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Abstract: Glucans like cellulose and starch are a major source of carbon for decomposer food webs, especially during early- and intermediate-stages of decomposition. Litter quality has previously been suggested to notably influence decomposition processes as it determines the decomposability of organic material and the nutrient availability to the decomposer community. To study the impact of chemical and elemental composition of resources on glucan decomposition, a laboratory experiment was carried out using beech (Fagus sylvatica, L.) litter from four different locations in Austria, differing in composition (concentration of starch, cellulose and acid unhydrolyzable residue or AUR fraction) and elemental stoichiometry (C:N:P ratio). Leaf litter was incubated in mesocosms for six months in the laboratory under controlled conditions. To investigate the process of glucan decomposition and its controls, we developed an isotope pool dilution (IPD) assay using (13)C-glucose to label the pool of free glucose in the litter, and subsequently measured the dilution of label over time. This enabled us to calculate gross rates of glucose production through glucan depolymerization, and glucose consumption by the microbial community. In addition, potential activities of extracellular cellulases and ligninases (peroxidases and phenoloxidases) were measured to identify effects of resource chemistry and stoichiometry on microbial enzyme production. Gross rates of glucan depolymerization and glucose consumption were highly correlated, indicating that both processes are co-regulated and intrinsically linked by the microbial demand for C and energy and thereby to resource allocation to enzymes that depolymerize glucans. At early stages of decomposition, glucan depolymerization rates were correlated with starch content, indicating that starch was the primary source for glucose. With progressing litter decomposition, the correlation with starch diminished and glucan depolymerization rates were highly correlated to cellulase activities, suggesting that cellulose was the primary substrate for glucan depolymerization at this stage of decomposition. Litter stoichiometry did not affect glucan depolymerization or glucose consumption rates early in decomposition. At later stages, however, we found significant negative relationships between glucan depolymerization and litter C:N and AUR:N ratio and a positive relationship between glucan depolymerization and litter N concentration. Litter C:N and C:P ratios were negatively related to cellulase, peroxidase and phenoloxidase activities three and six months after incubation, further corroborating the importance of resource stoichiometry for glucan depolymerization after the initial pulse of starch degradation.

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A B S T R A C T

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1. Introduction

Cellulose and starch represent a major source of carbon (C) for microbial decomposer communities, constituting about one-third of total plant biomass (Somerville, 2006). Cellulose is the major structural component of the plant cell wall (Brett and Waldron, 1996), which can be found in nearly all plant tissues, and has therefore been denoted the most abundant biopolymer on earth (Perez et al., 2002). Starch is an osmotically inactive storage molecule, which is synthesized during photosynthesis and stored in form of starch granules in plastids (Taiz and Zeiger, 2002). Cellulose is considered more recalcitrant than starch because of its...
internal composition of β-linked glucose units resulting in long fiber structures (Perez et al., 2002) and its interconnection with lignin forming the so called “lignocellulose” complex. The ratios of easily degradable to recalcitrant compounds have been reported to substantially influence the rate of litter decomposition (Aber et al., 1990; Austin and Ballare, 2010; Berg and Agren, 1984; Couteaux et al., 1995; Fioretto et al., 2005).

The enzymatic depolymerization of glucans by extracellular enzymes is considered to be the rate-limiting step in glucan decomposition (Perez et al., 2002). Extracellular enzymes are produced and excreted by microbial decomposers to depolymerize macromolecules into smaller, soluble compounds that can subsequently be taken up by microorganisms. The production of extracellular enzymes is regulated by nutrient availability and is thought to reflect the demand of the microbial community for nutrients and energy (Sinsabaugh et al., 2009). As enzyme production requires nutrients, especially nitrogen (N) (Schimel and Weintraub, 2003), the elemental composition of plant litter (i.e., its elemental stoichiometry) may have a major impact on decomposition rates. Activities of extracellular C-acquiring enzymes like cellulases and amylases have previously been suggested to be N-limited (Berg, 2000; Berg and Matzner, 1997; Fogg, 1988) and should therefore be enhanced if N is readily available, or at least not limiting, which could result in a higher glucan depolymerization rates in litter with lower C:N ratios.

Up to now, decomposition of cellulose and starch in plant litter could only be examined by observations of the decrease in the respective pool size compared to absolute litter mass loss (e.g., Fioretto et al., 2005; Papa et al., 2008; Sariyildiz and Anderson, 2003; Tagliavini et al., 2007), or by measuring potential activities of glucan-depolymerizing enzymes i.e. cellulases and amylases (e.g., Fioretto et al., 2005; Papa et al., 2008) via photometric and fluorimetric assays (Koenig et al., 2002; Marx et al., 2001; Sinsabaugh et al., 1999). However, changes in pool sizes only provide insight into long-term depolymerization rates, and the determination of potential enzyme activities has previously been criticized as they do not represent the actual rates of decomposition, which are dependent on concentration and accessibility of substrates as well as on enzyme activity and environmental conditions (Wallenstein and Weintraub, 2008). To overcome these challenges, we have established a new method to estimate actual glucan depolymerization rates based on the contents of free glucose, which are an important class of biopolymers, including the glucose disaccharides (e.g. sucrose), oligosaccharides (e.g. raffinose) or polymeric glucose (starch, cellulose). The enzymatic control of glucan depolymerization by extracellular enzymes has been extensively studied and is thought to strongly influence the demand of the microbial community for nutrients and energy (Sinsabaugh et al., 2009). As enzyme production requires nutrients, especially nitrogen (N) (Schimel and Weintraub, 2003), the elemental composition of plant litter (i.e., its elemental stoichiometry) may have a major impact on decomposition rates. Activities of extracellular C-acquiring enzymes like cellulases and amylases have previously been suggested to be N-limited (Berg, 2000; Berg and Matzner, 1997; Fogg, 1988) and should therefore be enhanced if N is readily available, or at least not limiting, which could result in a higher glucan depolymerization rates in litter with lower C:N ratios.

To examine the controls of litter chemistry, including the concentrations of starch, cellulose and lignin as well as the stoichiometry of C and N availability, on glucan depolymerization, we conducted a laboratory incubation experiment under controlled conditions using beech (Fagus sylvatica L.) litter of varying elemental stoichiometry (C:N and C:P ratios) and chemical composition. The litter had been sterilized by ɣ-irradiation and re-inoculated with a beech forest soil inoculum (organic horizon) to establish a comparable initial microbial community for each litter type, and was then incubated for six months in mesocosms at constant temperature and humidity (Wanek et al., 2010).

2. Material and methods

To clarify the use of terminology we define carbohydrates, being synonymous with saccharides, as organic compounds with the general formula \( C_n(H_2O)_m \), i.e. polyhydroxy aldehydes or polyhydroxy ketones. Saccharides are divided into monosaccharides ([\( C_n(H_2O)_m \) with \( n > 3 \), e.g. glucose, fructose, xylose, arabinose], disaccharides (e.g. sucrose), oligosaccharides and polysaccharides. The term sugars is commonly used for mono- and disaccharides. Oligosaccharides typically contain between three and ten monosaccharide units, and polysaccharides more than ten units. The latter are an important class of biopolymers, including the glucose homo-polymers starch and cellulose (glucans, i.e. polysaccharides of α-glucose monomers linked by alpha- or beta-glycosidic bonds) and the hetero-polymers hemicellulose (e.g. xylans, arabinoxylans, galactomannans). Hemicelluloses in beech contain only traces of glucose (<1%) and are mainly composed of xylose (65%), arabinose (20%) and galactose (15%), and therefore are not subsumed under glucans (Schaedel et al., 2010). Free glucose comprises the free, mostly cytosolic and vacuolar pool of unbound dissolved glucose, as contrasted by bound glucose such as that contained in disaccharides (e.g. sucrose, maltose, cellobiose), oligosaccharides (e.g. raffinose) or polymeric glucose (starch, cellulose). Decomposition of homo-polymeric glucans therefore releases a range of glucose-based oligosaccharides, disaccharides of glucose (maltose, cellobiose) and ultimately free glucose.

2.1. Litter decomposition experiment

A litter decomposition experiment was carried out over six months to explore the impact of litter quality on glucan decomposition. Freshly fallen beech (F. sylvatica L.) leaf litter with different litter chemistry and elemental stoichiometry (referred to as ‘litter type’) was collected in autumn 2008 from four sampling sites in Austria: Achenkirch (A), Klausenleopoldsdorf (K), Ossiach (O) and Schottenwald (S). Site characteristics and preparation of the litter types were described by Wanek et al. (2010). In short, leaves were dried at 40 °C for 48 h, shredded to pieces between 1 and 20 mm and sterilized twice with gamma rays of 35 kGy with one week between irradiation events. Then all litter types were inoculated with a suspension of an O-horizon litter mixture (1:1 (w:w)) collected from Klausenleopoldsdorf in December 2008 to obtain an identical initial microbial community structure on all four litter types. For the inoculation, each litter type and the inoculum suspension amounting to 1.5% dry weight of litter were thoroughly mixed. After homogeneous distribution of the inoculum, mesocosms (n = 5), which had been prepared from PVC plastic tubing (10 cm length, 12.5 cm diameter), were filled with 60 g of litter fresh weight for all four litter types (A, K, O and S) and three harvest time points (two weeks, three and six months after inoculation), giving 60 mesocosms in total. Mesocosms were placed on humid sponge cloth in a climate chamber at 15 °C and watered weekly with autoclaved tap water to keep the water content stable at 60% fresh weight. Additional mesocosms were prepared for each harvest and litter type to determine the size of the free glucose pool one week before the actual harvests. The litter from each mesocosm was homogenized before analysis by putting it into a plastic bag and thoroughly mixing it.
2.2. Isotope pool dilution assay

To determine rates of gross glucan depolymerization and gross glucose immobilization, a new isotope pool dilution (IPD) assay was developed. Isotope pool dilution is a common method to determine gross transformation rates of N, P and S in soil (Di et al., 2000) by labeling a target pool and subsequently measuring the dilution of the label and the change in pool size. We developed an IPD method using $^{13}$C-labeled glucose (99 atom % $^{13}$C, D-glucose, Isotec Inc.) as a tracer. What this IPD assay therefore measures are gross influx rates into the labeled free glucose pool (which is by glucose production from glucan decomposition) and gross efflux rates (which in this experiment is mainly due to microbial uptake/immobilization). Glucose production from glucan decomposition leads to input of unlabelled glucose and therefore to isotope pool dilution of the free glucose pool, while microbial uptake/immobilization consumes glucose with the $^{13}$C/$^{12}$C ratio as present at any time point in the free glucose pool. For the assay, 1.5 g of litter fresh weight were filled into 50 ml HDPE centrifuge tubes in triplicates. To start the assay, a maximum of 50% of the free glucose pool in the sample was added in form of $^{13}$C-labeled glucose (30 atom% $^{13}$C) dissolved in high purity water (MilliQ, >18.2 MΩm, Millipore), ranging from 0.01 to 5 mg $^{13}$C-Glc l$^{-1}$ in 5 ml MilliQ water. The size of the free glucose pool was determined for each litter type prior to the start of the IPD assay. The vials were shaken vigorously to distribute the label homogeneously. The amount of liquid added via the tracer solution was selected to form a thin water film on the leaf particles that assured a homogeneous tracer distribution without causing anoxic conditions. After shaking the vials, they were re-opened, sealed loosely with cotton wool to enable gas exchange and then incubated at 15 °C for 5 min at 10,845 g. The supernatant was then decanted into 30 ml syringes that contained a plug of cotton wool on the top of OnGuard II A, volume 1 cc, bicarbonate form; mixed with NaOH (sodium persulfate (sodium peroxodisulfate purum p.a., >99%, Fluka, Sigma–Aldrich) and 1.7 M phosphoric acid (orthophosphoric acid puriss. p.a., crystallized, >99%, Fluka, Sigma–Aldrich) and were added to the column effluent at a flow rate of 50 μl min$^{-1}$ each. In a gas separation unit, the CO$_2$ was transferred over gas-permeable membranes to a counter flow of helium as carrier gas. This gas stream was dried over NaI-on tubes and before entering the IRMS via an open split, excess oxygen was removed as described by Hettmann et al. (2007) inside a reduction reactor to improve both filament lifetime and reproducibility of the analysis.

2.3. Isolation of glucose from litter

Immediately after filtration, the solution was applied to coupled cation and anion exchange cartridges (OnGuard II H, volume 1 cc, H$^+$ form, on top of OnGuard II A, volume 1 cc, bicarbonate form; both from Dionex) which had been soaked by flushing with 10 ml of MilliQ water for two hours prior to sample application. After the sample solution was passed slowly through the ion exchange cartridges, they were eluted with 5 ml of MilliQ water to collect non-adsorbed neutral compounds (including glucose). The flow-through was collected and transferred into 250 ml vacuum proof round bottom flasks, frozen at −20 °C and freeze-dried for 24 h. The residue was dissolved in 3 ml of MilliQ water, transferred into 20 ml HDPE vials, frozen again and freeze-dried over night. The dried extract was dissolved in 0.5 ml MilliQ water and stored frozen until analysis.

2.4. Isotopic analysis

The amount and δ$^{13}$C value of glucose in the samples was measured via compound-specific isotopic analysis on a high performance liquid chromatography-isotope ratio mass spectrometer (HPLC-IRMS) system as described by Wild et al. (2010). The HPLC system consisted of an ICS3000 pump, an AS50 autosampler with a 25 μl injection loop and an Ultimate 3000 column compartment (all provided by Dionex). The separation column was a HyperREZ XP Carbohydrate Că$^+$ 8 μm column (Thermo Fisher Scientific, USA), run at 85 °C with 0.5 ml min$^{-1}$ MilliQ water as eluent. The HPLC was connected to the IRMS (Finnigan Delta V Advantage Mass Spectrometer, Thermo Fisher Scientific, USA) via a Finnigan LC IsoLink Interface (Thermo Fisher Scientific, USA), where the glucose was oxidized to CO$_2$ via acid persulfate digestion inside an oxidation reactor at 99.9 °C. As oxidant, a 0.5 M solution of sodium persulfate (sodium persulfate purum p.a., >99%, Fluka, Sigma–Aldrich) and 1.7 M phosphoric acid (orthophosphoric acid puriss. p.a., crystallized, >99%, Fluka, Sigma–Aldrich) were added to the column effluent at a flow rate of 50 μl min$^{-1}$ each. In a gas separation unit, the CO$_2$ was transferred over gas-permeable membranes to a counter flow of helium as carrier gas. This gas stream was dried over NaI-on tubes and before entering the IRMS via an open split, excess oxygen was removed as described by Hettmann et al. (2007) inside a reduction reactor to improve both filament lifetime and reproducibility of the analysis.

2.5. Spiking of low concentration samples

The limit of isotope determination of glucose at precision better than 0.25% (SD) with the HPLC-Isolink-IRMS system was approximately 20 mg Glc l$^{-1}$. Samples with glucose concentrations below this limit had to be measured through spiking of the samples. A standard stock solution with a concentration of 10 g l$^{-1}$ D-glucose (Merck, Vienna, Austria) in MilliQ water was prepared and a working solution was prepared freshly every day by diluting the stock solution 1:10. Then, 10 μl of the working solution were pipetted into 250 μl glass inserts for GC vials and 90 μl sample were added. The concentration of the standard in the spiked sample therefore constituted 100 mg Glc l$^{-1}$. The δ$^{13}$C value of the spiked sample was then measured on the HPLC-IRMS system. Additionally, the glucose concentration of unspiked samples was determined on a high performance anion exchange chromatography-pulsed amperometric detection system (HPAEC-PAD), which has a much lower detection limit than the HPLC-IRMS system (0.024 mg l$^{-1}$). The HPAEC-PAD system consisted of an ICS3000 SP-1 Pump, an AS50 Autosampler with a 10 μl injection loop and an ICS3000 DC-2 Detector/Chromatography Module (all provided by Dionex, Vienna, Austria). As separation column a CarboPac PA20 (3 × 150 mm Analytical Column with a CarboPac PA20, 3 × 30 mm Guard Column, Dionex) was run with 0.5 ml min$^{-1}$ 20 mM NaOH as eluent. For calibration, the glucose stock solution was used in concentrations between 0.1 and 50 mg l$^{-1}$. The δ$^{13}$C value of the glucose in the sample could then be determined using an isotopic mixing model (1):

$$\delta_{\text{sample}} = \frac{c_{\text{all}} \times \delta_{\text{all}} - c_{\text{spike}} \times \delta_{\text{spike}}}{c_{\text{sample}}}$$

(1)

$$c_{\text{all}} = c_{\text{sample}} + c_{\text{spike}}$$

(2)

where $c_{\text{sample}}$ is the concentration of glucose in the sample as measured by the HPAEC-PAD system. $c_{\text{spike}}$ is the concentration of the glucose standard, which was 100 mg l$^{-1}$, and $c_{\text{all}}$ is the...
The potential activity of β-1,4-cellobiosidase as a representative of the large class of cellulases ('cellulase') was determined using a microplate fluorimetric assay based on MUF-β-o-cellobioside (4-methylumbelliferyl-β-o-cellobioside, 0.5 mM in 100 mM sodium acetate buffer) (Kaiser et al., 2010; Marx et al., 2001). The activities of β-1,4-cellobiosidase and β-glucosidase have previously been used as a proxy for total cellulase activity in soils (e.g., Geisseler and Horwath, 2009; Kaiser et al., 2010; Sinsabaugh et al., 2009). β-1,4-cellobiosidase is an exo-cellulase and releases the disaccharide cellobiose from cellulose and cellootetraose, which then is cleaved by β-glucosidase into two glucose monomers to be taken up by microbes (Perez et al., 2002). Activities of oxidative enzymes (peroxidase and phenoloxidase) were measured photometrically according to standard assays (Sinsabaugh et al., 1999) with small modifications as described by Kaiser et al. (2010). As substrate, L-3,4-dihydroxyphenylalanin (L-DOPA, 20 mM) was used for both assays of oxidative enzymes.

2.9. Litter and microbial stoichiometry, respiration and litter chemistry

Litter C (C_{lit}) and N (N_{lit}) content were measured after drying at 80 °C and grinding in a ball mill with an elemental analyzer (EA 1110, CE Instruments). Phosphorus content of the ground litter (P_{lit}) was determined after acid digestion (Henschler, 1988) by inductively coupled plasma atomic emission spectrometry (ICP-AES). Microbial biomass C (C_{mic}) and N (N_{mic}) content were estimated in fresh litter by the chloroform fumigation-extraction technique (Schinner et al., 1996) using a TOC/TN analyzer (TOC-CPH and TNM-1, Shimadzu). Ratios of litter and microbial C:N, C:P and N:P as well as litter cellulose:N and AUR:N were calculated on a mass basis. C:N imbalance as a measure of the imbalance between microbial and litter stoichiometry was calculated as follows:

\[
C : N_{imbalance} = \frac{C_{lit}}{C_{mic} : N_{mic}}
\]
chamber (SRC-1 Soil Respiration Chamber, PP systems) which was placed on top of the mesocosm and the bottom was closed with a gas tight lid. Carbon dioxide concentration in the headspace was continuously recorded at 5-s intervals for 70 s, and the averaged CO₂ increase per second was used to calculate respiration rate. Respiration was measured one day prior to the harvesting of the mesocosms. Additionally, respiration was monitored throughout the entire 6 months incubation period in weekly respiration measurements using the same system (Keiblinger KM, unpublished data).

Starch content was determined as described by Goettlicher et al. (2006) by determination of glucose released after addition and incubation with heat-stable α-amylase (Sigma–Aldrich, Vienna, Austria) from Bacillus licheniformis (500 U ml⁻¹ MilliQ water) and amyloglucosidase (Roche Diagnostics, Vienna, Austria) from Aspergillus niger (10 U in 0.5 ml 20 mM sodium acetate buffer, pH 4.6) by HPAEC-PAD as described above. Cellulose and acid unhydrolyzable residue (AUR) content were determined following a modification of the acid detergent method (Rowland and Roberts, 1994) as published in the manual “Use of isotope and radiation methods in soil and water management and crop nutrition” by the International Atomic Energy Agency (http://www-pub.iaea.org/MTCD/publications/PDF/TCS-14.pdf). Aliquots of finely ground plant litter (0.5 g, dried at 60°C) containing the remaining fraction (AUR and ash) were ashed at 50 to 260°C in a gas tight lid. Carbon dioxide concentration in the headspace was measured one day prior to the harvesting of the litter. Kolmogorov-Smirnov-test was performed to test for normal distribution and Levene’s test to test for homogeneity of variance. Data points with a distance from the arithmetic mean of more than twice the standard deviation were defined as outliers and excluded, and if necessary, the data was log(x + 1)-transformed to obtain normal distribution and homogeneity of variances. Relations between gross rates and litter chemistry and elemental stoichiometry as well as enzymatic activities were examined through simple Pearson correlation analysis on untransformed data. Statistical analyses were performed with Statgraphics 5.0 (Statistical Graphics Inc.) and Statistica 7.1 (StatSoft Inc.).

3. Results

3.1. Litter chemistry and elemental stoichiometry

Litter chemistry was significantly affected by harvest and litter type (Table 1). As expected, starch content (Fig. 1) decreased rapidly in the first three months from initial concentrations ranging between 0.11 and 0.29% d.w. to between 0.06 and 0.18% d.w. at harvest two, but did not further decrease between harvests two and three. Cellulose concentration (Fig. 1) ranged from 18.0 to 20.7% d.w. in the initial litter, then increased in the first three months of decomposition to values between 19.7 and 25.8% d.w., and then slightly decreased until finally reaching values between 18.1 and 24.4% d.w. after six months. Acid unhydrolyzable residue (AUR) concentrations (Fig. 1) were lowest in litter collected from Ossiach and Schottenwald, which also had the lowest AUR:N ratios (11.3–19.6). The Klausenleopoldsdorf and Achenkirch litter had the highest AUR:N ratios, ranging from 16.0 to 37.7. Cellulose and AUR content showed a strong positive relationship (r = 0.79, p < 0.001, Supplementary Table 2). In litter from Klausenleopoldsdorf and Achenkirch, AUR concentrations increased during the first three months of the experiment, and then only slightly decreased reaching final values around 26% d.w. after six months of litter decomposition. The large increase in the proportion of cellulose and AUR during the incubation may be due to the loss of mass

| Harvest × litter type | F     | p   | F     | p   |
|-----------------------|-------|-----|-------|-----|
| C:N                  | 8.45  | 0.0007 | 360.99 | 0.0001 | 1.58 | 0.1749 |
| C:P                  | 1.25  | 0.2958 | 251.45 | 0.0001 | 3.10 | 0.0120 |
| N:P                  | 3.37  | 0.0425 | 184.66 | 0.0001 | 1.82 | 0.1152 |
| Glucose concentration | 112.70 | 0.0001 | 26.90  | 0.0001 | 16.43 | 0.0001 |
| MRT Glucose          | 230.31 | 0.0001 | 48.01  | 0.0001 | 28.01 | 0.0001 |
| Starch concentration | 51.08  | 0.0001 | 35.94  | 0.0001 | 7.61  | 0.0001 |
| AUR concentration    | 29.20  | 0.0001 | 24.84  | 0.0001 | 3.41  | 0.0079 |
| Harvest               | 20.18  | 0.0001 | 37.11  | 0.0001 | 4.56  | 0.0012 |
| Gross glucan         | 4.21   | 0.0216 | 10.35  | 0.0001 | 6.47  | 0.0001 |
| Gross glucose        | 22.22  | 0.0001 | 22.86  | 0.0001 | 10.01 | 0.0001 |

2.10. Statistical analyses

Significant differences (p < 0.05) between litter types and harvests were analyzed by two-way ANOVA followed by Fisher’s LSD post-hoc test or, if sample sizes were unequal, Scheffé post-hoc test. Kolmogorov-Smirnov-test was performed to test for normal distribution and Levene’s test to test for homogeneity of variance. Data points with a distance from the arithmetic mean of more than twice the standard deviation were defined as outliers and excluded, and if necessary, the data was log(x + 1)-transformed to obtain normal distribution and homogeneity of variances. Relations between gross rates and litter chemistry and elemental stoichiometry as well as enzymatic activities were examined through simple Pearson correlation analysis on untransformed data. Statistical analyses were performed with Statgraphics 5.0 (Statistical Graphics Inc.) and Statistica 7.1 (StatSoft Inc.).
during decomposition of the litter, due to accumulation of microbial products or be related to inaccuracies of the acid detergent methodology used to measure cellulose and AUR. Mass loss after 3 and 6 months was small, ranging between 1.2% and 2.2% of initial mass after 3 months and between 2.8% and 4.6% after 6 months. Mass loss therefore cannot account for the large increase in AUR and cellulose concentration. The technique to quantify AUR and cellulose using solvent/acid extractions has been criticized for its unspecificity (Hatfield and Fukushima, 2005). PyGCMS is much more amenable to analyze the macromolecular composition of environmental samples such as soils and litter, where pyrolysates peaks are quantified and assigned by mass spectrometry to major C fractions (glucan, lignin, protein, fatty acid etc). Lignin contents by pyGCMS initially ranged between 28.9% (A), 29.9% (K), 30.5% (S) and 31.2% (O) while carbohydrates (glucan) comprised 25.9% (A), 26.1% (K), 26.9% (S) and 29.2% (O) of dry matter. PyGCMS did not show a consistent change in lignin over the first three or six months or if an increase was found it was marginal (A -0.2%, K +0.7%, O +1.7%, S +2.9%) compared to the increases found with solvent/acid extractions. Cellulose decreased slightly (by 1.5–2.4%) in two litter types (Ossiach, Klausenleopoldsdorf) but not in the others. Moreover, pyGCMS analysis of the AUR preparation from this experiment clearly showed that lignin biomarkers comprised only a small fraction of AUR, with fatty acids, cutin and other materials contributing. We assume that unknown changes in the chemistry of the AUR fraction, most presumably an accumulation of fatty acids, cutin and microbial products, have led to the above-mentioned large increase in this fraction with progressing litter decomposition. Correlations presented in the paper were nonetheless calculated for solvent/acid extraction data of cellulose and AUR.

Elemental stoichiometry of the initial litter used in this experiment has already been described elsewhere (Wanek et al., 2010). Litter chemistry including elemental contents and ratios, pH and concentrations of \( \text{NH}_4^+ \), \( \text{NO}_3^- \), \( \text{PO}_4^{3-} \) and macro- and micronutrients of the initial litter are given in Table 2. Differences in C:N_{lit}, C:P_{lit} and N:P_{lit} were significant for the four litter types (Table 1) and these differences persisted across all three harvests.

![Fig. 1. Litter concentrations of starch, cellulose and acid unhydrolyzable residue (AUR) in the initial litter and after 3 and 6 months of decomposition, respectively (A, Achenkirch; K, Klausenleopoldsdorf; O, Ossiach; S, Schottenwald). Given are means of 5 mesocosms ± SE with exception for the initial litter (n = 4). Lower case letters indicate significant differences between litter types, upper case letters indicate significant differences between harvests (two-way ANOVA followed by Scheffé test, \( p = 0.05 \)).](image-url)
Table 2
Litter element contents, elemental stoichiometry, pH and concentrations of ammonium, nitrate and phosphate and macro- and micronutrients in four beech litter types (A, Achenkirch, K, Klausenleopoldsdorf, O, Ossiach, S, Schottenwald) measured after 2 weeks of decomposition. Given are means (±SE) (n = 5).

| Element | A | K | O | S |
|---------|---|---|---|---|
| pH      | 6.5 (±0.0) | 6.5 (±0.0) | 6.4 (±0.1) | 6.4 (±0.1) |
| C (mg g⁻¹ d.w.) | 508 (±4) | 494 (±5) | 481 (±4) | 489 (±3) |
| N (mg g⁻¹ d.w.) | 8.79 (±0.12) | 9.40 (±0.13) | 8.03 (±0.13) | 11.71 (±0.16) |
| C/N (mass ratio) | 57.9 (±0.6) | 52.6 (±0.5) | 60.0 (±0.7) | 418 (±8.8) |
| C/P (mass ratio) | 1282 (±21) | 1548 (±25) | 905 (±15) | 699 (±9) |
| N/P (mass ratio) | 22.2 (±0.5) | 29.5 (±0.6) | 15.1 (±0.3) | 168 (±0.4) |
| NH₄ (μg N H₄–N g⁻¹ d.w.) | 2.54 (±0.20) | 10.39 (±0.68) | 6.70 (±0.51) | 13.18 (±1.21) |
| NO₃ (μg N O₃–N g⁻¹ d.w.) | 2.24 (±0.07) | 2.33 (±0.07) | 2.09 (±0.09) | 3.12 (±0.16) |
| PO₄ (μg PO₄–P g⁻¹ d.w.) | 16.9 (±2.3) | 17.1 (±3.8) | 37.5 (±4.6) | 779 (±26.3) |
| K⁺ (mg g⁻¹ d.w.) | 2.58 (±0.02) | 5.36 (±0.04) | 2.08 (±0.01) | 5.48 (±0.04) |
| Ca²⁺ (mg g⁻¹ d.w.) | 13.3 (±0.1) | 12.6 (±0.2) | 16.3 (±0.1) | 12.3 (±0.2) |
| Mg²⁺ (mg g⁻¹ d.w.) | 2.72 (±0.02) | 1.40 (±0.01) | 1.96 (±0.01) | 1.50 (±0.01) |
| Fe (μg g⁻¹ d.w.) | 209 (±2) | 208 (±4) | 453 (±12) | 192 (±4) |
| Mn (μg g⁻¹ d.w.) | 172 (±3) | 1429 (±10) | 776 (±9) | 2137 (±51) |
| Zn²⁺ (μg g⁻¹ d.w.) | 30.6 (±0.4) | 33.0 (±0.3) | 35.8 (±1.0) | 422 (±0.6) |

3.2. Gross rates of glucan depolymerization and glucose consumption

Gross rates of glucan depolymerization and glucose consumption (Fig. 2) varied significantly between harvests and litter types (Table 1). Glucan depolymerization showed a different time course for each of the four litter types. After two weeks, litter from Ossiach had the highest rates of all four litter types (1390 μg C g⁻¹ d.w. d⁻¹), but rates decreased to intermediate levels after three months (507 μg C g⁻¹ d.w. d⁻¹). The Achenkirch litter, which had the lowest rates of all four litter types in the beginning (153 μg C g⁻¹ d.w. d⁻¹), showed a slight increase in glucan depolymerization until reaching levels similar to those of the other litter types six months after inoculation (291 μg C g⁻¹ d.w. d⁻¹). Glucan depolymerization in Schottenwald litter was relatively low in the beginning (433 μg C g⁻¹ d.w. d⁻¹), then peaked after three months, where Schottenwald litter had the highest rates of all four litter types (963 μg C g⁻¹ d.w. d⁻¹), and finally decreased to levels similar to the beginning (281 μg C g⁻¹ d.w. d⁻¹). Glucan depolymerization rates of litter from Klausenleopoldsdorf were intermediate and constant throughout the experiment (between 636 and 562 μg C g⁻¹ d.w. d⁻¹). After six months of incubation, differences in glucan depolymerization rates between litter types were no longer significant.

Gross rates of glucose consumption (Fig. 2) also showed significant differences between harvests and litter types (Table 1). Three of the four litter types (Ossiach, Schottenwald and Klausenleopoldsdorf) showed a strong decrease in glucose consumption rates over time with exception for litter from Achenkirch, which had generally very low rates at all time points that did not change over time. After two weeks of decomposition, glucose consumption rates of litter from Klausenleopoldsdorf, Ossiach and Schottenwald were at their highest levels (between 1690 and 2580 μg C g⁻¹ d.w. d⁻¹), being approximately 10-fold higher than the rates of Achenkirch litter (237 μg C g⁻¹ d.w. d⁻¹). After three months glucose consumption rates had declined until reaching very low levels at the end of the incubation after six months (between 331 and 574 μg C g⁻¹ d.w. d⁻¹), varying only slightly between the four litter types.

3.3. Glucose concentration and mean residence time

Glucose concentrations (Fig. 2) in litter from Klausenleopoldsdorf, Ossiach and Schottenwald were highest after two weeks (between 160 and 354 μg g⁻¹ d.w.), then decreased by 80% after three months (ranging from 25.3 to 56.5 μg g⁻¹ d.w.) and did not change further. Litter from Achenkirch exhibited very low glucose concentrations from the beginning (16.7 μg g⁻¹ d.w.), which did not change throughout the experiment. Mean residence times (MRT) for glucose (Fig. 2), calculated by dividing the pool size by the mean of influx and efflux rates, were highest at harvest one (0.23–0.82 d) and declined toward harvests two (0.11–0.16 d) and three (0.08–0.16 d), demonstrating an accelerating turnover of the glucose pool with declining glucose concentration.

3.4. Correlations between glucan depolymerization, glucose consumption, litter stoichiometry and litter chemistry

Linear regressions were performed to explore the influence of litter chemistry and elemental stoichiometry on glucan depolymerization and glucose consumption rates. Correlation coefficients are shown in Supplementary Table 1 for harvest one and Supplementary Table 2 for the combined harvests two and three. We decided to split the dataset, examining harvest one (n = 20) separately from the combined data of harvests two and three (n = 40), because our data suggested that initially starch was the main glucan being degraded, while at later stages of the experiment cellulose was the primary substrate for glucan depolymerization. This was indicated by a correlation between litter starch content and glucan depolymerization at harvest one (r = 0.63, p < 0.05) and a rapid decrease of starch content in the first three months of our experiment. In contrast, at harvests two and three glucan depolymerization was no longer related to starch content, but instead showed a very robust relationship (Fig. 3, r = 0.80, p < 0.001) to cellulose activity. Additionally, cellulases were almost not present at harvest one (Fig. 4), further corroborating starch as the main substrate for glucan depolymerization at early stages of decomposition.

After two weeks of decomposition litter stoichiometry was not correlated with glucan depolymerization and glucose consumption rates. For the combined data of harvests two and three, however, we found significant relationships of C:N (Fig. 5) with glucan depolymerization (r = –0.44, p < 0.01) and glucose consumption (r = –0.47, p < 0.01). The relationship was also significant for harvest two when investigated independently (p < 0.001, r < –0.72), but not for harvest three (p < 0.25). C:P and N:P were not correlated with glucan depolymerization and glucose consumption rates. At harvest one, C:N was negatively correlated with glucose mean residence time (r = –0.86, p < 0.001). At harvests two and three we found significant positive relationships...
of MRT Glc with C:Plit ($r = 0.52, p = 0.001$) and N:Plit ($r = 0.62, p < 0.001$).

The pattern of correlations between litter chemistry and glucan depolymerization also changed over time: after two weeks of decomposition, glucan depolymerization was positively correlated with starch content ($r = 0.63, p < 0.05$), and negatively with cellulose ($r = -0.65, p = 0.01$) and AUR ($r = -0.55, p < 0.05$) content. After three months, however, glucan depolymerization was not correlated with starch, cellulose or AUR concentration, but we found significant negative relationships of glucan depolymerization with litter cellulose:N ($r = -0.40, p < 0.05$) and AUR:N ($r = -0.38, p < 0.05$) ratios. Glucose concentration was not correlated with glucan depolymerization and glucose consumption rates at harvest one, but after three and six months we found highly significant positive relationships of glucose concentration with glucan depolymerization ($r = 0.73, p < 0.001$) and glucose

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**Fig. 2.** Gross rates of glucan depolymerization and glucose immobilization and concentrations and mean residence times (MRT) of free glucose at the three sampling time points (after 2 weeks and 3 and 6 months of decomposition, respectively) of the litter decomposition experiment (A, Achenkirch; K, Klausenleopoldsdorf; O, Ossiach; S, Schottenwald). Given are means of 5 mesocosms ± SE. Lower case letters indicate significant differences between litter types, upper case letters indicate significant differences between harvests (two-way ANOVA followed by Fisher’s LSD, $p = 0.05$).
consumption ($r = 0.90, p < 0.001$) (Fig. 6). Glucose mean residence time was negatively correlated with starch content ($r = -0.75, p < 0.01$) and cellulose:N ratios ($r = -0.71, p < 0.01$) at harvest one, but we did not find any correlations between litter chemistry and MRT Glc after three and six months.

Cellulases (Fig. 4) were only detectable three and six months after inoculation, where they were negatively correlated with C:N$_{lit}$ ($r = -0.55, p < 0.001$) and C:P$_{lit}$ ($r = -0.45, p < 0.01$) as well as with cellulose:N ($r = -0.52, p = 0.001$) and AUR:N ($r = -0.55, p < 0.001$). Furthermore, we found close positive relationships of cellulases with lignolytic peroxidases ($r = 0.58, p < 0.001$) and phenoloxidases ($r = 0.63, p < 0.001$). Lignin-degrading enzymes (Fig. 4) were present at all three harvests and were generally very closely related ($r = 0.90, p < 0.001$).

Glucose consumption was strongly positively related to glucan depolymerization (Fig. 7) at harvest one ($r = 0.76, p < 0.001$) and harvests two and three ($r = 0.94, p < 0.001$). However, we found no correlation whatsoever between glucose consumption and respiration rate.

4. Discussion

The aim of our study was to develop a new isotope pool dilution method, which enabled us to closely examine the influence of litter quality and elemental stoichiometry on rates of glucan depolymerization and glucose consumption during early stage litter decomposition (Fig. 8).

4.1. Rationale of experimental set-up

To test this we used an experimental set-up that was rather artificial: we sterilized four different beech litter types differing in elemental and biochemical composition, re-inoculated them with a beech litter/soil suspension from one site to obtain a uniform initial microbial metacommunity on all four litter types, and incubated the litter in the laboratory for six months. Elemental variation can be large in litter of one plant species collected from different sites while macromolecular composition (cellulose, lignin content) is expected to vary less (see Results). Although the process of sterilization and re-inoculation destroyed the native microbial community of the litter, we deliberately chose this approach because it allowed us to observe the development of distinct microbial communities and the related process dynamics based on differences in litter quality only, excluding site-specific differences in microbial inocula, soils, temperature and humidity. Our results may be difficult to extrapolate to natural environments, but our simplified, controlled experimental approach allowed us to investigate basic relationships between resource quality (elemental stoichiometry, C chemistry), process dynamics and the related enzymatic activities. This simplified controlled experimental set-up together with a newly developed isotope pool dilution assay allowed us for the first time to dissect the interactions between resource quality, enzyme activity and gross rates of glucan depolymerization and glucose consumption.

4.2. Temporal dynamics of glucan depolymerization

We demonstrate that litter composition, i.e. the availability of starch and cellulose, plays an important role in the temporal regulation of glucan depolymerization. Initially, glucan depolymerization rates were very high and positively correlated with starch content, suggesting that starch was the primary substrate for glucan depolymerization at this early stage of decomposition. This finding was supported by the lack of cellulolytic enzyme activity at this time point and the decrease of starch concentration in the litter in the first three months of decomposition. Furthermore, glucan depolymerization was correlated with mass loss at the first harvest, suggesting that glucan decomposition contributed substantially to litter mass loss at this stage. After three months, the correlation between glucan depolymerization and starch content diminished, in line with the often reported rapid degradation of starch in the first weeks of litter decomposition (e.g., Berg and McClaugherty, 2008). Amylase activity was not measured in our experiment, so we cannot directly link glucan depolymerization to amylase activity. However, amylase activity in decaying litter has previously been observed to decrease after the first weeks of decomposition (Fioretto et al., 2005; Papa et al., 2008). In addition, during the rapid depletion of the starch pool, glucan depolymerization rates declined and thereafter showed a strong linear correlation with cellulase activity. We therefore assume that glucose production three and six months after inoculation derived mostly from cellulose degradation. Nevertheless, we found no correlation between glucan depolymerization and cellulose content at harvests two and three; we therefore assume that cellulose depolymerization was enzyme-limited but not substrate-limited at this stage. These findings are also supported by the observation that cellulose content hardly changed over time. As cellulose is present in high concentrations in plant litter (Osono and Takeda, 2005; Papa et al., 2008; Pauly and Keegstra, 2008; Perez et al., 2002; Preston et al., 2009), a cellulose-limited situation would only be encountered at more advanced stages of degradation (Herman et al., 2008). Furthermore, we found a negative relationship between glucan depolymerization and cellulose:N ratio, pointing toward a N-limitation of the glucan depolymerization process (see below).

4.3. Stoichiometric controls of glucan depolymerization

The way litter stoichiometry influenced glucan depolymerization rates changed over the course of our experiment. Two weeks after inoculation of the litter, when starch was the main substrate for glucan depolymerization, no relationship of glucan depolymerization with either C:N$_{lit}$, C:P$_{lit}$, N:P$_{lit}$, or N and P content of the litter was found. We therefore assume that litter N or P content did not notably influence glucan degradation at this early
stage of decomposition, probably because labile N or P containing substances (e.g., amino acids, RNA or inorganic N and P) were still available. At later stages of our experiment, however, when cellulose became the primary substrate, glucan depolymerization was correlated with C:Nlit and Nlit, indicating that glucans were preferably decomposed in litter with a high N content. Furthermore, we found negative relationships between cellulase activity and C:Nlit and C:Plit, probably indicating that low C:Nlit and C:Plit ratios resulted in increased production of cellulolytic enzymes (Fig. 8). These findings are in line with the previously reported accelerated degradation of cellulose in leaf litter (Allison et al., 2010; Berg and Matzner, 1997; Sjoberg, 2004) and increased activities of cellulases in litter (Carreiro et al., 2000; Keeler et al., 2008; Saiya-Cork et al., 2002; Weand et al., 2010) after N fertilization. Sinsabaugh et al. (2009) pointed out that the ratio of activities of C- to nutrient-acquiring enzymes is relatively constant across scales and ecosystems with the mean C:N:P ratio of global extracellular enzyme activities approximating 1:1:1, reflecting the equilibrium between microbial growth efficiency and the elemental compositions of substrate and microbial biomass. Considering this premise and assuming that the differences in C:Nlit that we observed in our experiment were caused by variation in Nlit not Clit, we propose that in litter with high N content more resources were allocated to C-acquiring enzymes like cellulases, resulting in higher glucan depolymerization rates in litter with low C:Nlit (Fig. 8). This was also supported for harvest 2 and 3 by the negative correlation between glucan depolymerization and C:Nimbalance, i.e. the imbalance between microbial and litter stoichiometry (the ratio of C:Nlit to C:Nmic). A high C:Nimbalance implies that C:Nlit is high compared to C:Nmic, thus microbes experience N-limitation. Otherwise, if C:Nlit is very close to C:Nmic, C:Nimbalance is low and microbes might be rather C-limited. The negative relationship between glucan depolymerization and C:Nimbalance therefore suggests that glucans, which are rich in C and only contain minute amounts of associated N in plant cell walls (Brett and Waldron, 1996) or starch granules, are depolymerized to a greater extent if the microbial community is sufficiently supplied with N.

4.4. Enzymatic relationships

Examining the relationships between enzymatic activities, we found close relationships between cellulases and oxidative...
enzymes (peroxidases and phenoloxidases) involved in lignin degradation after three and six months of decomposition, probably indicating a synergistic action between cellulose- and lignin-degrading enzymes. This was confirmed by the correlation between glucan depolymerization and the lignin-degrading enzymes peroxidase and phenoloxidase. As cellulose fibers are tightly associated with lignin molecules (Perez et al., 2002), a concerted degradation of the lignocellulose complex seems plausible as previously suggested (Berg and McClaugherty, 2008; Cooke and Whipps, 1993; Herman et al., 2008; Perez et al., 2002; Romani et al., 2006).

4.5. Microbial consumption of glucose

Gross glucose consumption was highly correlated with glucan depolymerization, although both processes are thought to have different controls: glucan depolymerization may be driven by the activities of extracellular enzymes secreted into the soil environment whereas glucose is taken up directly by soil microorganisms. However, both processes are intrinsically linked by the demand of microbes for glucose and the resource allocation to enzymes that degrade glucans and thus liberate glucose (Sinsabaugh et al., 2009). Our results suggest that extracellular glucan depolymerization and microbial glucose consumption are tightly co-regulated and that glucan depolymerization is a ‘fine-tuned’ process meeting the momentary microbial demand for glucose. As the production of extracellular enzymes is highly nutrient- and energy-demanding and therefore expensive for microorganisms, it is very likely that cellulolytic enzymes are only produced to an extent necessary to satisfy the microbial demand for C (Schimel and Weintraub, 2003; Sinsabaugh et al., 2008). In support of this hypothesis we found significant relationships of glucose consumption with C:N_lit and with N_lit, suggesting that a higher N content of the substrate resulted in an improved N supply of the microbial community and a subsequently higher microbial demand for C. Furthermore, we found significant correlations between glucose consumption and glucose concentration of the litter, indicating that the demand of the microbial community for glucose was not saturated. As glucose is an easily accessible carrier of C and energy, it should be readily taken up by microbes. Nevertheless, we did not find any correlation between glucose consumption and respiration throughout the experiment, suggesting that glucose was not the only substrate used for microbial respiration. A correlation between glucose
consumption and respiration would only be expected, if all (or
a constant proportion) of the consumed carbon source (i.e.,
glucose) were actually respired. However, it is likely that a variable
part of the C acquired by glucose uptake was assimilated and used
for buildup of new microbial biomass.

4.6. Conclusions

In summary our results suggest that plant litter chemistry and
elemental stoichiometry constitute important controls of glucose
production and glucose consumption by microorganisms during
litter decomposition (Fig. 8). Glucan depolymerization was clearly
controlled by the availability of suitable substrates, which changed
over the course of experiment: at initial stages of decomposition
starch was the primary substrate for depolymerization, while at
later stages of decomposition cellulose was the main substrate.
Interestingly, potential cellulase activities were highly correlated
with glucan depolymerization, indicating that at later stages of
litter decomposition glucan depolymerization was controlled by
size of the enzyme pool, not by the content and accessibility of

Fig. 7. Linear regressions of glucose consumption and glucan depolymerization after 2 weeks (n = 20) and after 3 and 6 months (combined data, n = 40). Shown are data of four
litter types (●, Achenkirch; ▲, Klausenleopoldsdorf; ■, Ossiach; and ●, Schottenwald) and two time points (black, 3 months and white, 6 months of litter decomposition). Letters
indicate correlation coefficients (r) and p-values.

Fig. 8. Conceptual diagram of sources and controls of glucan depolymerization in decomposing beech litter. At the beginning of decomposition (a), starch is the primary source of
glucan depolymerization. At this stage, labile N-rich compounds (e.g., amino acids), which derive from the litter material, serve as easily accessible N-source for the microbial
community. After depletion of the starch pool after a few weeks of litter decomposition (b), cellulases are produced and the more recalcitrant cellulose pool in the plant cell walls is
decomposed. At this stage of decomposition, labile N-sources are mostly depleted and microbial decomposers have to degrade protein to obtain N. The ratio of lignocellulose
contains most of litter C) to protein (contains most of litter N) corresponds roughly to the total C:N of the litter. Cellulose depolymerization is facilitated by the activity of oxidative
linolytic enzymes like peroxidases and phenoloxidases, which attack the lignocellulose complex in plant cell walls and thus provide access to cellulose fibers for cellulases. In
general, production of glucan-degrading enzymes is controlled by the microbial C demand. The C demand, in turn, is related to N supply of the microbial community because
microorganisms can only use C for growth and enzyme production if they have N. The C and N supply to microbial decomposers ultimately depends on the amount and availability
of C and N present in plant litter. Squares represent substrate and product pools, ellipses represent enzyme pools, arrows represent fluxes, valves represent controls of fluxes. Size of
pools is not represented in quantitative relationships.
cellulose. We were further able to demonstrate that the N content and C:N ratio of the litter strongly affected glucan depolymerization at later stages of decomposition, suggesting an increased C demand of the microbial community and subsequently an increased resource allocation to C-acquiring enzymes with increasing N availability. These are the first data published to date on gross rates of glucose dynamics in decomposing organic matter, which allowed to discern the controls of gross glucan depolymerization and gross rates of microbial glucose use. The work therefore stands in contrast to previously published studies that either measured net changes in cellulose or other carbohydrate pools during litter decomposition, or focused on potential enzyme activities involved in carbohydrate breakdown. The wider application of isotope pool dilution assays in litter and soil organic carbon decomposition studies will represent a leap forward in our understanding of microbial carbon use, of in situ activities of extracellular enzymes and the regulation of microbial partitioning of energy (carbon) into extracellular enzymes, biomass production and respiration.

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Appendix. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.soilbio.2012.03.012.

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