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

We would like to thank the anonymous reviewer for the constructive comments. We agree with the reviewer that the manuscript will improve with the suggestions made by the anonymous reviewer.

To describe the nature, size and setup of microcosms we will add two more References (Mooshammer et al., 2011;Wanek et al., 2011) where this set up was already described. Additionally we will add the following text:

Page 11833 line 4:

“After homogeneous distribution of the inoculum, microcosms which had been prepared from PVC plastic tubing (10 cm length, 12.5 cm diameter, bottom lid perforated plastic grid, Wanek et al., 2011) were filled with 60 g of litter fresh weight for all three litter types (KL, OS and SW), treatments, controls and sampling time points, with five replicates each giving 90 microcosms in total. The microcosms were laced in trays, lined with wet cloth and were capped with lids of micromesh cloth to ensure CO_2 exchange during incubation (Mooshammer et al., 2011). For respiration measurement, microcosms were closed with airtight rubber on the bottom and a reduction gadget on the top to use the SRC-1 chamber (PPSystems, USA).”

The perforated bottom lid enabled nutrient leaching from the microcosms which therefore represent an open system. This was proven by the fact that no significant changes in litter C:N:P were observed over the entire experiment (6 months). Therefore we think that the linear correlations we found stem from different behaviour of the three litter types. To address the reviewer’s concerns about calculating correlations with ratios C/N and C/P and not with C, N and P concentrations, Table 3 was recalculated for the different treatments separately now including correlations with concentrations of C, N and P. We referred to nutrient stoichiometry instead of nutrient concentrations to link nutrient availability from the substrate with consumer requirement as a conceptual way used in ecological theory. We try to understand patterns and processes in terrestrial habitats and to elucidate elemental imbalances, via ecological stoichiometry as a measure of dissimilarity in relative supply of an element between organism and its resource according to Sterner and Elser (2002). This experiment is part of a project consortium where studies on stoichiometric effects were a major issue (Mooshammer et al.,

2011;Wanek et al., 2011;Hall et al., 2011b;Hall et al., 2011a;Keiblinger et al., 2010). For reasons of continuity we would prefer to leave the term “stoichiometry” in the manuscript.

Concerning the natural relevance of the treatments used, we know that rises in temperate to 20-40℃ are usually only temporal especially in spring time when the forests are leafless in the Vienna area (Bruckner, 1998). However, future scenarios forecast an increase of the Earth’s surface temperature by 1.5 – 6.4℃ by the end of the 21 st century (IPCC 2007). Furthermore, the frequency and intensity of extreme weather events such as heat-waves have been projected to increase considerably in the forthcoming decades (Christensen and Christensen, 2007;Fischer and Schar, 2010). In Austria beech is a common tree species in forests, and in this area hot periods defined after Kysely et al (2000) will appear more frequently and this will also increase forest surface temperature.

As we used a model system (i.e., microcosms to test the connectivity of beech litter stoichiometry, microbial community structure and activity as well as stress tolerance), we decided to use rather harsh temperature stress conditions, which might not yet occur in the field. Heat and frost stress are common during leaf litter decomposition, although maybe not with these intensities. However, we think that even if the applied temperature treatments were rather extreme, we are convinced that our study results provide valuable insights into microbial stress response during litter decomposition.

Therefore we will add into the introduction section:

Page 11831 line 23:

“These “perturbations” are thought to become more frequent and the intensity of extreme weather events such as heat-waves have been projected to increase considerably in the forthcoming decades (Christensen and Christensen, 2007;Fischer and Schar, 2010). These future scenarios forecast an increase of the Earth’s surface temperature by 1.5 – 6.4℃ by the end of the 21 st century (IPCC 2007).”

Page 11832 line 13:

“in a model system under harsh stress conditions.”

Additional changes within the Discussion section: we will add:

Page 11846 line 16:

“Increases in Earth’s surface temperature and therefore forest floor was tested in soil warming field experiments. Heat effects on microbial community structure were difficult to detect although microbial function (i.e. respiration rates were largely altered (Schindlbacher et al., 2011).”

Interestingly the suggested new table 3 revealed different changes in the relationship of nutrient to enzyme activities under stable (constant temperature as in control samples) conditions and non-stable (heat and freezing treatment) conditions.

The paragraph in the results section will be adapted to changed table 3:

Page 11840 line 3:

"In order to identify the main site/litter properties that drive microbial activity SLR was applied (Table 3). SLR was performed for each treatment separately. Generally, litter C:N ratios and litter N concentrations were 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 under stable conditions (control treatment). Furthermore, enzyme activities were clearly influenced by nutrients contained in the microbial biomass under stable conditions. The activities of the C-acquiring enzymes cellulases, peroxidases and phenoloxidases correlated positively with C_{mic} for the control treatment. In addition, N_{mic} correlated positively with peroxidase and phenoloxidase activity, two enzymes which release N that is bound in plant cell walls due to their ligninolytic action (Table 3), but this correlation did not persist for peroxidase after heat treatment. On the other hand, cellulase activity showed stronger relationships with N contents and C:N ratios after heat and stress treatments. Chitinase activity strongly related to C:N ratios and N content after freezing, while for heat and control treatments this effect was medium and weak, respectively. The correlation of between phosphatase activity and litter C:N ratios was much stronger for the freezing treatment (R^2~39) than for heat and control treatments (R^2~18). Protease showed no significant relationship (Table 3) with resource nutrients / stoichiometry after freezing, although in heat and control treatments protease was weakly related to litter P content. Concentrations of NH_4^+, NO_3^- and PO_4^{3-} were related to N_{mic} (Table 3), with the exception of nitrate after freezing. The correlations between litter C:N ratios and NH_4^+ concentrations ranged from $0.53 < R^2 < 0.71$ and for NO_3^- concentrations from $0.18 < R^2 < 0.23$, for litter C:P ratios and PO_4^{3-} concentrations a range from $0.78 < R^2 < 0.83$ was observed for control, heat and freezing respectively."

We will discuss this as follows:

Page 11844 line 2:

"In the present study a weak positive impact of litter P content on N-acquiring enzyme activities (protease and chitinase activity) was observed under stable conditions, while Olander and Vitousek

(2000) found that P addition had no impact on N-acquiring enzymes. Generally, nutrients favoured high enzyme activities although the freezing resulted in stronger disturbances (Table 3) of enzyme activities as can be seen in Figure 1 as well."

Total mass loss of the system was recorded, but we did not observe significant mass losses after duration of six months of our experiment and therefore we did not present the data. The total mass loss was calculated of separate microcosms, which were sampled 15 months after the experiment, and this resulted in significant differences in mass loss according to litter site (Mooshammer et al., 2011). In addition we calculated accumulated respiration over the whole experiment.

We will add in the Methods section:

Page 11833 line 12: "Accumulated respiration was calculated assuming linear transition between weekly measurements."

We will add in the results section:

Page 11840 line 21:

"Total mass of the system did not result in significant mass losses after duration of six month, but separate microcosms, which were sampled after duration of 15 months, resulted in significant differences in mass loss according to litter site (Mooshammer et al., 2011). Accumulated respiration was greater in treated leaf litter compared to control samples, especially after freezing at the second sampling."

Page 11846 line 23:

"Additionally accumulated respiration was greater in treated leaf litter compared to control samples, especially after freezing at the second sampling."

We agree on the reviewer's opinion that proteins might have persisted after the treatments as they might be stable. Based on our data we cannot show if a protein was synthesized before or after the treatment but we are able to draw conclusions from the fact that relative abundances of microbial groups or enzymes change in response to the treatment. While RNA based approaches would most likely miss this information because degradation of instable RNA might be influenced by the temperature treatments in our dataset the changes of abundances of proteins that sustained the treatments and might be still active are incorporated. This links our data closely to enzymatic activity and provides specific information that cannot be given by other large-scale approaches investigating

the activity of the entire microbial community. DNA/RNA microbial community analyses are also considered in this project consortium but so far are still under construction, by our project partners, and are beyond the scope of this paper. To clearly identify the use of proteomics instead of DNA/RNA in the present MS we will added the following:

Page 11843 line 7:

“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 (Fierer et al., 2007). However, in the present study we focused on active members of a community, the active products, the proteins, which are still present and active in the ecosystem, although RNA might be degraded and not detectable anymore due to very short half-life time of RNA (Nocker and Camper, 2009). Metaproteomics has the potential to link processes/functions to microbes on a detailed phylogenetic level 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).”

Concerning the evaluation of proteomic data, the reason why we decided to use the single value data instead of the average of different litter samples undergoing the same treatment, was that the interconnection of litter-types could not be recorded if averages were used; however we take the reviewers point that this approach weakens the robustness of discussions due to missing statistical analysis.

We propose to delete Figure 3 (Microbial community distribution) from the manuscript. Instead, we will include a new Figure 3 with a heavier focus on metaproteomic replicates to combine samples of different litter types undergoing the same treatment and recording the direction of their development. We propose to change the discussion section by the outcomes of this data analysis.

We will add the following to the results section:

Page 11841 line 11:

“To test whether treatment had a significant influence on phylogeny, we grouped the sites together to deal with replicates for the treatments at the respective sampling time points. We observed that bacteria significantly decreased after freezing compared to the control, which was a transient effect because there was no significant difference in bacterial abundance between freezing treatments and control at the second sampling. Fungi were significantly increased after freezing compared to control

and heat treatment, which again was a short term effect. In contrast to bacteria and fungi, both treatments significantly increased affect metazoal abundance only in the long term (Fig. 3)."

Fig. 3 was changed by using different sites as replicates for treatments to do statistical analysis of the impact of heat and freezing during decomposition. This resulted in similar outcomes as discussed earlier, but with a stronger data background which will be discussed in detail. We believe that the metaproteome data are worth including in the MS, as they help to illustrate the point we are making in the paper that freezing had a stronger impact on the decomposition process.

In original Table 3 the number of data points used were from three litter types, two treatments and two sampling time points in five replicates which results in 90 data points used for each SLR. Given that the samples were independent replicates of the litter types (5 replicates from 3 different litter types) and treatments (5 replicates from 3 different treatments) each represented by one independent microcosm sampled at a specific time point (no repeated measure), we therefore used all data points in the single linear regression analysis as suggested in Motulsky and Christopoulos (2004). Given the natural variability in elemental stoichiometry between samples of the same litter type the data presented can also be seen to be representative of a continuum of C:N:P stoichiometric ratios within litter of one species (European beech) instead of viewing them as distinct litter types. To better represent the treatments separately this was considerate with the data, presented in Table 3 and therefore it was changed to separate the SLR for the different treatments.

Tables and Figures

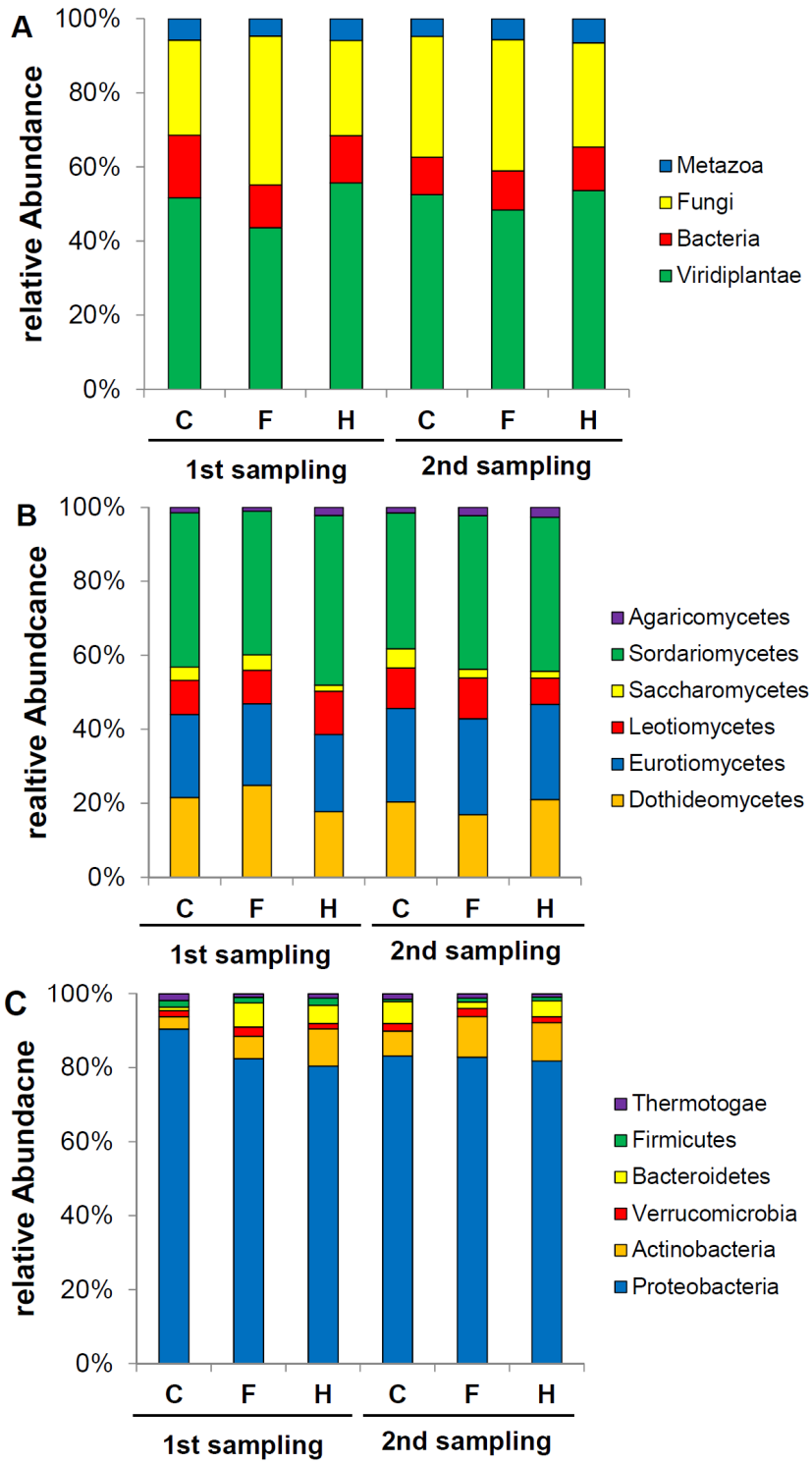

Fig. 3. Microbial community distribution comparing treatments (by grouping sites): (A) general groups, (B) fungal lineages of Basidiomycota (*Agaricomycetes*) and Ascomycota (*Leothiomycetes, Dothideomycetes, Saccharomycetes*), and (C) bacterial lineages. Given are relative abundances of proteins observed at the different sampling time points comparing all treatments ("C" control; "F" freezing; "H" heat). The values displayed are means of the three different sites to obtain replicates for

analysis of treatments. 1st sampling was two weeks after treatments and 2nd sampling was three months after treatments.

Table 3. Single linear regression (SLR) of litter C, N and P concentrations as well as microbial biomass C_{mic}, N_{mic} and P_{mic} concentrations with ammonium, nitrate, phosphate, respiration activity (CO_2) and cellulase, chitinase, phosphatase, protease, peroxidase and phenoloxidase activity. The value shown is R^2 and stars indicate the significance (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.0001$).

	n=30	N		C		P		C:N		C:P		C_{mic}		N_{mic}		P_{mic}	
control	NH_4^+	0.55	***	0.16	*	0.22	**	0.53	***	0.08		0.55	***	0.67	***	0.47	***
	NO_3^-	0.25	**	0.26	**	0.00		0.25	**	0.02		0.09		0.3	**	0.14	*
	PO_4^{3-}	0.07		0.23	**	0.22	**	0.04		0.83	***	0.32	**	0.13	*	0.20	*
	CO_2	0.39	**	0.30	**	0.03		0.36	**	0.00		0.20	*	0.35	**	0.28	**
	Cellulase	0.24	**	0.00		0.25	**	0.23	**	0.2	*	0.31	**	0.00		0.01	
	Chitinase	0.39	**	0.02		0.24	**	0.39	**	0.16	*	0.30	**	0.00		0.00	
	Phosphatase	0.18	*	0.20	*	0.03		0.18	*	0.00		0.18	*	0.20	*	0.13	
	Protease	0.00		0.03		0.15	*	0.00		0.13		0.07		0.13		0.19	*
	Peroxidase	0.58	***	0.06		0.46	***	0.53	***	0.26	**	0.76	***	0.51	***	0.47	***
	Phenoloxidase	0.60	***	0.09		0.33	**	0.55	***	0.19	*	0.66	***	0.38	**	0.37	**
heat	NH_4^+	0.77	***	0.12		0.20	*	0.71	***	0.13		0.28	*	0.55	***	0.66	***
	NO_3^-	0.36	**	0.09		0.07		0.33	**	0.04		0.09		0.20	*	0.15	*
	PO_4^{3-}	0.00		0.26	**	0.70	***	0.00		0.78	***	0.41	***	0.23	**	0.24	**
	CO_2	0.00		0.13		0.28	*	0.01		0.40	**	0.16	*	0.03		0.08	
	Cellulase	0.78	***	0.08		0.41	**	0.76	***	0.26	**	0.44	***	0.82	***	0.42	***
	Chitinase	0.40	**	0.06		0.10		0.37	**	0.07		0.12		0.31	**	0.55	***
	Phosphatase	0.19	*	0.08		0.00		0.19	*	0.00		0.02		0.07		0.12	
	Protease	0.14	*	0.00		0.26	*	0.16	*	0.17	*	0.13		0.38	**	0.09	
	Peroxidase	0.25	**	0.05		0.04		0.23	*	0.04		0.00		0.09		0.22	**
	Phenoloxidase	0.63	***	0.12		0.18	*	0.61	***	0.11		0.16	*	0.44	***	0.31	**
freezing	NH_4^+	0.67	***	0.13		0.27	**	0.61	***	0.14	*	0.34	**	0.50	***	0.39	**
	NO_3^-	0.15	*	0.17	*	0.08		0.18	*	0.21	*	0.06		0.01		0.03	
	PO_4^{3-}	0.03		0.00		0.74	***	0.01		0.87	***	0.27	**	0.29	**	0.20	*
	CO_2	0.12		0.05		0.38	**	0.06		0.42	***	0.30	**	0.21	*	0.36	**
	Cellulase	0.81	***	0.17	*	0.63	***	0.75	***	0.41	**	0.60	***	0.89	***	0.31	**
	Chitinase	0.72	***	0.22	**	0.45	***	0.66	***	0.27	**	0.40	**	0.71	***	0.28	**
	Phosphatase	0.40	**	0.15	*	0.04		0.39	**	0.00		0.03		0.15	*	0.05	
	Protease	0.01		0.01		0.09		0.01		0.05		0.08		0.07		0.01	
	Peroxidase	0.48	***	0.11		0.15	*	0.43	***	0.06		0.28	**	0.30	**	0.24	**
	Phenoloxidase	0.7	***	0.1		0.30	**	0.71	***	0.12		0.52	***	0.59	***	0.19	**

Response to other minor comments not covered by above:

We will change the term “freeze” to “freezing” over the whole MS.

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