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Short communication

Microbial functionality as affected by experimental warming of a temperate mountain forest soil—A metaproteomics survey

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ABSTRACT

Soil microbes play an important role in terrestrial carbon (C) cycling, but their functional response to global warming remains yet unclear. Soil metaproteomics has the potential to contribute to a better understanding of warming effects on soil microbes as proteins specifically represent active microbes and their physiological functioning. To quantify warming effects on microbial proteins and their distribution among different functional and phylogenetic groups, we sampled forest soil that had been artificially warmed (+4 °C) during seven consecutive growing seasons and analyzed its metaproteomic fingerprint and linked to soil respiration as a fundamental ecosystem service.

Bacterial protein abundances largely exceeded fungal abundances at the study site but protein abundances showed only subtle differences among control and warmed soil at the phylum and class level, i.e. a temperature-induced decrease in *Firmicutes*, an increase in *Agaricomycetes* and *Actinobacteria*, and a decrease in the Asco/Basidiomycota ratio. Community function in warmed soil showed a clear trend towards increased proteins involved in microbial energy production and conversion, related to the increased CO_2 efflux from warmed soil as a result of stress environmental conditions. The differences in community function could be related to specific phyla using metaproteomics, indicating that microbial adaptation to long-term soil warming mainly changed microbial functions, which is related to enhanced soil respiration. The response of soil respiration to warming (+35% soil CO_2 efflux during sampling) has not changed over time. Accordingly, potential long-term microbial adaptations to soil warming were too subtle to affect soil respiration rates or, were overlaid by other co-varying factors (e.g. substrate availability).

1. Introduction

Soils are a huge reservoir of organic Carbon (C), and the biotic CO_2 efflux from soil (=soil respiration) is one of the largest fluxes in the global C cycle. Global warming stimulates C flux from soil to the atmosphere by increased decomposition of SOM and consequently increases respiration rates of decomposer microbes (Cox et al., 2000; Qian et al., 2010; Trumbore et al., 1996). Alpine regions are strongly affected by global warming (Pepin et al., 2015) and C stocks of calcareous forest soils in the study region have been identified as particularly vulnerable (Prietzel et al., 2016). Among all natural environmental compartments, soils likely contain the greatest microbial biomass and diversity, which classifies them as one of the most challenging habitats for microbiologists (Mocali and Benedetti, 2010; Nannipieri et al., 2003; Torsvik and Øvreås, 2002). How these complex communities respond to a warmer climate remains poorly understood, since the long-term response of decomposer microbes may deviate from their short-term response to rising temperatures. There is evidence that long-term warming potentially alters decomposer community physiology and accordingly the CO_2 efflux from soil (Allison et al., 2010;

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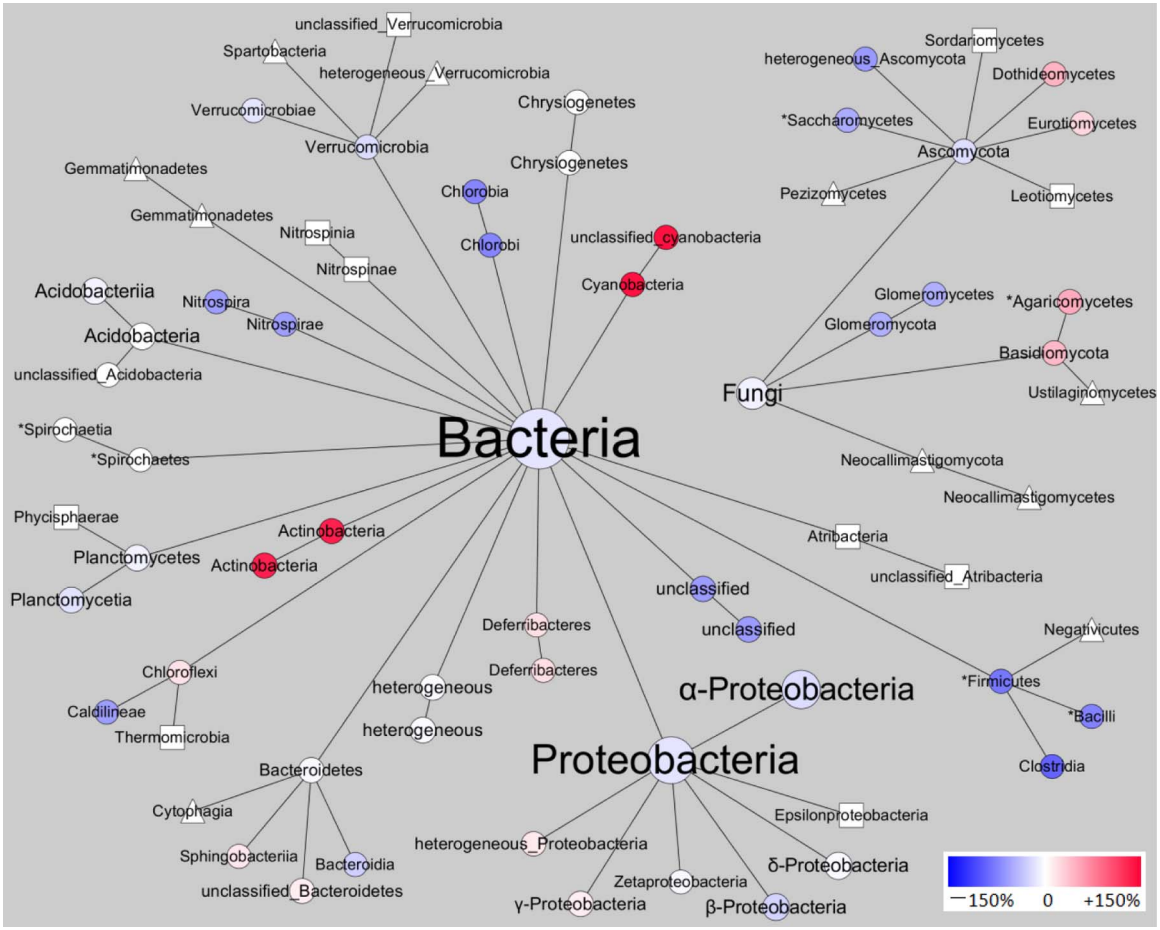

Fig. 1. Phylogenetic network of proteins. Relative abundances calculated from the sum of the normalized spectral abundance factor (NSAFs) found for the warmed plot. The size of the nodes and labels represents the abundance of the corresponding taxonomic group. Labels are class-level assignments and shape-indicators are: Triangle, only abundant in control; Rectangle, only abundant in warmed plot; Circle, abundant in both. For the proteins that were abundant in both treatments (shown in circles) the gradient of colour represent the increase (red) or decrease (blue) of the % change in relative abundance of the treatment plots compared with control. Values are the mean of three biological replicates. *indicates independent-samples *t*-test at a significance level of 0.05. Taxonomic networks were generated using the perfuse force directed layout in Cytoscape 3.3.0 (free download: http://www.cytoscape.org/download.php) and manual adjustments.

Bardgett et al., 2008; Rousk et al., 2012). Long-term warming may provide competitive advantage for species adapted to higher temperatures (Rinnan et al., 2009; Yergeau et al., 2012), which are supposed to allocate more of a given C substrate into their biomass and less into respiration (Fierer et al., 2009; Keiblinger et al., 2010). This could, in turn, mitigate warming effects through reduced CO_2 emissions from soil over time (Allison et al., 2010; Knorr et al., 2005). Therefore, together with warming induced changes in C substrate availability and quality, microbial adaptation mechanisms may be the primary drivers of future soil C dynamics and soil respiration rates (Craine et al., 2013; Frey et al., 2013; Wei et al., 2014). Warming driven changes in microbial functioning are however difficult to detect and it is even more difficult to assign them to specific changes in microbial community structure. New metaproteomic approaches can contribute to a better understanding of this link. While, amplicon sequencing approaches provide information on microbial community structure, they lack information on functional changes. Meta-omics approaches, especially metaproteomics, allow the simultaneous examination of various protein functions (e.g., transcription, signal transduction) and responses (such as changes in relative abundances of functional proteins), and therefore help to unravel the complex interplay between soil respiration dynamics, microbial community structure, and physiological functioning of soil microbes in a changing environment (Aylward et al., 2012; Bastida and Jehmlich, 2016; Bastida et al., 2009; Kim et al., 2010).

We sampled soil from a long-term soil warming experiment (Achenkirch) and performed a metaproteomic survey along with a characterization of the topsoil C. We hypothesized that the protein abundances would shift towards a microbial community that is dominated by oligotrophic lifestyle in warmed plots, as has been observed in other long-term warming of temperate forest soils (DeAngelis et al., 2015). Because soil respiration rates were still strongly accelerated after 7 years of warming, we further hypothesized increasing functional proteins towards more expenses in energy production (i.e. towards respiration related processes) when compared to microbial growth and other metabolism-related functions.

2. Material and methods

In the present study, soil samples were collected from the warming experiment located in Achenkirch, Austria (11° 38′ 21″ East; 47° 34′ 50″ North). The study site is located at 910 m a.s.l. in the North Tyrolean Limestone Alps. The 130-year-old mountain forest consists of Norway spruce (*Picea abies*) with inter-spread of European beech (*Fagus sylvatica*) and silver fir (*Abies alba*). Mean annual air temperature and precipitation were 6.9 °C and 1506 mm, respectively (1992–2012, ZAMG). Soils were shallow Chromic Cambisols and Rendzic Leptosols with high spatial variability (FAO, 1998). More details on the study site and soil characteristics are given in Supplementary Table S1. Soil was warmed 4 °C above ambient continuously during snow-free seasons since 2005 using resistance heating cables which were buried ~ 2–3 cm into the mineral soil (Schindlbacher et al., 2009). Three experimental control and warmed plots with subplots size of 2 × 2 m each were

imposed in this study. A homogenized composite soil sample (10 subsamples obtained with a 2 cm diameter corer) was taken from the Ah horizon (0–5 cm) from each of the three warmed and three control plots at 3rd of October 2012. The samples were stored in cooling boxes and transported to the laboratory in Vienna where the soil samples were homogenized with 2 mm mesh sized sieves and stored frozen at −80 °C for soil metaproteome analysis as suggested in Keiblinger et al. (2016).

Total soil C and N were measured by combustion using a C/N analyzer (Thermo Finnigan Flash EA 1112). Soil carbonate content was determined by treating soil samples with 10% HCl, connected to a Scheibler apparatus according to Austrian Standards (OENorm-L1084, 2006). Organic carbon was determined by subtracting the inorganic C content from the measured total C (Blume et al., 2015). Dissolved organic C (DOC) was determined through soil extraction (2 h shaking with a soil to distilled water ratio of 1:5 w/v), followed by centrifugation and analysis of the extract solution on a Multimode Plate Reader (PerkinElmer, EnSpire) at a wavelength of 254 nm (Brandstetter et al., 1996). Soil CO_2 efflux in the field was measured using an infrared gas analyzer as described in (Schindlbacher et al., 2009).

In order to extract proteins (with three biological replicates for each treatment) from forest soil samples, frozen soil samples were ground in liquid N_2 prior to extract proteins based on the SDS–phenol method previously described by Keiblinger et al. (2012). For more details on extraction as well as sample processing & mass spectrometry see electronic Supplementary file 1. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaíno et al., 2016) partner repository with the dataset identifier PXD004428 and 10.6019/PXD004428.

The obtained MS/MS data were searched against NCBI entries, downloaded from NCBI server webpage https://www.ncbi.nlm.nih.gov/at the 25th June 2014. The new version of PROteomics results Pruning and Homology group ANotation Engine (PROPHANE) workflow (www.prophane.de) was used to assign proteins to phylogenetic (Fig. 1) and functional (Fig. 2B & C) groups. Protein abundances were calculated based on the normalized spectral abundance factor (NSAF, Zybailov et al., 2006). The obtained proteins were classified into cluster of orthologous groups (COG; prokaryotic proteins) and TIGR (main role and subrole) categories based on their protein assignments (Fig. 2B & C).

Diversity of microbial taxa and functional categories was calculated at class-level with Shannon diversity index (H′) by considering the proportion of each class/functional category in NSAF values (i) relative to the total number of classes/functional categories in NSAF value (p_i), and then multiplied by the natural logarithm of this proportion ($\ln p_i$). The products are summed across the total number of classes/functional categories (S) and multiplied by (−1) see Eq. (1).

$$H' = -\sum_{i=0}^{S} p_i \ln(p_i) \quad (1)$$

Significance of the diversity index between the two treatments was calculated by independent-samples *t*-test at a significance level of 0.05. Cytoscape 3.3.0 was used for visualization of the community structure and the NSAF values of microbial class level were taken to create taxonomic networks.

3. Results and discussion

3.1. Taxonomic changes

Bacterial proteins dominated the microbial community compared to the amount of fungal proteins as expressed by the sum of the NSAF values (Table 1). This high bacterial abundance in the topsoil confirms a previous assessment (Schindlbacher et al., 2011) and is likely associated with the comparatively high pH at the calcareous study site (Bárcenas-Moreno et al., 2011; Rousk et al., 2009). However, the observed small and almost equal amount of fungal proteins in warmed and control plots could also be attributed to a generally poor representation of environmentally-relevant eukaryotes (including fungi) in genomic databases (Kollmar et al., 2014). Two hits on archaeal proteins were detected in only one replicate of our control samples, which might be due to the fact that the present site is characterized by nearly neutral pH, whereas archaeal abundance was mentioned to be high in acidic temperate forest soils (Kemnitz et al., 2007), and to decrease with rising pH (Tripathi et al., 2013). Data shown here are based on the level of phylum and class, and to some extent on the level of order. With the present data set, statistical analysis on a deeper level of resolution for microbial taxa was not possible with high confidence. However, the resolution level for soil metaproteomics is generally lower compared to metagenomics and −transcriptomics partly due to stabilization of proteins from the soil extracellular matrix (Keiblinger et al., 2016; Nielsen et al., 2006). Microbial diversity at class-level as revealed by the averaged Shannon index (H′) based on NSAF values for bacteria and fungi was slightly (albeit not significantly) lower in the warmed plot compared to the control (Table 1). This was surprising because environmental parameters did differ between control and warmed plots: Concentrations of total C, dissolved organic C (DOC) and total organic C (TOC) were significantly lower in the warmed plot than in the control ($P < 0.05$), while respiration was higher (Table 1). These parameters have been considered as part of a multifunctionality index (Bastida et al., 2016; Wagg et al., 2014), which is basically an index for the active diversity of an ecosystem. This would suggest that event though microbial diversity at class-level was not significantly different in warmed plots, the functional diversity did in fact change.

However, when examining microbial community structure on the phylum level (Fig. 1), we observed a few significant differences between control and warmed soil. For instance, the relative abundance of the phylum of *Firmicutes* was approx. 80% lower ($P < 0.05$) in warmed soil (Fig. 1). Within the phylum of *Firmicutes*, the class *Bacilli* was less abundant ($P < 0.05$, Fig. 1) with soil warming. *Bacilli* have a strong ability to secrete extracellular enzymes (McSpadden Gardener and Driks, 2004) and a large versatility for carbohydrate utilization (Yadav et al., 2011). Therefore, the low abundance of this class might explain the low concentrations of DOC in warmed plots (Table 1), indicating a decrease in SOC decomposition and a deficiency in easily available C.

The most abundant bacterial phylum over all samples were *Proteobacteria* (> 60%), dominated by the class of α-*Proteobacteria* (40% of total bacteria). Within the *Proteobacteria*, warming induced changes on the resolution of orders, such as *Rhizobiales* and *Rhodospirallales* significant declined ($P < 0.05$; Fig. 2A). The decline in relatively fast growing *Rhodospirallales* had also been found in other long-term warming experiments, which was suggested to be related to their copiotrophic life strategy (DeAngelis et al., 2015; Deslippe et al., 2012). While DeAngelis et al. (2015) found that *Rhizobiales* mostly increased with warming, we found a decline in the present study. This might be explained by the fact that *Rhizobiales* abundance is associated with high DOC concentrations (Bastida et al., 2015, 2016), but DOC was low in warmed plots.

Among the phyla that most strongly increased with warming were *Actinobacteria* and *Cyanobacteria* (Fig. 1). *Cyanobacteria* are known for their capacity to thrive in soils of low resource availability, which might constitute an advantage in sites that are depleted in available C. In addition, *Cyanobacteria* are a key ecological phylum in drylands (Bastida et al., 2016), another potential benefit in warmed soils, as lower water content is often a secondary effect of soil warming (e.g. Conant et al., 2004). In this study, soil water content was slightly lower in the warmed soils, albeit not significantly (Table 1).

The class of *Actinobacteria* dominated by the order of *Actinomycetales* increased most strongly (Fig. 1), albeit not significantly. Increases in *Actinobacteria* abundance in response to warming is generally acknowledged, it has been reported after FAME analysis in the temperate Harvard forest site after 12 years of warming (Frey et al.,

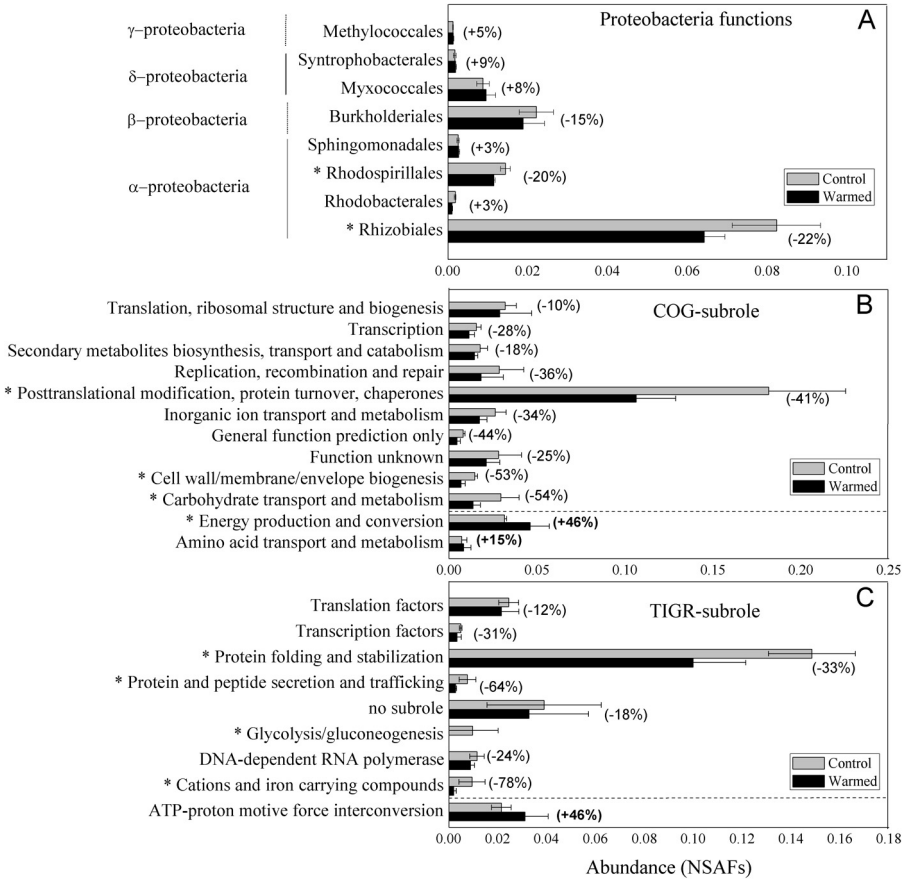

Fig. 2. (A) Changes in Proteobacteria diversity on the level of orders based on normalized spectral abundance factors (NSAFs); (B) Bacterial community functionality, based on the classification of orthologous groups (COG; prokaryotic proteins) and (C), TIGR (subrole) categories based on their protein assignments. The x-axis shows absolute numbers of normalized spectral abundance factors (NSAFs). Data represent the mean ± SD of three biological replicates. *represent significant differences, $P < 0.05$, independent-sample *t*-test. Values in bracket show the percentage change of the warmed plot compared to the control.

Table 1
Chemical properties, CO_2 flux and water content, as well as the abundance of bacterial, fungal and plant proteins, Shannon diversity indices and ratios between microbial proteins. Data show mean and standard deviation; significant differences according to the Independent-Samples *t*-Test ($P < 0.05$) are indicated with *.

	Control	Warmed
Water content%$_{w/w}$	45.6 ± 3.4	42.1 ± 5.2
Total C (g 100 g^{-1})	13.53 ± 1.68	10.79 ± 1.33*
Total organic carbon (mg C kg^{-1}).	12.34 ± 0.90	10.51 ± 1.31*
Total N (g 100 g^{-1})	0.63 ± 0.11	0.54 ± 0.07
C/N ratio	21.70 ± 1.69	20.11 ± 1.15
Dissolved organic carbon (mg C kg^{-1})	1.08 ± 0.18	0.97 ± 0.04*
CO_2 flux (μmol m^{-2} s^{-1})	2.96 ± 0.56	4.58 ± 0.94*
Bacteria (NSAFs)	0.546 ± 0.029	0.461 ± 0.095
Fungi (NSAFs)	0.020 ± 0.005	0.020 ± 0.007
Ascomycota/Basidiomycota (NSAFs ratio)	2.11 ± 0.04	1.20 ± 0.05*
Plant proteins (Streptophyta) NSAFs	0.12 ± 0.09	0.06 ± 0.01
Shannon-B[a]	0.43 ± 0.37	0.22 ± 0.38
Shannon-F[b]	1.15 ± 0.48	0.71 ± 0.65

[a] The Shannon index of bacterial diversity.
[b] Shannon index of fungal diversity.

2008), in a metagenomics study after 20 years of warming (DeAngelis et al., 2015; Pold et al., 2016), and also in arctic soils warmed for 18 years (Deslippe et al., 2012). *Actinobacteria* are relatively slow-growing bacteria with filamentous growth form similar to fungi, and they contain many representative taxa that are able to decompose complex soil organic C (Barret et al., 2011). The latter is in accordance with the depletion of easily available C pools when copy numbers of carbohydrate-degrading *Actinobacterial* genes increased with warming (Pold et al., 2016). This often-observed dominance of *Actinobacteria* after long-term warming indicates changing niches in soil and different microbial contributions to C-cycling (DeAngelis et al., 2015).

There appeared a slight reduction in fungal NSAFs (Table 1) which was, however different on the level of phylum and class (Fig. 1). On the phylum level, we observed a shift from *Ascomycota* to *Basidiomycota* with warming, which resulted in a declined ratio of *Asco-/Basidomycota* ($P < 0.05$, Table 1). Ratios of *Asco-/Basidomyctoa* have been reported to decrease over the course of decomposition, where *Basidiomycota* are considered to decompose lignin and cellulose (Kuramae et al., 2013; Zhang et al., 2014). Within the *Basidiomycota*, the class of *Agaricomycetes* dominated by the order of *Agaricales* was significantly more abundant in warmed plots. The *Agaricales* include several button mushrooms that are dominant ectomyccorhizal species. In warmed soil, however, not all orders within the class of *Ascomycota* declined, as increasing relative abundances of *Eurotiomycetes* and *Dothideomycetes* were examined. Both have been shown to produce C-degrading enzymes, specifically cellulases (Schneider et al., 2012). On the other side, the class of *Saccharomycetes* dominated by the order of *Saccharomycetales* significantly ($P < 0.05$, Fig. 1) declined with warming. This class had been reported to prefer soils with organic amendments (Bastida et al., 2015), therefore the reduction in warmed plots in the present study was probably due to the low availability of DOC. Besides, *Saccharomycetales* were described as oligotrophs according to Starke et al. (2016). Decreasing ratios of *Asco-/Basidiomycota* with soil warming may consequently shift the fungal community towards more saprotrophic species.

In contrast to the present study, previous assessments of the microbial community structure using PLFA and bacterial DNA/RNA

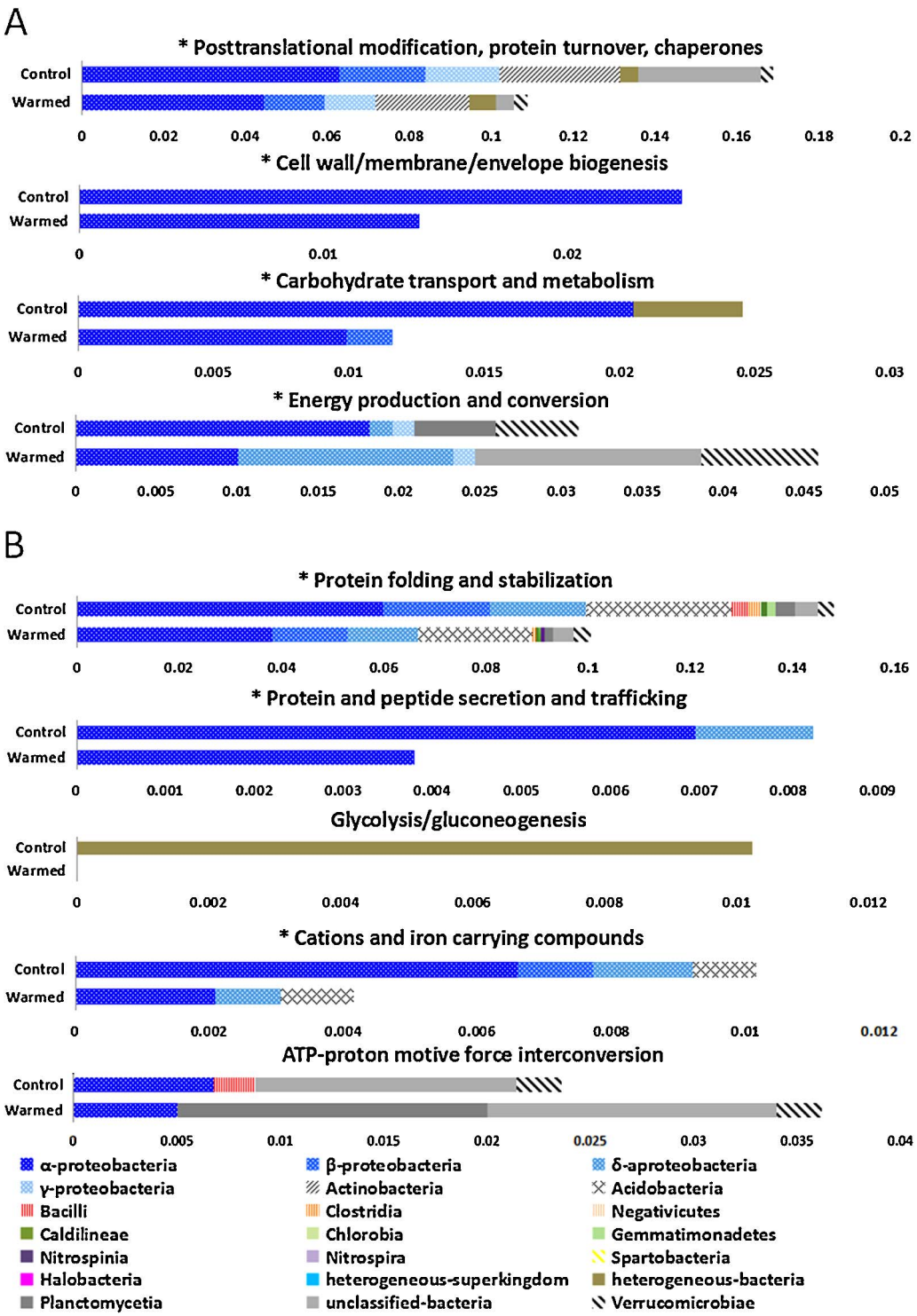

Fig. 3. Significant changed microbial community functionalities and its function assigned taxonomic groups based on normalized spectral abundance factors (NSAFs) of microbial proteins. (A), the clusters of orthologous group (COG) categories; (B), TIGR main/sub role; values on the x-axes are NSAF values of microbial functions assigned to their phylogentetic origin at class-level. * asterisks refer to the functions where the α-*Proteobacteria* functions are significantly reduced. To indicate their origin on the phylum level, shadings were included, dotted bars indicate Proteobacteria, *Firmicutes* are crosshatched, *Verrucomicrobia* are striped from bottom right to top left, *Actinobacteria* are striped from bottom left to top right, *Acidobacteria* are plaid and heterogenous.

at our site did not find significant changes in microbial community structure after four years of warming (Kuffner et al., 2012; Schindlbacher et al., 2011). However, when compared to these previous studies, the metaproteomics approach in the present study provided a better characterization of the soil microbial community that is actively involved in SOC decomposition, a process that is strongly related to soil temperature. Nevertheless, it has to be noted that our study represents only a snapshot in time, as–given to feasibility issues and the complexity of the metaproteomics approach–only soil samples from a single date were used. Microbial biomass and decomposition can change considerably throughout the seasons and microbial functionality may change as well (Castro et al., 2010; DeAngelis et al., 2015; Machmuller et al., 2016); such temporal variations, however, were not captured in the present study.

3.2. Functional changes and physiological adaptation

Compared to the taxonomic variation, community function was more strongly affected by soil warming (Fig. 2B & C). Two specific functions that are important for microbial energy status significantly increased with soil warming ("Energy production and conversion" and "ATP-proton motive force interconversion"; Fig. 2B & C). The higher soil respiration rates in warmed plots (on the date of soil sampling, soil CO_2 efflux was 35% higher in warmed plots compared to controls) could be attributed to the higher metabolic rate at higher temperatures, and consequently increasing abundance of proteins belonging to these two functional groups. Since we did not observe massive changes in SOC chemistry and availability at our forest site yet (Schnecker et al., 2016), the higher abundance of proteins related to energy production also fits with our observations of, generally higher respiration rates, lower substrate use efficiency at higher soil temperatures at our site (Schindlbacher et al., 2015) and elsewhere (e.g. Frey et al., 2013; Lehmeier and Billings, 2016; Tucker et al., 2013). Beside the increase in protein abundance related to energy production, a set of functions responsible for microbial growth and other metabolism-related functions showed a declining trend in warmed soil (Fig. 2B & C). Among them the identified proteins assigned to the three functional groups "Posttranslational modifications, protein turnover, chaperones", "Cell wall/membrane/envelope biogenesis" and "Carbohydrate transport and metabolism" were significantly lower in warmed than the control plots ($P < 0.05$; Fig. 2B), supporting lower carbon use efficiency under higher soil temperatures. The function "Cell wall/membrane/envelope biogenesis" was considered as an indicator for copiotrophy (Bastida et al., 2015). The reduction of this function indicates a shift towards a higher activity of oligotrophic phyla. This is consistent with a study by DeAngelis et al. (2015) who found lower numbers of rRNA copies in warmed soils, which was interpreted to be related to reduced microbial growth and hence an indicator for oligotrophic lifestyle. A shift towards more oligotrophic communities suggest that warming may cause an ongoing depletion in physically protected C pools that are usually preferably decomposed by microbes of oligotrophic life strategy (DeAngelis et al., 2015).

In a further step, we tried to link intracellular functions to their phylogenetic origin (Fig. 3), comparing the abundances of the classes that responded most strongly to warming. The significant decline in the aforementioned functions in response to warming is directly associated with a lower abundance of total and proteobacterial proteins (Fig. 3). The significant increase in functions assigned to "Energy production and conversion" was mainly related to a relative increase in the abundance of *δ-Proteobacteria*, (Fig. 3A), while their function for "protein and peptide trafficking" declined (Fig. 3B). Considering that *α-Proteobacteria* decreased in all functions in warmed plots, even if the total function increased, this further indicates the strong effect of warming on this specific class, which was highly abundant. The abundance of *Bacilli* was reduced in warmed plots and correlated with DOC concentrations, which also decreased with warming. Furthermore, their functions were related to "Energy metabolism" and "Protein fate", but only in control plots where DOC was not reduced (Fig. 3B).

4. Conclusion

In conclusion, the trend of changes in microbial community structure that were found in the present study does not fully support the development of microbial taxa towards a more oligotrophic life strategy with soil warming that was suggested by DeAngelis et al. (2015). We rather observed a development towards a more C depleted environment that still favored enhanced CO_2 release after 7 years of warming. It seems plausible that the observed changes on the phylum level in the present study were related to DOC changes, since many parts of the active soil microbial community had been shown to decrease with decreasing DOC concentrations (Bastida et al., 2016). Some exceptions to this are *Basidiomycota* and *Actinobacteria*, which both show hyphal growth forms and prefer C-depleted sites. These physiologically different phyla may have a lower contribution to biomass specific respiration than others due to a generally higher investment into growth/biomass production. Consequently, an increase in their abundance in warmed plots would only result in minor changes to soil respiration. Taken together, the observed shifts at phylum and class level could be a first sign for a microbial community composition response to longer-term soil warming and corresponding changing SOC availability. Furthermore, the link to specific changes in microbial function may indicate an ongoing microbial adaptation to warming. The present study suggests that long-term soil warming strongly affects the physiological adaptation of soil microbes, which leads to soil CO_2 emission rates that were still enhanced even after 7 years of soil warming.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.apsoil.2017.04.021.

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