SDS-PAGE (Laemmli, 1970) in a 12% polyacrylamide gel to clean samples from interfering substances (for example, humic acids) and to reduce sample complexity. Protein lanes were cut into seven slices, and gel slices were subjected to immediate in-gel tryptic digestion by employing sequencing grade-modified trypsin (Promega, Madison, WI, USA, reference V5111) (Shevchenko *et al.*, 1996). The resulting peptide mixtures were analyzed on a hybrid LTQ-Orbitrap mass spectrometer (ThermoFischer Scientific, Waltham, MA, USA) as described earlier (Schneider *et al.*, 2010).

Database searches

Using MASCOT (version no.2.2.04), MS and MS/MS data were searched against a database containing all proteins from UniRef100 (9808438 protein entries, downloaded from <http://www.ebi.ac.uk/uniref/> at the 26 January 2010) and protein sequence information from a farm silage soil metagenome (Tringe *et al.*, 2005) (184 374 entries, downloaded from <http://img.jgi.doe.gov> at 15 October 2009), as well as common contaminants such as keratin and trypsin (in total, 9 993 117 protein entries). The following search parameters were applied: (i) trypsin was chosen as protein-digesting enzyme and up to two missed cleavages were tolerated, (ii) carbamidomethylation of cysteine was chosen as fixed modification, and (iii) oxidation of methionine was chosen as variable modification. Searches were performed with a parent-ion mass tolerance of ± 5 ppm and a fragment-ion mass tolerance of ± 0.8 Da. A second database search was performed with the X!Tandem (version 2007.01.01.1, <http://www.thegpm.org/TANDEM/>) search engine (Craig and Beavis, 2004) with similar settings.

Data processing

Scaffold (version Scaffold 3.0, Proteome Software, Portland, OR, USA) was used to validate and quantify MS/MS-based peptide and protein identifications from both search engines. Protein identifications were accepted if they were established at $>95\%$ peptide probability and 90% protein

probability with at least one peptide uniquely assigned to a respective protein in one of our samples. Proteins that were identified with the same set of peptides were grouped to protein clusters to satisfy the principles of parsimony. False-discovery rate was determined according to Elias and Gygi (2007) by searching against a composite version of our protein reference database, created by concatenating the target protein sequences with reversed sequences (19 986 234 entries). Starting from the Scaffold output files, all obtained protein hits were assigned to phylogenetic and functional groups and assignments were validated by the PROPHANE workflow (<http://prophane.svn.sourceforge.net/viewvc/prophane/trunk/>). Higher protein abundance is represented by a higher number of MS/MS spectra acquired from peptides of the respective protein. Thus, protein abundances were calculated based on the normalized spectral abundance factor (NSAF; Florens *et al.*, 2006; Zybailov *et al.*, 2006). This number allows relative comparison of protein abundances over different samples (Bantscheff *et al.*, 2007).

Statistical analyses

The whole dataset was tested for normal distribution using the Shapiro–Wilk test and homogeneity of variances using Levene's test. When the assumption of normal distribution was violated, the data were transformed as indicated. Differences between sampling time and site were either checked by *t*-test or ANOVA, Tukey HSD. We performed single linear regression (SLR) analysis to evaluate which factors predict changes in microbial community structure and function. To identify key-parameters affecting structure and function of the decomposing community, we performed a PCA. All statistical analyses were conducted using PASW (version no. 18.0.0) statistical software packages.

Results

Litter from the sampling sites varied in nutrient content and environmental parameters

The four sampling sites AK, KL, OR and SW showed differences in their environmental conditions and leaf-litter quality. At all sampling sites, mean air temperature and precipitation increased from Feb to May. SW was the warmest of the four sites, whereas AK was the coldest (Table 2). Precipitation was highest in AK and lowest in KL at both sampling time points (Table 2).

As litter quality parameters, pH, WC and litter nutrient content were determined. WC was similar in litter of all sites in February (Table 3), which might be explained by the snow coverage of the litter. In May, WCs were generally lower, but showed site-specific differences. Litter pH was lowest in OR and highest in SW and KL. With respect to nutrient content, litter showed significant site-specific

Table 3 Beech leaf-litter water content, pH and nutrient content at the four sampling sites in February and May

	Achenkirch		Klausenleopoldsdorf		Ort		Schottenwald	
	Feb	May	Feb	May	Feb	May	Feb	May
	Water content (%)	73.07 ± 0.6 ^{ab}	42.2 ± 3.3 ^c	79.39 ± 2.0 ^a	63.7 ± 15.5 ^{ab}	57.0 ± 4.1 ^{bc}	12.0 ± 0.3 ^d	77.5 ± 1.6 ^a
pH	n.d.	4.9 ± 0.1 ^{cd}	4.7 ± 0.1 ^c	5.3 ± 0.1 ^e	4.5 ± 0.1 ^a	4.6 ± 0.1 ^b	5.2 ± 0.1 ^{de}	5.2 ± 0.1 ^{de}
C (%)	50.16 ± 0.17 ^d	50.49 ± 0.08 ^d	46.82 ± 1.45 ^{bc}	46.12 ± 0.68 ^{bc}	47.20 ± 0.20 ^{bc}	47.70 ± 0.26 ^c	45.47 ± 0.39 ^b	43.44 ± 1.13 ^a
N (%)	1.24 ± 0.03 ^d	1.27 ± 0.02 ^d	1.00 ± 0.02 ^b	1.14 ± 0.02 ^c	0.82 ± 0.02 ^a	1.07 ± 0.01 ^c	1.25 ± 0.04 ^d	1.45 ± 0.08 ^e
C:N ratio	40.31 ± 0.76 ^c	39.87 ± 0.63 ^c	46.76 ± 2.32 ^e	40.64 ± 0.97 ^c	57.75 ± 1.25 ^f	44.42 ± 0.49 ^d	36.43 ± 1.19 ^b	29.96 ± 1.30 ^a
P (%)	0.035 ± 0.001 ^a	0.039 ± 0.006 ^a	0.040 ± 0.002 ^a	0.040 ± 0.013 ^a	0.035 ± 0.006 ^a	0.056 ± 0.003 ^b	0.064 ± 0.004 ^b	0.104 ± 0.002 ^c
C:P ratio	1439.8 ± 4.8 ^t	1318.6 ± 197.9 ^d	1160.2 ± 86.7 ^{cd}	1237.4 ± 361.5 ^d	1361.2 ± 200.7 ^t	853.24 ± 42.4 ^{bc}	714.0 ± 43.0 ^{ab}	418.6 ± 17.3 ^a
N:P ratio	35.73 ± 0.55 ^e	33.03 ± 4.48 ^{de}	24.80 ± 0.84 ^{bcd}	30.33 ± 8.32 ^{cde}	23.62 ± 3.86 ^{bc}	19.22 ± 1.16 ^{ab}	19.59 ± 0.55 ^{ab}	13.99 ± 0.82 ^a
PO ₄ (ng g ⁻¹ DW)	11.51 ± 6.15 ^a	14.09 ± 1.55 ^b	14.87 ± 4.97 ^a	9.87 ± 4.14 ^a	22.73 ± 5.39 ^a	90.30 ± 6.93 ^b	9.38 ± 2.48 ^a	113.66 ± 6.29 ^c
NH ₄ (μg g ⁻¹ DW)	38.84 ± 10.21 ^b	21.81 ± 3.06 ^b	23.45 ± 3.18 ^a	40.05 ± 8.08 ^b	12.46 ± 0.95 ^a	46.02 ± 4.96 ^b	18.89 ± 5.54 ^a	70.21 ± 5.27 ^c
NO ₃ (μg g ⁻¹ DW)	4.39 ± 1.84 ^{abc}	1.00 ± 0.48 ^{ab}	7.31 ± 1.87 ^{bc}	0.40 ± 0.63 ^a	9.55 ± 4.74 ^c	1.75 ± 0.16 ^{ab}	5.09 ± 2.14 ^{abc}	17.37 ± 3.57 ^d
K (%)	0.102 ± 0.005 ^a	0.087 ± 0.008 ^a	0.185 ± 0.018 ^d	0.151 ± 0.007 ^{bc}	0.170 ± 0.011 ^{cd}	0.140 ± 0.003 ^b	0.163 ± 0.006 ^c	0.260 ± 0.009 ^e
Ca (%)	1.39 ± 0.08 ^{ab}	1.32 ± 0.001 ^a	1.42 ± 0.03 ^{ab}	1.51 ± 0.40 ^{ab}	1.35 ± 0.03 ^{ab}	1.47 ± 0.03 ^{ab}	1.76 ± 0.06 ^b	1.37 ± 0.01 ^{ab}
Mg (%)	0.209 ± 0.013 ^b	0.173 ± 0.002 ^{ab}	0.140 ± 0.008 ^{ab}	0.160 ± 0.069 ^{ab}	0.141 ± 0.003 ^{ab}	0.116 ± 0.002 ^a	0.182 ± 0.012 ^{ab}	0.163 ± 0.002 ^{ab}
Fe (ppm)	166.6 ± 26.8 ^a	343.8 ± 22.9 ^a	659.1 ± 486.7 ^a	818.5 ± 195.3 ^a	385.9 ± 162.1 ^a	573.6 ± 17.3 ^a	728.7 ± 238.7 ^a	1703.9 ± 391.9 ^b
Mn (ppm)	88.8 ± 2.5 ^a	109.6 ± 2.0 ^a	832.1 ± 89.6 ^b	1025.2 ± 417.2 ^b	1040.5 ± 82.8 ^b	1172.3 ± 16.5 ^b	1493.3 ± 73.9 ^c	2140.7 ± 63.7 ^d
Zn (ppm)	46.08 ± 3.42 ^b	160.00 ± 6.06 ^t	39.44 ± 4.23 ^b	40.83 ± 8.25 ^b	29.17 ± 1.44 ^a	41.17 ± 2.38 ^b	64.33 ± 2.52 ^c	67.19 ± 3.76 ^c

Abbreviations: Feb, February; n.d., not determined because of lack of material.

The different letters in a given line denote significant differences ($P < 0.05$) between values as determined by ANOVA followed by Student–Newman–Keuls multiple range test.

differences in C, N and P (Table 3). Carbon content was highest in AK and lowest in SW, N content was highest in SW and lowest in OR, and P content was highest in SW and lowest in AK. Huge differences were also found for the micronutrients Fe and Mn with highest values in SW and lowest in AK. Litter stoichiometry (C:nutrient and N:P ratios) differed strongly between sites. Generally, SW was the nutrient-richest litter (lowest C:N, C:P and N:P ratios), whereas AK was the nutrient-poorest litter (highest C:P, highest N:P).

Semi-quantitative metaproteome analysis

When litter samples prepared as described in the materials and methods section were analyzed by SDS-PAGE, no distinct bands were detected on the 1D-gels, but protein separation resulted in a smear covering the whole lane (Supplementary Figure 1). This might be a result of the high sample complexity and protein degradation in the litter material. Furthermore, humic substances, which interact with the Coomassie stain might be responsible for the intensive blue staining. Lanes were cut into seven equally sized gel slices and analyzed further by LC-MS/MS. Spectra were assigned to a total of 8895 proteins; protein hits that were identified based on the same set of peptides were subsequently merged to 1724 unique protein clusters. Decoy database searches resulted in a protein false-discovery rate below 1.5%. Protein assignments were validated using PROPHANE by testing cluster homology using multiple sequence alignment analyses. This resulted in 204 inconsistent clusters (amino-acid identity < 50%), which were therefore excluded from further analyses.

Subsequently, PROPHANE assigned the remaining protein clusters to phylogenetic and functional groups and quantified cluster abundance by spectral counting based on the NSAF that is an indicator of relative differences in protein cluster abundances. All functional or phylogenetic group abundances presented in the figures are based on NSAFs. Proteins belonging to the respective clusters and protein identification parameters are listed in Supplementary Table 1. Supplementary Table 2 shows NSAF-based cluster abundance and a list of representative proteins assigned to the different phyla and functional categories. Supplementary Table 3 shows the sequences and charge states of all peptides that were assigned to proteins or protein clusters. The complete MASCOT results dataset including MS/MS spectra information is provided on the PRIDE database (Vizcaino *et al.*, 2009) at <http://www.ebi.ac.uk/pride/>; accession number is 17171.

Litter microbial community differs between sampling sites and seasons

Independent of the sampling site, the majority of the spectra were assigned to three major phylogenetic

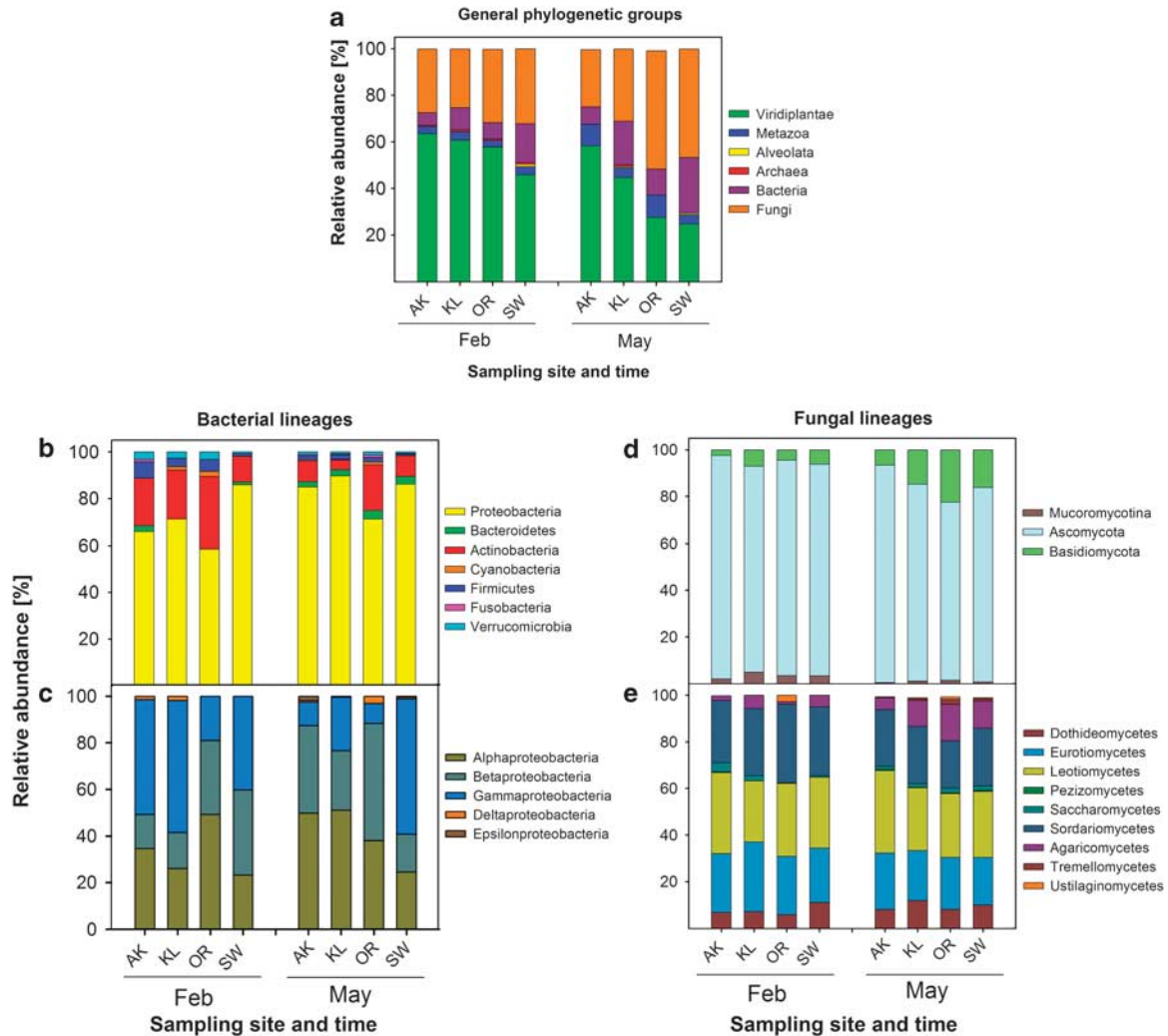


Figure 1 Assignment of spectra to taxonomic groups of organisms at different sampling times and sites. Relative abundances were calculated from the sum of NSAFs found for each group at the respective sampling sites and time. (a) General taxonomy, (b) Bacterial phyla, (c) Proteobacteria, (d) Fungal phyla, (e) Fungal classes. Groups are only presented if their relative abundance is >0.5% in the respective sample. Sampling sites are presented according to increasing mean air-temperature (from left to right).

groups: *Viridiplantae* (non-degraded leaf proteins, 45–64% of all assigned spectra), fungi (24–51%), and bacteria (5–24%). Moreover, spectra were assigned to *Metazoa* (3–9%), *Alveolata* (0–1%) and *Archaea* (0–0.8%) depending on sampling-site and -time (Figure 1a). We used the abundance of plant-derived spectra as a marker of the extend of litter decomposition. The obtained phylogenetic composition of the leaf-litter community is different from the proportions of database (DB) protein entries of the respective groups; in the DB, bacteria dominate the number of entries (62.2%) followed by *Metazoa* (16.7%), fungi (7.8%) and plants (6.3%).

When comparing samples collected in February and May, a significant decrease (*t*-test, $t = 2.64$; $P = 0.019$) of the spectra from plants was observed at all sampling sites. Vice versa, the number of fungal spectra increased significantly (*t*-test, $t = -2.29$; $P = 0.050$). The number bacterial spectra also increased, but this was not significant.

A comparison of samples from the four sampling sites revealed site-specific differences of the community composition between February and May. The smallest decrease in the proportion of plant spectra was observed in AK (64 to 58%) followed by KL (60 to 45%), SW (45 to 25%) and OR (57 to 27%; Figure 1a). Although the proportion of fungal spectra did not increase in AK, the proportion of fungi rose in KL from 25 to 31%, in SW from 32 to 46%, and in OR from 31 to 51%. A similar trend was observed for bacterial spectra (almost no changes in AK, 10 to 19% in KL, 7 to 11% in OR and 17 to 24% in SW). The ratio between the number of fungal and bacterial spectra (F/B-ratio) did not differ between February and May, but site-specific differences were observed. In February, the highest F/B-ratio (5.0) was found in AK and OR (4.5) followed by KL (2.6) and SW (1.9). In May the highest ratio was observed in OR (4.6) followed by AK (3.2), SW (2.4) and KL (1.6).

Fungi and bacteria are thought to be the main degraders of leaf litter and therefore their phylogeny was analyzed in more detail. Most of the bacterial spectra could be assigned to the *Proteobacteria* (between 59% and 90%) and the *Actinobacteria* (between 4% and 31%; Figure 1b). In February, γ -*Proteobacteria* dominated in AK (50%), in KL (57%) and in SW (37%), whereas in OR, the majority of proteobacterial spectra assignments belonged to the α -*Proteobacteria* (49%, Figure 1c). A significant (*t*-test, $t = -3.18$; $P = 0.0066$) absolute increase in α -proteobacterial spectra was observed from February to May in AK and KL, whereas in SW and OR, γ - and β -proteobacterial spectra dominated, respectively.

Detailed analysis of the fungal phylogeny revealed only minor differences in their community composition. A clear dominance (>80%) of ascomycotal spectra was observed at all sites and times. The proportion of basidiomycotal spectra increased slightly from February to May at all sites (Figure 1d) (*t*-test, $t = -2.99$; $P < 0.009$). Moreover, a significant decrease in the number of *Mucoromycotina*-spectra was observed from February to May (*t*-test, $t = 3.37$; $P = 0.007$) at all sites. No significant changes were observed for fungal classes when different sites and times were compared (Figure 1e); most spectra fit data for the classes *Leotiomycetes*, *Sordariomycetes* and *Eurotiomycetes*.

To validate data obtained from the metaproteomics analysis, PLFA analysis was performed and the phylogenetic origin of fungi present in the litter samples was investigated by cultivation and subsequent identification (Supplementary Information). To enable comparison of both datasets, spectra had to be assigned to the major taxonomic groups that can be distinguished by PLFA (Figure 2). A significant correlation of proteome and PLFA data for fungi (SLR, $r^2 = 0.380$; $P = 0.014$), total bacteria (SLR, $r^2 = 0.394$; $P = 0.009$) and Gram-negative bacteria (SLR, $r^2 = 0.352$; $P = 0.015$) was observed; Gram-positive bacteria and *Actinobacteria* showed similar but insignificant trends (Figure 2).

The assignment of spectra to different fungal groups was partly confirmed by cultivation experiments. The finding that the *Ascomycota* phylum dominates the cultivable fungi followed by *Basidiomycota* and *Mucoromycotina* is in good accordance with the metaproteome data (for details see Supplementary Information, Supplementary Table 4). Neither the metaproteome analysis nor the cultivation approach detected any members of the *Glomeromycota*. The absence of these arbuscular mycorrhizal fungi is in agreement with the assumption that they are only found in soil layers and in close association with plant roots (Domsch *et al.*, 2007).

Fungi are the main producers of litter-degrading enzymes

Our metaproteomics analyses enabled us to link structure and function of the complex microbial community present in the leaf litter. The obtained spectra were classified into COG (prokaryotic proteins) and KOG (eukaryotic proteins) categories based on their respective protein assignments (Supplementary Figure 2). The majority of bacterial protein spectra were assigned to functional categories such as translation, cell wall/membrane/envelope biogenesis, post-translational modifications/protein turnover and conversion, as well as energy production and conversion. Dominant functional groups of fungal proteins were translation, chromatin structure and dynamics, post-translational modifications/protein turnover and conversion and energy production and conversion. The proportion of proteins related to transport functions was higher in fungi than in bacteria (Supplementary Figure 2). When comparing samples from February and May a significant increase of proteins assigned to post-translational modification and protein turnover was observed for both bacteria (*t*-test, $t = -3.79$; $P = 0.002$) and fungi (*t*-test, $t = -2.51$; $P = 0.025$).

As we were mainly interested in litter-decomposing enzymes, the metaproteome was searched for

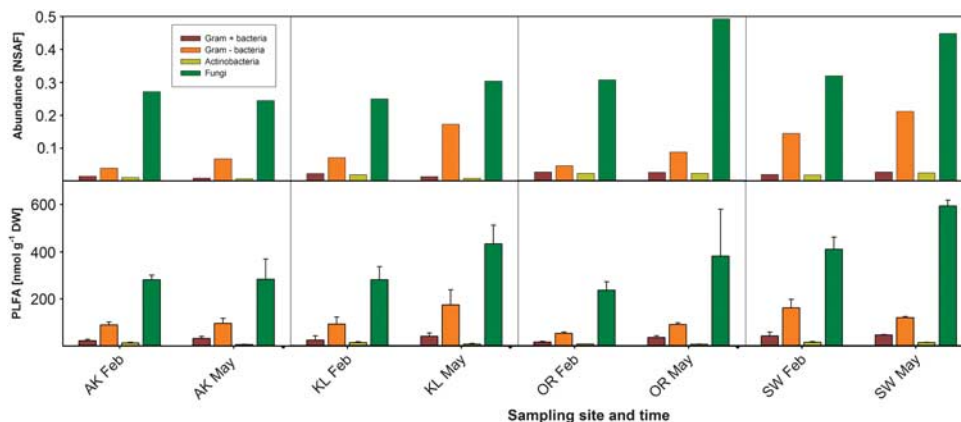


Figure 2 Comparison of community structure based on NSAF and PLFA analyses. The upper part of the figure shows community structure based on NSAF. Data represent the mean of two biological replicates. The lower part of the figure depicts community structure based on PLFA analysis. Data represent the mean \pm SD of three biological replicates.

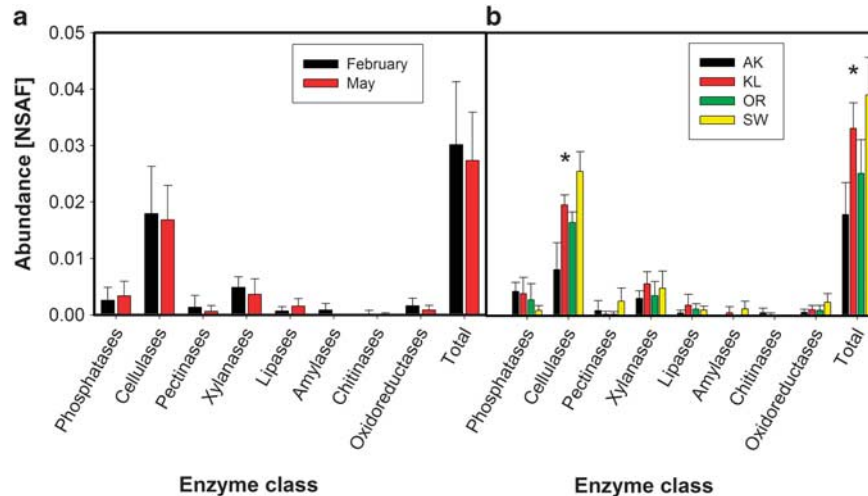


Figure 3 Relative abundance of hydrolytic enzymes at different sampling times and sites. (a) Abundance of different enzymatic classes at different sampling times. Data represent mean \pm SD of eight samples collected at the respective sampling time. No significant differences among sites were detected by *t*-test analysis. (b) Abundance of different enzymatic classes at different sampling sites. Data represent mean \pm SD of four samples collected at the respective sampling site. *Represent significant differences, $P < 0.05$, ANOVA followed by LSD-test.

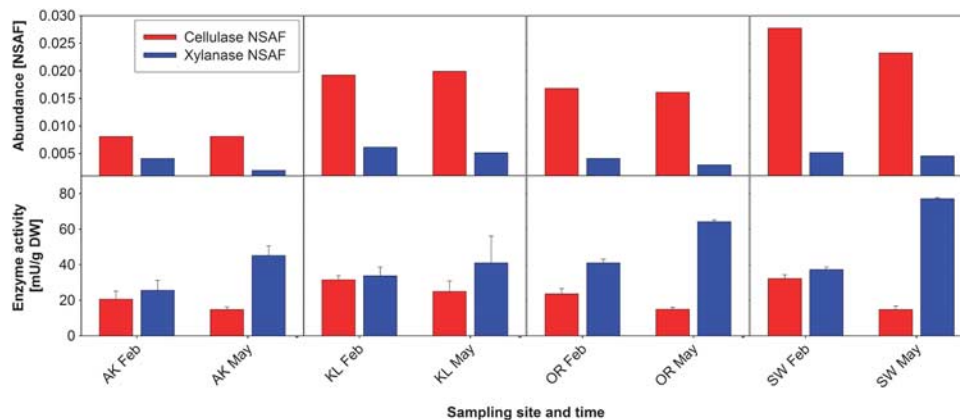


Figure 4 Comparison of cellulase and xylanase abundance and total cellulolytic and xylanolytic activities. The upper part of the figure shows enzyme abundances based on NSAFs. Data represent the mean of two biological replicates. The lower part of the figure shows enzyme activities based on photometric assays. Data represent the mean \pm SD of three biological replicates.

proteins that were assigned as extracellular hydrolytic enzymes. Moreover, total cellulolytic and xylanolytic activities were measured by photometric assays. Strikingly, spectra could only be assigned to enzymes of fungal origin, including phosphatases, cellulases, pectinases, xylanases, lipases, amylases, chitinases and oxidoreductases (Figure 3). The most prominent hydrolases were cellulolytic enzymes, that is, exo- and endo-glucanases as well as β -glucosidases.

The abundance of litter-degrading extracellular enzymes is affected by the sampling site but not by season

No significant changes were observed when the abundance of different fungal litter-degrading enzyme classes was compared between February and

May (Figure 3a). However, significant differences in enzyme abundances based on the NSAF between the sampling sites were observed (ANOVA, $F = 10.32$; $P < 0.01$) (Figure 3b), which was particularly evident for cellulases (ANOVA, $F = 20.24$; $P < 0.01$). Cellulases were most abundant in SW followed by KL, OR and AK. Furthermore, obvious trends in the abundance of phosphatases were detected between the sampling sites; fungal phosphatases were most abundant in AK followed by KL, OR and SW.

We did not observe any change in cellulolytic activity between February and May, which is in good agreement with cellulolytic enzyme abundance based on spectral counting (Figure 4). The comparison of cellulase abundance and total cellulolytic activity showed a significant but weak positive correlation (SLR, $r^2 = 0.285$, $P = 0.033$). In contrast, no correlation was observed when

abundance and activity of xylanase(s) were compared (SLR, $r^2 = 0.009$, $P = 0.279$). Whereas xylanase abundance did not differ between seasons; xylanase activity was higher in May.

The phylogenetic origin of cellulase-producing fungi depends on the sampling time and site

Cellulases represented the most abundant enzyme class involved in litter degradation at the investi-

gated sampling times; therefore, their phylogenetic origin was more precisely investigated (Figure 5). Although fungal community changed only slightly, dramatic changes in the cellulase-producing fungal taxa were observed when samples from February and May were compared. In February, *Leotiomyces* were the main cellulase producers, whereas in May a general trend to a broader spectrum of cellulase producers was observed. *Eurotiomyces'* cellulases were most abundant in AK, whereas *Dothideomyces'* (KL), *Leotiomyces'* (OR) and *Sordariomyces'* (SW) cellulases dominated the other sampling sites.

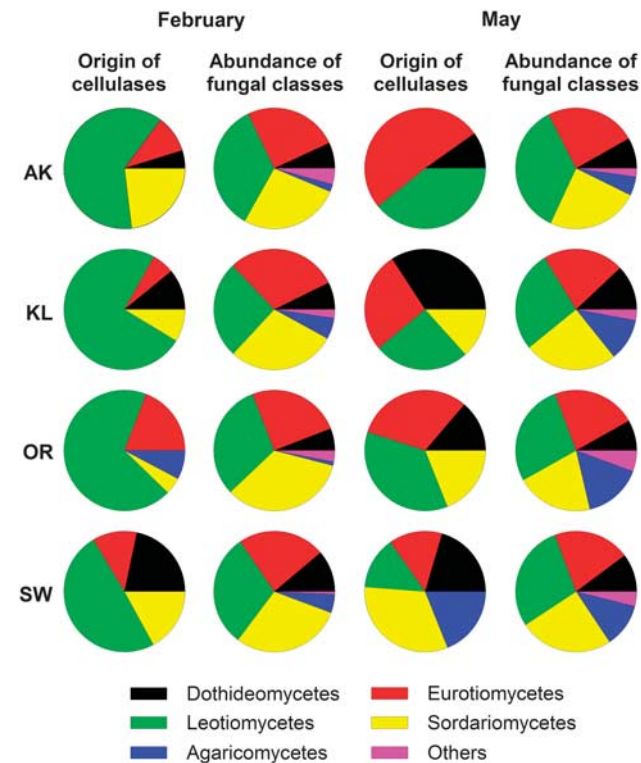


Figure 5 Phylogenetic origin of spectra assigned to fungal cellulases at different sampling times and sites compared with the overall distribution of fungal classes. Data represent the average of two biological replicates.

Community structure and function is influenced by season and leaf-litter nutrient content

SLR (Supplementary Figure 3) and PCA were performed to elucidate the influence of stoichiometry, season and leaf-litter nutrient content on community structure and functionality, as well as to identify the set of variables with the strongest relation to the decomposer community.

In our PCA biplots (Figures 6a, b and Supplementary Figures 4a–d), each axis (= principal component) is calculated from a set of variables, which together account for a high percentage of the variability of the data and can therefore be regarded as important drivers. The position of the sampling sites at the respective season within the biplot displays their relation to the measured variables. During litter decomposition, plant proteins decrease whereas fungal and bacterial proteins increase. On the x-axis (PC1) in Figure 6a, remaining plant proteins ('*Viridiplantae*'), which can be considered as markers for the amount of remaining litter material, are allocated on the left/negative side together with the remaining C and Ca content. Bacterial proteins ordinate together with pH, K and the micronutrients Fe and Mn. Fungal proteins, temperature (T) and N content ordinate together. These variables are

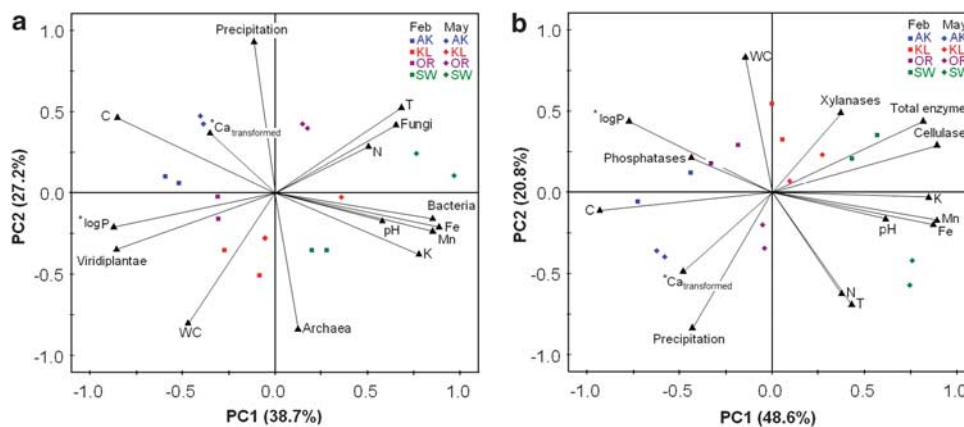


Figure 6 PCA biplots of community structure and function obtained in the metaproteomics approach as well as environmental data and leaf-litter nutrient content. (a) General taxonomy–community structure, (b) community function. Data points are from different sampling times (February—squares and May—diamonds) and four sampling sites. Stars indicate transformations of the variables to meet the assumption of normal distribution, P is log 10 transformed and Ca is $1/(1 + \log_{10}(\text{Ca}))$ transformed.; T, mean air-temperature; * indicates inversion of the vector orientation because of data transformation.

located opposite to the remaining plant proteins, indicating that they are related to a reduction in litter plant proteins. P content is also related to fungal growth as referred from the negative ordination of log P in the PCA (inversed direction results from log transformation). The *y*-axis (PC2) is dominated by the antagonistic variables precipitation and archaeal proteins. The positioning of sampling sites within the biplot and their shift from February to May towards the upper right reveals the effect of time/season factors on litter proteins: the reduction of plant proteins and the accumulation of (mainly) fungal proteins is positively correlated to warmer conditions. In addition, the individual sampling sites are separated according to their nutrient composition.

SLR (Supplementary Figure 3) and PCA were also performed to identify factors affecting enzyme abundance and activity at the different sampling sites. PCA of community function (represented by enzyme abundance) and time/season factors and nutrient content (Figure 6b) revealed relationships which support our findings on the community structure: the *x*-axis (PC1) can be regarded as 'decomposition' axis with remaining, not yet respired, litter C content on the left/negative side and total enzymes, pH, K and micronutrients on the right side. Xylanases and cellulases—the main C-degrading enzymes—are ordinated opposite to litter C content. Also, Ca was inversely related to total enzyme abundance. Similarly, phosphatases were inversely related to P content. The *y*-axis (PC2) is dominated by season factors, such as WC and T, as well as by N content. Sampling sites are clearly separated along the *x*-axis due to their different nutrient and enzyme concentrations. The relation between time/season and nutrient release is indicated by an allocation of the May samples towards the warmer, drier conditions in the direction of the lower right in this plot. As enzyme abundances did not change with season, this movement is independent of enzyme ordination.

Interestingly, bacterial abundance correlated positively with the total abundance of extracellular hydrolytic enzymes (Supplementary Figure 3; $r^2 = 0.33$, $P = 0.025$) and in particular with cellulases ($r^2 = 0.44$, $P = 0.007$). However, no significant correlation between fungal abundance and extracellular hydrolytic enzymes was found (Supplementary Figure 3; $r^2 = 0.05$, $P = 0.419$ for cellulases; $r^2 = 0.02$, $P = 0.619$ for total enzymes). The bacterial relationship to cellulase abundance resulted in a negative correlation of the F/B ratio and cellulase abundance (Supplementary Figure 3; $r^2 = 0.42$, $P = 0.002$).

Discussion

Community structure of litter decomposers as revealed by metaproteomics

Metaproteomics can provide detailed information on the succession of the active part of the leaf litter

inhabiting community (Nocker and Camper, 2009) and it thus enabled us to follow succession on a higher taxonomic resolution compared to e. g. PLFA (for a detailed review see Joergensen and Wichern, 2008). *Ascomycota* and *Basidiomycota* are commonly regarded as predominant fungal phyla in the soil-litter interface (Osono and Takeda, 2006). The metaproteomics data showed that the fungal community was dominated by *Ascomycota* and contained only a small proportion of *Basidiomycota* and *Mucoromycotina* at all sampling-sites and -times (Figure 1d). This is in agreement with the results of our cultivation approach (Supplementary Information). For fungal succession it has been observed that *Mucoromycotina* belong to the first colonizers followed by *Ascomycota* (Torres *et al.*, 2005). These fungi have limited ability to degrade lignin and are mainly regarded as cellulose decomposers or sugar fungi (Osono, 2007). *Basidiomycota*, with their ability to degrade the recalcitrant lignin-containing litter material, appear only later in the decomposition process (Osono, 2007; Lundell *et al.*, 2010). Successional analysis of the fungal community showed a decrease of *Mucoromycotina* and *Ascomycota* and an increase of *Basidiomycota* from February to May (Figure 1d). This is in good agreement with the observed fungal succession described by Torres *et al.* (2005) and Osono (2007).

For bacterial succession, an increase in the proportion of *Proteobacteria* from winter to spring was observed, whereas that of *Actinobacteria* and *Verrucumicrobia* decreased (Figure 1b). Changes in the respective group abundances were validated by a PLFA analysis, which showed similar trends (Figure 2). A reduction of *Actinobacteria* was unexpected, because they are known to be involved in decomposition of organic materials, and thus are important for organic matter turnover and C cycle (Kirby, 2006). In other studies, an increase in the abundance of *Actinobacteria* has been shown during later stages of litter decomposition (Torres *et al.*, 2005; Snajdr *et al.*, 2011). The same accounts for the absence of *Acidobacteria*; members of this bacterial phylum can degrade various polysaccharides including cellulose and xylan (Ward *et al.*, 2009). Based on RNA sequencing, Baldrian *et al.* (2012) found *Acidobacteria* to be the main bacterial group that was enriched in an active litter inhabiting community. The low number of acidobacterial proteins in our metaproteomics dataset might be explained by the limited number of *Acidobacteria* protein entries in the reference database (0.003% of database entries), which might have led to an underestimation of the contribution of *Acidobacteria* to the bacterial population.

Microbial succession is mainly influenced by leaf-litter quality

Previously, it has been shown that litter quality affects decomposing community and ecosystem

processes (Prescott, 2010; Strickland and Rousk, 2010). A major factor in terrestrial ecosystems is pH (Sinsabaugh *et al.*, 2008). Our results revealed that pH had an impact on the bacterial, but not on the fungal community, as seen from a strong negative correlation between pH and the F/B ratio. Bacterial abundance was higher at more basic pH values (Figure 6a, Supplementary Figures 3 and 4) as it has also been observed in other studies (Fierer and Jackson, 2006; Högberg *et al.*, 2007). The lower impact of pH on fungal diversity might be explained by the fact that fungi can grow over a wide pH range (Penalva *et al.*, 2008), whereas bacterial growth is restricted to a smaller pH range as has been shown in pure culture studies with isolates from natural soils (Baath, 1996).

Ecosystem processes are often controlled by the availability of N or P (Elser *et al.*, 2007). Although litter N varied only slightly in our approach, clear differences between litter P values were present (Table 3). The SLR and PCA analysis revealed that P is a major factor influencing the abundances of all major fungal and bacterial groups with higher abundances at higher P (Figure 6a, Supplementary Figures 3 and 4). Phosphorous is needed for DNA replication and transcription and, therefore, fast growing bacteria are particularly affected under P-limiting conditions (Elser *et al.*, 2000, 2003). Our data showing higher F/B ratios at nutrient and especially P poor sites (Supplementary Figure 3) are in good agreement with several earlier studies (Hieber and Gessner, 2002; van der Wal *et al.*, 2006; Güsewell and Gessner, 2009).

The function of fungi and bacteria in the initial phase of litter decomposition

Fungi are thought to be key players during litter decomposition because of their ability to degrade recalcitrant compounds such as lignin and their dominance in the decomposition of cellulose and hemicellulose (de Boer *et al.*, 2005; Meidute *et al.*, 2008). However, our knowledge about the contribution of bacteria and fungi to this process is still scarce. Recently, the analysis of ligninolytic and chitinolytic enzymes by a targeted metatranscriptomics approach of a forest soil (Kellner *et al.*, 2010) was performed with a focus on fungal enzymes, though neglecting the bacterial part of the community. Moreover, the analysis of a forest-soil metatranscriptome indicated that the active microbial community identified from RNA sequencing is dominated by *Ascomycota* and it is significantly different from the total community as indicated by DNA-based sequencing (Baldrian *et al.*, 2012).

Our analysis of the entire community revealed that only fungi produced extracellular enzymes in the investigated environments, corroborating with earlier findings that dealt with a secretome study of two model organisms growing on beech litter as substrate (Schneider *et al.*, 2010). Cellulases were

the most abundant enzymatic class. Interestingly, the phylogenetic origin of cellulases changed over time (Figure 5) with fungal classes (*Leotiomycetes*, *Dothideomycetes* and *Sordariomycetes* of the *Ascomycota*) growing in association with leaves before litter fall as pathogens or endophytes dominating cellulase production in February. In May, the contribution of ascomycotal classes like the *Eurotiomycetes* and basidiomycotal classes derived from soil and known for their saprotrophic lifestyle (Domsch *et al.*, 2007) increased. The generally low proportion of basidiomycotal cellulases might result from a low number of respective database entries. Only 21% of fungal cellulase entries are from *Basidiomycota*, whereas 73% belong to the *Ascomycota* and 6% to other fungal groups. Baldrian *et al.* (2012) were able to increase the number of *Basidiomycota*-derived transcripts of the exocellulase gene *cbh1* by sequencing of *Basidiomycota* species with a previously unknown genome, supporting the hypothesis that basidiomycotal cellulase entries are underrepresented in public protein databases.

No enzymes of bacterial origin were detected; this is likely not a result of a low number of bacterial cellulases in the reference database (almost 60% of cellulase DB entries are of bacterial origin), but might rather be explained by a low abundance of bacterial enzymes indicating the minor importance of these enzymes in the overall decomposition process. During the investigated stages of litter decomposition, bacteria might proliferate on low molecular weight carbohydrates provided by fungal enzymes. This idea is supported by the observed strong positive correlation between bacterial abundance and the abundance of fungal enzymes (Supplementary Figure 3) and might be referred to as 'cheating behaviour' (Velicer, 2003). Bacteria might contribute to the production of extracellular degrading enzymes later in the decomposition process as shown for *Actinobacteria*, a group known for its ability to utilize lignin-derived compounds (Kirby, 2006). Moreover, it was shown that bacteria dominate litter decomposition in particular micro niches where anaerobic or high temperature conditions prevail (Lynd *et al.*, 2002).

Extracellular enzymes and their influence on decomposition

Our data indicated that the production of extracellular enzymes was influenced by litter stoichiometry. Cellulases and total enzyme abundances were highest at lowest C:P and C:N ratios (Supplementary Figure 3). C:P ratios correlated positively with the remaining plant proteins (*Viridiplantae*) and negatively with proteins assigned to many microbial groups (that is, *Bacteroidetes*, *Ascomycota*, *Basidiomycota* (Supplementary Figure 3), which shows that protein degradation and microbial community were strongly impacted by P. Sterner and Elser (2002) observed that the availability of P might be a

rate-limiting factor in the synthesis of cellulolytic enzymes. The present study confirms this finding. Phosphatases ordinated opposite to P in our PCA (Figure 6b) supporting the idea that they are specifically produced when microbes have a certain demand for P (Sinsabaugh *et al.*, 2008).

The same holds true for the release of C from litter material. Different models for C-acquisition propose a sequential decomposition of polysaccharides, starting with hemicellulose and cellulose degradation followed by the removal of lignin (Berg and Mcclaugherty, 2008; Snajdr *et al.*, 2011). Recently, Klotzbücher *et al.* (2011) suggested that lignin is degraded preferentially in early phases of litter decomposition. In our approach, the dominance of xylanases and in particular cellulases indicates a decomposition phase in which hemicellulose and mainly cellulose can be considered as the major C-source. No insights into the degradation of highly recalcitrant phenolic lignin compounds were obtained because no lignolytic enzymes were detected. However, the increase of *Basidiomycota*, (known as main lignin decomposers) in May indicates that lignin degradation might start later in the decomposition process. Furthermore, our data imply that in litter with higher abundance of cellulose-degrading enzymes the amount of remaining degradable C is lower (Figure 6b) and the abundance of microorganisms is higher (Figure 6a, Supplementary Figure 3). This finding indicates that enzyme production might be regarded as the 'bottleneck' of C-degradation/mineralization from litter. Similar hypotheses were postulated for soil organic-matter decomposition by Kuzyakov *et al.* (2009) and Schimel and Weintraub (2003).

Conclusion

After litter fall, microorganisms grow on the litter using easily accessible compounds. Microbes are affected by litter quality and environmental factors such as temperature and pH. The investigation of microbial succession revealed that *Ascomycota* dominated the fungal decomposer community with respect to abundance (total protein abundance) and activity (production of extracellular enzymes) especially in early stages of litter decomposition. In May, contribution of *Basidiomycota* to community functionality had increased in comparison with February. This strongly supports the assumption that the active part of microbial community changed over time. Only fungal, but no bacterial, polymer-degrading enzymes were detected at all sampling sites and -times, a finding that strongly supports our conclusion that fungi are the main players in litter decomposition at the investigated sites. Furthermore, a strong relationship between leaf-litter nutrient content and microbial abundance as well as cellulase production was found, indicating that high N and P content stimulate microbial

decomposer activity. Accordingly, litter decomposition was fastest at sites with highest abundances of extracellular enzymes, suggesting that the production of extracellular enzymes is a rate-limiting step in the decomposition process.

We are fully aware that the metaproteomics-based approach suffers from certain limitations; the community structure and functionality assessed on the protein level might be influenced by the protein extraction efficiency, and hampered by false or missing assignments of MS and MS/MS data to protein entries in the reference database. Nevertheless, we believe that the metaproteome analysis, together with the validation by well-established complementary approaches, provides a deep insight into molecular details of the leaf-litter decomposition process. It opens up a new level of information in which both microbial succession and the activity of certain phylogenetic groups can be analyzed on the basis of their proteomes. The latter represent the active 'building-blocks' in the ecosystem. Thus, further proteome analyses, for example, the investigation of later phases during litter decomposition or other litter types, will help to answer open questions in the field of litter decomposition: how the decomposer community further develops, who contributes to the actual degradation and which factors influence the process.

Conflict of interest

The authors declare no conflict of interest.

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