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Effects of stoichiometry and temperature perturbations on beech litter decomposition, enzyme activities and protein expression

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Abstract

Microbes are major players in leaf litter decomposition and therefore advances in the understanding of their control on element cycling are of paramount importance. Our aim was to investigate the influence of leaf litter stoichiometry in terms of carbon (C) : nitrogen (N) : phosphorus (P) on the decomposition process, and to follow changes in microbial community structure and function in response to temperature-stress treatments. To elucidate how the stoichiometry of beech litter (*Fagus sylvatica* L.) and stress treatments interactively affect the decomposition processes, a terrestrial microcosm experiment was conducted. Beech litter from different Austrian sites covering C:N ratios from 39 to 61 and C:P ratios from 666 to 1729 were incubated at 15 °C and 60 % moisture for six months. Part of the microcosms were then subjected to severe changes in temperature (+30 °C and −15 °C) to monitor the influence of temperature stress. Extracellular enzyme activities were assayed and respiratory activities measured. A semi-quantitative metaproteomics approach (1D-SDS PAGE combined with liquid chromatography and tandem mass-spectrometry; unique spectral counting) was employed to investigate the impact of the applied stress treatments in dependency of litter stoichiometry on structure and function of the decomposing community. In litter with narrow C:nutrient ratios microbial decomposers were most abundant. Cellulase, chitinase, phosphatase and protease activity decreased after heat and frost treatments. Decomposer communities and specific functions varied with site i.e. stoichiometry. The applied stress evoked strong changes of enzyme activities, dissolved organic nitrogen and litter pH. Freeze treatments resulted in a decline in residual plant litter material, and increased fungal abundance indicating slightly accelerated decomposition. Overall, we could detect a strong effect of litter stoichiometry on microbial community structure as well as function. Temperature perturbations resulted in short- to medium-term alterations of microbial function, especially heat blocked decomposing enzymes.

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1 Introduction

By decomposing natural compounds microbes exert a major control on biogeochemical cycles, thereby affecting climate and nutrient availability in the biosphere. This is reflected by a large increase in litter decomposition investigations over the last decades (Prescott, 2010). Litter decomposition rates are strongly affected by litter quality and are limited by certain thresholds of lignin:N, polyphenols, phosphorus, temperature and moisture (Prescott, 2010). These processes are controlled by abiotic factors, such as temperature and precipitation, and by litter chemical composition and soil organisms (Couteaux et al., 1995). The biological degradation of litter is mainly carried out by microbial decomposers, including microbes (bacteria and fungi) which have lower C:nutrient values compared to the substrate they consume i.e. litter. There are different stoichiometric (C:N:P) demands and constraints on the decomposition process, based on microbial group and life strategy (Keiblinger et al., 2010). In terms of C cycling processes bacteria and fungi are major players, due to their inherent stoichiometry of C and N. While bacteria are dominating in aquatic ecosystems this is different for terrestrial ecosystems where fungi play an important role in nutrient cycling processes (de Boer et al., 2005). Fungi have the ability to degrade highly recalcitrant compounds by excreting extracellular enzymes and seem to dominate the production of a wide range of extracellular enzymes that break down complex high molecular weight organic matter (Romani et al., 2006). Moreover, it has been proposed that fungi and bacteria cycle C in a distinctly different way due to differences in their C-use efficiencies (Keiblinger et al., 2010). The C respired provides energy to decomposers, while increasing the relative amount of nutrients in their substrate. Decomposers excrete enzymes to degrade recalcitrant compounds in order to make these substrates available for microbial nutrition. Extracellular enzyme activities link nutrient demand of microbes by their elemental stoichiometry (i.e. the C:N:P ratio), with available substrate and biomass production (Sinsabaugh et al., 2008). In addition, they are involved in mineralization of P, humus formation, the degradation of microbial cell walls and plant material and therefore are

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of major importance during leaf litter decomposition (Allison et al., 2006). It is possible to link these activities to C sequestration on an ecosystem scale (Allison et al., 2006). Enzyme activities provide information about the biogeochemical and microbiological situation in an ecosystems, i.e. nutrient limitation, microbial activity, perturbation responses and organic matter characteristics (Allison et al., 2006).

Enzyme production is energy and N demanding for microbes; an effort which can be wasted due to loss of control over extracellular enzymes after excretion. To maximise their yield microorganisms are expected to regulate their enzyme production in relation to production costs, by a sensitive induction of enzyme production pathways by available substrates, and the microbial density effects (Mandels and Reese, 1960). The microbial community therefore is thought to greatly benefit from the presence of fungi as important providers of assimilable substrates and nutrients to the whole microbial community (Romani et al., 2006).

Numerous environmental factors may affect microbial community activity and structure (Nannipieri et al., 2003). Under steady environmental conditions it is proposed that a minimum number of species is necessary for ecosystem functioning, but during and after perturbations a larger number of species are essential to maintain stable process rates (Nannipieri et al., 2003). Although part of the terrestrial C pool is highly variable in time and space, large inert C pools might become active after perturbations in soils (De Deyn et al., 2008). Drought conditions favour fungal diversity and probably moderate fungal competition under periodical perturbations (Hawkes et al., 2011). Moisture may increase the variability in fungal responses in terms of metabolic activity, in water limited terrestrial systems (Hawkes et al., 2011).

Metaproteomics has been proven to be useful to investigate the active microbial community, in different habitats like leaf phyllosphere (Vorholt et al., 2009), soil and groundwater (Benndorf et al., 2007), activated sludge and wastewater (Wilmes et al., 2008) and soil (Wang et al., 2009, 2011). In comparison to studies of DNA and RNA, which inform on precursors of microbial functions, proteins and their modifications, have an intrinsic metabolic function which can be used to directly relate microbial activities to

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defined organisms in multispecies communities. Additionally, the measurement of microbial enzyme activities may relate leaf litter decomposition to specific extracellular enzymes acquiring C, N and P, as constrained by microbial biomass stoichiometry (Sinsabaugh et al., 2008). In this study we try to link microbial community structure as determined by metaproteomics (the study of indigenous proteins of an environmental sample, Schneider et al., 2010), and the microbial activity in terms of extracellular enzyme activities and respiration dynamics as affected by litter chemistry and temperature stress. To this end relating leaf litter stoichiometry, metaproteome and enzyme activities to the effects of temperature perturbations will provide new insights into the microbial processes during leaf litter decomposition. Our aim was to investigate changes of microbial decomposer structure and function during leaf litter decomposition as affected by leaf litter stoichiometry (C:nutrient ratios) and temperature perturbations (heat and freeze stress). Therefore we conducted a terrestrial microcosm experiment, using beech litter (*Fagus sylvatica* L.) from three different sites in order to ensure similar litter biochemistry (i.e. lignin content) and answer the following questions:

Q1: Which factor – resource stoichiometry or temperature treatments – has the strongest effect on microbial community structure and function?

Q2: How do extreme temperatures affect microbial function (enzyme activities)?

Q3: Is it possible to link microbial community structure using metaproteomics to its decomposer function and the contribution of major phylogenetic groups to the decomposition process?

2 Material and methods

2.1 Experimental setup

Beech (*Fagus sylvatica* L. 1753) litter with different elemental and stoichiometric composition (C:N:P, Table 1) was collected at three sites across Austria in autumn 2008

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(Wanek et al., 2011): Klausenleopoldsdorf "KL" (Kitzler et al., 2006), Ossiach "OS" and Schottenwald "S"). The collected litter was dried at 40 °C for 48 h, shred into pieces and sieved. A fraction of pieces between 2 mm and 1 cm size was sterilized by γ-radiation and inoculated with a soil-litter suspension (Wanek et al., 2011). Microcosms were incubated at 15 °C for 25 weeks. After 12 weeks treatments were performed as follows: the microcosms were either heat treated (3 days at 23 °C, 5 days at 30 °C, 1 day at 23 °C and 3 days before sampling back to 15 °C) or freeze treated (3 days at 4 °C, 5 days at −15 °C, 1 day at 4 °C and 3 days before sampling back to ambient temperature). Two weeks after begin of treatment the first sampling was performed, with a second sampling three months after treatment. Water content of the litter was maintained at 60 % fresh-weight by adding autoclaved tap water weekly. Litter samples were analysed in five replicated microcosms for each treatment (control, heat, and freeze).

2.2 Litter moisture and nutrient concentrations

Litter moisture was determined gravimetrically on an aliquot of litter (by drying at 65 °C). Dry samples were ground in order to obtain a fine powder. The total carbon and nitrogen contents of litter were analysed from the respective samples with an elemental analyser (Leco CN2000, LECO corp. St Joseph, MI, USA). Further, the ground samples were wet oxidized with an acid mixture (H_2SO_4+ HNO_3, Henschler, 1988) in a microwave oven and element concentrations (P, K, Mg, Mn, Ca, Fe) determined by inductively coupled plasma atomic emission spectrometry (ICP-AES). Litter C:N and C:P ratios are given in Table 1.

2.3 Microbial biomass carbon (C_{mic}), nitrogen (N_{mic}) and phosphorus (P_{mic})

Litter samples were analyzed for microbial biomass C and microbial biomass N and P contents using the chloroform fumigation-extraction method as described by Schinner et al. (1996). Control samples were not fumigated but extracted in the same way as fumigated ones. Samples were extracted in a ratio of 1:33 ($w : v$) with 0.5 M K_2SO_4-

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solution. The total amount of dissolved C and N was determined with a TOC-V CPH E200V, linked with a TN-unit TNM-1 (Shimadzu Corporation, Kyoto, Japan). The total amount of microbial P was determined with a persulfate digestion of the 0.5 M K_2SO_4 extracts based on Doyle et al. (2004). Phosphate was quantified photometrically at a wavelength of 882 nm based on the phosphomolybdate blue reaction (Schinner et al., 1996) with a microplate reader (BIO-TEK Instruments, Inc.). The 0.5 M K_2SO_4 extracts were kept frozen (−20 °C) until analysis.

2.4 Community function analysis

2.4.1 Respiration measurements

Respiration of the litter within the microcosms was monitored once a week using an infrared gas analyzer (IRGA, EGM4 with SRC1, PPSystems, USA). Measurements were conducted using the following settings: volume of the chamber 1551 cm^3, area of the chamber 115 cm^2, linear measurement, and respective temperature, of the incubation chamber. CO_2 concentration was measured over 70 s and the increase in CO_2 concentration per second was calculated based on gram litter dry-weight. Measurements of ambient air were performed before and after each measurement to assess possible leaks or base-line drifts of the IRGA system (EGM4 with SRC1).

2.4.2 Ammonium, nitrate and phosphate concentration

Extractable N and P were determined after extraction in a ratio of 1:33 (w:v) with 0.5 M K_2SO_4-solution. Ammonium was extracted and analysed by the Berthelot reaction according to Schinner et al. (1996). Nitrate was analysed by the VCl_3-Griess reaction according to Hood-Novotny et al. (2010). Phosphate determination was based on the phosphomolybdate blue reaction (Schinner et al., 1996). Colour intensities were determined photometrically using a microplate reader (BIO-TEK Instruments, Inc.). The 0.5 M K_2SO_4 extracts were kept frozen (−20 °C) until analysis.

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2.4.3 Enzyme activities

Potential extracellular enzyme activities were measured using microplate fluorometric and photometric assays. All activities were measured within 48 h after litter sampling.

Cellulase, chitinase, phosphatase and protease

Extracellular cellulase ("cellobiohydrolase"), chitinase, and phosphatase were measured fluorimetrically (Kaiser et al., 2010) using methylumbelliferyl (MUF) substrates in black microplates in three technical replicates. Plates were incubated for 140 min in the dark and fluorescence was measured at 450 nm emission wavelength and at an excitation wavelength of 365 nm using a Tecan Infinite M200 Fluorimeter (Werfen, Austria).

Phenoloxidase, peroxidase

Phenoloxidase and peroxidase activities were measured photometrically based on standard methods (Kaiser et al., 2010), using L-3,4-dihydroxyphenylalanin (L-DOPA, Sigma- Aldrich, Vienna, Austria) in microplates. For peroxidase activity 0.3 % H_2O_2 solution was added to the assay. At the beginning and after 20 h, absorption was measured at 450 nm. Enzyme activity was calculated from the increase in absorption over time divided by the molar extinction coefficient.

2.5 Community structure analysis

2.5.1 Metaproteome analysis

From the five replicates of each treatment and harvest, samples were stored at −80 °C, litter material from one replicate was analysed by metaproteomics resulting in a total of 18 samples. This was the upper limit of what could be achieved in this project as one metaproteomic sample takes at least five days of processing and analysis. Aliquots

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(3 g) of litter material were ground in liquid nitrogen and the resulting powder was 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 3 000 g at 4 °C for 10 min. Supernatants were removed and centrifuged for 5 min at 14 000 g and 4 °C. Supernatants were concentrated about 5-fold by vacuum-centrifugation (Eppendorf Vacuum Concentrator plus) at 30 °C. 25 µl of concentrated supernatants were then subjected to 1D-SDS-PAGE (Laemmli, 1970) in a 12 % polyacrylamide gel to clean samples from interfering substances (e.g. humic acids) and to reduce sample complexity. Protein lanes were cut into four slices and the gel slices subjected to in-gel tryptic digestion by employing sequencing grade modified trypsin (Promega, reference V5111) (Shevchenko et al., 1996). The resulting peptide mixtures were analyzed on a hybrid LTQ-Orbitrap mass spectrometer (ThermoFisher Scientific) interfaced with a nano-electrospray ion source as described earlier (Schneider et al., 2010).

2.5.2 Database searches

The MASCOT Search Engine (version no.2.2.04) was used for protein database searches. MS and MS/MS data were searched against a database containing all proteins from UniRef100 (9808438 entries, downloaded from the European Bioinformatics Institute webpage http://www.ebi.ac.uk/uniref/ at the 26 January 2010) and protein sequence information from a translated metagenome of the microbial community of a Minnesota farm silage soil (Tringe et al., 2005) (184 374 entries, downloaded from http://img.jgi.doe.gov at the 15 of October 2009) as well as common contaminants like keratin and trypsin (total no. of entries 9 993 117). The following search parameters were applied: (1) trypsin was chosen as protein-digesting enzyme and up to two missed cleavages were tolerated, (2) carbamidomethylation of cystein was chosen as fixed modification, and (3) oxidation of methionine was chosen as variable modification. Searches were performed with a parent-ion mass tolerance of ±5 ppm and a

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fragment-ion mass tolerance of ± 0.8 Da. A second database search was performed with the X!Tandem (version 2007.01.01.1) search engine (Craig and Beavis, 2004) with similar settings.

2.5.3 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. Peptide identifications were accepted if they were established at greater than 99 % probability as specified by the Peptide Prophet algorithm (Keller et al., 2002). Protein identifications were accepted if they were established at greater than 90 % probability and at least one peptide was uniquely assigned to a respective protein in one of our samples. Protein probability was assigned by the Protein Prophet algorithm (Nesvizhskii et al., 2003). Proteins that were identified with the same set of peptides and could not be differentiated by the MS/MS analysis were grouped to protein clusters to satisfy the principles of parsimony. A protein false discovery rate of 5.5 % was calculated by the Scaffold software.

2.5.4 Data validation and quantitative assignment of data to phylogenetic and functional groups

Starting from the Scaffold output files, all protein hits obtained by the database searches were assigned to phylogenetic and functional groups and assignments were validated by a newly developed perl-script based PROteomics result Pruning and Homology group ANotation Engine (PROPHANE) workflow (http://prophane.svn.sourceforge.net/viewvc/prophane/trunk/). To this end, protein clusters were checked for sequence homology at a 50 % identity threshold. Afterwards, phylogenetic information of each protein hit was retrieved from the Swissprot database. If Swissprot was not available the Entrez database was used instead. For functional assignments, proteins were blasted against the KEGG database, the cluster of orthologous group

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(COG) database and Swissprot. If a protein cluster contained more than one protein hit, clusters were checked for consistency in the phylogenetic and functional assignments (Schneider et al., 2011). Finally, protein abundances were calculated based on the normalised spectral abundance factor (Zybailov et al., 2006; Florens et al., 2006). In this process, the numbers of unique spectra assigned to each protein are divided by the number of the amino acid chain length of the longest candidate in the protein cluster giving the spectral abundance factor (SAF). The SAF allows the comparison of protein abundances in one sample with taking into account protein molecular weight. Afterwards each SAF is divided by the sum of all SAFs in the respective sample to allow cross-sample abundance comparison.

2.6 Data evaluation and statistical analyses

For individual analyses of the effects of litter stoichiometry on processes and microbial community structure we performed simple linear regression (SLR) analysis. For SLR we tested the residues to check if the assumption of normal distribution was met, by using the Shapiro-Wilk test. When the assumption of normal distribution was violated the data were log transformed to approach normal distribution. Individual tests between the parameters sampling time were evaluated using Student's *t*-test. To evaluate differences between multiple groups (i.e. treatment) we used one-way analysis of variance (ANOVA) and post-hoc Tukey HSD respectively. To evaluate which factor (litter type-"site", "time", "treatment") best predicted differences in decomposition we performed a principal component analysis (PCA). To evaluate the influence of treatment, site and time and their combination on microbial functions we performed a multivariate ANOVA (MANOVA). All statistical analyses were conducted using SAS Enterprise 4 (SAS Institute 1992) or PASW 18.0 (SPSS) statistical software packages.

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3 Results

The present experiment was designed to evaluate the impact of different factors on decomposition of beech litter. The factors were the different sampling "time" points (3 and 6 months after inoculation), "treatment" ("treat") of the microcosms (heat and freeze) and the litter types from three locations as the factor "site" which differed in nutrient stoichiometry. Potential extracellular enzyme activities were investigated in detail, as they are a measure of microbial community function and they play an important role in litter decomposition. Multivariate analysis of variance (MANOVA) revealed that the factor "treatment" had the most prominent influence on potential enzyme activity levels of cellulases, chitinases, phosphatases and proteases, and less so on phenoloxidase and peroxidase activities which were most strongly related to "site" i.e. litter stoichiometry and "time" respectively (Table 2). As shown in Fig. 1 there was a significant decline in cellulase, chitinase and phosphatase activity after heat or freeze treatments in comparison to controls. Protease activity declined as well, but not significantly. Heat resulted in a stronger decline of enzyme activities than freeze. Dissolved organic carbon (DOC) and nitrogen (DON) concentrations were significantly affected by "treatment" (Table 2) but in a time and site specific manner (Fig. 2). The impact of "treatment" on DON and DOC was most pronounced at the second sampling, with the strongest declines in litter from the nutrient rich Schottenwald (SW) site. The opposite was noticed for the poorer sites Ossiach (OS) and Klausenleopoldsdorf (KL) with enhanced DOC values three months after treatment. Generally heat showed stronger effects compared to freeze.

Interestingly, the microbial biomass (C_{mic}, N_{mic} as well as P_{mic}) was affected by "site" and therefore related to leaf litter stoichiometry (Table 2). Microbial biomass stoichiometry (C_{mic}:N_{mic} and C_{mic}:P_{mic}) declined over time, which indicates a microbial succession towards bacteria. Although litter NH_4^+ concentrations were more affected by "site", NO_3^- concentrations varied mostly with "time" (Table 2), which indicates that the microbial driven process of nitrification may change during succession. Respiration activity and PO_4^{3-} concentrations were both mainly impacted by the factor "site" i.e. litter stoichiometry (Table 2).

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The MANOVA showed strong influences of the factor "site" (besides "treatment") which indicates that litter quality has a major function in defining the response of the microbial community to the applied experimental conditions. In order to identify the main site/litter properties that drive microbial activity SLR was applied (Table 3). Litter C:N ratios were negatively related to activities of cellulase, chitinase, peroxidase and phenoloxidase. Interestingly litter *P*-concentration and litter C:P were not correlated with phosphatase activity, but were positively related to all other enzyme activities.

Generally, enzyme activities were clearly influenced by nutrients contained in the microbial biomass. The activities of the C-acquiring enzymes cellulases, chitinases, peroxidases and phenoloxidases correlated positively with C_{mic}. In addition N_{mic} correlated positively with peroxidase ($R^2 = 0.22$) and phenoloxidase ($R^2 = 0.31$) activity, two enzymes which release N besides C due to their ligninolytic action (Table 3). Microbial stoichiometry (C_{mic}:N_{mic}, and C_{mic}:P_{mic}) showed a weaker relation to enzyme activities compared to C_{mic} N_{mic} and P_{mic} (Table 3). Cellulase, chitinase and phosphatase activities were related to C_{mic}:N_{mic} while not to N_{mic}. Concentrations of NH_4^+, NO_3^- and PO_4^{3-} were positively related to N_{mic} (Table 3). An inverse relationship between litter C:N ratios and NH_4^+ concentrations ($R^2 = 0.58$) and NO_3^- concentrations ($R^2 = 0.25$) were noted, as found for litter C:P ratios and PO_4^{3-} concentrations ($R^2 = 0.82$). Dissolved organic carbon (DOC) and nitrogen (DON) were correlated with respiration activity, although SLR probability was weak (data not shown). This implies that easily available C is respired by microbes.

To evaluate how the microbial community is influenced by the investigated factors, its structure and function was investigated by a metaproteomics approach. In total 1020 protein clusters were identified from which 138 were excluded from further analysis because of a lack of sequence homology in the respective clusters. The remaining 882 clusters were assigned to phylogenetic and functional groups using PROPHANE. The normalized numbers of unique spectra act as marker for the abundance of the respective groups. Cluster identification parameters as well as quantitative information and functional assignments are provided in the Table S1 of the Supplement. The complete

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MASCOT results data set including MS/MS spectra information is provided as online material at the PRIDE database (Vizcaino et al., 2009), at http://www.ebi.ac.uk/pride/; accession number is 19501.

In all samples community structure was dominated by remaining plant proteins (designated as "*Viridiplantae*") followed by *Fungi* and *Bacteria* (Fig. 3). Plant litter protein (*Viridiplantae*) loss which can be used as a proxy for decomposition was lowest at the nutrient poorest site (KL) and highest at the nutrient richest site (SW). Fungal and bacterial contribution to the total community was higher at nutrient richer sites. The fungal community was dominated by *Ascomycota* (*Eurotiomycetes*, *Sordariomycetes*) and *Basidiomycota* (*Agaricomycetes*) while the bacterial community was dominated by *Proteobacteria*, *Actinobacteria* and *Firmicutes* (Fig. 3). Changes in the relative composition of phylogenetic groups were observed for different "sites" i.e. litter stoichiometry and stress treatments (Fig. 3), although it seems that stress had a stronger effect on the general groups (Fig. 5). To assign community structure to leaf litter quality parameters principal component analysis (PCA) was performed. Microbial community domains were separated by the different leaf litter "sites", as can be seen in the PCA biplots (Fig. 4). While fungal abundance was ordinated in direction of the site Schottenwald (SW) which had most narrow C:nutrient ratios, the remaining plant material (*Viridiplantae*) was grouped to the site Klausenleopoldsdorf (KL) (wide C:P). *Viridiplantae* abundance was directed opposite to P and P_{mic} (Fig. 4). *Bacteria* and *Metazoa* grouped to the site Ossiach (OS), opposite to leaf litter C concentration. Fungal abundance was closely related to leaf litter nutrients (N, P) and stoichiometry (C:N, C:P) as evident in the PCA analysis (Fig. 4a) and in Table 4.

Principal component analysis of the microbial community at the phylum level (Fig. 4b) indicated that *Proteobacteria* and *Basidiomycota* were delineated towards OS with a negative relation to litter C concentration. *Ascomycota* and *Actinobacteria* were related to litter types with narrow C:nutrient ratios. These findings are in agreement with the SLR analysis (Table 4) for *Ascomycota* which were negatively associated with leaf litter stoichiometry (C:N, C:P). The class of *γ-Proteobacteria* ordinated close to the

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site OS (Fig. 4c) and opposite to litter C. The fungal classes of *Dothideomycetes, Eurotiomycetes* and *Sordariomycetes* grouped to SW (Fig. 4d), and correlated negatively with C:N and C:P (Table 4). Also α- and β-*Proteobacteria* were grouped in direction of SW which had narrowest C:nutrient ratios. Leaf litter C concentration correlated negatively to *Bacteria*, and most prominently to *Proteobacteria* (Table 4).

Total fungal abundance as well as the abundances of all fungal subgroups correlated positively with C_{mic} and N_{mic} (Table 4). Furthermore, a negative correlation of plant protein abundance (*Viridiplantae*) with the accumulation of microbial nutrients (C_{mic}, N_{mic} and P_{mic}) was observed. Microbial stoichiometry (C_{mic}:N_{mic}) correlated negatively ($R^2 = 0.73$) to α-*Proteobacteria* (Table 4).

Leaf litter stoichiometry (C:P and C:N ratios) and nutrient concentrations predicted changes in microbial community structure after temperature extremes. Microbial community changed in response to stress treatments, where fungal abundance increased after freezing which seems to accelerate decomposition. A reduction in bacterial abundance after freeze treatment was only transient as they recovered after three months. The modifications in microbial community after temperature stress were different in SW at the second sampling, fungal abundance decreased and litter decomposition was reduced indicated by higher *Viridiplantae* abundance (Fig. 5).

Besides the determination of the community structure based on present proteins we were able to detect extracellular litter degrading enzymes in the metaproteomics approach. The main functional groups were xylanases, pectinases, cellulases and proteases. Both, fungi and bacteria, contributed to the enzyme production (Table 2 of the Supplement). A comparison of the microbial community with respect to the production of extracellular litter degrading enzymes showed a dominance of *Fungi* and in particular *Ascomycota* in all litter types and at both sampling times. A strong "site" i.e. litter stoichiometry effect in the production of extracellular enzymes was observed with the highest enzyme abundances at the nutrient richest site SW. Furthermore an increase of total enzyme abundances was observed over time. As the abundances of extracellular enzymes were still low at the second sampling time a deeper investigation

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of their phylogenetic origin was not possible because of generally low spectral counts. However, our results underpin that the applied method provides the possibility to investigate the link of microbial community structure to its decomposer function (Table 2 of the Supplement).

4 Discussion

We studied beech litter decomposition under controlled conditions with a metaproteomic approach in order to link microbial community structure and function. We suspected that the investigated litter samples with varying nutrient stoichiometry control the collective activity of microbial communities by differing nutrient availability (Moorhead and Sinsabaugh, 2006). As expected beech litter stoichiometry strongly affected potential enzyme activities and generally higher enzyme activities were found in beech litter with narrow C:nutrient stoichiometry. During the early stages of decomposition microbial N limitation can occur due to high microbial N demand (Moorhead and Sinsabaugh, 2006). Thus, N is proposed to accelerate decomposition during the early stages while it might have an inhibiting effect during the late stages (Gallo et al., 2004). Although beech litter can be completely decomposed within one year in the field e.g. at site SW, (Zechmeister-Boltenstern et al., 2002) we assume that within the six months of our study the litter was still in a relatively early stage of decomposition. This is indicated by the low abundance of extracellular enzymes and the dominance of pectin and xylan degrading enzymes. These enzymes are produced in the first stage of decomposition when easily accessible material is still present in the litter material. Litter C:N ratios declined over time, while extracellular enzyme activities and respiratory C losses increased. The increasing enzymes were targeting high-molecular weight organic C compounds e.g. cellulase, chitinase, peroxidase and phenoloxidase. A release of C substrates together with microbial growth reinforces microbial N demand (Moorhead and Sinsabaugh, 2006), and can aggravate microbial N limitation. Phosphorous (P)

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availability may also have an impact on the decomposition process as it may limit the production of cellulolytic enzymes (Sterner and Elser, 2002). In the present study a weak negative impact of litter C:P on N acquiring enzyme activities (protease activity) was observed, while Olander and Vitousek (2000) found that P addition had no impact on N acquiring enzymes. The relationships in our investigation could result from increased investment of abundant N into extracellular enzymes mining for P, to maintain stoichiometric N:P balance of the microbes. Carbon and nutrients are utilized in specific stoichiometric ratios to maintain balanced growth conditions (Cleveland and Liptzin, 2007), and therefore resource stoichiometry has been shown to influence decomposer communities (Fig. 4). The fungal relationship with C_{mic} and N_{mic} indicates that the microbial biomass mostly consisted of *Fungi*. In addition the strong "time" effect of C_{mic}:N_{mic} indicates that the microbial community is rather homeostatic in terms of C_{mic}:N_{mic} and varies with succession.

Recent advances in stream ecology (Gulis and Suberkropp, 2003) and terrestrial ecology (Baldrian et al., 2011) showed a fungal dominance of leaf litter-decomposing microbial communities, which we could corroborate in the present study. Fungi are highly abundant in litter compared to soil. This might be explained by the wider resource C:N values. In the present study, litter C:N ranged from ~40–60 while the respective soils had narrower C:N ratios of ~16 (Kitzler et al., 2006). This is consistent with the suggestion of Six et al. (2006) that high quality substrate (narrow C:N) favors *Bacteria* and low quality substrate favors *Fungi*. In addition, *Fungi* generally grow under aerobic conditions (leaf litter), while *Bacteria* can inhabit anaerobic microniches within the soil matrix. Therefore, we propose that in fungi-dominated leaf litter r-selected *Fungi* may function as the respective *Bacteria* in soils.

We investigated the microbial decomposer community in depth in order to find the main drivers of the decomposition process and how they are controlled. In all our samples *Fungi* were the dominant microbial decomposers followed by *Bacteria* . The fungal community was dominated by *Ascomycota*, which were favoured in sites with narrow C:nutrient ratios, as were their subphyla (*Dothideomycetes, Eurotiomycetes*

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and *Sordariomycetes*); *Ascomycota* also dominated decomposer activity i.e. the production of extracellular enzymes. *Viridiplantae* loss (which we used as a surrogate for litter decomposition) was significantly higher, and *Fungi* abundance was positively related to nutrient contents (N, P) with only minor impacts on *Bacteria*. Nutrient availability to microbes may be limiting the production of cellulolytic and other extracellular enzymes. In addition the nutrient composition of litter selects for certain microbial populations which are the producers of extracellular enzymes.

It has been proposed that the initial phase of decomposition is usually dominated by pioneer species like *Zygomycota* or so called "sugar fungi" (Osono, 2007), which metabolize easily available C and are fast growers (Torres et al., 2005) that start colonizing the leaf surface. The succession then continues with *Ascomycota* who have only limited ability to attack lignin (Torres et al., 2005). Although investigating the early stages of decomposition in this study, the metaproteomics approach revealed only a minor impact of *Zygomycota* (*Mucuromycotina*) and a strong dominance of *Ascomycota* in the fungal community. Later in the decomposition process *Basidiomycota* increased in their abundance (mean NSAF value of *Basidiomycota* increased 2.4-fold at the second sampling compared to the first sampling), presumably due to their ability to degrade the recalcitrant litter material (Torres et al., 2005) and to obtain nutrients by decomposing dead organic matter (Osono, 2007). As decomposition proceeds, fast growing opportunistic microbes (*Zygomycota* and *Ascomycota*) are usually succeeded by *Basidiomycota*, which are slower growing decomposers specialized on degradation of more recalcitrant substrate (Osono, 2007).

In the present study we found that in litter with narrow C:nutrient (SW) ratios decomposition was accelerated (determined as decrease in plant litter material – *Viridiplantae* abundance), that enzyme activities were inhibited by stress, which decreased decomposition rates, and DOC and DON concentrations. In sites with wider litter C:nutrient ratios (KL, OS) decomposition was slower and lower enzyme activities were observed. In low nutrient litter, stress obviously increased the availability of soluble organic substrates, partly by physical processes of freezing, which led to increased DOC and DON

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availabilities for microbes. Dissolved organic carbon and DON were well correlated with respiration, which underpins that easily available C was respired by microbes. Temperature perturbations decreased potential hydrolytic enzyme activity dramatically.

Stress of certain intensity can alter the microbial community, at any stage of decomposition (Fierer et al., 2010). In our study microbial community analysis using the metaproteomic approach revealed that freeze treatment had a stronger effect on the microbial community structure than heat treatment. Microbes can survive and remain active after temperature stress (heat, freeze) by physiological acclimation (Schimel et al., 2007). This might be the reason that *Fungi* were favoured after freeze treatment while the reduction in bacterial abundance was a transient effect. Abundance of *Viridiplantae* declined after freezing which indicates that freezing accelerated decomposition in the short term. For instance freezing involves physical processes of cell lysis and disruption by the formation of ice crystals which render substrates available for decomposers (Schimel et al., 2007), processes that do not occur in the heat treatment. Microbial community structure changed less in the heat treatment, especially the abundance of *Fungi* and *Viridiplantae* showed minor effects (Fig. 5). In field experiments of soil warming heat effects on microbial community structure were difficult to detect although microbial function (i.e. respiration rates) was largely altered (Schindlbacher et al., 2011). Although the microbial community might respond only minor to temperature stress, the acclimation and stress repair costs can have strong effects on the allocation of C and nutrients to growth respiration and extracellular enzymes. This can be deduced from the greatly altered activities of most investigated enzymes (notably cellulases, chitinases, phosphatases and proteases) in our study.

Linking taxon identity to function is a major challenge in microbial ecology (Nannipieri et al., 2003). Although metaproteomics is still in its infancy, this post-genomic approach overcomes some of the methodological limitations of genetic as well as cultivation-based methods (Schneider et al., 2010; Strickland and Rousk, 2010). The study of Romaniet al. (2006) deals with enzymatic activities and the impact of *Fungi* and *Bacteria* and their contributions to leaf litter decomposition based on DNA (DGGE), while

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the present study links microbial activity and microbial phylogeny, at the extracellular enzyme level. Generally, extracellular enzymes derived mainly from *Fungi*. The *Ascomycota* within the phylum of *Fungi* were most abundant and active. Interestingly, this is consistent with the findings of Baldrian et al. (2011) who investigated the litter layer of a Norway spruce forest soil using a meta-transcriptome approach but not addressing specific enzymes.

In conclusion we can answer our research questions (Q) as follows:

In respect to Q1 we state that resource stoichiometry had a strong influence on microbial community structure (metaproteomic results) whereas temperature most prominently affected community functions (enzyme activities). Extreme temperatures (Q2) strongly reduced all enzyme activities. It is possible (Q3) to at least qualitatively link microbial community structure using metaproteomics to the decomposition process. In addition the contribution of major phylogenetic groups can be linked to decomposer function.

Supplementary material related to this article is available online at: http://www.biogeosciences-discuss.net/8/11827/2011/bgd-8-11827-2011-supplement.zip.

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Table 1. Stoichiometry – C:N ratios and C:P ratios of the litter types from the respective locations at the different treatments and sampling time points.

		Klausenleopoldsdorf			Ossiach			Schottenwald		
		control	frost	heat	control	frost	heat	control	frost	heat
C:N	1st sampling	49.8 ± 1.74	50.2 ± 1.97	51.9 ± 3.33	60.7 ± 0.89	60.9 ± 0.71	61.2 ± 0.92	40.3 ± 0.58	41.3 ± 1.65	41.0 ± 2.18
	2nd sampling	50.9 ± 0.46	48.9 ± 1.47	48.3 ± 0.98	57.7 ± 2.54	57.6 ± 2.79	58.1 ± 2.13	39.7 ± 3.37	39.1 ± 1.52	38.9 ± 1.09
C:P	1st sampling	1486 ± 47	1389 ± 69	1372 ± 274	914 ± 41	900 ± 41	850 ± 34	695 ± 25	672 ± 24	666 ± 57
	2nd sampling	1729 ± 63	1551 ± 82	1551 ± 30	921 ± 48	933 ± 38	850 ± 19	713 ± 34	676 ± 35	692 ± 17

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Table 2. Multivariate ANOVA with the factors "site", "treat" (treatment) and "time" (sampling time), to evaluate their impact on different parameters like cellulase, chitinase, phosphatase, protease, peroxidase and phenoloxidase activity, respiration activity (CO_2), phosphate (PO_4^{3-}), nitrate (NO_3^-) and ammonium (NH_4^+) concentration in litter, and on microbial elements and stoichiometry as well as dissolved organic carbon (DOC), dissolved organic nitrogen (N) and pH value.

	site		treat		site*treat		time		time*site		time*treat		time*site*treat	
	F	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Cellulase	89.7	< .0001	**121.4**	< .0001	2.7	0.0459	30.8	< .0001	4.7	0.0149	57.6	< .0001		
Chitinase	142.7	< .0001	**329.2**	< .0001	21.1	< .0001	14.6	0.0005			89.6	< .0001	5.5	0.0015
Phosphatase	18.3	< .0001	**1237.5**	< .0001			33.2	< .0001						
Protease	10.7	0.0002	21.5	< .0001			1.5	0.226	15.9	< .0001	**31.0**	< .0001		
Peroxidase	**61.1**	< .0001	8.9	0.0007	4.8	0.0034	74.1	< .0001	19.0	< .0001	5.4	0.0092		
Phenoloxidase	**98.8**	< .0001	27.7	< .0001			5.3	0.0271	12.0	0.0001	4.2	0.0233		
CO_2	**6.3**	0.0049												
NH_4^+	**273.4**	< .0001	10.6	0.0002			175.3	< .0001	49.1	< .0001	3.6	0.0362		
NO_3^-	16.9	< .0001					**60.3**	< .0001	5.8	0.0064	4.4	0.0195		
PO_4^{3-}	**600.1**	< .0001					40.4	< .0001	13.9	< .0001				
C_{mic}	**164.1**	< .0001	7.7	0.0017	3.8	0.011	24.6	< .0001	14.6	< .0001	8.1	0.0012	4.2	0.0072
N_{mic}	**705.4**	< .0001	46.8	< .0001	14.6	< .0001	26.1	< .0001			30.7	< .0001	15.2	< .0001
P_{mic}	**92.1**	< .0001	12.1	< .0001			20.6	< .0001	31.0	< .0001	4.4	0.0197		
C_{mic}:N_{mic}	2.8	0.073	28.3	< .0001	9.8	< .0001	**207.0**	< .0001	15.2	< .0001	14.4	< .0001	7.9	0.0001
C_{mic}:P_{mic}	54.1	< .0001	7.9	0.0014	3.1	0.027	**99.3**	< .0001	9.1	0.0006				
DOC	128.8	< .0001	62.5	< .0001	87.0	< .0001	71.8	< .0001	**203.7**	< .0001	12.0	0.0001	72.3	< .0001
DON	49.3	< .0001	**204**	< .0001	47.2	< .0001	24.7	< .0001	150.5	< .0001	13.9	< .0001	99.4	< .0001
pH			**52.1**	< .0001			30.1	< .0001	6.3	0.0044	12.9	< .0001		

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Table 3. Single linear regression (SLR) of litter C, N and P concentrations and litter stoichiometry (C:N and C:P) as well as microbial biomass C_{mic}, N_{mic} and P_{mic} concentrations and stoichiometry (C_{mic}:N_{mic} and C_{mic}:P_{mic}), with phosphate (PO_4^{3-}), nitrate (NO_3^-) and ammonium (NH_4^+) concentration, respiration activity (CO_2), and cellulase, chitinase, phosphatase, protease, peroxidase and phenoloxidase activity. The value shown is R^2 and stars indicate the significance (*$p <= 0.01$; **$p <= 0.01$; ***$p <= 0.0001$), the grey colouring indicates negative correlation while no colour indicates positive correlation.

	N		C		P		C:N		C:P		C_{mic}		N_{mic}		P_{mic}		$C:N_{mic}$		$C:P_{mic}$	
NH_4^+	0.61	***	0.13	**	0.23	***	0.58	***	0.10	**	0.42	***	0.54	***	0.45	***	0.06	*	0.03	
NO_3^-	0.25	***	0.14	**	0.01		0.25	***	0.01		0.03		0.14	**	0.08	**	0.14	**	0.01	
PO_4^{3-}	0.02		0.12	**	0.73	***	0.01		0.82	***	0.30	***	0.20	***	0.19	***	0.02		0.10	**
CO_2	0.01		0.04		0.12	**	0.02		0.15	**	0.03		0.00		0.01		0.06	*	0.02	
Cellulase	0.22	***	0.02		0.16	***	0.21	***	0.10	**	0.26	***	0.03		0.12	**	0.23	***	0.11	**
Chitinase	0.18	***	0.02		0.08	**	0.18	***	0.04		0.17	***	0.00		0.11	**	0.25	***	0.04	*
Phosphatase	0.02		0.01		0.00		0.02		0.01		0.02		0.01		0.10	**	0.08	**	0.02	
Protease	0.02		0.01		0.08	**	0.01		0.07	*	0.09	**	0.07	*	0.10	**	0.00		0.00	*
Peroxidase	0.41	***	0.07	*	0.17	***	0.38	***	0.10	**	0.32	***	0.22	***	0.37	***	0.00		0.02	
Phenoloxidase	0.53	***	0.08	**	0.20	***	0.52	***	0.10	**	0.46	***	0.31	***	0.35	***	0.01		0.10	**
total enzymes	0.45	**	0.45	**	0.03		0.43	**	0.00		0.08		0.28	*	0.28	*	0.29	*	0.04	

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Table 4. Single linear regression (SLR) of litter C, N and P concentrations and litter stoichiometry (C:N and C:P) as well as microbial biomass C_{mic}, N_{mic} and P_{mic} concentrations and stoichiometry (C_{mic}:N_{mic} and C_{mic}:P_{mic}), with microbial community structure. The value shown is R^2 and stars indicate the significance (*$p <= 0.01$; **$p <= 0.01$; ***$p <= 0.0001$), the grey colouring indicates negative correlation while no colour indicates positive correlation.

	C		N		P		C:N		C:P		C_{mic}		N_{mic}		P_{mic}		C_{mic}:N_{mic}		C_{mic}:P_{mic}	
Viridiplantae	0.00		0.30	*	0.80	***	0.27	*	0.69	***	0.44	**	0.56	**	0.26	*	0.03		0.20	
Metazoa	0.11		0.12		0.00		0.15		0.01		0.08		0.06		0.03		0.01		0.40	**
Bacteria	0.26	*	0.18		0.00		0.21		0.02		0.02		0.00		0.00		0.02		0.07	
Fungi	0.10		0.61	***	0.48	**	0.60	*	0.31	*	0.45	**	0.51	**	0.16		0.02		0.33	*
Proteobacteria	0.30	*	0.16		0.01		0.30		0.04		0.00		0.00		0.01		0.01		0.03	
Actinobacteria	0.15		0.11		0.03		0.09		0.01		0.02		0.11		0.12		0.22		0.05	
Alphaproteobacteria	0.05		0.02		0.00		0.02		0.00		0.05		0.09		0.00		0.73	***	0.19	
Betaproteobacteria	0.05		0.01		0.01		0.00		0.01		0.00		0.03		0.16		0.22		0.26	*
Gammaproteobacteria	0.32	*	0.16		0.01		0.17		0.04		0.00		0.01		0.01		0.13		0.05	
Firmicutes	0.03		0.00		0.00		0.00		0.00		0.00		0.04		0.06		0.14		0.07	
Ascomycota	0.14		0.70	***	0.46	**	0.70	***	0.28	*	0.51	**	0.55	**	0.19		0.01		0.33	**
Basidiomycota	0.01		0.16		0.21		0.15		0.17		0.31	*	0.31	*	0.40	**	0.02		0.03	
Dothideomycetes	0.07		0.61	***	0.62	***	0.58	**	0.42	**	0.55	**	0.52	**	0.19		0.00		0.47	**
Eurotiomycetes	0.29	*	0.84	***	0.30	*	0.83	***	0.14		0.56	**	0.50	**	0.32	*	0.00		0.28	*
Sordariomycetes	0.12		0.54	**	0.41	**	0.53	**	0.27	*	0.31	*	0.47	**	0.20		0.07		0.11	

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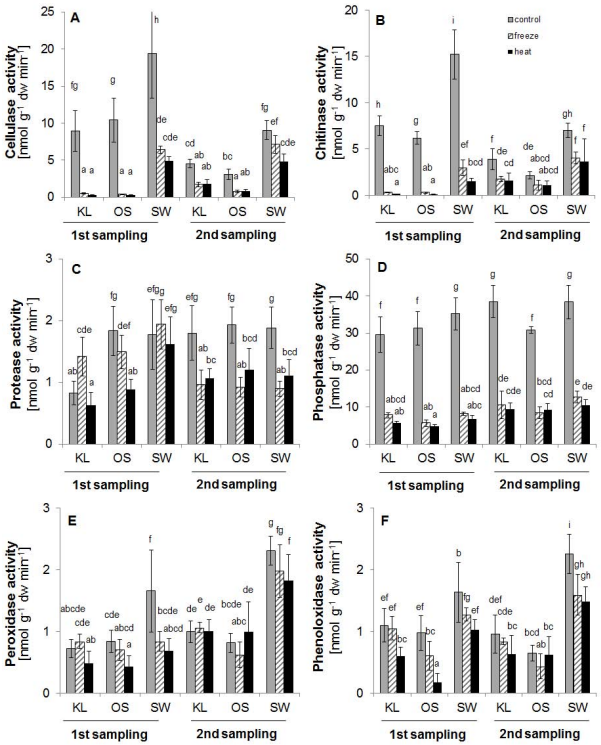

Fig. 1. Enzyme activities **(A)** cellulase activity, **(B)** chitinase activity, **(C)** protease activity, **(D)** phosphatase activity, **(E)** peroxidase activity and **(F)** phenoloxidase activity at different sampling times, treatments and sites. The different locations where the litter derived from were indicated as follows: Klausenleopoldsdorf (KL), Ossiach (OS) and Schottenwald (SW) and treatments are indicated as "control", "freeze" and "heat". Error bars indicate standard deviation. 1st-sampling was two weeks after treatments and 2nd-sampling was three months after treatments.

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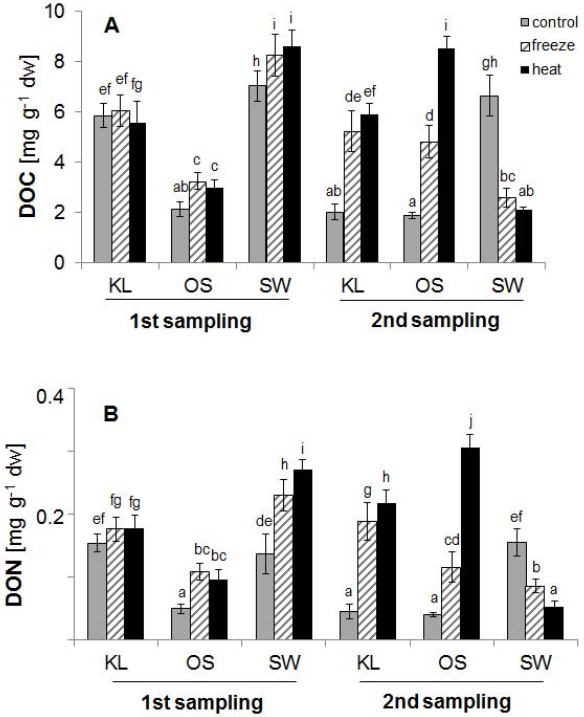

Fig. 2. (A) Dissolved organic carbon (DOC) and **(B)** dissolved organic nitrogen (DON) at different sampling times, treatments and sites. The different locations where the litter derived from were indicated as follows: Klausenleopoldsdorf (KL), Ossiach (OS) and Schottenwald (SW) and treatments are indicated as "control", "freeze" and "heat". 1st-sampling was two weeks after treatments and 2nd-sampling was three months after treatments.

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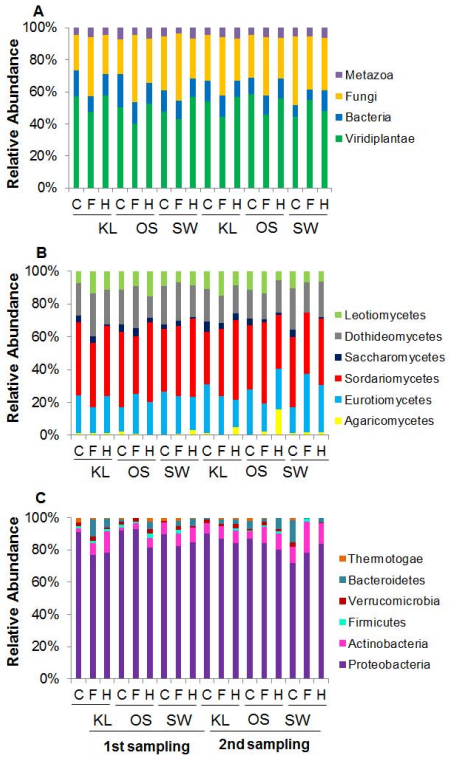

Fig. 3. Microbial community distribution, **(A)** general groups **(B)** fungal lineages of the *Basidiomycota* (*Agaricomycetes*), and the *Ascomycota* (*Leotiomycetes, Dothideomycetes, Saccharomycetes* and *Eurotiomycetes*) **(C)** bacterial lineages. The relative abundance of proteins observed at the different sampling time points include all treatments ("C": control, "F": freeze, "H": heat). The different locations where the litter derived from were indicated as follows: Klausenleopoldsdorf (KL), Ossiach (OS) and Schottenwald (SW). 1st-sampling was two weeks after treatments and 2nd-sampling was three months after treatments.

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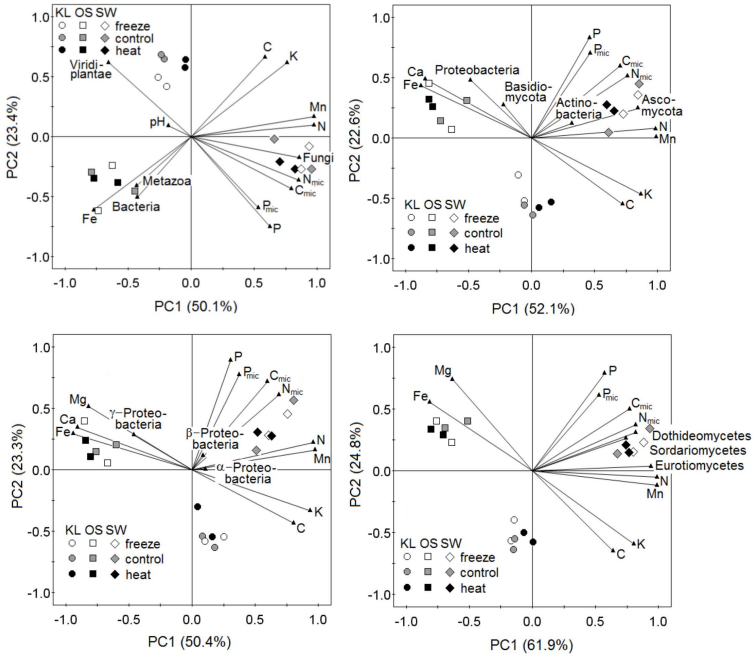

Fig. 4. Principal component analysis (PCA) of microbial community structure **(A)** two components of general domains *Bacteria*, *Fungi*, *Viridplantae* and *Metazoa* **(B)** two components of general bacterial and fungal phyla, **(C)** two components of the phylum of *Proteobacteria* **(D)** PCA of the phylum of *Ascomycota*, and the individual elements involved magnesium (Mg), calcium (Ca), iron (Fe), manganese (Mn), potassium (K), and phosphorus (P), carbon (C), nitrogen (N), and microbial biomass C (C_{mic}), microbial biomass N (N_{mic}) and microbial biomass P (P_{mic}). The number in brackets indicates the percentage of variance which is explained by the principal component. The different locations where the litter derived from were indicated as follows: circles: Klausenleopoldsdorf (KL), squares: Ossiach (OS) and diamonds: Schottenwald (SW). Varying colours were selected for the treatments, white for freeze treatment, grey for control and black for heat treatment.

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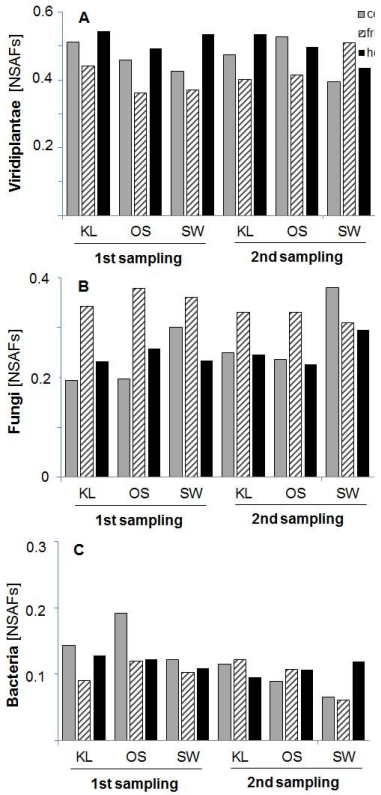

Fig. 5. Abundance of **(A)** *Viridiplantae*, **(B)** *Fungi* and **(C)** *Bacteria* at different sampling times, treatments and sites. The different locations where the litter derived from were indicated as follows: Klausenleopoldsdorf (KL), Ossiach (OS) and Schottenwald (SW) and treatments are indicated as "control", "freeze" and "heat". NSAF means normalized spectral abundance factor. 1st-sampling was two weeks after treatments and 2nd-sampling was three months after treatments.

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