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“I am a creature of the mud,

not of the sky.”

Donna Haraway, “When species meet”.

Contents

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Summary

Lignin is a major component of plant litter, and considered highly resistant against decomposition. Polymeric carbohydrates, in contrast, are more easily available carbon sources for microbes. Traditional concepts of litter decomposition propose that lignin remains unaltered during early litter decomposition and degraded only when its high content limits the degradation of other compounds. However, it has recently been shown that high lignin degradation rates may also occur during early decomposition. Nitrogen may strongly influence lignin degradation rates because nitrogen addition increases mass-loss in low-lignin litter, but decreases mass-loss in high-lignin litter. More specifically, it has also been demonstrated that lignin decomposition rates decreased when nitrogen was added to litter.

In this work, pyrolysis-GC/MS was used to estimate lignin and carbohydrate degradation during litter decomposition. Beech litter with different N and P content and C:N and C:P ratio was collected at 4 different sites in Austria, sterilized and inoculated with a litter/top-soil mixture from one site to ensure that all litter share the same initial microbial community. Litter was then incubated in mesocosms for up to 15 months under controlled environmental conditions. Lignin and carbohydrate decomposition rates from 2 periods (0-6 month and 6-15 months) were compared with potential activities of cellulases, phenoloxidases and peroxidases. Fungi/bacteria ratios obtained from meta-proteome analysis were used to characterize changes in the microbial community composition.

Carbohydrate decomposition rates were found to be positively correlated with litter N content and negatively with litter the C:N ratio during both periods, while lignin decomposition followed this trend only during the second period (6-15 months). Lignin decomposition during the first period was negatively correlated to litter P content and positively correlated to the litter C:P ratio. Lignin decomposition was best predicted by $C:P_{litter}/C:P_{microbial}$ ratios, indicating that early lignin decomposition was promoted by P limitation of the microbial community. Fungi were more successful than bacteria in using readily available carbon during early litter decomposition and were more successful in colonizing litter with high N and P content. However, fungi were less dominant in litter that had higher lignin degradation rates. The results demonstrate that substantial amounts of lignin can be degraded during early decomposition, presumably triggered by N and P limitation and the establishment of K-strategist microorganisms. How-

ever, early lignin decomposition did not depend on high abundance of fungi, which are commonly assumed to mediate lignin decomposition or stoichiometric conditions that favor fungal growth. Differences in lignin decomposition rates were lost through microbial succession during litter decomposition, and both lignin and carbohydrate decomposition rates increased with N availability during later decomposition stages.

Zusammenfassung

Lignin ist ein Hauptbestandteil von Pflanzenstreu, und wird als in der hoch resistent gegenüber Abbauprozessen eingeschätzt. Im Gegensatz dazu sind Kohlenhydratpolymere (Cellulose und Stärke) für Mikroorganismen verhältnismäßig leicht verfügbare Kohlenstoffquellen. Klassische Konzepte des Streuabbaus gehen davon aus, dass Ligin während frühen Abbaustadien angereichert und erst abgebaut wird, wenn hohe Ligninkonzentrationen den Abbau anderer Substanzen einschränken. Vor Kurzem konnte gezeigt werden, dass die Ligninabbaurate auch Beginn der Streuabbaus am höchsten Ligninabbauraten sein kann. Ein starker Einfluss des Stickstoffgehaltes auf den Streuabbau wird vermutet, da Stickstoffdüngung in ligninarmer Streu zu einem schnelleren, in ligninreicher Streu aber zu einem langsameren Masseverlust führen. Desweiteren konnte bereits gezeigt werden, dass Stickstoffzugabe den Ligninabbau verlangsamt.

In dieser Arbeit werden Lignin- und Kohlenhydratabbauraten während Streuabbau mit Pyrolyse-GC/MS bestimmt. Buchenlaub mit unterschiedlichem N- und P-Gehalt und unterschiedlicher C:N- und C:P-Stöchiometrie wurden an vier verschiedenen Orten in Österreich gesammelt, sterilisiert und mit einer Mischung aus Streu und oberstem Bodenhorizont eines Standortes innokuliert, um sicherzustellen, dass zu Beginn des Experiments stets die selbe mikrobielle Gemeinschaft vorhanden ist. Anschließend wurde das Buchenlaub in Mesokosmen bis zu 15 Monate lang unter kontrollierten Umweltbedingungen inkubiert. Lignin- und Kohlenhydratabbauraten wurden für zwei Perioden (0-6 und 6-15 Monate) bestimmt und mit potentieller Cellulase-, Phenol- und Peroxidaseaktivitäten verglichen. Das Pilz/Bakterien-Verhältnis aus Metaproteomanalysen wurde genutzt um die mikrobiellen Gemeinschaften zu charakterisieren.

Kohlenhydrateabbauraten waren währen des gesamten Experiments positiv mit dem N Gehalt und negativ mit dem C:N Verhältnis in der Streu korreliert, während der Ligninabbau diesem Trend nur in der zweiten Periode (6-15 Monate) folgte. Während der ersten Periode war der Ligninabbau negativ mit dem P-Gehalt und positiv mit dem C:P-Verhältnis des Laubs und korreliert. Der Ligninabbau wurde am besten durch das Verhältnis $C{:}P_{\text{Streu}}/C{:}P_{\text{Mikrobielle Biomasse}}$ beschrieben, was die Annahme nahelegt, dass der beobachtete frühe Ligninabbau durch P-Mangel gefördert wird. Pilze waren erfolgreicher als Bakterien in der Nutzung von leicht verfügbaren Kohlenstoffquellen und in der Kolonisierung von Streu mit

hohem N- und P-Gehalt. Ihre Dominanz war in Streu mit hohen Ligninabbauraten geringer ausgeprägt.

Diese Ergebnisse zeigen, dass bedeutende Mengen an Lignin bereits während der frühen Streuzersetzung abgebaut werden können. Dies wird durch N- und P-Limitierung und der Etablierung von mikrobiellen K-Strategen ausgelöst. Der frühe Ligninabbau war nicht an eine hohe Abundanz von Pilzen, die gewöhnlich als Träger des Ligninabbaus angenommen werden, oder stöchiometrische Bedingungen, die ihr Wachstum fördern, gebunden. Unterschiede in Ligninabbauraten gingen mit fortschreitender Sukzession verloren, sodass in späteren Abbaustadien sowohl Lignin- als auch Kohlenhydratabbau mit höheren N-Gehalten anstiegen.

1

General Introduction

1.1 The global carbon cycle and global climate change

Between 2000 and 2005, mean annual CO_2 emissions from fossil fuel burning and cement production accounted for 7.2 ± 0.3 Gt CO_2-C. Additionally, land use change caused annual emissions 1.6 ± 1.1 Gt CO_2-C. Since pre-industrial times, annual means of atmospheric CO_2 concentration increased from 280 to 379 ppm (v/v). The source of approximately 80% of this increase could be pinned down to fossil fuel usage by comparing the atmospheric CO_2 increase with changes in its ^{13}C signature (fossil fuel C is depleted in ^{13}C) and the corresponding decrease in atmospheric O_2 concentrations. Rising atmospheric CO_2 concentrations led to an increased interest in natural carbon cycles and their anthropogenic modifications (Treut et al., 2007).

Anthropogenic CO_2 emissions are tightly interconnected with the natural carbon cycles. Increased frequencies of disturbances (e.g. fire, drought, and other climatic extremes) and anthropogenic effects (e.g. climate change, land use change, fertilization) transforms natural ecosystem. This potentially affects the long-term carbon cycling in such systems, i.e. pools that are usually in a steady state (in-flux equals out-flux) might start to accumulate or loose stored carbon. These unknown feedback mechanism potentially slows down or multiplies global greenhouse gas accumulation. Only 45% of the CO_2 currently emitted accumulate in the atmosphere, 30% CO_2 are absorbed in oceans and 25% in terrestrial ecosystems. Oceans function as CO_2 sinks due to the export of particular and dissolved organic carbon and dissolved inorganic carbon species (HCO_3^-, CO_3^{2-}) to intermediate and deep water layers. Land sinks take up carbon into larger vegetation- and soil C pools, e.g. due to a nothward shift of climatic limits for vegetation or higher plant biomass caused by CO_2 and N fertilization. However, a large part (-2.6 Gt a^{-1}) of the terrestrial carbon sinks is yet unaccounted for (Denman et al., 2007). Finding this “missing sink” and predicting its future behavior challenges scientists to deepen their understanding

of large scale biotic carbon storage and cycling.

1.2 Litter decomposition and the global carbon cycle

The amount of carbon cycled through plants and soils is impressive: Globally, land plants assimilate 120 Gt C annually through photosynthesis (gross primary production). This is almost one sixth of the atmospheric CO_2 pool (750 Gt) and more than 15 times that of anthropogenic C emissions. Autotrophic (plant) respiration consumes one half of this assimilated carbon, while the other half (net primary production, NPP) - approximately 60 Gt C a^{-1} - is introduced into decomposition pathways as plant litter. Between 30 and 70% of this litter input are mineralized in the first year and further 20 to 30 % within another 5 to 10 years (Chapin et al., 2002). Respiration during litter decomposition accounts for approximately 50% of global soil respiration (Coûteaux et al., 1995).

Temperate forests are highly productive, with an average net primary production is estimated of 1550 g m^{-2} a^{-1}. They cover 1.7 * 10^7 km^2, which is approximately 1/15 of earth land surface, and account for 8.1 Gt a^{-1} NPP, approximately 1/8 of total terrestrial NPP (Chapin et al., 2002). European beech (*Fagus silvaticus* L.) is the dominant forest-forming tree species in the potential vegetation of western and central Europe.

1.3 Ecological stoichiometry

Carl Sprengel proposed in 1828 that crops rely on nutrients in a given ratio, and that their growth is limited by the nutrient that is least frequent compared to this given ratio. Only fertilization with the limiting nutrient increases plant growth and agricultural yields, while plants can not utilize an additional input of other nutrients in the form of growth. While since then plants - and other organisms - have been shown to have a certain plasticity in their nutrient requirement, there is a trade-off between adaptation to nutrient availability and competitive fitness.

Organisms do not only rely on nutrient inputs in a certain elemental ratio, they are also bound (within an adaptive range) to keep them these elemental ratios in their internal milieu in a specific range. An homeostatic organism keeps this internal milieu constant independent of their environmental conditions, while in non-homeostatic organisms, the internal milieu changes with the environment. Ecological stoichiometry focuses this perspective on ratios between carbon, nitrogen and phosphorus (Sterner and Elser, 2002).

By 1958, marine biologist Albert C. Redford published results from measurements of the elementel composition of marine biomass featuring a constant ratio between carbon, nitrogen, and phosphorous

(C:N:P = 106:16:1 (n/n)) in both living biomass and detritus. The high constancy of this ratio is based on controls over CO_2 assimilation by N and P availability and controls of the biogeochemical cycling of nutrients (i.e. export by sedimentation, N fixation and denitrification) by biological systems. A similar relationship was found for both soils and soil microbial communities. On a global scale, soil C and N ratios were tightly correlated. Soil P contents are more variable, but still significantly correlated to C and N contents, with an an average C:N:P ratio of 186:13:1. The stoichiometry of soil microbial biomass is even more strongly restricted (C:N:P 60:7:1), suggesting that soil microbiota are homeostatic at a community level (Cleveland and Liptzin, 2007).

Plants are able to assimilate carbon from atmospheric CO_2, but have to extract mineral nutrients from soils, and are therefore enriched in carbon. Temperate broadleaf foliage has an average C:N ratio of 35:1 and an C:P ratio of 920:1. A significant part of nitrogen and other nutrients is removed from senecenced leaves before abscission, resulting in an average C:N ratio of 58:1 and a C:P ratio of 1700:1 in plant litter. Both tropical and coniferous forests are poorer in N and P. Litter in lower latitudes is generally P poor while litter in high latitudes is N poor, reflecting general nutrient limitations of the corresponding ecosystems (McGroddy et al., 2004).

Along with lignin contents, litter C:N and C:P ratios are frequently used to describe litter quality. Both C:N and C:P ratios have a strong effect on the rate of litter decomposition. Nutrient rich litter decomposes faster than nutrient poor litter, with N and P content combined explaining 90 % of the variance of decomposition rates in a decomposition experiment covering detritus from phytoplancton to vascular plants (Enríquez et al., 1993). Fast-growing plants also produce litter with lower C:N and C:P ratios than that slowly growing plants. This leads to an feedback loop: litter of slow-growing plants is decomposed more slowely, thereby releasing less nutrients and promoting the growth of adapted plants, and vice versa (Enríquez et al., 1993).

1.4 Litter mass loss rates

Simple models of litter decomposition assume that litter mass loss follows an exponential function:

$$X_t = X_0 e^{-kt} \tag{1.1}$$

or

$$ln\left(\frac{X_t}{X_0}\right) = -kt \tag{1.2}$$

where X_0 stands for the initial amount of litter, and X_t the amount of litter which remains after time

t (Berg, B. & McClaugherty, 2008). The decomposition constant k allows comparing the speed in which litter decomposes, effectively describing the half-life time of litter. While this model can serve only as a first approximation, it provides a simple and efficient tool to compare the speed of litter decomposition between litter of different species, incubated in different climates or of different quality. On a global scale, k values increase with mean annual temperature and precipitation and decrease with latitude. However, climatic factors explain only 30 % of the total variance of the decomposition rate, indicating that they affect litter decomposition rather via control over litter quality than directly. In contrast, litter N content and C:N ratio together explain 70 % of the differences in degradation rates (Prescott, 2010; Zhang et al., 2008).

Prescott (2010) used a different approach and explained litter degradation rates not by correlation with possible controls, but by defining optimal and inhibitory ranges for regulatory factors. She suggested that for each controlling factor there is an optimal range where this factor does not interfere with decomposition rates and other factors control decomposition rates, and a inhibitory range, in which decomposition rates are uniformly low. Only between optimal range and inhibitory range and decomposition rates are correlated to the controlling factor. For example, litter decomposition is uniformly low at moisture contents above 80% or below 30%, or at temperatures below 10 °C. Optimal decomposition rates are between 60 and 70 % moisture and above 30 °C. Furthermore she suggested a hierarchical order of these controls, in which decomposition rates are primarily controlled by temperature and moisture, and litter chemistry becomse importent when if climatic conditions do not limit decomposition.

Prescott pointed out that decomposition rates are of subordinate importance for the carbon balance during litter decomposition, and emphasized that these balances are determined by the proportion of litter carbon ending up in soils as soil organic matter (SOM). However, the simple exponential model is not sufficient to understand this litter C sequestration, but more detailed knowledge of the chemical nature of litter and its transformation during decomposition is needed.

1.5 Chemical constituents of initial beech litter

The dry biomass of freshly fallen plant litter is chemically dominated by polymeric compounds. Nitrogen is present almost exclusively as protein. Among carbohydrates, cellulose is most common (10-50 % of litter dry mass). Other carbohydrates - referred to as hemicelluloses - together make up between 30 and 40 % of litter dry mass. Lignin, an irregular polymer that forms secondary plant cell walls, makes up 15-40% of litter dry mass. Along with the long chain aliphatic esters that form the leaf surface wax layer (cutin), they are considered most resistant to microbial decomposition (recalcitrant). Lignin can incorporate substantial amounts of protein and carbohydrates, thereby occluding them from decomposing

enzymes and lowering their bioavailability (Berg, B. & McClaugherty, 2008). Although lignin itself does not contain nitrogen, its contents in beech lignin can be twice as high as in bulk litter (Dyckmans et al., 2002).

Only a small fraction of foliar plant litter (approximately 25% for decidous litter, and less in conifer litter) is soluble in water (Berg, B. & McClaugherty, 2008), and dissolved organic matter (DOM) contents are below 1% (Don and Kalbitz, 2005). Therefore, decomposing microorganisms rely on the excretion of extracellular enzymes to break down plant biomass into soluble fragments (Klotzbücher et al., 2011). Hydrolases break down protein and carbohydrates to amino acids and sugars, while the degradation of lignin is mediated by oxidative enzymes (Sinsabaugh, 2010).

1.6 Changes of litter carbon chemistry during decomposition

1.6.1 The traditional model developed by B. Berg

Traditional models of chemical changes during litter decomposition describe three phases of litter decomposition. The first phase starts even before abscission and can last until up to 40% dry mass is lost. During this phase, microorganisms degrade easily available substrates like soluble compounds and non-lignified carbohydrates. Nutrient (N & P) levels are limiting during this phase, and decomposition rates increase with their content. Decomposition rates are also strongly affected by climatic factors. In the late phase, lignin content has reached a critical value at which it inhibits further decomposition. Mass loss rates are limited by lignin contents at this stage. Nitrogen, which represses lignin degradation, now slows down overall mass loss rates. Manganese is needed as a cofactor for lignin degrading enzymes, enhancing lignin decomposition and therefore increasing mass loss rates. As mass loss is limited by lignin decomposition, climatic controls on the decomposition rate are suppressed or totally disappear. During this phase, lignin contents reach a constant value. Finally, at the end of decomposition, mass loss of near-humus litter reaches a limit value, and remaining necromass becomes incorporated into soils (fig. 1.1, Berg, B. & McClaugherty (2008)).

1.6.2 Microbial nitrogen mining hypothesis

The "nitrogen mining hypothesis" is based on the concept that the breakdown of recalcitrant carbon allows soil microbiota to access recalcitrant nitrogen, while yielding little to no energy. This explains why nitrogen starvation triggers the excretion of enzymes degrading phenolic compounds (Craine et al., 2007; Moorhead and Sinsabaugh, 2006). In plant litter, nitrogen is often occluded within lignin molecules, which are also degraded by oxidative enzymes. Isolated lignin fractions from fresh beech leaves were

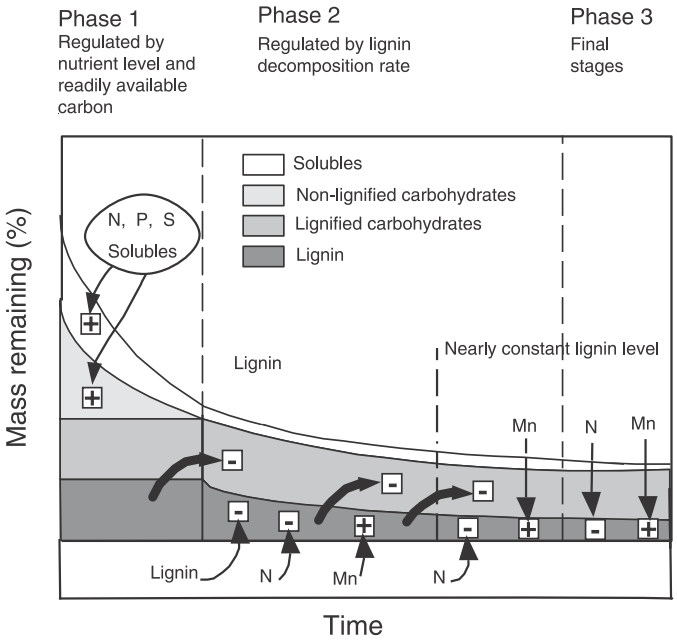

Fig. 6.1 Model for chemical changes and rate-regulating factors during decomposition (modified from Berg and Matzner 1997). The decomposition of water-soluble substances and unshielded cellulose/hemicellulose is stimulated by high levels of the major nutrients (early stage – *phase 1*). When all unshielded holocellulose is decomposed, only lignin-encrusted holocellulose and lignin remain. The early phase has been observed to last up to ca. 40% mass loss (case B in Table 6.1), with a very high mass-loss rate, or leaching. It may also be close to nonexistent, as in case C in Table 6.1. In the late stage (*phase 2*), the degradation of lignin controls the litter decomposition rate. Nitrogen hampers the degradation of lignin, and higher N concentrations suppress decomposition, whereas Mn appears to have a stimulating effect on lignin degradation. Finally, in the humus-near stage (*phase 3*), the lignin level is nearly constant, often at values of 50–55%, the litter decomposition rate is close to zero, and the accumulated mass loss also reaches its limit value

Figure 1.1: Litter decomposition model (taken from Berg, B. & McClaugherty (2008))

shown to contain twice as much nitrogen as bulk material (Dyckmans et al., 2002). Craine et al. (2007) incubated mixtures from 50 different soils and plant litter from 50 species, adding mineral nitrogen and phosphorus. They found that nitrogen, but not phosphorus addition lowered the amount of recalcitrant carbon decomposed. However, their study was not based on the direct measurement of carbon pools sizes but exclusively on mathematic modeling. They defined labile and recalcitrant carbon as components of litter exponential and linear declining respiration rates. respectively.

1.6.3 Carbon limitation of lignin decomposition

Recently, Klotzbücher et al. (2011) suggested that the degradation of lignin compounds depends on the availability of labile carbon sources. In a climate chamber experiment, the authors incubated samples previously decomposed in-situ for up to three years, recording microbial respiration, lignin content (based on CuO-oxidation and GC analysis), and the production of soluble carbon (DOC). Regardless of the previous field incubation time, samples showed the highest lignin degradation rates at the beginning

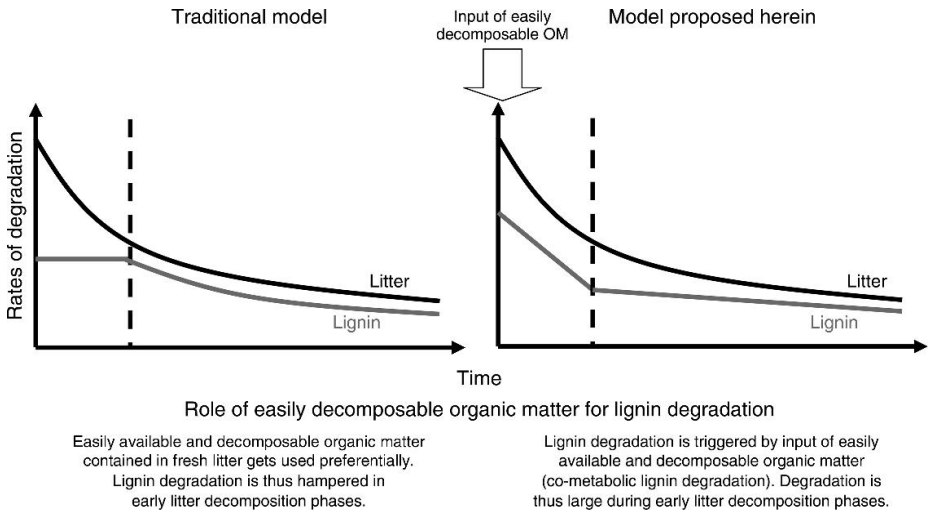

FIG. 5. Revised conceptual model for the fate of lignin during litter decomposition, based on data presented herein. The traditional model was proposed in Berg and Staaf (1980) and based on data from the decomposition of Scots pine needles. In our model, lignin will be degraded if easily degradable OM is available.

Figure 1.2: Lignin decomposition model (taken from Klotzbücher et al. (2011))

of the laboratory incubation, and a decrease of lignin contents over the first 200 days. Soluble carbon production was correlated to respiration rates thereafter. Therefore, the authors concluded that soluble carbon limited carbon mineralization rates in litter after an initial pool of labile carbon has been used up. This labile carbon pool can result from experimental manipulation i.e. draught-rewetting at the experimental setup. They also suggested, that lignin is not decomposed under such carbon-limited conditions. As lignin decomposition rates did not increase with previous field incubation time, they contradict the longstanding hypothesis that lignin is not degraded during early litter decomposition, until a critical lignin content is reached, but proposed a concept of early lignin decomposition, where lignin decomposition rates are highest during early litter decomposition, when plenty of labile carbon is available (fig. 1.2).

1.6.4 N control over priming effects

The priming effect is defined as the amount of recalcitrant carbon that is mineralized when a source of labile carbon is added to the system (e.g. a soil). It is determined by adding a labeled substrate (e.g. 13C cellulose) and determining the difference in respiration and the amount of respired label. Fontaine et al. (2011) conducted an experiment adding cellulose, nitrogen or both and calculating the priming effect of high-N and low-N treatments. Additional mineralization of SOM carbon was lower when soils where also N-fertilized. They proposed that in low-N soils, soil microbiota are more inclined to degrade N-rich SOM that yields less energy, and preferentially degrade low-N but energy-rich organic matter, e.g.

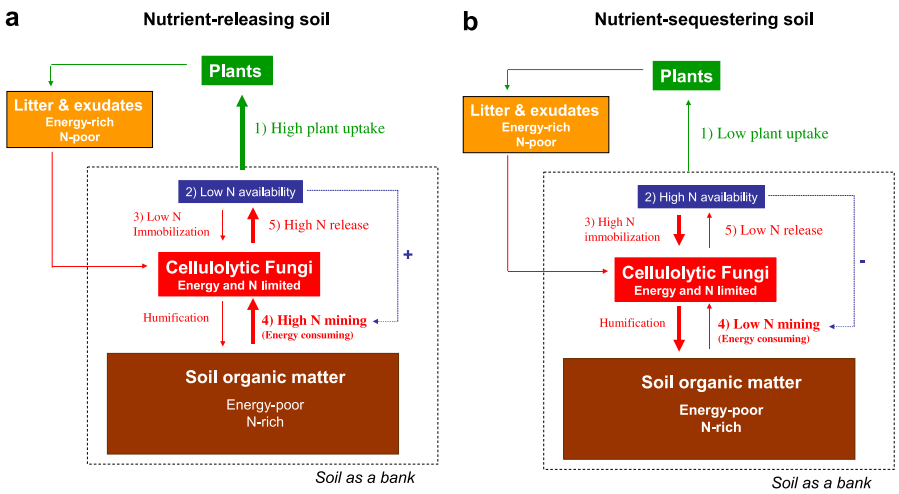

Fig. 1. The bank mechanism adjusting the sequestration of nutrients and carbon in soil organic matter (SOM) to the availability of nutrients in soil solution tested in this study. This mechanism is based on the assumption that microbial degradation of recalcitrant SOM (priming effect) is modulated by the concentration of nutrients in soil solution (Fontaine et al., 2003, 2004a). When nutrient availability is low (a), for example because the plant uptake of nutrient is high, the microbial mining of SOM could be intense and eventually exceed the formation of new SOM through humification of fresh-C, leading to net destruction of SOM and release of mineral nutrients. In contrast, when soluble nutrients are abundant (b), microbial immobilization of N should increase while mining of SOM should decrease, leading to a greater sequestration of nutrients in SOM. Given that plant demand

Figure 1.3: Influence of nitrogen on the decomposition of labile and recalcitrant soil carbon (taken from Fontaine et al. (2011))

plant litter leachates. The interaction of N and energy demand which directs soil microbes to degrade labile or recalcitrant organic matter might also be effective in litter. In litter, it could decide, whether microbes follow a "nitrogen mining" strategy or degrade labile carbohydrates (see fig. 1.3.

1.7 Determining lignin contents

The traditional model of lignin decomposition as described above (Berg and Staaf, 1980) was developed based on lignin contents determined as *acid unhydrolysable residue* (AUR). Thereby, lignin is defined as the part of the sample, that remains undissolved after treatment with 72 % H_2SO_4. Beside lignin. this fraction was shown to contain substantial amounts of cutin, surface waxes, and condensed tannins, along with secondary compounds that are produced by microbes during litter decomposition (Klotzbücher et al., 2011). Hatfield and Romualdo (2005) pointed out, that solvent extraction methods provides better results for woody material than for herbaceous samples, because the latter ones contain waxes (cutin and suberin) and often have high protein contents (up to 25 %), which both remain at least partially undissolved. These substances do not necessarily occlude cellulose, as does lignin, therefore their effect on litter decomposition might be different from that of lignin. However, while being not a specific method to determine lignin, AUR contents serve as a good indicator for litter quality. AUR and AUR:N ratios can serve as good predictors for litter decomposition rates (Prescott, 2010). Furthermore, a part of the lignin in the sample is acid soluble. This soluble fraction is often corrected for by collecting

the extractant and determining its lignin concentration photometrically (Hatfield and Romualdo, 2005).

Several other methods have been used to determine lignin content. Differences between the methods used are large, anŝd Hatfield and Romualdo (2005) reported differences of up to a factor 4, that furthermore depended on the sample material. Lignin can be determined by UV, IR and Near IR (NIRS) spectroscopy. However, specific measurements of lignin in the presence of other phenolic compounds is often impossible (especially in UV spectroscopy). Quantitative Cross-polarization/magic angle spin (CP/MAS) NMR can provide more specific lignin contents, but is not used routinely due to the high costs of instrumentation. All spectroscopic methods need small sample amount (mg to g range), but have the common disadvantage that different forms of lignin are present in plants, with different absorbance maxima. Therefore it is difficult to define suitable reference materials. Additionally, lignin can be derivatized into soluble forms by treatment with acetyl bromide or thioglycolic acid, mostly in combination with further cleansing procedures. Both the acetyl bromide and the thioglycolic acid method are suitable for small sample amounts (in the range of 10 mg). Lignins consume higher amounts of oxidants than carbohydrates during solubilization. This is used in the paper industry, where lignin is determined exposing the sample to a defined amount of $KMnO_4$, chlorine or hypochlorite and the excess reactant is measured (Hatfield and Romualdo, 2005).

Another method to determine lignin, which originates from soil sciences, is its oxidation with CuO under pressurized alkaline conditions at 170 °C (for a description of the procedure see e.g. Johansson et al. (1986)). Thereby, concentrations of different lignin monomers and the functional groups on its side chain can be quantified. This information allows to compare lignin originating from different plants, the relative turnover of different lignin monomers and the conversion of functional groups during decomposition (Thevenot et al., 2010). However, this method is not well suited to quantify the absolute lignin content of a sample, and rather compares lignin quality and decomposition trends within related materials, because conversion of macromolecular lignin into GC amendable compounds might not be quantitative. The quantification of lignin with analytical pyrolysis, as used in the current study, is subjected to similar limitations and described later. Absolute quantification by analytical pyrolysis was most successful using trimethoxybenzene or tribenzenecarboxylic acid trimethyl ester as internal standards (Bocchini et al., 1997; Steinbeiss et al., 2006). Recently, good accordance was reported for the relative amount of lignin products in pyrograms and Klason-lignin content (Alves et al., 2006).

1.8 Analytical Pyrolysis

Pyrolysis is the decomposition of complex organic compounds under elevated temperatures in the absence of oxygen (Moldoveanu, 1998). In the case of analytical pyrolysis, samples are typically heated to

temperatures above 500°C in an helium atmosphere. At this temperature, high molecular weight compounds break down to smaller, voluntile compounds. Most frequently, the helium atmosphere with the pyrolysis products is injected into a gas chromatography/mass spectrometry (GC/MS) system. Pyrolysis products are separated on a GC column, and identified and quantified on a MS detector. Analytical pyrolysis was first applied by G. Williams in 1860 to proof that natural rubber (caoutchouc) is a polymer of isoprene units. Currently, analytical pyrolysis is used to identify artificial polymers and plasticizers added in their production, natural polymers from plant material to soil and dissolved organic matter, and whenever only minimal sample amounts are available, like forensics or art history.

Three types of pyrolytic systems are frequently used:

(1) micro-furnace type pyromates have an oven that is constantly heated to the pyrolysis temperature, into which the sample is dropped,

(2) resistively heated systems use a metal filament (mostly platin) to electrically heat the pyrolysis chamber, and

(3) curie-point systems inductively heat a metal cup containing the sample to it's material specific curie-point, at which ferromagnetism is lost and the cup therefore is not further heated (Sobeih et al., 2008).

Detected pyrolysis products can be related to their polymer origin. While this interpretation is easy for artificial polymers and natural homopolymers, the analysis of pyrograms from complex organic polymers can be challenging to the point where pyrolysis is used as simple fingerprinting method which does not allow qualitative interpretation but still provides insight into differences between natural polymers found in different samples. Nevertheless, analytical pyrolysis usually provides a good overview over the monomers present in complex organic material.

1.9 Study aims

This study aims to elucidate the interaction between litter nutrient content and the degradation of high molecular weight compounds, especially lignin and carbohydrates. According to the nitrogen mining theory, we expect that a low N content triggers early lignin decomposition while a high N content delays lignin degradation until late decomposition stages. In contrast, we expect carbohydrate degradation to be enhanced by a high N content as litter microorganisms are able to produce more extracellular enzymes under this condition. We expect that a low N content favor fungi, which have wider C:N ratios, while bacteria have stoichiometric advantages when litter N contents are high.

To test our hypotheses, we conducted a litter incubation experiment with beech litter varying in N and P content, collected from 4 sites across Austria. Litter was sterilized and re-inoculated with

a litter/topsoil mixture from one site to ensure that all treatments share the same initial microbial community. Litter samples were incubated under controlled climatic condition for a duration between 3 and 15 months, and subsequently analyzed by pyrolysis-GC/MS to account for carbohydrate and lignin losses. Results were compared with the stoichiometric composition of the litter incubated and it's microbial community, potential activities of enzymes degrading cellulose and lignin, and gross N and P gross recycling rates and the composition of the microbial community (based on metaproteome analysis)

2

Manuscript

Controls of litter chemistry on early lignin decomposition in beech litter

The following persons contributed to this manuscript:

Ieda Hämerle and Lucia Fuchslueger were responsible for the mesocosm experiment and measurements of enzyme activities. Katherina Keiblinger (supervised by Sophie Zechmeister-Boltenstern) provided data on litter chemistry, litter microbial biomass and respiration rates. Sonja Leitner, Maria Mooshammer and Jörg Schnecker conducted the N, P, and glucose isotope pool dilution experiments used for correlative analysis. The group of Thomas Schneider, Katherina Riedl and Leo Eberl contributed meta-proteomic data. Quantitative PCR data was provided by Markus Gorfer and Sandra Moll.

The author's work included pyrolysis-GC/MS method development, sample measurement and data analysis.

2.1 Abstract

Lignin is a major component of plant litter and is considered highly resistant to decomposition. Polymeric carbohydrates, in contrast, are more easily accessible carbon sources. We studied the decomposition rates of these two compound classes, to which extent they are controlled by litter C:N:P stoichiometry, and whether this control changes over time. Therefore, we conducted a 15-months mesocosm experiment under controlled climatic conditions, comparing beech litter of different N and P contents, which was sterilized and re-inoculated with a litter/topsoil mixture from one of the sites to ensure identical microbial communities at the start of the experiment. Lignin and carbohydrate decomposition rates were estimated for 2 periods (0-6 months and 6-15 months) by pyrolysis-GC/MS.

Positive correlations of carbohydrate decomposition rates with litter N content were found during the entire experiment. Lignin decomposition rates during the initial period were highly variable and negatively correlated to litter P content and positively correlated to the microbial P demand ($C:P_{litter}/C:P_{microbial}$). During the later stage, lignin decomposition rates were positively correlated to N contents, respiration, and carbohydrate decomposition. Initial lignin decomposition rates were highest in litter with low fungi/bacteria ratios, which occurred in N and P poor litter.

Our results showed that a substantial amount of lignin can be degraded during early decomposition. In the present study, early lignin decomposition was coupled to low N and P availability, and the establishment of K-strategist microorganisms. However, early lignin decomposition rates did not depend on fungi, which are commonly assumed to mediate lignin decomposition, or stoichiometric conditions that favor fungal growth.

2.2 Introduction

Plant litter is quantitatively dominated by macromolecular compounds. In foliar litter, lignin and carbohydrate polymers together make up 40-60% of litter dry mass (Berg, B. & McClaugherty, 2008), while leachable substances ("DOM") account for only 1.5-6% (Don and Kalbitz, 2005). The breakdown of these high molecular weight compounds into smaller molecules accessible to microbes is mediated by extracellular enzymes and considered rate limiting for decomposition processes (Sinsabaugh, 2010)

Litter decomposition models generally follow the concept that organic compounds in litter form up to three independent pools of increasing recalcitrance, i.e. (1) soluble compounds, (2) cellulose and hemi-celluloses, and (3) lignin and waxes (cutin and suberin). Soluble compounds are most accessible to microbes and are usually consumed first, followed by regular polymers, such as cellulose. Lignin can be decomposed only by specialized fungi and is not degraded until accumulated to a certain, critical level when it inhibits the degradation of less recalcitrant compounds(Adair et al., 2008; Berg and Staaf, 1980; Coûteaux et al., 1995; Moorhead and Sinsabaugh, 2006). These pools are usually quantified by gravimetric determination of the amount of cellulose, hemi-celluloses and lignins after sequential extractions with selective solvents. These methods were repeatedly criticized for being unspecific for lignin determination (Hatfield and Romualdo, 2005). When analyzed with alternative methods (NMR, CuO-oxidation, Pyrolysis-GC/MS), extracted lignin fractions were shown to contain also many other substances (e.g. Preston et al. (1997)).

Recent studies based on more specific methods to determine litter lignin contents question the assumed intrinsic recalcitrance of lignin. Experiments using isotope labeling used to calculate mean residence times for lignin in soils and litter/soil mixtures in both laboratory and in-situ incubation reported lignin residence times no longer than that of other carbon compounds or bulk soil organic matter (Bol et al., 2009; Thevenot et al., 2010). Also, the capability to degrade lignin was demonstrated for several bacterial taxa in addition to fungi (Bugg et al., 2011).

For leaf litter, lignin depletion even at early stages of decomposition and lignin decomposition rates that decreased during decomposition were recently reported by Klotzbücher and colleagues (Klotzbücher et al., 2011). Based on these results, they proposed a new concept for lignin degradation in which fastest lignin degradation occurs during early litter decomposition when the availability of labile carbon is high. Lignin decomposition during late decomposition, in contrast, is limited by the availability of easily assimilated C and therefore slowes down. Additionally, the decomposition of lignin may also be dependent on the nutrient content of the litter and thus the status of the microbial community. During radical polymerization, significant amounts of cellulose and protein are incorporated into lignin structures (Achyuthan et al., 2010). In isolated lignin fractions from fresh beech litter, N contents twice as high as

in bulk litter were found (Dyckmans et al., 2002). It was therefore argued that, while yielding little C and energy, lignin decomposition makes protein accessible to decomposers that is occluded in plant cell walls, and that lignin decomposition is therefore not driven by C but by the N demand of the microbial community ("Nitrogen mining theory", Craine et al. (2007)).

In favor of the N mining theory, fertilization experiments indicated N exerts an important control on lignin degradation: N addition increased mass loss rates in low-lignin litter while slowing down decomposition in lignin-rich litter (Knorr et al., 2005) and decreased the activity of lignolytic enzymes in forest soils (Sinsabaugh, 2010). Moreover, cellulose triggered a stronger priming effect in fertilized than in unfertilized soils indicating that the mineralization of recalcitrant carbon may be controlled by an interaction of easily accessible C and N availability (Fontaine et al., 2011).

Addition of N has a different effect on litter decomposition than varying N levels in the litter Talbot et al. (2011). This is due to the fact that leaf litter N is stored in protein and lignin structures and not directly available to microorganisms, while fertilizer N is added in the form of readily available inorganic N (ammonium, nitrate or urea). N-fertilization experiments can thus simulate increased N-deposition rates but not the effect of litter N on decomposition processes.

Our study therefore aimed at analyzing the effect of variations in beech litter nutrient (N and P) content and stoichiometry (C:N and N:P ratios) on decomposition rates. Towards this end, we followed the breakdown of lignin and polymeric carbohydrates by pyrolysis-GC/MS (pyr-GC/MS) during a mesocosm experiment under constant environmental conditions over a period of 15 month. In order to exclude effects resulting from different initial microbial communities, we sterilized beech litter samples from 4 different locations in Austria and re-inoculated them prior to the experiment with an litter/top-soil inoculum from one of the sites.

We addressed the following questions in our study:

(1) Is lignin decomposition delayed until late decomposition stages or are significant amounts of lignin already degraded during early litter decomposition, and if the timing of lignin decomposition depended on litter stoichiometry? We hypothesized, that ligin decomposition is initially slower in litter with a narrow C:N ratio (higher availability of assimilable nitrogen), than in litter with a high C:N ratio.

(2) Are high lignin degradation rates related to a higher fungal activity? We hypothesized that wider C:N and C:P ratios favor lignin degradation by fungi while narrow C:N and C:P ratios favor carbohydrate degradation by bacteria.

2.3 Results

2.3.1 Initial litter chemistry

Initital litter chemistry (14 days after incubation) is presented in table 2.1. C:N ratios between 41:1 and 58:1 and C:P ratios between 700:1 and 1300:1 were found, N:P ratios ranged between 15:1 and 30:1. No significant changes occurred during litter incubation except a slight decrease of the C:N ratio (41.8:1 to 37.4:1) found in the most active litter type (SW) after 15 month. Fe concentrations were more than twice as high for OS (approx. 450 ppm) than for other litter types (approx. 200 ppm). Litter Mn also was highly variable between litter types, ranging between 170 and 2130 ppm. Changes of micro-nutrient concentrations during litter incubation were significant, but in all cases <15% of the initial concentration. In initial litter, lignin accounted for 28.9-31.2% and carbohydrates for 25.9-29.2% of the total peak area of all pyrolysis products.

2.3.2 Mass loss, respiration and extractable organic carbon

Litter mass loss was not significant after 2 weeks and 3 months, and significant for 2 litter types after 6 months. After 15 months, litter mass loss was significant for all litter types, ranged between 5 and 12% of the initial dry mass, and was strongly correlated to litter N content ($R=0.794$, $p<0.001$). Detailed results were reported by Mooshammer et al. (2011).

Highest respiration rates were measured at the first measurement after 14 days incubation (150-350 µg CO_2-C d^{-1} g^{-1} litter-C), which dropped to 75 to 100 µg CO_2-C d^{-1} g^{-1} litter-C after 3 months. After 6 and 15 months, respiration rates for AK and OS further decreased, while SW and KL showed a second maximum in respiration after 6 months days (fig 2.1). Accumulated respiration was correlated to litter mass loss ($r=0.738$, $p<0.001$, $n=20$).

Soluble organic carbon concentrations decreased between the first three harvests (14 days to 6 months), and strongly increased to 15 months (from 0.1 to 0.7 mg C g^{-1} d.w. to 1.5 to 4 mg C g^{-1} d.w. after 15 months, fig. 2.1). After 14 days and 3 months, the highest soluble organic C concentration was found in SW litter followed by AK. Soluble organic C concentrations were weakly correlated with litter N content after 14 days ($r=0.69$, $p<0.001$) and after 3 months ($r = 0.65$, $p<0.01$), but were strictly correlated after 6 months ($r=0.85$, $p<0.001$) and 15 months ($r=0.90$, $p<0.001$).

Potential enzyme activities

Potential extracellular enzyme activities were correlated with litter N, respiration and other decomposition processes (all $R>0.8$, $p<0.001$). Cellulase activity increased from first harvest onwards to 15 months, with a small depression after 6 months (Fig. 2.1), phenoloxidase and peroxidase activities reached their

maximum after between 3 and 6 months (fig. 2.1). For all enzymes and at all time points, SW showed the highest and AK the lowest activity. Differences between these two sites were more pronounced in cellulase activity (SW 10x higher than AK) than in oxidative enzymes (4x higher). Conversely, the phenoloxidase/cellulase ratio was highest for AK and lowest for SW at all time points and decreased during litter decomposition. This indicates that microbial communities in AK litter invested more energy and nitrogen into degrading lignin and less into degrading carbohydrates than in litter from other sites. (fig. 2.1).

Microbial biomass abundance and community composition

Microbial biomass contents ranged from 0.5 to 6 mg C g^{-1} d.w., 0.05 to 5.5 mg N g^{-1} d.w. and 0.05 to 3.5 mg P g^{-1} litter d.w (fig. 2.2). In KL and OS microbial biomass buildup reached a plateau after 3 months, AK and SW showed further microbial biomass growth reaching a maximum of microbial C and N contents after 6 months (AK also for P). Microbial C:N ratios ranged between 6 and 18, C:P ratios between 8 and 35, and N:P ratios between 0.5 and 3.5 (fig. 2.2).

Litter microbial biomass was stoichiometrically homeostatic during the first 6 months (no or marginally negative correlations between microbial C:N:P and litter C:N:P, see also Mooshammer et al. (2011)), but after 15 months (microbial C:N:P ratios were significantly correlated to resource stoichiometry: R=0.53-0.64, all $p<0.002$), when the homeostatic regulation coefficients (Sterner and Elser, 2002) $H_{C:P}=1.68$, $H_{C:N}=2.01$, and $H_{N:P}=2.29$ were found. Microbial C:N ratios were tightly constrained after 3 months (14.5 to 18.2) and 6 months (6.9 to 9.0), but significantly different between the two time points. Microbial C:P and N:P ratios were less constrained, with the highest variance between litter from different sites after 3 months incubation (fig. 2.2).

Fungi/bacteria ratios derived from metaproteomics data of the litter (one replicate per litter type and harvest) were highest after 14 days (5 to 12) and decreased during litter decomposition (1.7 to 3 after 15 months). The large differences in fungi/bacteria ratios between litter types decreased during decomposition. Fungal proteins were dominant in all litter types at all stages, but most prominent in SW and least pronounced in AK. The fungi/bacteria ratios were negatively correlated to the ratios of lignin/cellulose decomposition and to LCI change during the first 6 months. In contrast, lignin decomposition rates were positively correlated with fungi/bacteria ratios after 15 months but not to the ratios of lignin/cellulose decomposition (fig. 2.3). In addition, fungi: bacteria ratios were measured on a DNA basis (qPCR) the results showing a similar pattern between litter types and harvests but with a much larger fungal DNA dominance (ratios between 10-180). Fungi/bacteria ratios were highly correlated between protein- and DNA-based estimates ($r=0.801$, $p<0.001$, with log-transformed qPCR ratios).

2.3.3 Pyrolysis-GC/MS and Lignin content

In total 128 pyrolysis products were detected, quantified, identified and assigned to their origin (2.2 -2.4). We found only minor changes in the relative concentration of litter pyrolysis products during decomposition, and differences between sites were small but well preserved during decomposition. However, the high precision and reproducibility of pyrolysis GC/MS analysis of litter allowed tracing small changes in lignin and carbohydrate abundance during decomposition. Lignin-derived compounds made up between 29 and 31% relative peak area (TIC) in initial litter, and increased by up to 3% over the first 6 months. Carbohydrate-derived pyrolysis products accounted for 26 to 29% in initial litter and decreased by up to 2.6% during litter decomposition. The pyrolysis-based LCI index showed a small range between 0.517 and 0.533 initially (Fig. 4). During decomposition, LCI increased by up to 9% of the initial value, with SW showing the highest increase while in AK LCI decreased. The changes in LCI almost completely occurred over the first 6 months, with insignificant changes thereafter (fig. 2.4).

During the first 6 months of litter decomposition, between one and 6% of the initial lignin pool and between 4 and 17% of the initial carbohydrate pool were degraded (Fig. 2.5). Lignin decomposition was highest in AK and KL litter, while KL, OS and SW decomposed carbohydrates fastest. Lignin preference values (% lignin decomposed/%carbohydrates decomposed) were lowest in SW and highest in AK litter (Figure 5). In AK litter, lignin macromolecules were 50% more likely to be decomposed than carbohydrates, while in SW litter carbohydrates were 10 times more likely to be decomposed (fig. 2.5). Between 6 and 15 months, no further accumulation of lignin occurred, lignin and carbohydrates were both degraded at the same rates and their relative concentrations remained constant (fig. 2.5).

2.3.4 Correlations between lignin and carbohydrate decomposition and litter chemistry, microbial community and decomposition processes

Relationships between lignin and carbohydrate degradation, litter chemistry, microbial biomass and decomposition processes were tested after 6 and 15 months (tables 2.5 and 2.6) including data presented by (Mooshammer et al., 2011) and (Leitner et al., 2011). After 6 months, we found that the ratio of lignin/cellulose degradation was positively correlated with the ratio of phenoloxidase/cellulase ($R=0.599$, $p=0.005$) and peroxidase/cellulase ($R=0.734$ $p<0.001$, table 2.5). Carbohydrate decomposition was positively correlated with litter N content, and negatively with litter C:N ratios and litter-microbial C:N imbalances. In contrast, lignin decomposition was negatively correlated to litter P, but positively with litter C:P and N:P ratios, and litter-microbial C:P and N:P imbalances (fig. 2.6). After 15 months, the ratio of lignin/carbohydrate decomposition was not related to stoichiometry or elemental composition any more. Most interestingly, lignin and carbohydrate decomposition exhibited the same controls, being

positively correlated to soluble organic C, litter N and litter P (table 2.6). Mass loss and accumulated respiration were positively correlated to lignin and carbohydrate decomposition (table 2.6), a pattern that we did not find for lignin decomposition in the early decomposition phase (table 2.5).

2.4 Discussion

Our experimental approach allowed us to single out the effects of litter quality on the microbial decomposer community as well as decomposition processes, while excluding effects of fauna, climate and the initial microbial community. By exploiting intra-specific differences in beech litter stoichiometry, we were able to minimize differences in the chemical composition of initial litter (e.g. similar lignin and cellulose content, table 2.1), while exploring the effect of litter nutrient contents on lignin and carbohydrate decomposition. Therefore, we can attribute different rates of carbohydrate and lignin decomposition to the intrinsic qualities of litter collected at different sites, i.e. elemental and stoichiometric composition.

Contradicting the traditional concepts of litter decomposition, our results demonstrate that relevant but variable amounts of lignin were degraded during the first 6 months of incubation. During this early stage, lignin decomposition rates depended on litter quality (P) and ranged from non-significant to degradation rates similar to bulk carbon mineralization rates (i.e. no discrimination against lignin). We can therefore confirm that early lignin decomposition rates are by far underestimated, as recently proposed by Klotzbücher et al. (2011), based on a complementary analytic approach. Unlike them, we found no decreases but constant or increasing lignin decomposition rates during litter decomposition over 15 months. Additionally, we found a marked change in the controls of lignin decomposition during this period. While carbohydrate and lignin decomposition were differently controlled by litter chemistry (N versus P) during the first 6 months, these litter components were decomposed at similar rates thereafter and decomposition rates were only related to litter N availability.

Differences in initial lignin contents were marginal (29-31% relative peak area), and lignin contents of sites with high initial lignin decomposition rates were not higher than that of sites with low rates. Therefore, differences in early lignin decomposition did not result from high or low lignin contents as is suggested by traditional litter decomposition models. Low lignin decomposition rates were also not caused by a lack of Mn or Fe, the metals being important cofactors of oxidative lignin decay, which were suggested to be rate limiting during late lignin decomposition (Berg, B. & McClaugherty, 2008). While Mn and Fe concentrations strongly varied between litter collected at different sites, Mn and Fe concentrations were lowest in the litter with highest lignin decomposition rates (AK, see Table 2.1). Low contents of these elements would explain decreased but not enhanced lignin decomposition. Moreover, soluble organic C was suggested to be limiting for lignin decomposition since the process of lignin decomposition does not generate enough energy for survival of lignin decomposers (Klotzbücher et al., 2011). Soluble organic C apparently did not control lignin decomposition since we found highest concentrations in the two litter types that showed the highest and the lowest lignin decomposition rates.

We found strong evidence that litter C:N:P stoichiometry and litter element concentrations exerted a

major control on the extent of lignin decomposition during the initial decomposition phase. Carbohydrate decomposition was positively correlated with litter N contents and negatively to litter C:N ratios, as were the majority of decomposition processes (mass loss, respiration, potential extracellular enzymatic activities). In contrast, lignin decomposition rates were positively correlated with litter C:P ratios and negatively with dissolved and total litter P. The relationship was strongest when lignin decomposition rates were compared to litter-microbe C:P imbalances, i.e. the greater the imbalance between resource and consumer C:P became (greater P limitation) the lower lignin decomposition rates became.

Cultivation studies showed that lignin decomposition by fungi is triggered by nitrogen starvation, and that lignin does not provide sufficient energy to maintain the decomposer's metabolism without the use of other organic C i.e. energy sources (Janshekar and Fiechter, 1988). Moreover, lignin decomposition was found in wild-type *A. thaliana* litter containing abundant cellulose as a C source, but not in a low-cellulose mutant during a 12-month incubation experiment in a boreal forest (Talbot et al., 2011). In the N- and P-(co-)limited situation commonly encountered during early litter decomposition, we may speculate that lignin is degraded to access additional nutrients (mainly N) or to use a C surplus by decomposing a less C efficient but nutrient enriched substrate (nutrient mining hypothesis). However, a stimulation of lignin decomposition by low P availability or microbial P limitation, as indicated by the strong negative correlations to P pools that we found, has not been reported yet. Though lignified materials have been reported to be N-rich and decomposition of these materials may therefore enhance N supply to microbial communities, lignins are not expected to contain quantitative important amounts of P.

In order to decompose litter lignin and carbohydrates, microbial decomposers rely on the production and excretion of hydrolytic and oxidative extracellular enzymes. While the absolute amounts, in which these enzymes are produced, were largely controlled by N availability, the ratio in which they were produced was strongly related to differences in the ratio of cellulose/lignin decomposition. Talbot et al. (2011) suggested that lignin decomposition comprises a strategy of slow-growing microbes to evade competition through colonizing more lignin-rich and nutrient-poor substrates. Indeed we found lignin decomposition in low quality litter (low N and P) with microbial communities that were subject to large imbalances in C:N and C:P between resource and consumer, pointing to N and P limitation or high N and P uns efficiency of these communities. Low P availability may limit fast growth of microbial populations and select for slow-growing lignin-degrading microbes during early decomposition and provide K-strategists (slow growing on recalcitrant carbon) an advantage over r strategists (fast growing on labile carbon). Indeed we found that lignin decomposition was highest in litter, where resource C:P and N:P were highest, i.e. low P supply may have limited microbial growth generally or the establishment of r strategists in particular.

While the mode of negative P regulation on lignin decomposition remains unknown, we found differences in the composition of the microbial decomposer communities on litter with fast and slow lignin decomposition. Unlike predicted by ecological stoichiometry theory, not bacteria but fungi were more successful in colonizing high N and high P litter during initial decomposition. Fungi colonized litter faster than bacteria and therefore dominated early litter decomposition, however the fungi: bacteria ratios decreased over the entire incubation period pointing to increasing population sizes of bacteria with time. Fungi-rich communities more efficiently used high litter N to produce extracellular enzymes that degrade carbohydrates immediately after inoculation (fungi: bacteria ratios were correlated to litter N 14 days after inoculation) and high litter P to build up microbial biomass on a longer time scale (fungi: bacteria ratios were correlated to litter P after 6 months). Interestingly, bacteria-rich communities (AK) were more active in decomposing lignin than those being dominated by fungi. This does not necessarily indicate that bacteria play the key role in lignin decomposition, though bacteria were also reported to produce oxidative enzymes that can decompose lignified materials in litter (Bugg et al., 2011). However, decreases in fungi/bacteria ratios may be superimposed on the increase of smaller subpopulations of e.g. fungi that are key mediators of lignin decomposition, or alternatively general increases in the size of microbial communities with declining fungi/bacteria ratios may as well mask stable fungal populations when bacterial abundance increases. The fungal communities were dominated by Ascomycetes (Dothideomycetes, Eurotiomycetes, Leotiomycetes and Sordariomycetes), with smaller contributions by Saccharomycetes and Basidiomycetes (Agaricomycetes and Tremellomycetes). It is particularly the latter, Basidiomycetes, that catalyze the cellulolytic and lignolytic decomposition of dead plant material, however they comprised less that 5% of the fungal protein ensemble. The bacterial community in contrast was dominated by Proteobacteria (mainly γ, declining, and α- and β-Proteobacteria, increasing with litter decomposition), Actinobacteria and Bacteroidetes both of which strongly increased with time. Actinobacteria are also known as important decomposers of plant detritus, with the potential to excrete oxidative enzymes and being oligotrophic, and Bacteroidetes also excrete a broad range of hydrolytic enzymes targeting cellulose and other polymers. Since the metaproteomic approach did not find oxidative extracellular enzymes we so far cannot dissect the contributions of bacteria and fungi to the lignin decomposition process.

While the microbial communities were strictly homeostatic during the first 6 months, substrate stoichiometry had a minor, but significant influence on microbial stoichiometry after 15 months. Together, these changes indicate that the microbial communities were able to compensate for differences in substrate quality by adjusting their C-, N- and P-use efficiency (Mooshammer et al. 2011) which was coupled to differences in substrate preference (lignin/carbohydrate) and occurred at the expense of microbial community growth and overall decomposition speed. However, stoichiometric compensation of

the microbial communities was limited after 6-15 months which points to larger stoichiometric differences between the microbial populations dominating the later stage decomposition processes.

2.5 Conclusions

Our results contradict the traditional concept that lignin decomposition is slow during early litter decomposition. While traditional litter decomposition models propose that lignin decomposition mainly occurs during late decomposition stages, we found that variable but in some cases substantial amounts of lignin were decomposed during the first 6 months. The extent to which lignin was decomposed was controlled by litter P during the first 6 months, but by litter N thereafter as was carbohydrate decomposition. Our results further question that recalcitrance is intrinsic to lignin as a chemical compound, but suggests that lignin decomposition also depends on litter chemistry and environmental conditions, which both affect microbial community structure including the abundance of fungal and bacterial groups that are key to decomposition of plant debris by excretion of hydrolytic and oxidative extracellular enzymes.

2.6 Material and methods

2.6.1 Litter decomposition experiment

Beech litter was collected at four different sites in Austria (Achenkirch (AK), Klausenleopoldsdorf (KL), Ossiach (OS), and Schottenwald (SW); referred to as litter types) in October 2008. Litter was cut to pieces of approximately 0.25cm^2, homogenized, sterilized twice by γ-radiation (35 kGy, 7 days between irradiations) and inoculated (1.5% w/w) with a mixture of litter and soil to assure that all litter types share the same initial microbial community. From each type, four samples of litter were taken immediately after inoculation, dried and stored at room temperature. Batches of 60g litter (fresh weight) were incubated at 15 °C and 60% relative water content in mesocosms for 15 months. For each litter type 5 replicates were removed and analyzed after 14, 97, 181 and 475 days. A detailed description of the litter decomposition experiment was published by Wanek et al. (2010).

2.6.2 Bulk litter, extractable, and microbial biomass nutrient content

To calculate litter mass loss, litter dry mass content was measurement in 5 g litter (fresh weight) after 48 h at 80 °C. Dried litter was ball-milled for further chemical analysis. Litter C and N content was determined using an elemental analyzer (Leco CN2000, Leco Corp., St. Joseph, MI, USA). Litter phosphorus content was measured with ICP-AES (Vista-Pro, Varian, Darmstadt, Germany) after acid digestion (Kolmer et al., 1951)). To determine dissolved organic C, dissolved N and P, 1.8 g litter (fresh weight) were extracted with 50 ml 0.5 M K_2SO_4. Samples were shaken on a reciprocal shaker with the extractant for 30 minutes, filtered through ash-free cellulose filters and frozen at -20 °C until analysis. To quantify microbial biomass C, N and P, further samples were additionally extracted under the same conditions after chloroform fumigation for 24 h (Brooks et al., 1985). Microbial biomass was determined as the difference between fumigated and non-fumigated extractions . C and N concentration in extracts were determined with a TOC/TN analyzer (TOC-VCPH and TNM, Schimadzu), P was determined photometrically as inorganig P after persulfate digestion (Schinner et al., 1996).

Substrate to consumer stoichiometric imbalances $C{:}X_{imbal}$ were calculated as

$$C : X_{inbal} = \frac{C : X_{litter}}{C : X_{microbial}} \tag{2.1}$$

where X stand for the element N or P.

2.6.3 Microbial Respiration

Respiration was monitored weekly during the entire incubation in mesocosms removed after 6 month and on the last incubation day for all mesocosms using an infrared gas analyzer (IRGA, EGM4 with SRC1, PPSystems, USA). CO2 concentration was measured over 70 seconds and increase per second was calculated based on initial dry mass. Accumulated respiration after 6 month was calculated assuming linear transition between measurements, accumulated respiration after 15 month was estimated from respiration rates after 181 and 475 days.

2.6.4 Potential enzyme activities

Potential activities of β-1,4-cellubiosidase ("cellulase"), phenoloxidase and peroxidase were measured immidiately after sampling. 1 g of litter (fresh weight) was suspended in sodium acetate buffer (pH 5.5) and ultrasonicated. To determine cellulase activity, 200 μl suspension were mixed with 25 nmol 4-methylumbelliferyl-β-D-cellobioside (dissolved in 50 μl of the same buffer) in black microtiter plates and incubated for 140 min in the dark. The amount of methylumbelliferyl (MUF) set free in by the enzymatic reaction was measured flourimetrically (Tecan Infinite M200, exitation at 365 nm, detection at 450 nm). To measure phenoloxidase and peroxidase activity litter suspension was mixed 1:1 with a solution of L-3,4-dihydroxyphenylalanin (DOPA) to a final concentration of 10 mM. Samples were incubated in microtiter plates for 20h to determine phenoloxidase activity. For peroxidase activity, 1 nmol of H_2O_2 was added before incubation. Absortion at 450 nm was measured before and after incubation. All enzyme activities were measured in three analytical replicates. The assay is described in detail in Kaiser et al. (2010).

2.6.5 Metaproteome analysis and quantitative PCR

For metaproteome analysis, 3 g of each sample were gounded in liquid nitrogen and extracted with Tris/KOH buffer (pH 7.0) containing 1% SDS. Samples were sonicated for 2 min, boiled for 20 min and shaken at 4 °C for 1 h. Extracts were centrifuged twice to remove debris and concentrated by vaccum-centrifugation. An aliquot of the sample was applied to a 1D-SDS-PAGE and subjected to in-gel tryptic digestion. The resulting mixtures were analyzed on a hybrid LTQ-Orbitrap MS (Thermo Fisher Scientific). Protein database serach against the UniRef library, the translated metagenome of the microbial community of a Mennesota farm silage soil (Tringe et al., 2005) and known contaminants was performed using the MASCOT Search Engine. A detailed description of the extraction procedure and search criteria was published by Schneider et al. (2010). Additionally, fungi/bacteria ratios were determined with quantitative PCR as described recently (Inselsbacher et al., 2010).

2.6.6 Pyrolysis-GC/MS

Pyrolysis-GC/MS was performed with a Pyroprobe 5250 pyrolysis system (CDS Analytical) coupled to a Thermo Trace gas chromatograph and a DSQ II MS detector (both Thermo Scientific) equipped with a carbowax colomn (Supelcowax 10, Sigma-Aldrich). Between 2-300 µg of dried and finely ground litter (MM2000 ball mill, Retsch) was heated to 600 °C for 10 seconds in a helium atmosphere. GC oven temperature was constant at 50 °C for 2 minutes, followed by an increase of 7°C/min to a final temperature of 260 °C, which was held for 15 minutes. The MS detector was set for electron ionization at 70 eV in the scanning mode (m/z 20 to 300).

Peaks were assignment was based on NIST 05 MS library after comparison with measured reference materials. 128 peaks were identified and selected for integration either because of their abundance or diagnostic value. This included 28 lignin and 45 carbohydrate derived substances. The pyrolysis products used are stated in tables 2.2 -2.4 For each peak between one and four dominant and specific mass fragments were selected, integrated and converted to TIC peak areas by multiplication with a MS response coefficient (Kuder and Kruge, 1998; Schellekens et al., 2009). Peak areas are stated as % of the sum of all integrated peaks.

A pyrolysis-based lignin to carbohydrate index (LCI) was calculated to derive a ratio between these two substance classes without influences of changes in the abundance of other compounds .

$$LCI = \frac{Lignin}{Lignin + Carbohydrates} \tag{2.2}$$

Accounting for carbon loss, we estimate % lignin and cellulose degraded during decomposition according to equation 2.3, where $\%_{init}$ and $\%_{act}$ stand for initial and actual %TIC area of lignin or cellulose pyrolysis products, C_{init} for the initial amount of C and R_{acc} for the accumulated CO_2-C respired by a mesocosm.

$$\%_{loss} = 100 \cdot \frac{\%_{init} - \%_{act}}{\%_{init}} \cdot \frac{(1 - R_{acc})}{C_{init}} \tag{2.3}$$

2.6.7 Statistical analysis

All statistical analyses were performed with the software and statistical computing environment R (R Development Core Team, 2008). If not mentioned otherwise, results were considered significant when $p < 0.05$. Due to frequent variance inhomogeneities Welch ANOVA and paired Welch's t-tests with Bonferroni corrected p limits were used. All correlations mentioned refer to Pearson correlations.

Figures

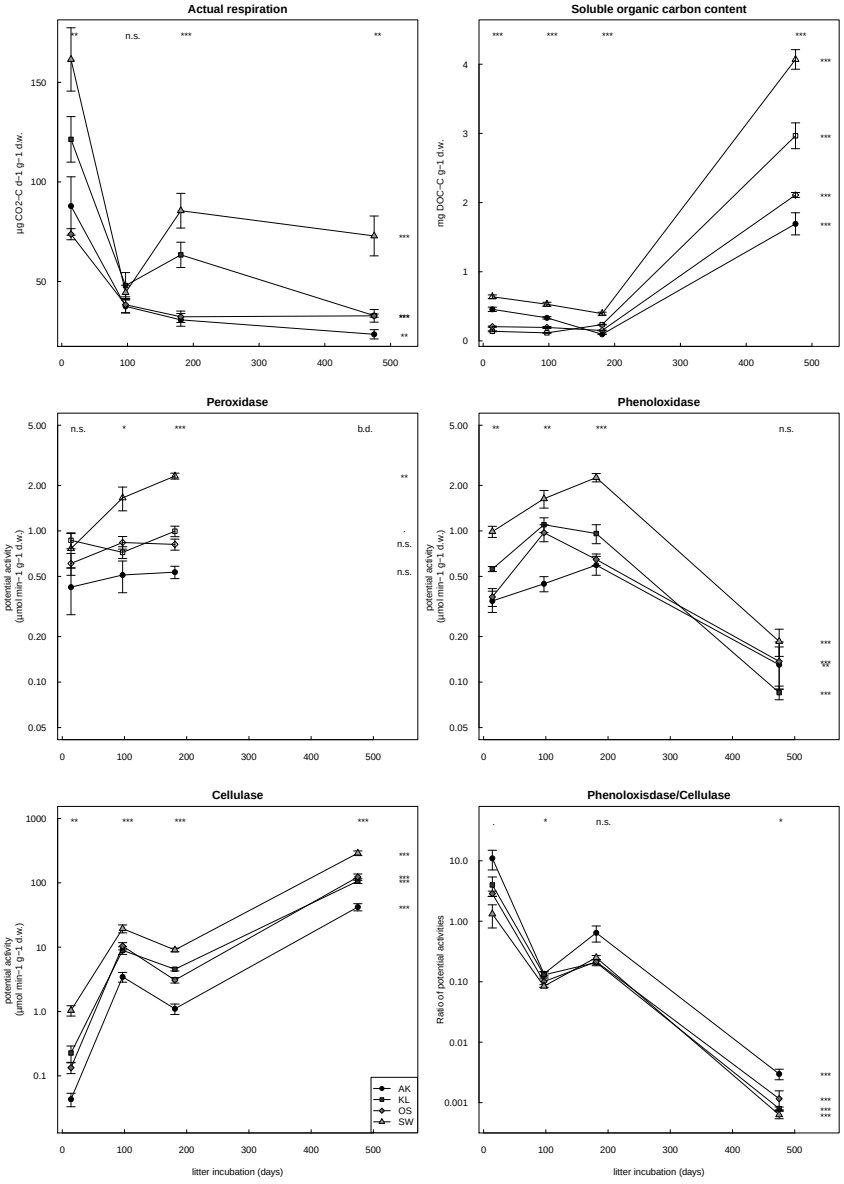

Figure 2.1: **Respiration rates, concentration of soluble organic C and potential extracellular enzyme activities** in decomposing beech leaf litter from a mesocosm experiment. Beech litter was collected in: triangles, Schottenwald (SW); diamonds, Ossiach (OS); squares, Klausenleopoldsdorf (KL); circles, Achenkirch, AK. Error bars indicate standard errors (n=5). Significant differences between litter types are presented by asterisks above the symbols, significant differences between time points by asterisks to the right of the curves. *, P<0.05, **, P<0.01, ***, P<0.001, b.d. - below detection limit.

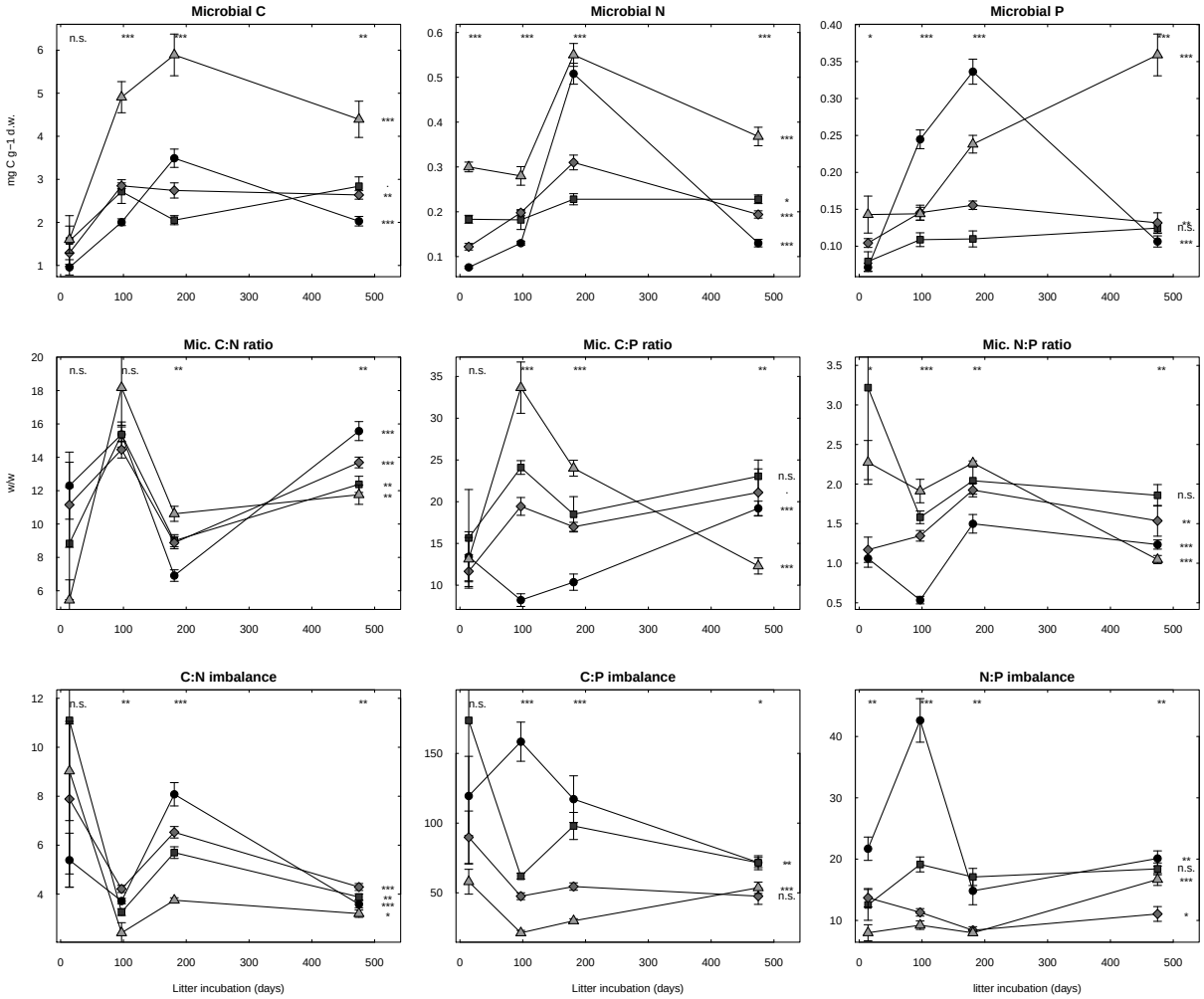

Figure 2.2: **Microbial biomass C, N and P, microbial C:N:P stoichiometry and resource/consumer stoichiometric imbalance in these elements**in decomposing beech leaf litter from a mesocosm experiment. Beech litter was collected in: triangles, Schottenwald (SW); diamonds, Ossiach (OS); squares, Klausenleopoldsdorf (KL); circles, Achenkirch, AK. Error bars indicate standard errors (n=5). Significant differences between litter types are presented by asterisks above the symbols, significant differences between time points by asterisks to the right of the curves. *, P<0.05, **, P<0.01, ***, P<0.001.

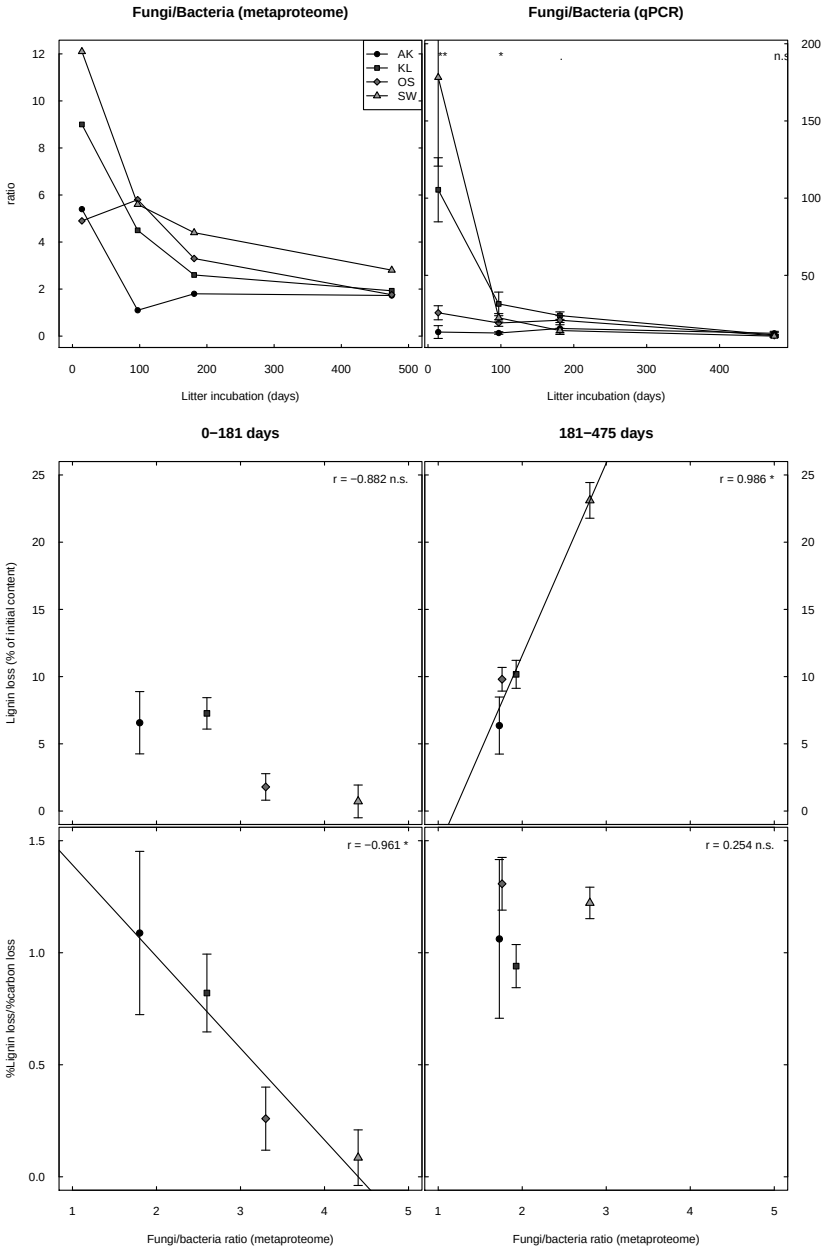

Figure 2.3: **Fungi/Bacteria ratios and their correlations with LCI change:** Top: Fungi/bacteria ratios derived from metaproteome (left) and qPCR (right) analysis. Bottom: The correlations between metaproteomic fungi/bacteria ratios with Lignin decomposition rates (mid) and lignin loss / carbon loss (bottom) for 0-6 months (left) and 6-15 months (right..Errorbars indicate standard errors (n=4-5). Beech litter was collected in: triangles, Schottenwald (SW); diamonds, Ossiach (OS); squares, Klausenleopoldsdorf (KL); circles, Achenkirch, AK. Error bars indicate standard errors (n=5). Significant differences between litter types are presented by asterisks above the symbols, significant differences between time points by asterisks to the right of the curves. *, P<0.05, **, P<0.01, ***, P<0.001.

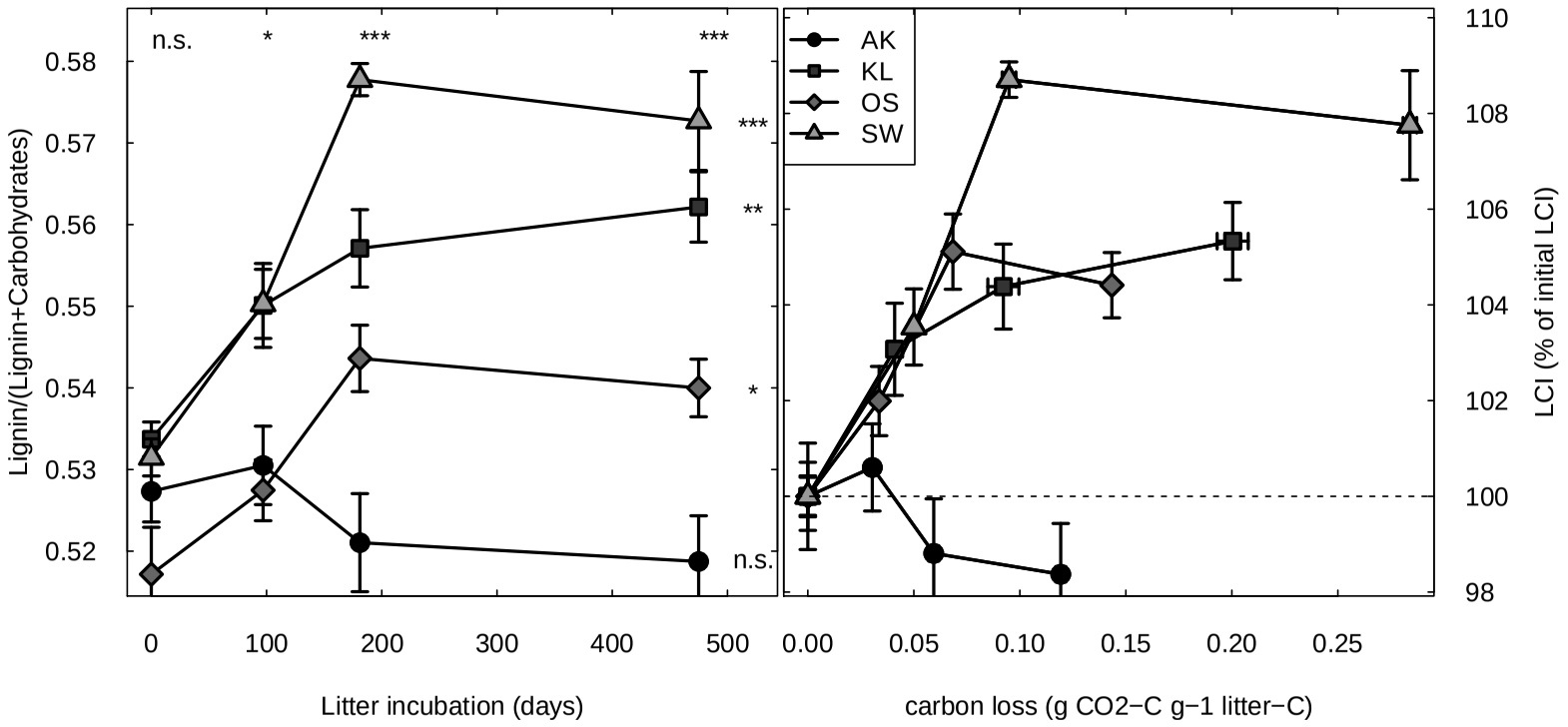

Figure 2.4: **Develoment of the LCI (lignin/(lignin+carbohydrates))** during time of beech litter decomposition (A) or plotted against cumulative C loss (B). Errorbars indicate standard errors (n=4-5). The dashed line indicates a constant ratio between lignin and carbohydrates (i.e. no preferential decomposition of carbohydrates. Beech litter was collected in: triangles, Schottenwald (SW); diamonds, Ossiach (OS); squares, Klausenleopoldsdorf (KL); circles, Achenkirch, AK. Error bars indicate standard errors (n=5). Significant differences between litter types are presented by asterisks above the symbols, significant differences between time points by asterisks to the right of the curves. *, P<0.05, **, P<0.01, ***, P<0.001.

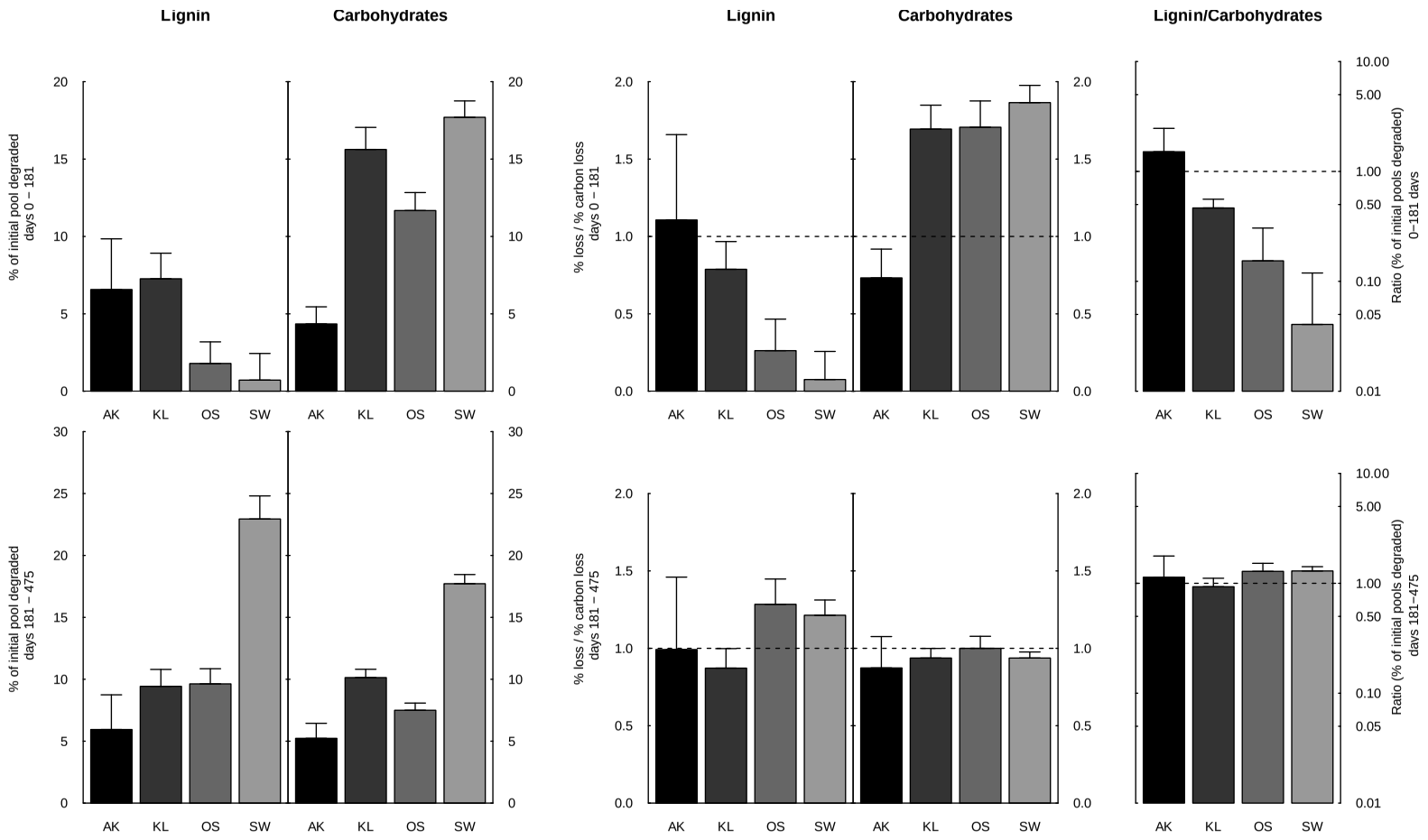

Figure 2.5: **Carbon loss corrected amounts of lignin and carbohydrates** degraded in beech litter collected in Achenkirch (AK), Klausenleopoldsdorf (KL), Ossiach (OS) and Schottenwald (SW). Carbon loss was calculated based on accumulated respiration for each mesocosm. Error bars indicate standard errors (n=4-5). The dashed line marks no discrimation during decomposition between lignin, carbohydrates and bulk carbon

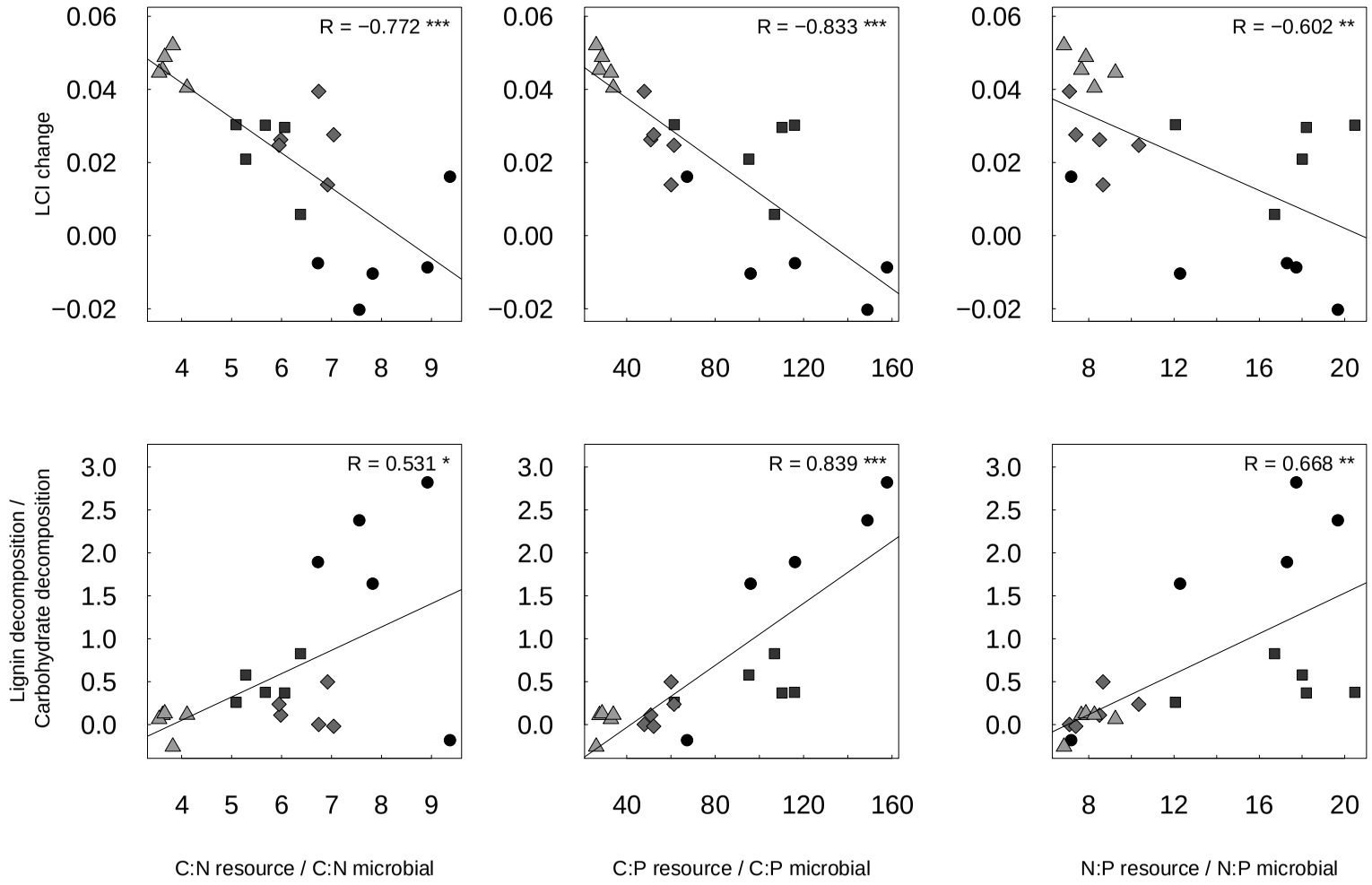

Figure 2.6: **Correlation between the LCI change or the ratio of lignin/carbohydrate decomposition ratio during the first 6 months of litter decomposition correlate to litter/microbe stoichiometric imbalances.** and change and Correlations between lignin accumulation during the first 6 month of litter incubation and stoichiometric resource:consumer imbalances. LCI is calculates as of lignin/(lignin+Carbohydrates). Beech litter was collected in: triangles, Schottenwald (SW); diamonds, Ossiach (OS); squares, Klausenleopoldsdorf (KL); circles, Achenkirch, AK. *, P<0.05, **, P<0.01, ***, P<0.001.

Tables

Table 2.1: Element concentrations, elemental stoichiometry and cellulose and lignin concentrations in beech litter measured after 14 days incubation. Standard errors are given in brackets (n=5). C extr represents for soluble organic carbon. Beech litter was collected in AK, Achenkirch, KL, Klausenleopoldsdorf, OS, Ossiach, and SW, Schottenwald.

	AK	(SE)	KL	(SE)	OS	(SE)	SW	(SE)	p value
C (% d.w.)	50.86	(0.39)	49.41	(0.53)	48.15	(0.39)	48.90	(0.34)	0.002
C extr (mg g-1)	0.46	(0.03)	0.14	(0.01)	0.21	(0.01)	0.64	(0.03)	<0.001
N (% d.w.)	0.878	(0.012)	0.938	(0.012)	0.806	(0.013)	1.172	(0.016)	<0.001
P (% d.w.)	0.040	(0.000)	0.030	(0.000)	0.052	(0.002)	0.070	(0.000)	<0.001
C:N (w/w)	57.86	(0.57)	52.60	(0.49)	59.97	(0.72)	41.78	(0.76)	<0.001
C:P (w/w)	1282	(21)	1548	(25)	905	(15)	699	(9)	<0.001
N:P (w/w)	22.17	(0.47)	29.45	(0.60)	15.10	(0.29)	16.75	(0.39)	<0.001
K (mg g-1)	0.26	(0.00)	0.54	(0.00)	0.21	(0.00)	0.55	(0.00)	<0.001
Ca (mg g-1)	1.33	(0.01)	1.26	(0.01)	1.63	(0.01)	1.23	(0.01)	<0.001
Mg (mg g-1)	0.27	(0.00)	0.14	(0.00)	0.20	(0.00)	0.15	(0.00)	<0.001
Fe (ppm)	210	(2)	208	(4)	453	(12)	192	(4)	<0.001
Mn (ppm)	172	(2)	1430	(10)	776	(9)	2137	(51)	<0.001
Zn (ppm)	30.8	(0.4)	33.0	(0.3)	36.0	(1.0)	42.4	(0.7)	<0.001
Lignin	28.9	(28.9)	29.9	(29.9)	31.2	(31.2)	30.5	(30.5)	<0.001
Carbohydrates	25.9	(25.9)	26.1	(26.1)	29.2	(29.2)	26.9	(26.9)	<0.001

Table 2.2: Lignin derrived and other phenolic pyrolysis products

Name	RT	MW	integrated framents	Origin	Class
Guaiacol	18.87	124	109+124	Lignin	Guaiacyl
Methylguaiacol	20.32	138	123+138	Lignin	Guaiacyl
Ethylguaiacol	21.40	152	137+152	Lignin	Guaiacyl
Propenylguaiacol	23.29	164	149+164	Lignin	Guaiacyl
Vinylguaiacol	23.69	150	135+150	Lignin	Guaiacyl
Propenylguaiacol	24.48	164	149+164	Lignin	Guaiacyl
Syringol	24.58	154	139+154	Lignin	Syringyl
Propenylguaiacol	25.66	164	149+164	Lignin	Guaiacyl
Methylsyringol	25.67	168	153+168	Lignin	Syringyl
Ethylsysringol	26.39	182	167+182	Lignin	Syringyl
Propenylsyringol	27.97	194	179+194	Lignin	Syringyl
Vinylsyringol	28.37	180	165+180	Lignin	Syringyl
Guaiacolaldehyde	28.40	152	109+152	Lignin	Guaiacyl
Propylguaiacol	28.72	166	137+166	Lignin	Guaiacyl
Oxo-hydroxy-etylguaiacol	28.77	182	182	Lignin	Guaiacyl
Propenylsyringol	28.91	194	179+194	Lignin	Syringyl
Oxo-ethylguaiacol	29.20	166	151+166	Lignin	Guaiacyl
Oxo-propylguaiacol	29.36	180	137+180	Lignin	Guaiacyl
Propenylsyringol	30.16	194	194+179	Lignin	Syringyl
Syringolaldehyde	32.68	182	139+182	Lignin	Syringyl
Oxo-hydroxy-ethylsyringol	32.80	212	212	Lignin	Syringyl
Guaiacolacetic acid	32.88	182	137+182	Lignin	Guaiacyl
Propylsyringol	33.15	196	181+196	Lignin	Syringyl
Oxo-propylsyringol	33.32	210	167+210	Lignin	Syringyl
Oxopropenylguaiacol	35.30	178	135+178	Lignin	Guaiacyl
Hydroxypropenylguaiacol	37.10	180	137+180	Lignin	Guaiacyl
Syringolacetic acid	38.78	212	212	Lignin	Syringyl
Oxo-propenylsyringol	43.06	208	165+208	Lignin	Syringyl
Phenol	21.02	94	65+66+94	Phenolic	
4-Methylphenol	22.11	108	107+108	Phenolic	
3-Methylphenol	22.22	108	107+108	Phenolic	
Ethylphenol	23.38	122	107+122	Phenolic	
Propenylphenol	26.93	134	133+134	Phenolic	
Propenylphenol	27.76	134	133+134	Phenolic	
Propylphenol	31.11	136	151+166	Phenolic	
Butylphenol	31.86	150	107+150	Phenolic	
4-Hydroxybenzaldehyde	32.70	122	121+122	Phenolic	
Hydroquinone	33.40	110	81+110	Phenolic	

Table 2.3: Carbohydrate derrived pyrolysis products

Name	RT	MW	integrated framents	Origin	Class
Acetaldehyde	2.06	44	29+44	Carbohydrates	
Furan	2.35	68	39+68	Carbohydrates	Furan
Methylfuran	2.74	82	81+82	Carbohydrates	Furan
Methylfuran	2.91	82	81+82	Carbohydrates	Furan
Dimethylfuran	3.43	96	95+96	Carbohydrates	Furan
Dimethylfuran	3.66	96	95+96	Carbohydrates	Furan
Vinylfuran	5.01	94	65+94	Carbohydrates	Furan
Unknown furan	6.36	108	107+108	Carbohydrates	Furan
Cyclopentanone	6.99	105?	84+105?	Carbohydrates	Cyclopentenone
Methylfuran	7.62	82	53+82+83	Carbohydrates	Furan
2-Oxopropanoic acid, methylester	7.92	102	43+102	Carbohydrates	
1-Hydroxypropanone	9.24	74	43	Carbohydrates	
2-Cyclopenten-1-one	10.26	82	53+54+52	Carbohydrates	Cyclopentenone
2-Methyl-2-cyclopenten-1-one	10.51	96	53+96	Carbohydrates	Cyclopentenone
1-Hydroxy-2-propanone	10.69	88	57+88	Carbohydrates	Cyclopentenone
Unknown	11.38	unk	65+66+94	Carbohydrates	
3-Furaldehyd	11.57	96	95+96	Carbohydrates	Furan
2(5H)Furanon	11.69	98	55+98	Carbohydrates	Furan
Propanoic acid, methylester	12.10	102	43+102	Carbohydrates	
2-Furaldehyd	12.22	96	95+96	Carbohydrates	Furan
Acetylfuran	12.99	110	95+110	Carbohydrates	Cyclopentenone
3-Methyl-cyclopentanone	13.31	96	67+96	Carbohydrates	Cyclopentenone
Dimethylcyclopentenone	13.69	110	67+95+110	Carbohydrates	Cyclopentenone
5-Methyl-2-furancarboxaldehyde	14.23	110	109+110	Carbohydrates	Furan
2-Cyclopenten-1,4-dione	14.44	96	54+68+96	Carbohydrates	Cyclopentenone
Butyrolactone	15.22	86	56+86	Carbohydrates	
Unknown	15.56			Carbohydrates	
Furanmethanol	15.61	98	98	Carbohydrates	Cyclopentenone
5-Methyl-2(5H)-furanone	16.06	98	55+98	Carbohydrates	Furan
Unknown	16.17	unk	110	Carbohydrates	
1,2-Cylopentandione	17.51	98	55+98	Carbohydrates	Cyclopentenone
Unknown	17.67	unk	42+70	Carbohydrates	
2-Hydroxy-3-methyl-2-cyclopenten-1-one	18.14	98	98	Carbohydrates	Cyclopentenone
3-Methy-l1,2-cyclopentanedione	18.42	112	69+112	Carbohydrates	Cyclopentenone
Unknown	19.06		58+86+114	Carbohydrates	
Unknown	19.35		98+126	Carbohydrates	
Unknown	21.77		116	Carbohydrates	
Unknown	22.33		44	Carbohydrates	
Unknown	26.18		57+69	Carbohydrates	
5-Hydroxymethylfuran-1-carboxaldehyde	27.51	126	97+126	Carbohydrates	Furan
Unknown	31.67		73+135	Carbohydrates	
Laevoglucosan	40.44	172	60+73	Carbohydrates	

Table 2.4: Other pyrolysis products quantified

Name	RT	MW	integrated framents	Origin	Class
25:0 Alkan	27.74	352	57+71	aliphatic	Alkan
25:1 Alken	28.34	350	57+69	aliphatic	Alken
27:0 Alkan	30.04	380	57+67	aliphatic	Alkan
27:1 Alken	30.63	378	57+65	aliphatic	Alken
29:0 Alkan	32.20	408	57+63	aliphatic	Alkan
29:1 Alken	32.82	406	57+61	aliphatic	Alken
Myristic acid (14:0)	2.35	68	39+68	Lipid	Fatty Acid
Palmitic acid (16:0)	2.74	82	81+82	Lipid	Fatty Acid
Stearuc acid (18:0)	2.91	82	81+82	Lipid	Fatty Acid
N-methyl-pyrrol	6.15	81	80+81	Protein	Pyrrol
Pyridine	6.90	95	52+79+95	Protein	Pyridine
Methylpyridine	7.50	93	66+92+93	Protein	Pyridine
Methylpyridine	7.54	93	66+92+93	Protein	Pyridine
methylpyridine	9.02	93	66+93	Protein	Pyridine
Pyrrol	13.11	67	39+41+67	Protein	Pyrrol
Methylpyrrol	13.81	81	80+81	Protein	Pyrrol
Methylpyrrol	14.10	81	80+81	Protein	Pyrrol
3-Hydroxypyridine	26.52	95	67+95	Protein	Pyridine
Indole	26.85	117	89+117	Protein	Indole
Methylindole	27.42	131	130+131	Protein	Indole
Toluene	4.54	92	91+92		Aromatic
Xylene	5.94	106	91+105+106		Aromatic
Xylene	6.09	106	91+105+106		Aromatic
Xylene	6.20	106	91+105+106		Aromatic
Xylene	6.99	105?	84+105?		Aromatic
Methoxytoluene	11.78	122	121+122		Aromatic
Indene	12.64	116	115+116		Aromatic
Benzaldehyde	13.35	106	77+106		Aromatic
Dihydrobenzofuran	26.19	120	91+119+120		Aromatic
Limonene	7.22	136	93		Terpene
Phytol	20.00	276	95+123	Chlorophyll	Terpene
Unknown aliphatic	22.82		58+71		aliphatic
Aceton	2.46	58	43		
2-Propenal	2.60	56	55+56		
Methanol	2.88	32	29+31+32		
3-Buten-2-one	3.39	70	55+70		
2,3-Butandione	3.67	86	69+86		
3-Penten-2-one	3.89	86	69+86		
2-Butanal	4.56	70	69+70		
2,3-Pentadione	4.77	100	57+100		
Hexanal	5.16	82	56+72+82		
1-Penten-3-one	11.28	84	55+84		
Hexan-2,4-dion	23.92	114	56+84+114		
unknown	15.98		119+134		
Unknown	20.85		81		
Unknown	20.86		82+95		
Unknown	22.43		98+128		
Unknown	27.76		138		

Table 2.5: Results of correlation analysis (R) between lignin and carbohydrate decomposition and other decomposition processes (mass loss, respiration), extracellular enzyme activities, litter chemistry, and litter and microbial biomass C:N:P stoichiometry. Significant ($p<0.05$) correlations are presented in bold. Data taken from Leitner et al. (2011); Mooshammer et al. (2011). Changes in litter chemistry (lignin and carbohydrate decomposition) were calculated between 0 and 181 days, other data were measured after 181 days. L acc - lignin accumulation, Ch acc - Carbydrate accumulation, LCI - LCI difference, L dec - lignin decomposition rate, C dec - carbohydrate decomposition, rate, L resp - lignin loss / carbon loss, C resp - carbohydrate loss / carbon loss, L/C dec - lignin loss / carbohydrate loss, Per/Cell - Potetial peroxidase activity / potential cellulase activity, Phen/Cell - Potetial phenolo activity / potential cellulase activity.

	L acc	Ch acc	LCI diff	L dec	C dec	L resp	C resp	L/C dec	Per/Cell	Phen/Cell
Massloss	0.291	-0.150	0.245	-0.328	0.106	-0.201	0.125	-0.081	0.048	0.0534
Actual respiration	0.333	**-0.723**	**0.606**	-0.082	**0.771**	-0.195	**0.594**	-0.368	-0.268	-0.362
Accumulated Respiration	**0.494**	**-0.704**	**0.688**	-0.132	**0.856**	-0.332	**0.557**	**-0.525**	**-0.506**	**-0.534**
Cellulase activity	**0.657**	**-0.760**	**0.803**	-0.431	**0.801**	**-0.497**	**0.664**	**-0.589**	-0.436	**-0.539**
Protease activity	0.186	-0.296	0.264	-0.132	0.274	-0.157	0.301	-0.270	-0.260	-0.180
Chitinase activity	0.409	**-0.749**	**0.663**	-0.170	**0.795**	-0.312	**0.677**	**-0.559**	**-0.490**	**-0.607**
Phosphatase activity	**0.549**	**-0.813**	**0.776**	-0.302	**0.851**	-0.407	**0.702**	**-0.556**	-0.418	**-0.522**
Phenoloxidase activity	**0.632**	**-0.669**	**0.737**	-0.415	**0.719**	**-0.449**	**0.552**	**-0.484**	-0.305	-0.356
Peroxidase activity	**0.599**	**-0.588**	**0.677**	-0.412	**0.639**	-0.438	**0.470**	-0.435	-0.173	-0.302
N mineralization	**0.466**	**-0.664**	**0.650**	-0.167	**0.739**	-0.299	**0.527**	-0.387	-0.282	-0.367
Nitrification	**0.587**	**-0.707**	**0.732**	-0.380	**0.740**	-0.432	**0.621**	**-0.499**	-0.369	-0.450
P mineralization	**0.665**	**-0.550**	**0.684**	**-0.544**	**0.596**	**-0.576**	0.414	**-0.478**	-0.212	-0.255
C litter	**-0.545**	**0.506**	**-0.578**	**0.604**	-0.368	**0.643**	**-0.618**	**0.698**	**0.525**	**0.581**
extractable C	**0.609**	**-0.766**	**0.782**	-0.370	**0.814**	**-0.446**	**0.658**	**-0.540**	-0.392	**-0.484**
N litter	0.354	**-0.517**	**0.503**	-0.140	**0.587**	-0.187	0.366	-0.203	-0.119	-0.159
P litter	**0.682**	-0.222	**0.517**	**-0.747**	0.175	**-0.680**	0.188	**-0.491**	-0.0728	-0.160
C:N litter	-0.405	**0.586**	**-0.570**	0.175	**-0.654**	0.234	-0.440	0.273	0.195	0.242
C:P litter	**-0.636**	0.174	**-0.453**	**0.754**	-0.082	**0.649**	-0.176	0.418	0.049	0.080
N:P litter	**-0.512**	-0.029	-0.264	**0.714**	0.147	**0.577**	-0.020	0.316	-0.031	-0.019
C:N mic	**0.666**	**-0.758**	**0.799**	-0.430	**0.798**	**-0.515**	**0.678**	**-0.609**	**-0.584**	**-0.596**
C:P mic	**0.692**	**-0.787**	**0.834**	**-0.476**	**0.814**	**-0.562**	**0.726**	**-0.672**	**-0.564**	**-0.648**
N:P mic	**0.582**	**-0.729**	**0.740**	-0.415	**0.729**	**-0.508**	**0.715**	**-0.670**	**-0.545**	**-0.671**
C:N imbalance	**-0.560**	**0.810**	**-0.772**	0.288	**-0.859**	0.391	**-0.710**	**0.531**	**0.564**	**0.560**
C:P imbalance	**-0.817**	**0.663**	**-0.833**	**0.757**	**-0.610**	**0.799**	**-0.668**	**0.839**	**0.575**	**0.670**
N:P imbalance	**-0.724**	0.351	**-0.602**	**0.810**	-0.253	**0.764**	-0.397	**0.668**	0.301	0.410
Fungi/bacteria(qPCR)	0.002	-0.122	0.079	-0.024	0.087	-0.066	0.135	-0.072	0.199	-0.033
Fungi/bacteria (metaproteome)	**0.998**	-0.854	**0.958**	-0.882	0.801	**-0.961**	0.824	-0.873	-0.679	-0.676

Table 2.6: Results of correlation analysis (R) between lignin and carbohydrate decomposition and other decomposition processes (mass loss, respiration), extracellular enzyme activities, litter chemistry, and litter and microbial biomass C:N:P stoichiometry. Significant ($p<0.05$) correlations are presented in bold. Data taken from Leitner et al. (2011); Mooshammer et al. (2011). Changes in litter chemistry (lignin and carbohydrate decomposition) were calculated between 181 and 475 days, other data were measured after 475 days.L acc - lignin accumulation, Ch acc - Carbydrate accumulation, LCI - LCI difference, L dec - lignin decomposition rate, C dec - carbohydrate decomposition, rate, L resp - lignin loss / carbon loss, C resp - carbohydrate loss / carbon loss, L/C dec - lignin loss / carbohydrate loss, Per/Cell - Potetial peroxidase activity / potential cellulase activity, Phen/Cell - Potetial phenolo activity / potential cellulase activity.

	L acc	Ch acc	LCI diff	L dec	C dec	L resp	C resp	L/C dec	Per/Cell	Phen/Cell
Massloss	0.246	0.156	0.068	**0.582**	**0.708**	0.005	0.279	-0.137	-0.444	0.403
Actual respiration	-0.0114	0.244	-0.212	**0.860**	**0.856**	0.122	0.192	-0.044	-0.403	0.290
Accumulated Respiration	0.283	0.354	-0.009	**0.852**	**0.968**	0.015	0.298	-0.177	**-0.608**	**0.486**
Cellulase activity	0.073	0.218	-0.137	**0.848**	**0.881**	0.148	0.295	-0.081	**-0.575**	0.414
Protease activity	0.004	0.054	-0.086	0.448	0.455	0.160	0.316	-0.110	**-0.456**	0.381
Phosphatase activity	0.256	0.310	0.069	0.298	0.373	-0.102	-0.014	-0.115	-0.152	0.017
Chitinase activity	0.163	0.339	-0.086	**0.643**	**0.671**	0.167	0.253	-0.026	**-0.580**	0.395
Phenoloxidase activity	0.319	-0.389	0.436	-0.248	-0.003	-0.221	**0.505**	-0.443	**-0.483**	**0.692**
Peroxidase activity	-0.277	0.379	-0.385	0.173	-0.049	0.160	**-0.510**	0.382	**0.546**	**-0.708**
N mineralization	0.246	0.337	0.077	0.009	0.062	-0.191	-0.113	-0.167	0.062	0.089
Nitrification	-0.0272	**0.567**	-0.320	**0.630**	**0.567**	0.090	-0.148	0.114	-0.105	-0.023
P mineralization	-0.0165	0.202	-0.138	**0.507**	**0.508**	-0.136	-0.062	-0.128	0.043	-0.027
C litter	0.123	-0.065	0.177	-0.325	-0.264	-0.204	-0.289	0.024	**0.501**	-0.348
extractable C	0.231	0.435	-0.0861	**0.828**	**0.890**	0.074	0.218	-0.109	**-0.538**	0.409
N litter	0.210	0.356	-0.0654	**0.816**	**0.896**	-0.004	0.172	-0.120	-0.431	0.349
P litter	-0.117	-0.037	-0.182	**0.764**	**0.762**	0.161	0.318	-0.074	**-0.464**	0.325
C:N litter	-0.272	-0.365	0.016	**-0.794**	**-0.901**	0.027	-0.207	0.155	**0.490**	-0.404
C:P litter	0.329	0.122	0.315	**-0.645**	**-0.541**	-0.276	-0.218	-0.0672	0.283	-0.162
N:P litter	**0.471**	0.289	0.328	-0.336	-0.179	-0.293	-0.113	-0.148	0.048	0.034
C:N mic	-0.184	-0.408	0.092	**-0.658**	**-0.703**	-0.032	-0.318	0.250	**0.570**	**-0.513**
C:P mic	0.237	-0.060	0.312	**-0.609**	**-0.505**	-0.192	-0.072	-0.063	0.233	-0.223
N:P mic	0.336	0.127	0.290	-0.373	-0.247	-0.180	0.048	-0.157	-0.002	-0.009
C:N imbalance	-0.145	-0.014	-0.076	-0.354	-0.447	0.061	0.044	-0.050	0.027	0.020
C:P imbalance	0.021	0.246	-0.074	-0.137	-0.200	-0.020	-0.241	0.095	0.160	-0.032
N:P imbalance	0.025	0.231	-0.085	0.040	-0.007	0.003	-0.268	0.172	0.160	-0.080
Fungi/bacteria(qPCR)	-0.03	-0.008	0.017	-0.236	-0.254	-0.089	-0.115	-0.003	0.161	-0.219
Fungi/bacteria (metaproteome)	0.158	0.570	-0.369	**0.986**	**0.972**	0.254	0.484	-0.274	-0.601	0.550

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