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"High molecular weight compounds in beech litter decomposition"

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Contents

Introduction

This work is composed of three major parts that serve different perposes and are aimed at different readers:

General introduction gives a broad introduction to litter decomposition and explains its importance in biogeochemical carbon and nutrient cycles. Plant polymers present in beech litter briefly presented. Furthermore, models and concepts used to describe litter decomposition and stoichiometrical approaches to describe decomposition processes are briefly reviewed. This chapter aims to give non-experts an compact overview over the background of this work. Finally, the experiment, of which this work forms part, is presented.

Methodological comments aims at future users of analytical pyrolysis shares experiences with this technique. It explaines the ratio behind choices taken in the implemention of our pyrolysis-GC/MS methods, points out alternatives, pitfalls encountered and possible further developments. It is not written not so much for professional analytical chemists as for biologists and other amateurs working with the method.

[**name**] Is a manuscript written to be submitted to Soil Biology and Biochemistry, and aims to become a state-of-the-art original research article in a peer-reviewed international journal.

1

General Introduction

1.1 Litter decomposition and the global carbon cycle

Rising atmospheric CO_2 concentrations and global climate changes caused by them (Treut et al., 2007) lead to an increased interest in natural carbon cycles and their anthropogenic modifications. Since pre-industrial times, annual means of athmospheric CO_2 concentration increased from 280 to 379 ppm (v/v) (Treut et al., 2007). The source of approximately 80% of the increase was be pined down to fossil fuel usage by comparing athmospheric CO_2 concentration to its ^{13} C signature (fossil fuel C is depleted in ^{13} C) or correnspondingdecrease in athmospheric O_2 concentrations(Treut et al., 2007). Furthermore, land use change and cement production are accounted for additional CO_2 emissions. Between 2000 and 2005, mean annual CO_2 emissions from fossil fuel burning and cement production accounts for 7.2 $\pm$ 0.3 Gt CO_2-C. Additionally, land use change causes the annual emmissions 1.6 $\pm$ 1.1 Gt CO_2-C. Together with other greenhouse gases, elevated CO_2 concentrations are prognosted to raise earth mean surface temperature by nn degree by 20nn (IPCC?).

Anthropogenic CO_2 emissions are tightly interconnected with natural carbon cycles. Only 45% of the emissions are found in the athmosphere, 30% of the emmited CO_2 is absorbed in oceans and 25% in terrestrial ecosystems . Oceanic CO_2 absorption is based on export of particular and dissolved organic carbon and dissolved inorganic carbon (HCO_3^- , CO_3^{2-}) to intermediate and deep water layers. Land sinks take up carbon into larger vegetation- and soil C pools, i.e. due to a nothward shift of climatic limits for vegetation and CO_2 and N fertilization. However, a large part (-2.6 Gt a^{-1}) of this terrestrial carbon sinks is unaccounted for (Denman et al., 2007, p. 515).

Finding this “missing sink” and prognosting feedback mechanisms of CO_2 emissions challenged scientists to strife for depthening their understanding of large scale biotic carbon transformation processes and storage. Globally, land plants assimilates 120 Gt C annually (gross primary production). This is

almost one sixth of the global atmospheric CO_2 pool (750 Gt a-1) and more than 15 times more than antropogenic C emissions. Autotrophic (plant) respiration consumes one half of the assimilated carbon, the other half is introduced into decomposition process as plant litter. Animal biomass and herbivory form only a neglectable part of the total biomass [lit.], but can wield key controls on vegetation and its succession.

Ecosystem carbon balances are determined by the difference between carbon assimilation (photosynthesis) and respiration. While controls on photosynthesis rates are well understood, knowledge about decomposition processes is by far more limited. This is due to the fact that organisms capable of photosynthesis generally are green, seesile and grow aboveground, are therefore easy to find and study, while a large part of heterotrophic respiration is conducted by soil microbial communities of microscopic scale that dwell belowground, are hard to identify, and live in a chemically complex environment. Due to the complex nature of soils, studying chemical transformation processes and chemical controls over microbial communities and physiology is easier in aquatic than in terrestrian habitates (for example, differences between nutrient contents and bioavailable nutrient amounts are smaller in aquatic environments, facilitating studies of nutrient control on microbial communities). However, research interest in terrestrial decomposition processes, especially litter decomposition, which sees the highest biomass turnover, is enormous, with more than one peer-reviewed research article per day published on litter decomposition between 2005 and 2009 (Prescott, 2010).

Global litterfall summs up to for approximately 60 Gt C a^{-1}. Frequently between 30 and 70% of this mass are lost in the first year and further 20 to 30 % within another 5 to 10 years (Chapin et al., 2002, p.157).

Temperate forests are highly productive, average net primary production is estimated for 1550 g m^{-2} a^{-1} (1/3 of which is allocated into belowground biomass). They cover 1.7 * 10^7 km^2 (1/15th of earth land surface) and account for 8.1 Gt a-1 NPP (1/8th of total terrestrial NPP) (Chapin et al., 2002, p?). European beech (*Fagus silvaticus* L.) is the dominant tree species in potential western and central Europe.

1.1.1 Ecological stoichiometry

Carl Sprengel proposed in 1828 that crops rely on nutrints in a given ratio, and that growth is limited by the nutrient that is least frequent compared to this given ratio. While since then plants - and other organisms - were shown to be capeable of a certain plasticity in their nutrient requirement, there is a tradeoff between addaption to nutrient availability and competitive fitness. [1]

But organisms do not only rely on elements in a certain ratio, they are also bound (within an adaptive

[1] citation

range) to keep them in specific range within their internal mileu. An homeostatic organism keeps this internal mileu constant independent of their ambiental conditions, while in on that is not homeostatic, the internal mileu changes with the elemental ratio in their substrate (Sterner and Elser, 2002).

By 1958, marine biologist Albert C. Redford pusblished 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 and dead biomass. The high constancy of this ratios 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) by biological systems (Cleveland and Liptzin, 2007).

Several attempts to find similar ratios in terrestrian ecosystem mostely failed due to the complex nature of terrestrian soils and difficulties to determine actual bioavailability of nutrients. [2]

Plants are able to assimilate carbon from atmosphaeric CO_2, but have to sequestrate other elements from soils. Furthermore, a significant part of nitrogen and other nutrients is removed from senecenced leaves before abscission. Therefore, plant detritus is enriched in carbon and depleted in nitrogen when compared to soils or decomposer organisms. C:N ratios (w/w) found in fresh beech litter are between 1:40 and 1:50 (Mooshammer et al., 2011), while soil C:N ratios are in the order of 1:20 [3]. Therefore, during litter decomposition, the part of carbon mineralized is higher than the part of nitrogen. Microbial decomposer communities found on early decomposition litter have biomass C:N ratios between 1:6 and 1:18, indicating microbes live in a environment characterized by a carbon surplass and a lack of nitrogen. Litter decomposition rates were found to correlate with detritus C:N and C:P rates (**?**).

1.2 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 in for of protein (Wanek et al., 2010), among carbohydrates, cellulose (the β - 1-4 glycosidic polymer of glucose) is most common (10-50 % of litter dry mass). Other carbohydrates - refered to as hemicelluloses - together make up between 30 and 40 % of litter dry mass. A wide variety of carbohydrate monomeres and glycosidic bindings occur in are leaf litter. Lignin, forms 15-40% biomass, is an aromatic polymer fromed through the radicalic polymerisation of several different phenylpropanoid monomers. (Berg, B. & McClaugherty, 2008, pp. 54f). The polymerization process of lignin can incorporates protein and carbohydrates into lignin polymers, thereby occluding them to decomposing enzymes and lowering their bioavailability. Nitrogen content of beech lignin was found twice as high as in bulk litter (**?**). Furthermore, cutin waxes (ester-bound long chain aliphaic compounds with aromatic components) and tannins are present.

[2] paper "redfield ratio for soils" here!
[3] citation

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). 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 facilitated by oxidoreductases (**?**).

1.2.1 Micronutrients

Litter micronutrient content varies and depends in Transition metals, especially manganese and iron, are important co-factors of oxidoreductases. The aerobic degradation of complex aromatic compounds is facilitated by reactive oxigen species generated by such enzymes. Therefore, a lack of their cofactors can limit the degradation of complex material (especially phenolic compounds like lignin) in the decomposition processes of litter and other complex organic material like soil orgnic matter or dissolved organic matter. [4]

1.3 Changes of litter carbon chemistry during decomposition

1.3.1 The traditional model developed by B. Berg

Traditional models of chemical changes during litter decomposition describe three phases of litter decomposition. In the early phase, which can expand until 40% of dry mass are lost, availability of labile carbon sources like soluble compounds and non-lignified carbohydrates is high. In this phase, mass loss rate were usually found to be nitrogen limited and - more generally - enhanced by high levels of nitrogen, phosphorous and dissolved carbon. In the late phase, lignin content inhibits further decomposition and mass loss rates are repressed by lignin and nitrogen contents, but enhances by high manganese contents. 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 biomass becomes incorporated into soils (fig. 1.1).

Berg, B. & McClaugherty (2008)).

1.3.2 Microbial nitrogen mining hypothesis

The “nitrogen mining hypothesis” is based on the idea, that the breakdown of recalcitrant carbon allows soil microbia 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

[4] references needed!!

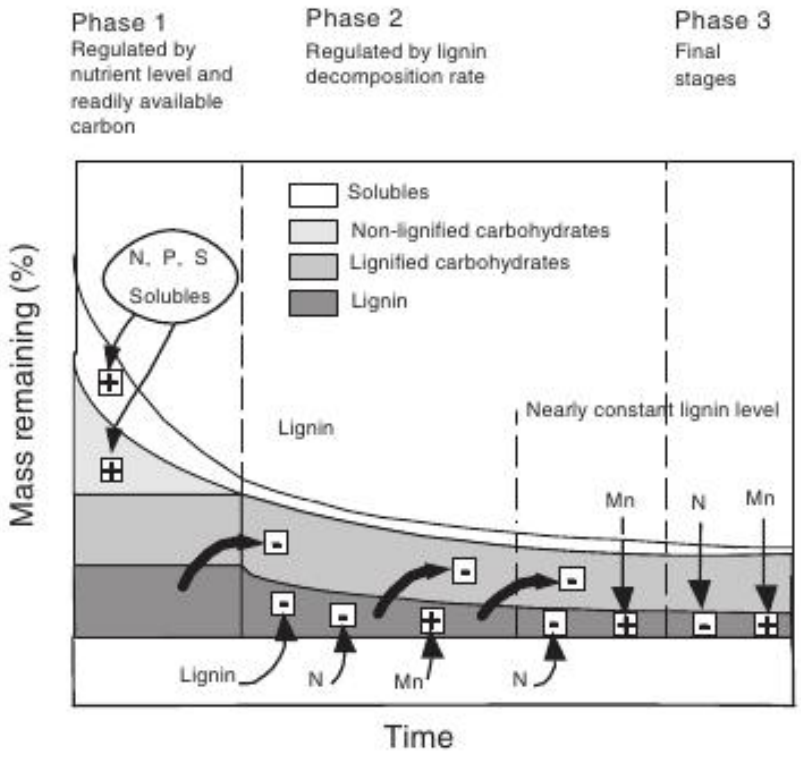

Fig. 6.1 Model for chemical changes and rate-regulating factors during decomposition (modified from Berg and Matzner 1997). The decomposition of water-soluble substances and unshielded cellulose/hemicellulose is stimulated by high levels of the major nutrients (early stage – *phase 1*). When all unshielded 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))

and Sinsabaugh, 2006). In plant litter, nitrogen is often occluded within lignin molecules, which are degraded by similar enzymes. Isolated lignin fractions from fresh beech leaves were shown to contain twice as much nitrogen as bulk material (Dyckmans et al., 2002). Moorhead and Sinsabaugh (2006) incubated mixtures from 50 different soils and plant litter from 50 species, adding mineral nitrogen and phosphorous. They

1.3.3 Carbon limitation of lignin decomposition

Recently, Klotzbücher et al. (2011) suggested that the degradation of lignin depends on the availability of labile carbon sources. In a climate chamber experiment, the authors incubated samples previously decomposed in-sitio for up to three years, recording microbial respiration, lignin content (based on CuO-

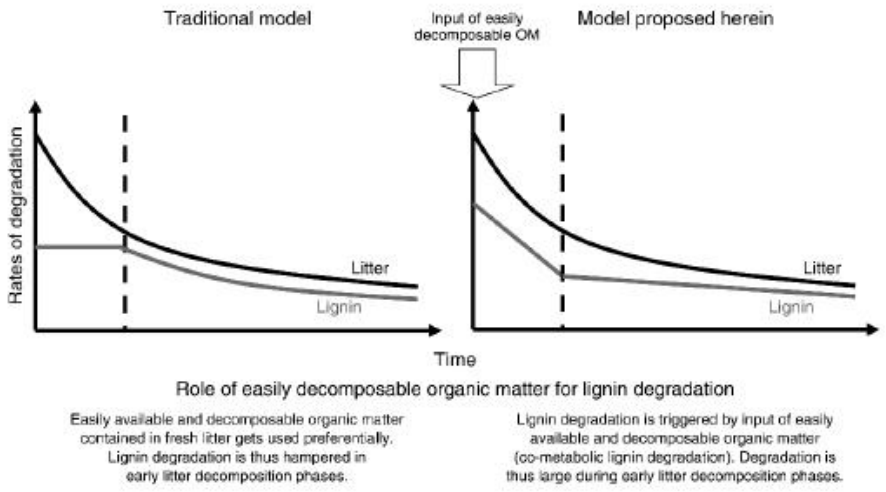

FIG. 5. Revised conceptual model for the fate of lignin during litter decomposition, based on data presented herein. The traditional model was proposed in Berg and Staaf (1980) and based on data from the decomposition of Scots pine needles. In our model, lignin will be degraded if easily degradable OM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

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

oxidation), and the production of soluble carbon (DOC). Regardless of the previous field incubation time, samples showed the highes lignin degradation rates at the beginning 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 conclude that soluble carbon limits carbon mineralization rates in litter after an initial pool of lable carbon (which can result from experimental manipulation (i.e. draught-rewetting) at the experimental setup) are degraded. They also suggest, that lignin is not decomposed under such carbon-limited conditions. As lignin decomposition rates do not increase with the 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 propose a concept of early lignin decomposition, where lignin degradation rates are highest during early litter decomposition, when plenty of labile carbon is available (fig. 1.2).

1.4 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 athmosphere. 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 GC/MS-system. Pyrolysis products are seperated 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 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 (forensics, art historym, ..).

Three types of pyrolytic systems are frequently used:

(1) microfurnance type pyromates have an oven that is constantly heated to the pyrolysis temperature, into which the sample is droped,

(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 is a straightforward interpretation for artificial polymers and natural homopolymers, the analysis of pyrograms from complex organic polymers can be challanging to the point where pyrolysis is used as simple fingerprinting method, allowing not qualitative interpretation but provide insight into difference between humic substances found in different samples. Nevertheless, analytical pyrolysis usually provides a good overview over the monomers present in complex organic material.

2

Methodological comments

2.1 Column choice and temperature programm

This work uses a Carbowax column (Supelcowax 10) for seperation. The column was chosen for better peak seperation after comparing several measuremts on this column with a RTX 35 (Restec) column. A good part of the published Pyrolysis-GC/MS studies use simple HP-5/SP-5 or similar standard GC columns.

However, during analysis, limitations of the column became evident, especially the limited temperature range (maximum temperature 280°C) of the column. Due to this and probably long retention of polar substances on the column, we were not able to detect several interesting compounds: long chained (C18+) n-alkyl-alcohols, ω - hydroxy - n-alkyl-fatty acids, and $\alpha - \omega$ - n-alkyl-dicarboxylic acids (all common in cuticulary waxes). Our detection of n-alkanes and alkenes was limited to C27 compounds (C29 compounds could be detected, but strong discrimination against them was suspected). Among the carbohydrate products, only traces of leavoglycosan and no other dehydroxysugars. Laevoglycosan is usually among the major decomposion products of cellulose, and among anhydrosugars, products originating from different sugar monomeres can be differenciated, especially between pentoses and hexoses. Among the lignin products, pyrolysis products with functional groups in the side chain and syringol derivatives in general were discriminated against.

The GC temperature program was designed to freeze - trap pyrolysis products at the beginning of the column. Therefore a low initial temperature was chosen (50 °C). The maximum temperature of the column according to the producer is 280 °C. Again, reaching a higher temperature would be of advantage, because larger molecules (which have high diagnostic value) would be detected.

2.2 Internal standards and absolute quantification

Quantifying pyrolysis products can be a challange itself: Beside the high number of complex products to be quantified, comercial availability of these substances is limited. Due to the low sample amounts (100-500 µg) exact balancing of the sample is difficult, especially as pyrolysis vials are usually not optimized for balancing of to avoid sample losses. For the GSG Pyromat instrumentation, recovery rates strongly varied between samples, suposedly due to gas leakage in the Pyr-GC interface. Generally, reproductivity of recovery rates and balancing is not sufficiently high enough to relate absolute peak areas to sample inweight for quantitative analysis.

Other chromatographic applications commonly exclude this "injection bias" by the use of an internal standard. Until now, this is not common in pyr-GC/MS analysis. Two recent publication add an internal standard to the sample: Steinbeiss et al. (2006) uses p-methoxyphenone, Bocchini et al. (1997) tests several substances and conclude that xx is most suited as an internal standard for lignin determination. In both approaches the internal standard is not chemically modified during pyrolysis but evaporated ("themal desorption") and results in a single peak in the pyrogram. Internal standard amounts found can account for losses of pyrolysis products. It does not account for losses during the pyrolysis process itself, i.e. incomplete pyrolysis of the sample is not throughoutly heated to the intented temperature. Adding the internal standard to the sample in a known ratio is also difficult: usually the internal standard is applied by pipetting a small amount of a solution onto the sample (1-5 µL). Larger volumes do no fit into the pyrolysis vials and often provoce leakage of the solution from the bottom-open vials.

A substancial part of the products formed by the pyrolysis of natural organic polymers are not or not exactly identified, commercial availability of pyroylsis products is limited. Also, if their thermal stability is insufficient, these substances can not be induced to the chromatic system by thermal desorption in the pyrolysis unit. Due to this problems and the high number of compounds produced, no publication quantifying single pyrolysis was published yet.

Quantifying substances of origin of pyrolysis products is even harder than quantifying the products themselves. For plant material, the most 1important classes of compounds analyzed - carbohydrates and lignin - are present in different forms in plant litter. However, especially for Carbohydrates can not be distinguished by pyr-GC/MS, but it has to be assumed that during pyrolysis they do not produce the same product in the same ratios. Lignin components different among plant families, reference material for angiosperm is scare. Chemical alternations in lignin structures are unavoidable during preparaation.

Due to the reasons above, commonly, analytical pyrolysis studies do not aim for an absolute quantification of pyrolysis products or their substances of origin.

2.3 Peak assignment

Peak assignment is the crucial step in the analysis of pyr-GC/MS data. Usually not the whole dataset, but a small number of representative files are screened.

For the current litter analysis, one replicate of initial litter and litter after 15 month incubation (from two different litter types) were analyzed. However, it was known from previous studies that litter types were highly similar in their composition. For more heterogenous samples, at least one replicate for each treatment should be analyzed.

The following steps were applied:

A List all peaks over a certain area treshhold was compiled. This is done by (1) automatic integration with the Xcalibur Qual Browser and (2) manual screening of print-outs of the chromtogram. Initial air contamination peaks are excluded. These are usually between 0.8 and and 2 minutes GC runtime, have characteristic molecule (M+) ions at m/z 28 (N2) 32 (O2) and 44 (CO2) and are often by far the highest peaks in the pyrogram.

An attempt to identify peaks with a relative peak area over a critical treshhold (i.e. 0.1 % total peak area).

When one substance class is detected, missing pyrolysis products from the same substance of origin are looked for, usually using their most abundant MS fragments.

Finally, critical diagnostic peaks can be found when looked for (specific ion traces)

For plant material, **?** present the most relevant data for the identification of pyrolysis products. They confirm the identity of over 100 pyrolysis products by standard addition. Recently, several studies supervised by Peter Buurman (Buurman and Roscoe, 2010; Schellekens et al., 2009; **?**) feature (1) up-to-date lists of peaks found and (2) good examples for information to be extracted from large datasets based on 100+ peaks in soil organic matter fractions.

2.4 Peak classification

Lignin pyrolysis products are 2- and 6- methoxylated and dimethoxylated phenols with alkyl groups of up to three carbon atoms in position 4. The peak list for lignin markers presented in this work is extensive and reliable. Other peaks of potential lignin origin include non-methoxylated phenoles with similar side chains. However, while lignin is expected to be accounted as source of a large part of free phenol produced during pyrolysis, it can also be a product of protein, carbohydrate and non-lignin phenolic compounds.

Carbohydrates products are derrivatives of furan and cyclopentenone with methyl-, oxomethyl and hydroxymethyl sidechains. Furan and Cyclopentenonederrivatives often show different trends. Some

authors attribute cyclopentenones to lipids in soils. Additionally, carbohydrates produce a large number of smaller molecules, including short chained aldehydes and carboxylic acids. In the current work, an important part of carbohydrate peaks could not be identified by their MS spetrum, but were assigned based on the measurement of reference carbohydrates (cellulose, glucose, xylan).

Protein is decomposed to pyridin and pyrrol and their methylated derrivatives during pyrolysis. Additionally, indole and methylindole were found, which are characteristic decomposition products of tryptophan. In literature, a number of small aromatic compounds (i.e. toluene) are described as pyrolysis products of individual amino acids.

Lipids

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3

Manuscript

Controls of litter chemistry over early lignin decomposition in beech litter

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Abstract

Lignin is considered the most recalcitrant component of plant litter, accumulated during early decomposition and degraded only during late decomposition stages when its concentration limits litter decomposition rates. A recent study based on the more specific methods challenges this concept, reporting highest lignin decomposition rates during early litter decomposition. Until now, no further studies exploring early lignin decomposition were published, and its potential controls remain unknown.

We follow lignin and carbohydrate decomposition during early litter decay with analytical pyrolysis in a climate-chamber decomposition experiment, focusing on resource control over microbial carbon substrate preferences. Beech litter with different C:N:P stoichiometry but identical initial microbial communities was incubated to identify the control of litter chemistry on the developing microbial community and its decomposition activity.

During the first 6 month fundamental differences in lignin degrading activities were found between sites. Lignin discrimination in litter decomposition ranges from insignificant amounts of lignin decomposed to lignin decomposition at the same rate bulk litter, leading to different niveaus of lignin accumulation. Between 6 and 15 month, no lignin discrimination was found, but different lignin contents aquired earlier reminded.

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