



## **Interim Report**

**IR-13-041**

### **Seasonal variation in functional properties of microbial communities in beech forest soil**

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## Abstract

Substrate quality and the availability of nutrients are major factors controlling microbial decomposition processes in soils. Seasonal alteration in resource availability, which is driven by plants via belowground C allocation, nutrient uptake and litter fall, also exerts effects on soil microbial community composition. Here we investigate if seasonal and experimentally induced changes in microbial community composition lead to alterations in functional properties of microbial communities and thus microbial processes. Beech forest soils characterized by three distinct microbial communities (winter and summer community, and summer community from a tree girdling plot, in which belowground carbon allocation was interrupted) were incubated with different  $^{13}\text{C}$ -labeled substrates with or without inorganic N supply and analyzed for substrate use and various microbial processes. Our results clearly demonstrate that the three investigated microbial communities differed in their functional response to addition of various substrates. The winter communities revealed a higher capacity for degradation of complex C substrates (cellulose, plant cell walls) than the summer communities, indicated by enhanced cellulase activities and reduced mineralization of soil organic matter. In contrast, utilization of labile C sources (glucose) was lower in winter than in summer, demonstrating that summer and winter community were adapted to the availability of different substrates. The saprotrophic community established in girdled plots exhibited a significantly higher utilization of complex C substrates than the more plant root associated community in control plots if additional nitrogen was provided. In this study we were able to demonstrate experimentally that variation in resource availability as well as seasonality in temperate forest soils cause a seasonal variation in functional properties of soil microorganisms, which is due to shifts in community structure and physiological adaptations of microbial communities to altered resource supply.

## **Keywords**

C and N availability, Extracellular enzyme activities, Microbial community composition, Microbial processes, PLFA, Priming effect, Respiration, Carbon use efficiency.

## **1. Introduction**

Microbial decomposition of soil organic matter (SOM) plays a key role in global C and N cycling (Davidson and Janssens, 2006). It is well known that microbial decomposition processes are controlled, amongst other factors, by substrate quality (e.g. lignin content) and the availability of labile C and nutrients (Chapin et al., 2002; Schmidt et al., 2011). Resource availability influences decomposition processes via effects on microbial physiology, e.g. production of extracellular enzyme activities. Microbial production of extracellular enzymes is stimulated by substrate supply or if available nutrients or C are scarce (Allison and Vitousek, 2005; Hernandez and Hobbie, 2010; Olander and Vitousek, 2000). Enhanced availability of labile C substrates may also increase the decomposition of recalcitrant SOM ('priming effect'), which is ascribed to either microbial activation by the labile C source or enhanced degradation of SOM for the acquisition of limiting nutrients (Blagodatskaya and Kuzyakov, 2008; Fontaine et al., 2011).

Apart from changing the physiology of microbial communities, alterations in resource availability may also influence microbial processes indirectly. As different species of microbes differ in substrate use efficiency and biomass composition and thus demand for C and nutrients (Degens, 1999; Gusewell and Gessner, 2009), changes in resource supply have been shown to induce shifts in microbial community composition (Eilers et al., 2010; Fierer et al., 2012; Griffiths et al., 1999; Waldrop et al., 2004). Changes in microbial community composition may in turn strongly affect microbial processes since certain classes of enzymes are produced by specific

groups of microorganisms. This is especially true for phylogenetically 'narrow' processes, i.e. processes performed by a relatively small number of specialized species, such as polyphenol degradation, while a higher functional redundancy between different microbial communities may be found for processes performed by a broad array of soil microorganisms, such as C mineralization or protein depolymerization (Balsler and Firestone, 2005; Schimel et al., 2005).

While the relation between microbial community composition and function has already been demonstrated in several studies comparing functional properties of microbial communities from different ecosystems (Balsler and Firestone, 2005; Brant et al., 2006; Paterson et al., 2011; Strickland et al., 2009; Waldrop and Firestone, 2004), few studies exist investigating the effects of microbial community changes at a single site, e.g. community shifts following increased or decreased plant inputs, on microbial functions (Brant et al., 2006; Paterson et al., 2011; Wickings et al., 2011). It is still not fully understood if and how changes in microbial community composition resulting from seasonal and experimental induced variation in resource availability affect the functional properties of microbial communities, a topic which is especially important in the light of ongoing global change.

In temperate forest ecosystems a seasonal pattern of microbial processes has been observed which is related to a seasonal variation in availability of different substrates, as well as variation in soil temperature and moisture (Kaiser et al., 2011; Kaiser et al., 2010b). The seasonal variation in resource availability is mainly driven by plants via belowground C exudation and nutrient uptake during the growing season and litter fall in autumn. These seasonal changes in resource availability have also been shown to induce shifts in microbial community structure (Kaiser et al., 2011; Kaiser et al., 2010b).

This study aimed at elucidating whether changes in the composition of soil microbial communities related to seasonal and experimentally induced variation in resource availability lead to altered functional properties of these microbial communities. We

hypothesized (1) that distinct microbial communities that are adapted to different substrates vary in their physiological capacities and in their functional response to addition of various organic substrates and (2) that the functional response of different microbial communities to addition of C substrates is influenced by microbial nutrient limitation.

In order to test these hypotheses we performed an incubation experiment with soils from a beech forest study site (Kaiser et al., 2010b) characterized by three different microbial communities. We chose winter and summer microbial communities as we assumed microbial adaptation to high availability of labile C in summer and to more recalcitrant substrates (litter) in winter. In summer we also collected soils from a tree girdling plot in which belowground carbon allocation had been interrupted which promoted the establishment of a more saprotrophic community. Collecting soils from a single site ensured that soils were comparable in soil properties. We incubated the different soils with a range of labile and complex substrates (glucose, protein, microbial cell walls, cellulose and plant cell walls) and analyzed changes in various microbial processes and pools of labile C and N in response to substrate addition and experimentally enhanced inorganic N supply. By using  $^{13}\text{C}$  labeled substrates we were also able to directly monitor microbial substrate utilization and analyze how the various amendments led to changes in the utilization of soil organic matter (i.e. priming).

## **2. Material and Methods**

### *2.1. Origin of soil*

The soil for the incubation experiment originated from a 65-year-old beech forest (*Fagus sylvatica*) about 40 km southwest of Vienna (48°07' N 16°03' E, 510 m a.s.l.). Soils were classified as Dystric Cambisols over flysh (pH in  $\text{CaCl}_2$  between 4.5 and 5.1) with a mean organic carbon content of 7.45% and nitrogen content of 0.48% in

the A horizon. The study site and experimental setup in the field was described in detail previously (Kaiser et al., 2010b), microbial communities were characterized by Kaiser et al. (2010b) and Rasche et al. (2010). Girdling of beech trees had been performed in May 2006 by removal of the bark over 10 cm sections around the circumference of the stems. Soils for the incubation experiment were collected in February 2008 (winter community) and in June 2008 (summer community and community from girdling plots). 4 subsamples of mineral soil (5 cm depth, A-horizon) were collected from each of 6 replicate plots in winter and summer, respectively, and from 6 replicate girdled plots in summer. Soils from replicate plots were pooled, sieved (5 mm) and stored at 4°C (winter) and 12°C (summer) until the start of the incubation experiment. Half of the winter soil was transferred to 12°C for equilibration 3 days before the incubation.

## 2.2. Substrates

Five <sup>13</sup>C-labelled substrates differing in complexity and C and N content were used for the incubation experiment: Glucose, protein, microbial cell walls, cellulose and plant cell walls, containing 20 atom % <sup>13</sup>C, except for cellulose (16 atom %) and protein (98 atom %). Glucose (99 atom % <sup>13</sup>C, from Sigma) and cellulose (97 atom % <sup>13</sup>C, from IsoLifeBV) were diluted with the respective unlabelled substances, algal protein extract (98 atom % <sup>13</sup>C, from Sigma) was applied undiluted.

<sup>13</sup>C-labelled microbial cell walls were prepared as follows: Two bacterial species (*Pectobacterium carotovorum* and *Verrucomicrobium spinosum*) and one fungal species (*Aspergillus nidulans*) were grown on <sup>13</sup>C-glucose (20 atom % <sup>13</sup>C). Growth conditions were described by Keiblinger et al. (2010). Microbial biomass was dried and then resuspended in NaCl-solution. After mechanical destruction of cell walls by ultrasonic treatment and bead beating, residues were repeatedly extracted with NaCl-solution, water, methanol/chloroform (5:3), hexane and pure water to remove

all labile cell constituents. The remaining residues were dried, homogenized (ball mill) and stored frozen.

$^{13}\text{C}$ -labelled plant cell walls were prepared as follows:  $^{13}\text{C}$ -labelled wheat roots (IsoLiveBV, U-60402) and unlabelled, dried wheat roots were finely ground and homogenized in a ball mill. The material was then incubated with  $\alpha$ -amylase solution to remove starch (Richter et al., 2009) and further extracted repeatedly with methanol/chloroform/water (12:5:3) to remove other labile substances.

### 2.3. *Experimental setup*

The respective substrate (1 mg substrate  $\text{g}^{-1}$  soil of glucose and protein, 4 mg  $\text{g}^{-1}$  soil of the other substrates) was amended to each soil in a dry form. A subset of the summer soils (from control and girdling plots) amended with either cellulose or plant cell walls, was also amended with inorganic N (3 mg  $\text{NH}_4\text{NO}_3$   $\text{g}^{-1}$  soil). We added N to these treatments in order to test the effects of increased N availability on the degradation of C substrates (one of them lignin containing), assuming microbial N limitation in summer.

Incubation of soils (22 g) was performed in a microcosm system with 5 replicates for each substrate and soil (Inselsbacher et al., 2009). For each soil controls without added substrate were prepared (2 x 5 replicates per soil). The microcosms were loosely closed by moist cotton wool and incubated in the dark for 2 days (glucose and protein incubations) or 6 days (microbial cell walls, cellulose and plant cell walls incubations). Incubation temperature was 12°C; additionally soils from the winter community were incubated at 4°C. The process rates of the winter communities relative to their control incubations were not significantly different between 12°C and 4°C incubation temperature, thus only the results of 12°C winter incubations are presented here. At the end of the incubation microcosms were destructively harvested for determination of C and N pools, gross N mineralization, enzyme activities and phospholipid fatty acids.

#### 2.4. Microbial respiration

Microbial respiration rates were measured at 5 time points during the incubation period. At the measurement, incubation tubes were sealed at the bottom, cotton wool was removed and instead polypropylene tubes closed by airtight rubber caps were mounted on the incubation tubes (Inselsbacher et al., 2009). 15 ml of headspace gas was sampled by syringe immediately after closing the tubes and replaced by 15 ml of air (ambient CO<sub>2</sub> concentration). A second gas sample was taken after 30 minutes. Concentration and carbon isotope ratio of CO<sub>2</sub> (relative to VPD) was determined via a GasBench II interfaced to continuous-flow isotope ratio mass spectrometry (IRMS; Delta V Advantage, Thermo Fisher, Germany). Respiration from substrate was calculated according to the following equation:

$$R_{\text{substrate}} = \text{APE}^{13\text{C}}_{\text{resp}} / \text{APE}^{13\text{C}}_{\text{substrate}} * 100 * R_{\text{total}}$$

Where APE<sup>13</sup>C means atom % excess <sup>13</sup>C in respiration and substrates, respectively, and R<sub>total</sub> is total respiration.

The change in C mineralization from SOM (including microbial biomass C) induced by addition of organic substrates (= 'priming effect') was calculated as follows:

$$\text{PE (\%)} = ((R_{\text{total}} - R_{\text{substrate}}) - R_{\text{control}}) / R_{\text{control}} * 100$$

Where R<sub>total</sub> is the total respiration from incubations with substrates, R<sub>substrate</sub> is the respiration from substrate and R<sub>control</sub> is the respiration from control incubations.

Microbial carbon use efficiency (CUE) was calculated according to the following equation:

$$\text{CUE} = {}^{13}\text{C}_{\text{mic}} / ({}^{13}\text{C}_{\text{mic}} + {}^{13}\text{C}_{\text{resp}})$$

where <sup>13</sup>C<sub>mic</sub> is the amount of substrate <sup>13</sup>C in the microbial biomass and <sup>13</sup>C<sub>resp</sub> is the cumulative respired substrate <sup>13</sup>C.

### 2.5. Gross N mineralization

Gross N mineralization was assessed using the pool dilution technique (Kaiser et al., 2005; Myrold and Tiedje, 1986).  $^{15}\text{NH}_4\text{Cl}$  was applied to subsamples of fresh soil, incubated for 4h or 24h, then extracted with 2M KCl. Ammonium was isolated by the microdiffusion method and  $^{15}\text{N}$  values of  $\text{NH}_4^+$  were analyzed by an elemental analyzer coupled to an IRMS (DeltaPLUS, Thermo Finnigan). Gross N mineralization rates were calculated according to the following equation:

$$m = (A_t - A_0) / t * ((\ln(APE_0 / APE_t) / \ln(A_t / A_0))$$

where  $m$  is gross mineralization,  $A_t$  is the  $\text{NH}_4^+$ -N pool after time  $t$ ,  $A_0$  is the initial  $\text{NH}_4^+$ -N pool, APE (atom % excess) is atom %  $^{15}\text{N-NH}_4^+$  sample – atom %  $^{15}\text{N-NH}_4^+$  natural abundance

### 2.6. C and N pools

Inorganic N was determined from water extracts by chemically suppressed ion-chromatography (Dionex HPAEC on a AS11 column for  $\text{NO}_3^-$  and Dionex HPCEC on a CS16 column for  $\text{NH}_4^+$ ) (Kaiser et al., 2005). Organic C and total N were analyzed in water extracts by a TOC/TN analyzer (TOC-V CPH E200V / TNM-1 220V, Shimadzu). Microbial biomass C and N was determined by the chloroform fumigation extraction method (Amato and Ladd, 1988). Microbial C and N was estimated from the difference of organic C and total nitrogen measured by a TOC/TN analyzer in KCl extracts of fumigated and unfumigated soils.

### 2.7. Extracellular enzyme activities

Potential enzyme activities were measured by microplate fluorimetric and photometric assays according to Kaiser et al. (2010b). 4-methylumbelliferyl- $\beta$ -D-cellobioside, 4-methylumbelliferyl- $\beta$ -D-N,N',N''-triacetylchitotrioside and L-leucine-7-amino-4-methyl coumarin, respectively, were used as substrates for the fluorimetric

detection of  $\beta$ -1,4-cellobiosidase, endochitinase and leucine amino-peptidase activities. Phenoloxidase and peroxidase activities were measured photometrically after addition of L-3,4-dihydroxyphenylalanine (DOPA).

Actual polysaccharide degradation rates were measured without substrate addition but with the addition of toluene to inhibit microbial uptake of enzymatic products, leading to their accumulation in the soil suspension (Boschker et al., 1995; Kaiser et al., 2010b). Glucose accumulation in the soil solution (analyzed by HPLC) was used for estimation of combined cellulase and amylase activities.

### 2.8. *Phospholipid fatty acids*

Phospholipid fatty acids (PLFAs) were extracted by a mixture of methanol, chloroform and citrate buffer (2:1:0.8, v/v/v), then separated from neutral lipids on silica columns and finally subjected to alkaline methanolysis (see Koranda et al. (2011) for details). Dried fatty acid methyl esters were re-dissolved in isooctane and concentrations and carbon isotope ratios of PLFAs were determined by a Trace Ultra GC (Thermo Fisher) interfaced with an IRMS (Delta V Advantage, Thermo Fisher) via a combustion unit (GC combustion II/TC, Thermo Fisher). A mixture of FAMES (Supelco, nr. 47080-U and 47885-U) was used as a qualitative standard. An internal standard (19:0) was used for calculation of FAME concentrations, as well as for correction of  $\delta^{13}\text{C}$  values.  $\delta^{13}\text{C}$  values of PLFAs were also corrected for  $\delta^{13}\text{C}$  values of the C added during methanolysis. We used the sum of the fatty acids i15:0, a15:0, i16:0, i17:0, a17:0 as indicator of Gram-positive bacteria, the sum of 16:1 $\omega$ 9, 16:1 $\omega$ 7, 18:1 $\omega$ 7, 18:1 $\omega$ 5, cy17:0, cy19:0, cy18:0 as indicator of Gram-negative bacteria, and all these together with 17:0, 17:1 $\omega$ 6, 17:1 $\omega$ 7 as a measure for total bacteria. The quantity of 18:2 $\omega$ 6,9 was used as an indicator of fungal biomass (Kaiser et al., 2010a).

We estimated  $^{13}\text{C}$  incorporation into microbial biomass by calculating the weighted average  $\delta^{13}\text{C}$  value of PLFAs and multiplying it with the amount of microbial biomass C, keeping in mind that  $\delta^{13}\text{C}$  values may vary between different cell components.

## 2.9. *Statistics*

Data were transformed prior to analysis to achieve normality and homogeneity of variances (natural logarithmic transformation was applied for absolute concentrations and process rates, square root transformation for relative values). Characteristics of the three soils were compared by one-way ANOVA. Multivariate statistics were performed with standardized data. We applied a principal component analysis (PCA) and analysis of similarity (ANOSIM), which is a permutation-based test, to evaluate differences in the functional response of microbial communities to substrate addition. Relative values of microbial processes, C and N pools and microbial groups in percent of control incubations were analyzed for differences from 100 % using student's t-test. Apart from significance level  $p < 0.05$  we also considered  $p < 0.1$  as indicating a marginal significance. Differences between microbial communities in incubations of one substrate were assessed using one-way ANOVA and Tukey's post-hoc test, differences between incubations of complex C substrates with and without added inorganic N, respectively, were analyzed using student's t-test. Two-way ANOVA was applied for determination of separate effects of substrate and community type on incubations of different substrates. Additionally, three-way ANOVA was applied for determination of the effect of N addition in incubations of complex C substrates. Univariate statistical analyses were performed using Statistica 6.0, multivariate statistics were run with Primer 6.

## 3. **Results**

### 3.1. *Characterization of soil and functional microbial communities*

Soils collected either in winter or in summer from control plots and from girdled plots were characterized by distinct microbial communities (description of seasonal changes in microbial community composition and effects of tree girdling in Kaiser *et al.* 2010b) and exhibited differences in availability of C and N and microbial biomass size (Table 1). Nitrate concentrations were significantly higher in soils from the winter harvest than from the summer harvest, while no significant differences could be determined for  $\text{NH}_4^+$ . Availability of  $\text{NH}_4^+$ , however, doubled in control incubations of soil collected in winter during the experiment (from  $0.21 \pm 0.039$  to  $0.43 \pm 0.070 \mu\text{mol g}^{-1} \text{DW}$ ) but decreased in summer soil (from  $0.19 \pm 0.005$  to  $0.11 \pm 0.009 \mu\text{mol g}^{-1} \text{DW}$ ) indicating generally higher inorganic N availability in winter than in summer. Total dissolved N concentrations, on the other hand, were highest in soils of girdled plots and lowest in soils collected in winter. Winter soil also exhibited a significantly lower concentration of DOC than the summer soil. Microbial biomass was lowest in girdled plots and had the highest C/N ratio.

Microbial process rates were generally lower in winter than in summer (for detailed description of seasonal variation in microbial processes see Kaiser *et al.* (2010b). Actual cellulase / amylase, peptidase and endochitinase activities were lower in girdled plots than in summer control plots, while oxidative enzyme activities were not significantly different (Table S1). If enzyme activities were calculated per unit biomass, however, oxidative enzyme activities were significantly higher in girdled plots than in control plots.

### 3.2. *Changes in C and N pools by substrate addition*

Addition of glucose significantly increased microbial C and C/N ratio in soils collected in winter, in summer and from girdled plots (Table S2). Incubations of microbial cell walls, cellulose and plant cell walls exhibited reduced microbial biomass (both C and N) in soil collected in summer from control plots while the first two substrates

revealed the opposite effect in soil from girdled plots. Microbial C was not affected by N addition in either of the communities. Addition of cellulose and plant cell walls significantly increased microbial N in winter (148 and 156 % of control incubations, respectively), which translated into a marked decrease in C/N ratio of microbial biomass (63 and 66 % of controls, respectively;  $p < 0.001$ ).

$\text{NH}_4^+$  concentrations significantly increased with protein addition in soils collected in winter, summer and from girdled plots (Table S2). Glucose and cellulose incubations of winter soil exhibited markedly reduced  $\text{NH}_4^+$  concentrations compared to control incubations (43 and 33 %, respectively;  $p < 0.001$ ), while in soil from girdled plots  $\text{NH}_4^+$  availability was enhanced by the addition of cellulose.

### *3.3. Changes in microbial processes by substrate addition / Microbial substrate utilization*

A principal component analysis of 7 microbial process rates (C mineralization, SOM mineralization, substrate  $^{13}\text{C}$  in respiration, actual cellulase/ amylase, cellobiosidase, peptidase, and chitinase activities) relative to unamended controls revealed that the functional response of microbial communities to substrate addition depended on the type of added substrate, glucose and protein incubations being set apart from incubations of microbial cell walls and complex C substrates (Fig. 1). While the three different microbial communities responded similarly to the addition of microbial cell walls and protein, clear functional differences between microbial communities were observed for the C substrates. Winter community significantly differed from the summer community in the response to addition of both labile C (glucose) and complex C substrates (cellulose, plant cell walls; ANOSIM  $p < 0.05$ ). Significant differences between communities from summer control plots and girdled plots were found in incubations of complex C substrates ( $p < 0.05$ ). Enhanced inorganic N availability clearly influenced the functional response of the community from control plots to addition of complex C substrates ( $p < 0.05$ ), while a significant effect of N on

the community from girdled plots was found in incubations of cellulose only. The first PCA axis mainly separating the glucose and protein incubations from the other substrates was negatively correlated to concentrations of microbial C and microbial C/N ratio (relative to control incubations) and positively correlated to  $\text{NH}_4^+$ ,  $\text{NO}_3^-$  and total dissolved N. The second PCA axis separating the winter community as well as the community from girdled plots from the summer community showed negative correlations to concentrations of microbial C and N,  $\text{NH}_4^+$  and also the abundance of fungi (relative to control incubations). Factor loadings on PCA 1 were most negative for respiration, APE  $^{13}\text{C}$  in respiration and cellulase/ amylase activity, while cellobiosidase, endochitinase and peptidase activities showed highest loadings on PCA 2.

Respiration was more than doubled by the addition of labile C in incubations of all microbial communities, the increase being significantly higher in winter than in summer (Fig. 2a). Addition of a complex C substrate, plant cell walls, however, stimulated respiration in summer only, while a significant decline in cumulative respiration was observed in winter ( $p < 0.05$ ). Increased inorganic N availability did not affect cumulative respiration in soils from either control or girdled plots. The different response in respiration of winter and summer communities mainly reflected differences in mineralization of soil organic matter rather than respiration of the added substrates. In winter a marked priming effect of SOM decomposition by glucose was observed ( $p < 0.001$ ) (Fig. 3a), while addition of plant cell walls reduced mineralization of SOM ( $p < 0.05$ ). In summer, however, a positive priming effect by plant cell walls was found ( $p < 0.05$ ). ANOVA results hence revealed a highly significant community x substrate interaction for both cumulative respiration and priming of SOM decomposition but no main effect of community (Table 2). Differences in carbon use efficiency between the different communities may also contribute to the observed pattern of respiration. Winter community exhibited lower

efficiency of glucose utilization than summer community but higher efficiency in the use of plant cell walls (Fig. 3b). As indicated by substrate  $^{13}\text{C}$  concentrations in respiration and microbial biomass (Table 3), glucose, protein and microbial cell walls were generally more intensively used by microbes than the polymeric C substrates. Uptake of added glucose was higher in summer than in winter, while the opposite was found for protein. The microbial community in soils from girdled plots tended to use the complex C substrates more intensively than the summer control community, a tendency considerably enforced by the addition of inorganic N. Enhanced inorganic N availability strongly increased the concentration of  $^{13}\text{C}$  in respiration in both soils from control and girdled plots ( $p < 0.001$ ). A comparably strong effect of N addition on substrate  $^{13}\text{C}$  in microbial biomass was only found in cellulose incubations of soils from girdled plots.

ANOVA results for gross N mineralization revealed significant effects of community, substrate and community x substrate interaction (Table 2). Glucose addition significantly stimulated N mineralization, with the highest increase being found in winter (Fig. 2b). Interestingly, protein exhibited no or a negative effect, while microbial cell walls, cellulose and plant cell walls increased gross N mineralization, especially in incubations of the summer community.

Significant effects of community, substrate as well as community x substrate interaction were also observed for all of the measured extracellular enzyme activities (Table 2).

Actual cellulase/ amylase activities were strongly stimulated by glucose, the enzymatic product, as well as by microbial cell walls (Fig. 4a). Winter community significantly differed from summer community in the response to complex C substrates. While in winter actual cellulase activity was stimulated by cellulose and plant cell walls amendments ( $p < 0.001$ ), no effect was found in summer. The pattern of cellobiosidase activity, indicating potential cellulolytic activity, differed considerably from actual cellulolytic activity, with an increase in plant cell wall incubations of the

summer community ( $p < 0.05$ ) but a decrease in cellulose incubations of the community from girdled plots ( $p < 0.01$ ) (Fig. 4b). Both actual cellulase/ amylase and potential cellobiosidase activities, however, showed the same trends in the response of communities to inorganic N addition, separating summer (control) community from the community from girdled plots. While in soils from control plots cellulolytic activity decreased in plant cell wall incubations with high N availability compared to plant cell wall incubations without added N ( $p < 0.05$ ), the community from girdled plots showed a significantly positive response to inorganic N addition in cellulose incubations ( $p < 0.05$ ), which was also depicted by a significant community x N interaction for cellulolytic enzymes (Table 2).

Differences between summer and winter communities in response to complex C substrates were also revealed by peptidase activities (Fig. 4c). In summer peptidase activity was significantly enhanced by addition of cellulose ( $p < 0.05$ ) and plant cell walls ( $p < 0.001$ ) while in winter the opposite effect was observed. The community from girdled plots tended to exhibit a lower response to these substrates than the community from control plots. Addition of inorganic N significantly reduced peptidase activity ( $p < 0.001$ ). Endochitinase, responsible for the depolymerization of N-rich microbial cell walls, showed a similar, although less significant response to N addition and similar differences between communities in plant cell wall incubations (Fig. 4d). Oxidative enzyme activities were significantly enhanced by the addition of plant cell walls in both soils from control and girdled plots, as well as by microbial cell walls in soil collected in winter (Fig. 4e and 4f). Stimulation of phenoloxidase activity by plant cell walls was higher in soil from girdled plots than from control plots ( $p < 0.05$ ). Inorganic N addition exerted little effect on oxidative enzyme activities, only a marginally significant decrease in peroxidase in soil from control plots was found ( $p < 0.1$ ).

#### 3.4. *Changes in abundances of microbial groups by substrate addition*

Substrate addition generally had a stronger influence on fungal abundance than on bacterial abundance (Table S3). We observed a marginally significant increase in fungal abundance after glucose addition ( $p < 0.1$ ) and a strong decrease after addition of cellulose and plant cell walls in soil collected in summer from control plots (79 and 49 % of control incubations, respectively;  $p < 0.05$ ), while in soil from girdled plots a slightly positive response to cellulose was observed. Enhanced inorganic N supply increased the abundance of fungi in soils from both control and girdled plots ( $p < 0.05$ ). A positive effect of organic N, however, was observed in soil from control plots only. The response of fungal abundance to substrate addition was negatively correlated to changes in peroxidase activity in incubations of the summer community ( $p < 0.05$ ), while in incubations of the community from girdled plots a positive correlation of fungal abundance with peroxidase activity ( $p < 0.05$ ) and negative correlations with peptidase and endochitinase activities ( $p < 0.05$ ) were observed. In contrast to fungal abundance, the total bacterial abundance was not significantly affected by substrate addition (Table S3).

#### **4. Discussion**

Previous studies have demonstrated a seasonal variation in microbial decomposition processes in temperate forest soils which was related to a seasonal shift in availability of substrates and a seasonal variation in soil temperature and moisture (Kaiser et al., 2011; Kaiser et al., 2010b). In these studies the reduction of belowground C allocation by tree girdling strongly affected microbial processes, as also reported by others (Ekberg et al., 2007; Högberg et al., 2001; Subke et al., 2004). Seasonal and experimentally induced alterations in resource availability and abiotic factors, however, have also been shown to induce changes in microbial community composition (Fierer et al., 2012; Kaiser et al., 2010b; Lauber et al., 2008;

Yarwood et al., 2009). Here we demonstrate experimentally that microbial community changes due to alterations in resource availability result in functional differences between microbial communities implying that the distinct microbial communities differ in their physiological capacities. In agreement with our expectations, the functional response of microbial communities to substrate addition depended on the type of added substrate (Fig. 1, Table 2), presumably since extracellular enzyme activities as well as C and N mineralization rates vary with resource supply and stoichiometry (Allison and Vitousek, 2005; Geisseler and Horwath, 2009; Hernandez and Hobbie, 2010). In addition, we also observed a significant influence of community type on relative process rates (Fig. 1, Table 2) underlining functional differences between microbial communities, especially in the decomposition of complex C substrates. This is in line with other studies also reporting functional differences between distinct microbial communities (Brant et al., 2006; Fierer et al., 2012; Paterson et al., 2011). When interpreting these results, it has to be kept in mind, however, that the soils collected at different seasons and from girdled plots differed in both microbial community composition and availability of labile C and N, which implies that effects of resource availability and effects of community structure or physiological adaptations of microbes may combine here. We experimentally enhanced inorganic N availability in summer soils, which facilitates separation of these effects.

Soil collected in winter was characterized by lower availability of DOC but higher availability of inorganic N than summer soil (Table 1). The relatively low C/N ratio of microbial biomass in summer soil was probably due to the fact that soil was sampled at the beginning of summer, prior to the sharp decrease in microbial biomass N usually observed at this site in July (Kaiser et al., 2011). Regardless of differences in resource availability our results revealed considerable functional differences between summer and winter communities in response to addition of C substrates, reflecting microbial adaptation to availability of different types of C sources in summer and winter. The winter community responded to the addition of complex C substrates with

significantly enhanced actual cellulase/amylase activity (Fig. 4a) and reduced mineralization of soil organic matter (Fig. 3a). Both suggest adaptation of the winter community to degradation of complex C substrates, such as plant litter, which is also reflected by the high carbon use efficiency for plant cell walls in this community (Fig 3b). In summer, on the contrary, actual cellulase/amylase activity was not influenced by the addition of plant cell walls and was decreased by the additional enhancement of inorganic N availability. This may indicate that the effects on cellulolytic enzyme activities (and SOM mineralization) observed in plant cell wall incubations of the winter community were not due to higher N availability in winter but reflected a functional property of the winter community. The marked increase in microbial biomass N and decrease in biomass C/N ratio in response to addition of polymeric C substrates in winter (Table S2) is consistent with our previous results showing a phase of enhanced microbial N uptake and N storage in microbial biomass during winter months (Kaiser et al., 2011). Glucose addition caused a considerably stronger increase in C mineralization in winter than in summer (Fig. 2a). This was mainly due to a strong priming effect of glucose on SOM mineralization in winter (Fig. 3a), which may reflect a depletion of labile carbon in winter that may have been overcome by the pulse of labile C. Enhanced turnover of microbial C pools after microbial activation may also contribute to this effect ('apparent priming effect'; (Blagodatskaya and Kuzyakov, 2008)). The summer community, on the other hand, seemed to be more adapted to utilization of labile C sources (which are known to be supplied to microbes during summer by plant roots (Dennis et al., 2010; Jones et al., 2009)), as exemplified by higher uptake rates of glucose compared to the winter community (Table 3) and higher carbon use efficiency for glucose (Fig. 3b). Activity of peptidase (and chitinase; Fig. 4c/d) was increased by complex C substrates in summer (contrasting to negative effects in winter), while inorganic N addition reduced peptidase and chitinase activities, as reported from other studies (Allison and Vitousek, 2005; Olander and Vitousek, 2000). The increase in N-acquiring enzymes

by the addition of cellulose and plant cell walls in summer may indicate microbial N limitation during the plant growing season and can be related to a positive priming effect of SOM decomposition (Fig. 3a), suggesting 'microbial N mining' (Fontaine et al., 2011).

In a previous study it was demonstrated that tree girdling significantly reduced the abundance of mycorrhizal fungi. Increased input of dead fine root biomass resulted in enhanced saprotrophic activity and high biomass turnover two years after girdling (Kaiser et al., 2010b). This is also indicated by oxidative enzyme activities and high levels of dissolved (organic) N, as well as high gross N mineralization in soils from girdled plots in our study (Table 1 and S3). Differences in the response to substrate addition between microbial communities from girdled and control plots are thus likely to reflect the different functional properties of a purely saprotrophic community and a microbial community shaped by plant roots.

Oxidative enzyme activities were enhanced in both soils from control and girdled plots by the addition of plant cell walls, but the increase in phenoloxidase activity was significantly higher in the soil from girdled plots. Enhanced oxidative enzyme activities in plant cell wall incubations may arise from the degradation of the lignin-containing substrate or from enhanced degradation of SOM (priming) (Fig. 3a). Increased inorganic N concentration showed little effect on oxidative enzyme activities, in contrast to other studies reporting negative (Carreiro et al., 2000; Sinsabaugh et al., 2002) but also positive effects (Saiya-Cork et al., 2002) of N fertilization or additions. However, as revealed by  $^{13}\text{C}$  concentrations in respiration and microbial biomass (Table 3), microbial utilization of complex C substrates was markedly enhanced by inorganic N addition and was significantly higher in soils from girdled plots than from control plots, which suggests a higher capacity of the saprotrophic community in girdled plots for degradation of these substrates, reflecting microbial adaptation to high availability of dead root biomass in girdled plots.

Interestingly, cellulolytic enzyme activities responded differently to N addition in soils from girdled and control plots. In soil from girdled plots N addition increased cellulolytic enzyme activity in cellulose incubations (Fig. 4a and 4b), consistent with results from previous studies (Allison and Vitousek, 2005; Geisseler and Horwath, 2009; Saiya-Cork et al., 2002; Sinsabaugh et al., 2002), whereas activity of these enzymes was decreased by inorganic N in soil from control plots. N limitation of the microbial community in girdled plots is unlikely to be the cause since lower peptidase and chitinase activities in these incubations indicate higher N availability in soils from girdled plots. Differences in the response of cellulolytic enzyme activities to N addition might hence suggest that different species of microbes were responsible for the degradation of complex C substrates in control plots and girdled plots, as indicated by their different response to N. It also suggests that N addition caused a community shift which was different in soils from control and girdled plots. Relating enzyme activities to changes in the abundances of different microbial groups, especially the abundance of fungi (Table S3) may help to clarify this. Counter to the current paradigm, the abundance of fungi was strongly decreased by the addition of complex C substrates in soil from control plots (pointing to cellulose-degrading bacteria outcompeting those fungal taxa adapted to labile C sources), while the opposite trend was observed in incubations of soil from girdled plots. But in both soils, abundance of fungi was enhanced by inorganic N addition. The fact that, unlike soil from girdled plots, soil from control plots exhibited a different response of cellulolytic enzyme activities and the abundance of fungi to N addition might indicate that fungi may be less involved in degradation of polymeric C substrates in control plots and that instead other members of the microbial community play an important role in cellulolytic activity. Differences in physiological capacities of fungi in control and girdling plots, respectively, are also indicated by positive correlations between relative peroxidase activity and fungal abundance in soil from girdled plots, but negative correlations in soil from control plots.

In summary our results revealed that microbial communities in soils collected at different seasons and from experimentally altered plots clearly differed in their response to substrate addition. The observed pattern in microbial processes reflects distinct physiological capacities of winter and summer communities. The winter community revealed a higher capacity for degradation of complex C substrates (cellulose, plant cell walls) but lower utilization of labile C sources (glucose) than the summer community. The saprotrophic community established in girdled plots exhibited a significantly higher utilization of complex C substrates compared to the community from control plots if additional nitrogen was provided.

It can thus be concluded that the plant-induced variation in resource availability in temperate forest soils leads to a seasonal variation in functional properties of soil microorganisms, resulting from seasonal changes in microbial community structure and physiological adaptations of microorganisms.

## **5. Acknowledgments**

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## Legends to figures

### Figure 1

Results of a principal component analysis from 7 microbial process rates (C mineralization, priming effect of SOM mineralization, substrate  $^{13}\text{C}$  in respiration, actual cellulase/ amylase, cellobiosidase, peptidase, and chitinase activities) relative to control incubations without substrate addition in soils collected in winter (triangles), in summer (circles) and in summer from girdling plots (squares), which were incubated with five organic substrates with or without inorganic N supply. Values are means  $\pm$  SE (n = 4).

### Figure 2

Changes in (a) cumulative C mineralization and (b) gross N mineralization rates (relative to unamended controls) in response to addition of different organic substrates and inorganic N in soils collected in winter (hatched bars), in summer (black bars) and in summer from girdling plots (grey bars). Values are means  $\pm$  SE. (a) n = 4, (b) n = 5. Significant differences between communities in incubations of a certain substrate (determined by ANOVA,  $p < 0.05$ ) are indicated by asterisks (\*). Values of unamended controls are given in Table S1. Incubation time was 2 days for glucose and protein incubations and 6 days for incubations of microbial cell walls, cellulose and plant cell walls.

### Figure 3

(a) Cumulative priming effect of SOM respiration induced by addition of different organic substrates and inorganic N and (b) microbial carbon use efficiency calculated from respired substrate  $^{13}\text{C}$  and substrate  $^{13}\text{C}$  in microbial biomass (see 2.4) in soils collected in winter (hatched bars), in summer (black bars) and in summer from girdling plots (grey bars). Values are means  $\pm$  SE (n = 4). Significant differences between communities in incubations of a certain substrate (determined by ANOVA or t-test,  $p < 0.05$ ) are indicated by asterisks (\*).

#### Figure 4

Changes in extracellular enzyme activities (relative to unamended controls) in response to addition of different organic substrates and inorganic N in soils collected in winter (hatched bars), in summer (black bars) and in summer from girdling plots (grey bars). Values are means  $\pm$  SE (n = 5). Significant differences between communities in incubations of a certain substrate (determined by ANOVA or t-test,  $p < 0.05$ ) are indicated by asterisks (\*). Values of unamended controls are given in Table S1. Incubation time was 2 days for glucose and protein incubations and 6 days for incubations of microbial cell walls, cellulose and plant cell walls.

Table 1 Characterization of soils collected in winter, in summer and in summer from girdling plots. Values from soil incubations without substrate addition (winter: 4°C, summer 12°C). Mean values (n = 5). Significant differences (by Tukey's post hoc test;  $p < 0.05$ ) are indicated by different letters.

	Winter	Summer	Summer - Girdling
NH <sub>4</sub> <sup>+</sup> (μmol g <sup>-1</sup> DW)	0.21 <sup>a</sup>	0.19 <sup>a</sup>	0.15 <sup>a</sup>
NO <sub>3</sub> <sup>-</sup> (μmol g <sup>-1</sup> DW)	1.73 <sup>b</sup>	0.65 <sup>a</sup>	0.78 <sup>ab</sup>
Total dissolved N (μmol g <sup>-1</sup> DW) <sup>†</sup>	2.5 <sup>a</sup>	3.3 <sup>b</sup>	4.6 <sup>c</sup>
Dissolved organic C (μmol g <sup>-1</sup> DW)	8.4 <sup>a</sup>	13.6 <sup>b</sup>	14.4 <sup>b</sup>
Microbial C (μmol g <sup>-1</sup> DW)	31.7 <sup>ab</sup>	37.0 <sup>b</sup>	24.2 <sup>a</sup>
Microbial N (μmol g <sup>-1</sup> DW)	5.1 <sup>ab</sup>	6.9 <sup>b</sup>	3.4 <sup>a</sup>
Microbial C/N ratio	6.2 <sup>ab</sup>	5.5 <sup>a</sup>	7.2 <sup>b</sup>
Net N mineralization (nmol NH <sub>4</sub> -N g <sup>-1</sup> DW h <sup>-1</sup> )	-6.1 <sup>a</sup>	-1.8 <sup>a</sup>	3.0 <sup>a</sup>

<sup>†</sup> measured in KCl-Extracts

Table 2 ANOVA results showing effects of community type, substrate and inorganic N addition on alterations of various microbial processes. n.d. means 'not determined'.

	dF	C mineralization		Gross N mineraliz.		Priming effect		C use efficiency		Cellulase / Amylase	
		F	p	F	p	F	p	F	p	F	p
Community	2	0.14	0.8684	6.57	<b>0.0027</b>	1.00	0.3747	2.66	0.0842	15.68	<b>0.0000</b>
Substrate	4	193.50	<b>0.0000</b>	15.23	<b>0.0000</b>	9.22	<b>0.0000</b>	69.39	<b>0.0000</b>	147.82	<b>0.0000</b>
Comm. x Substrate	8	12.77	<b>0.0000</b>	1.75	0.1066	11.27	<b>0.0000</b>	12.84	<b>0.0000</b>	18.44	<b>0.0000</b>
+ N	1	0.52	0.4789	n.d.	n.d.	1.84	0.1881	0.91	0.3515	1.37	0.2497
Comm. x N	1	0.05	0.8181	n.d.	n.d.	0.00	0.9812	1.14	0.2977	6.76	<b>0.0140</b>

	dF <sup>†</sup>	Cellobiosidase		Peptidase		Endochitinase		Phenoloxidase		Peroxidase	
		F	p	F	p	F	p	F	p	F	p
Community	2	6.44	<b>0.0029</b>	10.68	<b>0.0001</b>	11.08	<b>0.0001</b>	6.81	<b>0.0136</b>	5.85	<b>0.0214</b>
Substrate	4	3.55	<b>0.0115</b>	2.89	<b>0.0294</b>	6.88	<b>0.0001</b>	6.67	<b>0.0013</b>	9.91	<b>0.0001</b>
Comm. x Substrate	8	4.70	<b>0.0002</b>	4.73	<b>0.0002</b>	3.80	<b>0.0012</b>	4.96	<b>0.0062</b>	4.59	<b>0.0088</b>
+ N	1	0.64	0.4307	101.99	<b>0.0000</b>	13.05	<b>0.0010</b>	0.05	0.8202	1.03	0.3259
Comm. x N	1	9.65	<b>0.0039</b>	0.00	0.9582	0.01	0.9190	0.59	0.4553	0.10	0.7595

<sup>†</sup> dF Oxidative Enzyme activities : 1, 3, 3, 1, 1 (Summer only).

Table 3 Microbial utilization of <sup>13</sup>C-labeled substrates by the winter, summer and summer-girdling community indicated by concentrations of substrate <sup>13</sup>C in respiration and microbial biomass (estimated from PLFAs). Mean values; n = 4 (respiration), n = 3 (microbial biomass). Significant differences (by Tukey's post hoc test; p<0.05) are indicated by different letters. n.d. means 'not determined'.

	Substrate <sup>13</sup> C in respiration (atom % excess <sup>13</sup> C)			Substrate <sup>13</sup> C in microbial biomass (atom % excess <sup>13</sup> C)		
	W	S	G	W	S	G
Glucose	10.4 <sup>a</sup>	12.6 <sup>b</sup>	10.9 <sup>ab</sup>	0.84 <sup>a</sup>	1.14 <sup>b</sup>	1.13 <sup>ab</sup>
Protein	11.4	8.9	8.9	1.40 <sup>b</sup>	1.00 <sup>a</sup>	1.12 <sup>a</sup>
Microbial cell walls	2.7 <sup>a</sup>	3.4 <sup>b</sup>	3.7 <sup>b</sup>	1.91	1.51	2.09
Cellulose	0.31	0.31	0.41	0.06 <sup>ab</sup>	0.04 <sup>a</sup>	0.09 <sup>b</sup>
Cellulose + N	n.d.	0.59 <sup>a</sup>	1.07 <sup>b</sup>	n.d.	0.14 <sup>a</sup>	0.42 <sup>b</sup>
Plant cell walls	0.29 <sup>a</sup>	0.36 <sup>ab</sup>	0.43 <sup>b</sup>	0.11	0.11	0.08
Plant cell walls + N	n.d.	0.65 <sup>a</sup>	1.00 <sup>b</sup>	n.d.	0.11 <sup>a</sup>	0.16 <sup>b</sup>

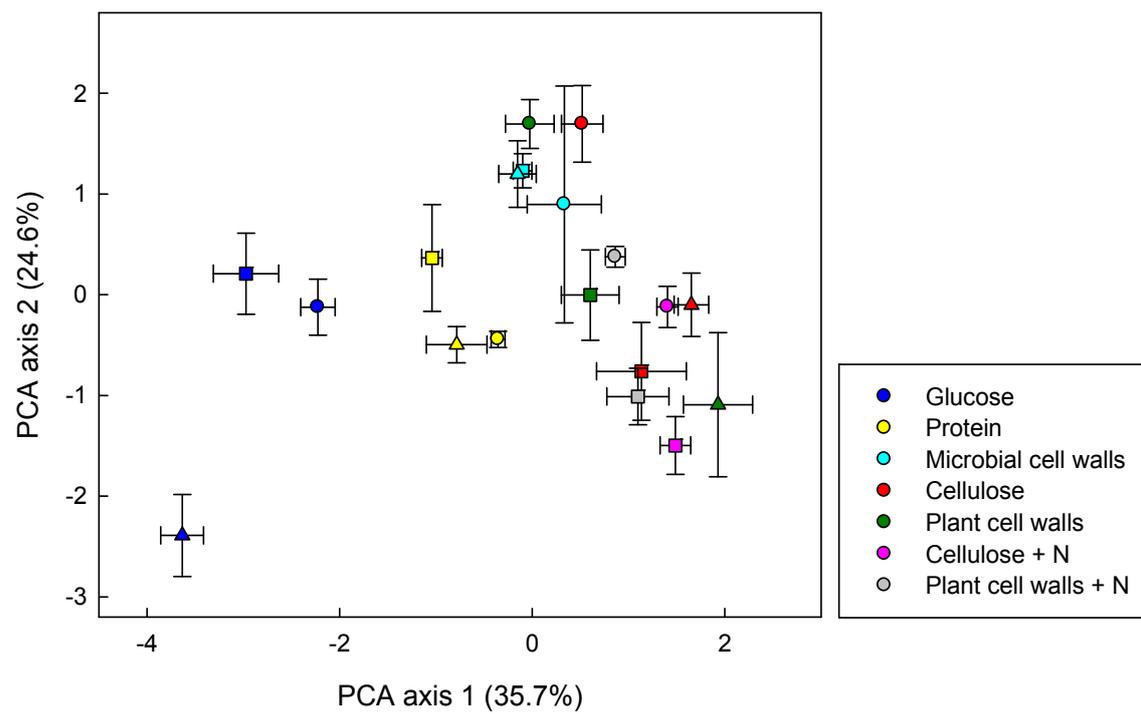


Fig. 1

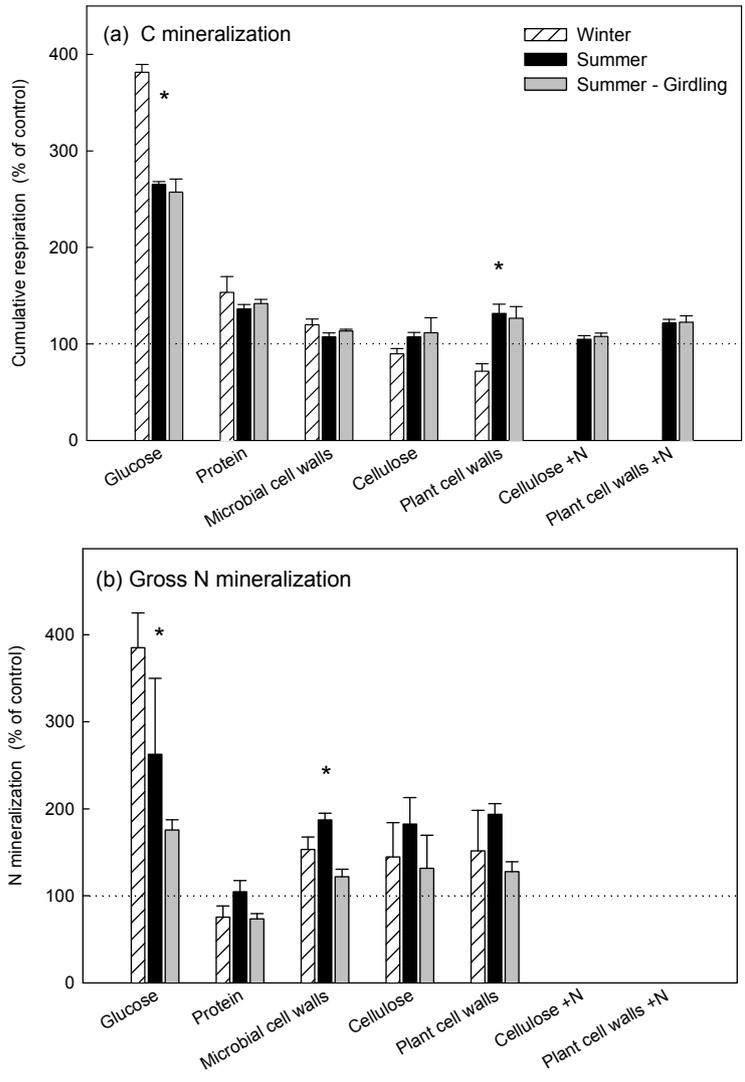


Fig. 2

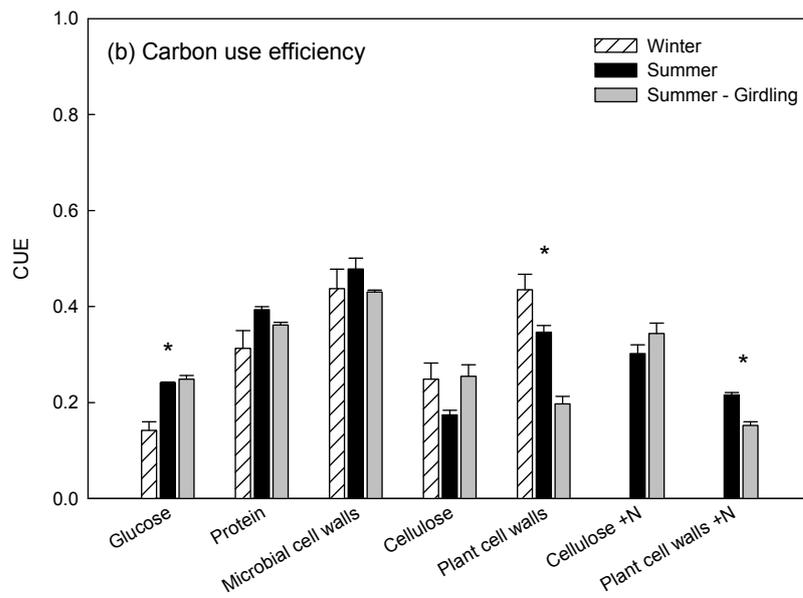
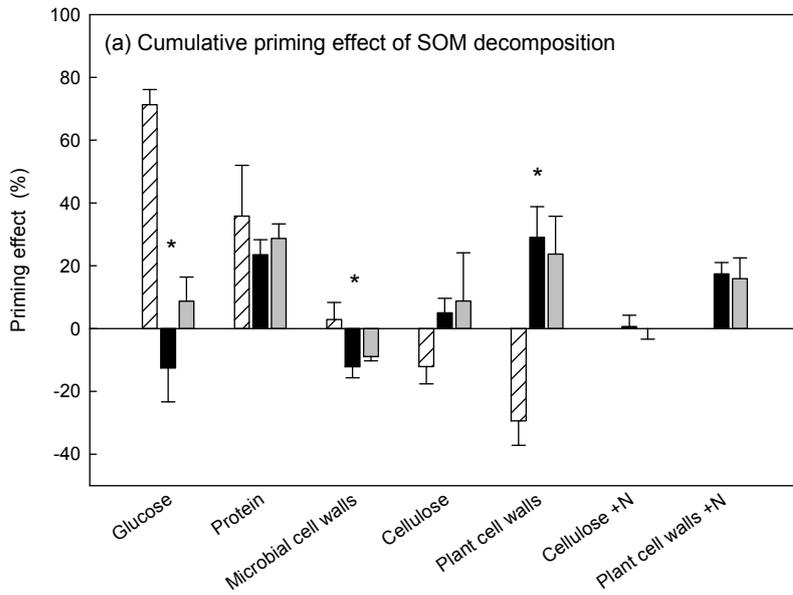


Fig. 3

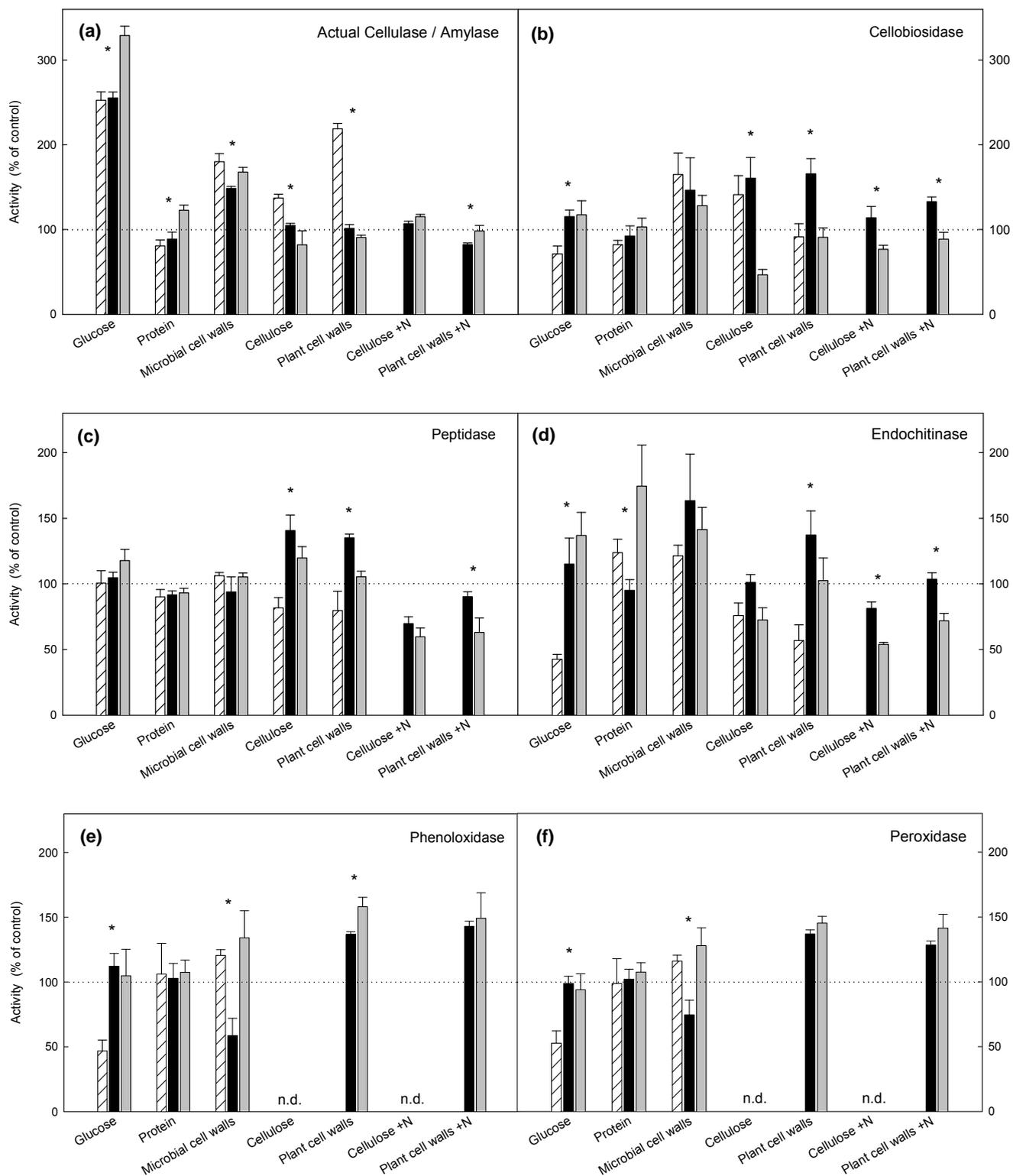


Fig. 4