Reply on

Interactive comment on "Effects of stoichiometry and temperature perturbations on beech litter decomposition, enzyme activities and protein expression" by K. M. Keiblinger et al.

We want to thank the anonymous reviewer for the constructive comments. We tried to substantially improve the manuscript with the suggestions made by the anonymous reviewer.

Anonymous Referee #2

In their methods the author's should mention which enzymes were used to examine enzyme activities. They only provide the generic term cellulose activity, where a substrate should be included. Additionally a time zero should have been calculated to examine the effects of the treatments

To provide more detailed information on how we examined enzyme activities, we changed the ***Material and Methods section*** *as follows:*

Deleted former page 11835 lines 5-10:

"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 450nm emission wavelength and at an excitation wavelength of 365nm using a Tecan Infinite M200 Fluorimeter (Werfen, Austria)."

Replaced by:

"Extracellular β-1,4-cellobiosidase ('cellulase'), β-1,4-N-acetylglucosaminidase, chitinase/lysozyme ('chitinase') and leucine amino-peptidase ('protease') were measured fluorimetrically (Kaiser et al., 2010). In short, 200 µl of litter suspension were pipetted in microplates and 50 µl substrate were added. The substrate used for measuring cellulose activity was 4-methylumbelliferyl-β-d-cellobioside (MUF-cellobioside). For chitinase activity two different substrates were used MUF-N-acetyl-β-d-glucosaminide and MUF-β-d-N,N′,N″-triacetylchitotrioside to cope the possibility of steric hindrance for polymer degradation. For phosphatase, MUF-P served as substrate. Activity was measured by following the release of 4-methylumbelliferone (MUF) from the respective substrate (cellulase, chitinases and phosphatase). For the protease assay L-leucine-7-amido-4-methyl coumarin (AMC) was used as substrate, andthe released coumarin adduct was measured with fluorescence. For calibration, methylumbelliferyl (MUF) was used for cellulose, chitinase and phosphatise activity; whereas AMC was used for calibration of protease activity. 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)."

Deleted former page 11835 line 14-15:

"For peroxidase activity 0.3% H_2O_2 solution was added to the assay."

Added former page 11835 line 14:

"Litter suspension was mixed with a 20 mM L-DOPA solution (1:1). After shaking of the samples for 10 min they were centrifuged, and pipetted into microplates. For peroxidase measurement wells additionally received 10 µl of a 0.3% H_2O_2 solution."

*To examine the effects of the treatments **Figure 1** was changed to include the baseline where enzyme activities were measured at time zero (before the treatment). For better readability of the figure we replaced letters indicating significant differences between enzyme activities with „stars" that indicate whether freezing or heat treatment were significantly ($p<0.05$) different from the control at the respective sampling time point.*

New Figure legend Figure 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", "freezing" and "heat". Error bars indicate standard deviation. 1st sampling was two weeks after treatments and 2nd sampling was three months after treatments. Respective enzyme activities at time zero are shown as horizontal lines which are dashed for KL, solid for OS and dotted and dashed for SW. Stars indicate a significant difference of the treatment to control at the respective sampling time."

*In addition we included the data of C:N and C:P values measured at the initial time point zero (baseline) to **Table 1.***

The authors fail to address implications of statistical significance. They indicate particular variables to be driving the system however neglect to state that all variables were significant. For example p11839 lines 8-12 the authors indicate that "treatment" was the most prominent influence on three enzymes and less so on others; however all enzymes were significant for treatment (Table 2). Furthermore they ignore interaction terms, which when provided were significant for all site*treatment with $p=0.05$ and 6 of the 9 variables presented. The authors need to either include the F statistic for other variables or explain explicitly why they were removed when F statistics for both site and treatment were present.

*We fully agree with the reviewers' critique that the implications of statistical significance were not presented and interpreted as it should be. To this end we focused on this particular limitation in our manuscript and changed the following in our **Results section**:*

Deleted former Page 11839 line 8-12:

"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)."

Deleted former page 11840 line 1-21

"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."

Deleted former Page 11839 line 22-29:

"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)."

*Furthermore we **added** the missing F-statistics into **Table 2**, to provide the whole information of interaction terms in the new version of the MS.*

New legend to Table 2:

"Multivariate ANOVA with the factors "site", "treat" (treatment) and "time" (sampling time) and their interaction terms, to evaluate their impact on different parameters like cellulase, chitinase, phosphatase, protease, peroxidase and phenoloxidase activity, respiration activity (CO_2), and on microbial elements and stoichiometry as well as pH value."

*In addition the **Results section** was changed as follows:*

Added former Page 11839 line 2:

"The factors were the different "treatments" ("treat") of the microcosms (heat and freezing), the litter types from three locations as the factor "site", which differed in nutrient stoichiometry, and sampling "time" points (2 weeks and 3 months after treatment). To evaluate our research questions and which of the above mentioned factors had the most pronounced effects on beech litter

decomposition a multivariate analysis of variance (MANOVA) was conducted. 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. MANOVA revealed that the interaction of all three factors "site*time*treat" was significant for chitinase activity, which means that a combination of all factors predicted chitinase activity. Similar results were observed for microbial biomass carbon and nitrogen (C_{mic} and N_{mic}), and their stoichiometric relationship (C_{mic}:N_{mic}). However, P_{mic} showed significant results for "time*site" and to a lesser extent "time*treat". Only two measures, phosphatase and respiration (CO_2), did not show significant results for an interaction term. Each factor itself was highly significant ($p<0.0001$) for phosphatase activity, with the highest F value observed for treatment, which therefore seemed to be most predictive for phosphatase. Respiration was predicted only by "site". All other parameters had significant interaction for at least one of the interaction terms with two factors. While for cellulase activity and protease activity the interaction term "time*treat" showed highly significant results, peroxidase and phenoloxidase were highly significant when combining the factors "time" and "site". Nevertheless, enzyme activities were all significant for each single factor except for protease activity and the factor "time"."

Added former Page 11839 line 15:

"The MANOVA showed strong influences of the factors "time*site" (besides "time*site*treat") which indicates that all factors had 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) separately for each treatment. Interestingly, there was no correlation of N_{mic} with chitinase under equilibrium temperature conditions but a strong linear regression to N_{mic} after heat and freezing perturbations, and similar but only moderate linear regression for P_{mic} (Table 3). Cellulase activity was more strongly significantly related to nutrients (N and P) after application of treatments compared to controls. After freezing treatment, peroxidase activity decreased although it was still significantly related to leaf litter N and P. Heat seemed to have a stronger impact on peroxidase activity, where only weak SLR with leaf litter N and P_{mic} was observed. Respiration was significantly related to leaf litter C and N for controls, but after temperature perturbation, significant linear regression was observed for leaf litter P and CO_2."

Discussion section changed

former page 11847 line 9

"In respect to Q1 we state that resource stoichiometry had a strong influence on microbial community structure (metaproteomic results) whereas community functions (enzyme activities) were generally affected by the interaction of at least two factors."

For me the key message of the paper was the effect of microbial community on decomposition dominates the discussion section, but is only briefly mentioned in the results. In the discussion the authors do an excellent job overviewing existing studies on the dominance of particular taxa over others while relating their data to the discussion. If this is the main take-home message then greater emphasis needs to be placed on the proteins earlier

in the paper. Finally they need to include greater detail on the methods of protein extraction and identification. Overall their methods for determining false discovery are accurate.

To focus stronger towards the key messages we added to the ***Introduction section:***

Added former page 11831 line 23:

"To examine these influences (i.e. stoichiometry, perturbations) on the microbial community in environmental samples a new generation of molecular methods (metagenomics, metatranscriptomics and metaproteomics) can be applied. While metagenomics studies the microbial community structure on the DNA level, metatranscriptomics analyses the prevailing RNA pool and, by examining these transcriptomes, aims at providing the gene activity profile of a community (Warnecke and Hess, 2009). To gain information on a deep phylogenetic level, this information can be provided by metagenomics and -transcriptomic approaches. Protein molecules and their modifications represent the final result of genetic expression (Ogunseitan, 2006) and have an intrinsic metabolic function, which can be used to directly relate microbial activities to defined organisms in multispecies communities. Therefore, metaproteomics, a post-genomic approach in nature, is considered to be a powerful tool in microbial ecology as it deals with finally processed proteins active for allocation of nutrients for microbial nutrition and allows for linking the structure and physiology of complex microbial consortia (Nannipieri et al., 2003;Nannipieri, 2006;Ogunseitan, 2006). Metaproteomics in environmental samples is challenging in terms of yields of proteins and to obtain high-quality resolution. However, it has been proven 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;Wang et al., 2011). Recently, Schneider et al., (2012) demonstrated the applicability of metaproteomics in combination with common approaches (e.g. enzyme activities, PLFAs) to analyse the community structure and function in leaf litter samples ."

In addition we changed the discussion section by putting the part with enzymes first and continued with metaproteomic discussions, as it is in the results section. Finally, we deleted parts from the ***Discussion section*** *to sharpen it up:*

Deleted former page 11844 lines 14-24:

"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 micro-niches within the soil matrix. Therefore we propose that in fungi-dominated leaf litter r-selected *Fungi* may function as the respective *Bacteria* in soils."

Deleted former page 11844 lines 11-13:

"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."

We agree with the reviewers comment that we did not give a lot of emphasis on the proteins in the ***Results section*** *therefore we added the following into the results section:*

Added former page 11847 lines 11 as described in response to Reviewer #1:

"To test whether treatment had a significant influence on phylogeny, we grouped the sites to provide replicates for the treatments at the respective sampling time points. We observed that bacteria transiently decreased after freezing compared to the control at the first sampling, while there was no significant difference in bacterial abundance at the second sampling. Fungi were significantly increased after freezing compared to control and heat treatment, which again was a short term effect. The treatments did not significantly affect metazoa shortly after application, but in the long term at the second sampling the abundance of metazoa was significantly increased for heat and freezing (Figure 3)."

To strengthen the focus of the manuscript on the key messages made in the former version, we skipped some parts of the original version, which are deleted in the new version of the MS. We deleted phosphate, nitrate and ammonium concentrations in Table 2 and 3 and in the ***Material and Methods section*** *as they are not part of the discussion.*

Deleted former page 11834 lines 19-25:

"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℃) until analysis."

In addition we deleted DOC and DON values in Table 2, and deleted Figure 2, as well as Figure 5 which is redundant with new Figure 2. To this end the following parts were deleted:

Results section

Deleted former page 11839 lines 15-21:

"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."

Discussion section

Deleted from former page 11845 line 25 page 11846 line 2:

"Enzyme activities were generally 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 availabilities for microbes. Dissolved organic carbon and DON were well correlated with respiration, which underpins that easily available C was respired by microbes."

All replicates should have been assayed for proteins, however due to the cost constraints it is understandable why they were not included. Yet proteomics is an emerging science and the authors rely heavily on proteomics for a key conclusion in the paper on the microbial community. They should have supplemented the proteomics data with some other established analysis of the microbial community. Granted that is most likely not feasible, the authors should explicitly elucidate the potential pit-falls of using proteomics data for the readers. Overall the combination of using proteomics for microbial community assessment coupled with the extensive study of enzyme kinetics make this an innovative study linking community to function. However the presentation and interpretation of some of the results limits the study.

Due to the fact that there was no possibility of replication due to cost limitation we followed a suggestion by Reviewer#1 who suggested combining treatments of different sites and we thereby created a new Figure, which is now Figure 3.

We are aware of the issue concerning replication in proteome studies. To this end we included the potential pitfalls of proteomics in the present manuscript in the ***Introduction section*** *to address the concerns of Reviewer#2 as follows:*

Added former page 11831 line 23:

"To examine these influences (i.e. stoichiometry, perturbations) on the microbial community in environmental samples a new generation of molecular methods (metagenomics, metatranscriptomics and metaproteomics) can be applied. While metagenomics studies the microbial community structure on the DNA level, metatranscriptomics analyses the prevailing RNA pool and, by examining these transcriptomes, aims at providing the gene activity profile of a community (Warnecke and Hess, 2009). To gain information on a deep phylogenetic level, this information can be provided by metagenomics and -transcriptomic approaches. Protein molecules and their modifications represent the final result of genetic expression (Ogunseitan, 2006) and have an intrinsic metabolic function which can be used to directly relate microbial activities to defined organisms in multispecies communities. Therefore, metaproteomics, a post-genomic approach in nature, is considered to be a powerful tool in microbial ecology as it deals with finally processed proteins active for allocation of nutrients for microbial nutrition and allows for linking the structure and physiology of complex microbial consortia (Nannipieri et al., 2003;Nannipieri, 2006;Ogunseitan, 2006). Metaproteomics in environmental samples is challenging in terms of yields of proteins and to obtain high-quality resolution. 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;Wang et al., 2011) metaproteomics has been proven to be useful. To analyse the

community structure and function in leaf litter samples using metaproteomics in combination with common approaches (e.g. enzyme activities, PLFAs) has been demonstrated by Schneider et al., (2012)."

In addition the following was added to the former version of the ***Discussion section*** *(including suggestions made by Reviewer#1):*

Former Page 11843 line 7 (now in front of the part which was former starting at page 11844 line 25):

"A metaproteomic approach was applied in order to determine microbial community structure and link it to microbial function. Nucleotide-sequencing techniques that typically target 16S or 18S rRNA or even the entire metagenome that are usually applied for community analysis are well suited to analyse community structure on a detailed phylogenetic level (Fierer et al., 2007). However, these techniques are still limited by the fact that DNA is present in both active and inactive cells and thus sequencing might not be best suited for determining the active members of a community (Nocker and Camper, 2009). RNA-based approaches which represent only the active part of the microbial community (e.g. (Baldrian et al., 2011) suffer from the fact that RNA half-life time is very short (Nocker and Camper, 2009). RNA might be degraded and not detectable anymore while the active products, the proteins, are still present and active in the ecosystem. Active metabolic pathways can be targeted with metatranscriptomics; however, there is a lack of correlation between protein levels and mRNA (Siggins et al., 2012). Due to these limitations we decided to use metaproteomics which provides functional - metabolic - information particularly at the intracellular level (Keiblinger et al., 2012;Bastida et al., 2012). Metaproteomics has the potential to link processes/functions to microbes on the basis of the active building blocks in the system, namely the proteins, as was shown recently for litter-inhabiting microbial communities collected in the field (Schneider et al., 2012). However, data on microbial abundance obtained in the present study should be considered with respect to the fact that the respective metagenome data was not available for the generated mass spectra and that their assignment to proteins was thus based on general databases. The obtained results as well as data interpretation might be improved with data from the corresponding metagenome analysis by using related genome sequences from the respective litter samples for advanced assignment of spectra. However, increasing numbers of pyro-sequencing studies have already improved the database situation, and additional database information will be available in the future by a continued sequencing effort."

We deleted in the ***Introduction section*** *the following to improve the readability and to focus the MS more towards metaproteomics.*

Deleted former page 11830 lines 20-23:

"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."

Deleted former page 11831 lines 1-2

“It is possible to link these activities to C sequestration on an ecosystem scale (Allison et al., 2006).”

Deleted former page 11831 lines 6-10:

“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).”

Moved from former page 11831 lines10-113 to former page 11830 line 20:

“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).”

Deleted former page 11831 lines 18-23:

“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).”

Deleted former page 11831 line 27 – former page 11832 line 1:

“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 defined organisms in multispecies communities.”

Finally, we think that the presentation of the data has greatly improved with the new tables and figures as well as the restructured Results and Discussion sections and now provides compelling statements of the impact of driving factors on leaf litter decomposition.

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