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Lignin degradation dynamics in boreal forest soils

Influence of chemical composition and nitrogen on lignin degradation

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Cover: Scots pine forest (from a litter bag point of view), Norrliden, Sweden (Photo by Aswin Thirunavukkarasu, 2021)

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Abstract

Soil organic matter (SOM) is a critical component of the global carbon cycle, storing more organic carbon than the atmosphere and global vegetation combined. Plant-derived organic inputs are the primary carbon source for SOM formation, which arises through the microbial decomposition processes. Among these inputs, lignin, a complex biopolymer abundant in plant biomass, is often considered a key contributor to SOM due to its resistance to degradation. However, the influence of lignin's moieties on SOM stability remains poorly understood. In this thesis, I have investigated the lignin decomposition and its contribution to SOM formation using Aspen wood (*Populus tremula*) genotypes with varying lignin content and composition as model substrates. Laboratory incubations (Paper I-II) and field experiments (Paper III), coupled with advanced two-dimensional Nuclear Magnetic Resonance (2D NMR) spectroscopy, were systematically employed to characterize the degradation of lignin moieties. In Paper I, lignin content did not affect overall decomposition, with approximately 60% mass loss across substrates. While the lignin decomposed at a similar rate to carbohydrates, oxidized syringyl units exhibited resistance to degradation compared to other lignin monomers. Paper II demonstrated that nitrogen addition altered fungal community structure, suppressing the ligninolytic fungus *Renatobasidium notabile* and increasing fungal diversity. This shift enhanced the degradation of more resistant lignin moieties, such as guaiacyl units and their linkages. Paper III demonstrated that lignin composition, rather than content, is crucial in early-stage decomposition across pine and spruce forest stands. Long-term nitrogen fertilization enhanced mass loss but selectively degraded syringyl units and their linkages. These findings highlight that lignin composition and microbial community dynamics regulate decomposition rather than lignin content alone. This emphasizes the need to focus on microbial and compositional factors to better understand SOM dynamics and carbon cycling.

Keywords: SOM, Lignin degradation, 2D NMR, Early-stage decomposition.

Ligninnedbrytning i boreal skogsmark – Inverkan av kemisk sammansättning och kväve på ligninets nedbrytning

Sammanfattning

Organiskt material i marken är en viktig del av den globala kolcykeln och lagrar mer organiskt kol än atmosfären och den globala vegetationen tillsammans. Organiska föreningar som härrör från växter är den primära källan för bildning av markorganiskt material, som uppstår genom mikrobiella nedbrytningsprocesser. Bland dessa ämnen anses ofta lignin, en komplex biopolymer som förekommer rikligt i växtbiomassa, vara en viktig bidragsgivare till inlagring av markkol på grund av dess motståndskraft mot nedbrytning. Det är dock fortfarande dåligt känt hur ligninets beståndsdelar påverkar stabiliteten hos markkolet. I denna avhandling undersöktes ligninnedbrytningen och dess bidrag till markkol med hjälp av genotyper av aspved (*Populus tremula*) med varierande ligninninnehåll och sammansättning. Laboratorieinkubationer och fältexperiment (artiklar I-III), i kombination med avancerad tvådimensionell NMR-spektroskopi (2D NMR), användes för att karakterisera nedbrytningen av lignin. I artikel I påverkade ligninninnehållet inte den totala nedbrytningen, med en massförlust på cirka 60 % för alla substrat. Ligninet bröts ned i samma takt som kolhydrater, men oxiderade syringylenheter uppvisade motståndskraft mot nedbrytning jämfört med andra ligninmonomerer. Artikel II visade att kvävetillförsel förändrade svampsamhällets struktur genom att undertrycka den ligninolytiska svampen *Renatobasidium notabile* samtidigt som svampdiversiteten ökade. Detta skifte ökade nedbrytningen av mer resistenta ligninfraktioner, såsom guaiacylenheter och deras kopplingar. Artikel III visade att ligninsammansättningen, snarare än innehållet, är avgörande för nedbrytningen av ved i tall- och granskogsbestånd. Långvarig kvävegödsling ökade massförlusten men försämrade selektivt nedbrytning av syringylenheter och deras kopplingar. Dessa resultat visar att ligninsammansättning och mikrobiell populationsdynamik, snarare än enbart ligninninnehåll, reglerar nedbrytning och bildning av markorganiskt material. Denna forskning understryker behovet av att fokusera på mikrobiella och kompositionella faktorer för att bättre förstå dynamiken hos markorganiskt material och kolcykling.

Nyckelord: SOM, Ligninnedbrytning, 2D NMR, Tidig nedbrytning.

You have the right to work, but never to the fruit of work.
You should never engage in action for the sake of reward, nor should you long for inaction.

—The Bhagavad Gita.

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. Thirunavukkarasu, A.*, Hedenström, M., Sparrman, T., Nilsson, MB., Schleucher, J., Öquist, M. (2024). Unraveling the dynamics of lignin chemistry on decomposition to understand its contribution to soil organic matter accumulation. Plant and Soil. https://doi.org/10.1007/s11104-024-07066-y

II. Thirunavukkarasu, A.*, Liu, T., Hedenström, M., Sparrman, T., Nilsson, MB., Schleucher, J., Bertilsson, S., Lindahl, B., Öquist, M. Impact of nitrogen on decomposition: microbial community shifts over lignin content as primary drivers (manuscript)

III. Thirunavukkarasu, A.*, Hedenström, M., Sparrman, T., Nilsson, MB., Schleucher, J., Öquist, M. Long-term nitrogen fertilization effects on the initial stages of lignin degradation in spruce and pine stands (manuscript)

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Scots Pine forest, Norrliden, Sweden

1. Introduction

Soil organic matter (SOM) holds the second largest amount of organic carbon in the biosphere after oceans. It is estimated that around 2400 gigatons (Gt) of organic carbon are stored in the top 3 meters of soil, with approximately 828 Gt of organic carbon stored in the top 30 cm (Stockmann et al. 2013; Friedlingstein et al. 2020; Beillouin et al. 2023; Lin et al. 2023). The microorganisms in the soil degrade and transform plant materials, creating SOM. This process, known as decomposition, is a key biogeochemical mechanism in carbon cycling within and between ecosystems (Bradford et al. 2016).

The complexity of SOM arises from the physical, chemical and biological processes involved in the transformation of plant materials into organic products (Lehmann & Kleber 2015). In the early development of foundational paradigms in ecology, environmental factors such as temperature, moisture and chemical recalcitrance were seen as the regulators of plant litter (leaves, twigs, branches and roots) decomposition rates at global and regional scales (Meentemeyer 1978; Parton et al. 1988; Wall et al. 2008). However, later studies suggested that plant litter quality is a significant driver at smaller spatial scales, where variations in climatic conditions are less pronounced (Bradford et al. 2016; Fanin et al. 2019). Understanding the factors that contribute to the chemical complexity of decomposing litter and the resulting SOM is crucial for elucidating the mechanisms of long-term carbon storage in soil and aiding in the better comprehension of carbon cycling (Wickings et al. 2012; Cotrufo et al. 2015; Djukic et al. 2018; Hoffland et al. 2020).

Over the years, our understanding has taken major strides in explaining the fate of plants organic inputs into SOM. Previously, the existence of SOM

was linked to the humification concept that assumed the initial decomposition products from the plant input have transformed into a chemically more complex and decomposition resistant compound (Waksman 1926; Kononova & Kononova 1961). However, there was no evidence of the physical existence of humic substances (such as humin, humic and fulvic acids) in natural systems (Lehmann & Kleber 2015). Later decomposition studies of plant materials formed the concept of selective preservation. According to this concept, the plant inputs comprise both labile and recalcitrant compounds where the latter would accumulate and only be used by the microorganisms when the former is exhausted (Lützow et al. 2006). However, evidence suggests that under suitable conditions, the microorganisms can even decompose recalcitrant compounds more quickly than previously anticipated (Gramss et al. 1999; Hamer et al. 2004; Wiesenberg et al. 2004; Klotzbücher et al. 2011). Thus, the current emerging view about SOM is that it is continuous in nature and consists of plant organic fragments of all molecular sizes at various stages of decomposition with a significant amount of microbial biomass and necromass (Cotrufo et al. 2013; Lehmann & Kleber 2015; Liang et al. 2019).

Boreal forests are one of the major global sinks for carbon accounting for approximately 14.5 % of total land area and 30 % of the forest area on the Earth (Gauthier et al. 2015; Liu et al. 2021). Geographically, boreal forests contain 272 ± 23 Gt of carbon with biomass contributing only 20% while the soil serves as the primary reservoir, storing 60% of this carbon (Pan et al. 2011; Dalsgaard et al. 2016). In boreal forests, a significant portion of soil carbon accumulates in the surface moor layer, known as the O-horizon. One notable feature of this moor layer is the presence of high abundance of carbohydrate polymers (e.g. cellulose) which typically make up 40-50% of the mass of SOM, even in SOM that has undergone decomposition for decades to centuries (Erhagen et al. 2013).

Lignin is widely believed to exert significant control over the accumulation of carbohydrates (Cornwell et al. 2008). As a complex aromatic polymer, lignin constitutes roughly 30% of plant biomass. Lignin biosynthesis in plants primarily involves three hydroxycinnamyl alcohol monomers, also known as monolignols: p-coumaryl-, coniferyl-, and sinapyl alcohols. These monolignols, differing in their degree of methoxylation, are polymerized to form lignin polymers. Once incorporated into lignin polymers, they are commonly referred to as *p*-hydroxyphenyl (H), guaiacyl

(G), and syringyl (S), respectively. The linking of monolignols occurs through radical coupling reactions, where new monolignols are coupled in an end-wise manner to the growing polymer (Adler 1977; Boerjan et al. 2003a; Vanholme et al. 2010). During the lignification process, the monolignols are connected by either ether (C–O–C) or carbon-carbon (C–C) linkages. The most prevalent linkage is the β–O–4 linkage, with other linkages including β–5, β–β, 5–5, 5–O–4, and β–1 (Ralph et al. 2004; Brown & Chang 2014).

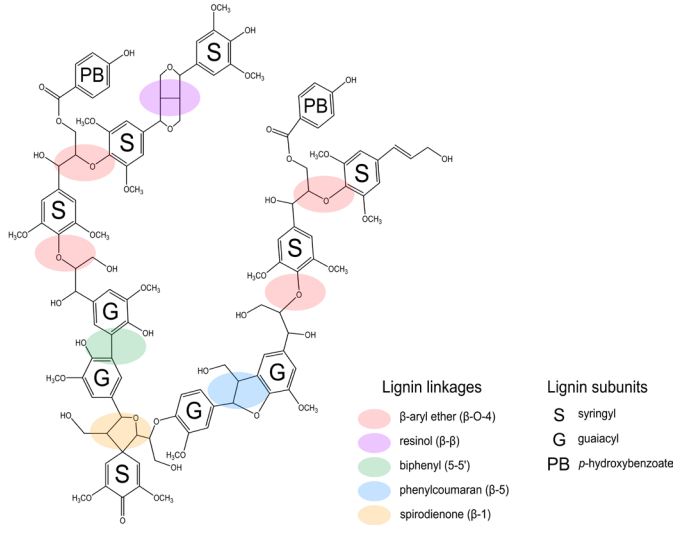


Figure 1: Schematic representation of lignin structure in Aspen (*Populus tremula*). This figure illustrates the different subunits (*p*-hydroxybenzoate (H), guaiacyl (G), and syringyl (S)) and various potential linkages (represented by colors) that can exist in lignin. It is important to note that this depiction does not reflect the actual abundance of specific subunits or linkages in Aspen lignin.

Lignin occurs in complexes containing hemicellulose-protein matrices, within which cellulose microfibrils are embedded (Boerjan et al. 2003a). Lignin acts as a binding material within the lignocellulose biomass of plants by being involved in the cross-linking of hemicellulose and cellulose to provide rigidity and stiffness to the cell wall (Boer et al. 2005; Datta et al. 2017). As a result, lignin could physically protect the labile cell wall polysaccharides from microbial attack or the presence of covalent bonds between lignin and the labile cell wall polysaccharides could potentially protect hemicellulose and cellulose chemically from hydrolysis during the

decomposition (Cadisch et al. 1997; Boerjan et al. 2003a; Talbot et al. 2012). Thus, lignin could be the important control over the decomposition and transformation of plant inputs. However, the biogeochemical mechanisms by which saprotrophic decay of lignin occurs remain unclear.

Microbes (such as fungi and bacteria) use a broad range of extracellular enzymes like peroxidase and phenol oxidase, each employing distinct mechanisms to degrade lignin. For instance, phenol oxidase catalyzes non-specific reactions, including the oxidation of Mn^{2+} and Fe^{2+}, which can lead to the depolymerization or transformation of lignin. In contrast, peroxidase can directly oxidize the carbon-carbon (C-C) bonds between lignin monomers, facilitating lignin breakdown through a more targeted approach (Sinsabaugh 2010; Janusz et al. 2017). These varying enzymatic strategies highlight the complexity of lignin degradation pathways. However, recent advancements in Nuclear Magnetic Resonance (NMR) techniques, such a $^{1}H–^{13}C$ Heteronuclear Single Quantum Coherence (2D HSQC), enables the study of lignin degradation at the molecular level.

Traditionally, lignin was analyzed as acid unhydrolysable residue (AUR, i.e. Klason Lignin) after applying different extraction protocols. However, this fraction is known to contain also other constituents, such as condensed tannins, waxes and cutin (Preston et al. 1997) leading to biased results and potentially erroneous conclusions. Studies that are more recent use CuO-oxidation, pyrolysis, or tetramethyl ammonium hydroxide (TMAH) thermoschemolysis methods to quantify lignin content. These methods focus on a few defined lignin derived monomers, leaving out the lignin linkages that can vary largely in their degradability and may influence lignin decomposition. For instance, syringyl is mostly associated with β-O-4 linkages that are more labile than the phenylcoumaran (β-5) and aryl-aryl linkages (5-5') that polymerize guaiacyl (Fig. 1) (Kim & Ralph 2010). In addition, these methods involve a massive physio-chemical disruption of the sample matrix and may result in potentially incomplete extraction or contamination by undesired organic fractions. Thus, the lack of understanding in the biogeochemical mechanisms by which saprotrophic decay of lignin occurs is partly due to the difficulties in analytical capabilities in analysing complex chemical structures of lignin in soils at adequate resolution.

Many concepts regarding lignin degradation exist today. The conventional rationale behind why lignin degradation is hampered is that

microorganisms tend to receive less carbon and energy compared to the resources invested, due to the energetic cost of producing enzymes for lignin degradation. Due to this low return on investment of energy, microorganisms typically do not view lignin as their primary source of energy or carbon. As a result, lignin decomposition is expected to proceed more slowly compared to the other major cell wall constituents such as cellulose and hemicellulose (Schimel & Weintraub 2003; Moorhead & Sinsabaugh 2006). Nevertheless, later studies challenged this conventional view on lignin degradation and suggested that when the input of an easily decomposable fraction is available e.g. carbohydrates during the early stage of decomposition lignin degradation is triggered. In this way, microorganisms will have sufficient carbon and energy to produce lignin-degrading enzymes. On the other hand, the lignin degradation will be hampered at the later stages of decomposition due to the decline of easily decomposable resources (Klotzbücher et al. 2011; Lehmann & Kleber 2015; Angst et al. 2021).

Boreal forests are considered nitrogen limited due to poor plant nitrogen availability (Persson & Bockheim 1981; Galloway et al. 2004). Given the nutrient scarcity, long-term nitrogen fertilization provides a way to boost forest productivity. However, this practice has significantly altered nitrogen storage mechanisms within these ecosystems, thereby impacting the carbon pool dynamics (Kicklighter et al. 2019). Nitrogen addition is believed to decrease the lignin degradation. One plausible reason is that microorganisms perceive lignin degradation as a step toward accessing the organic nitrogen pool embedded in plant biomass. However, if nitrogen is readily available in mineral forms then there is less incentive for the microbes to invest resources in producing enzymes that degrade lignin (also known as the N mining hypothesis) (Keyser et al. 1978; Carreiro et al. 2000; Craine et al. 2007; Talbot & Treseder 2012). Resistance to lignin degradation can lead to decrease in the net mass loss by preventing microbial accessibility to the more labile cell wall components such as polysaccharides. Specifically, the N additions were found to accelerate the decomposition of litter with high lignin content, while exerting a comparatively suppressive effect on litter with lower lignin content (Janssens et al. 2010; Perakis et al. 2012; Marshall et al. 2021). Moreover, lignin does not exhibit fixed molecular structure and microbial suppression of lignin degradation likely affects different lignin components to varying degrees, but such controls remain unknown and require further investigation.

Nitrogen availability also plays a crucial role in shaping a wide range of interactions, from mutualistic to commensal and antagonistic interactions. Such interactions occur not only between fungi and bacterial communities but also within each group itself (Johnston et al. 2016). For instance, saprotrophic fungi from the phylum Basidiomycota, recognized, as the primary decomposers of wood, are especially important. Among these, white-rot fungi are pivotal in deadwood decomposition, largely due to their ability to break down lignin through a suite of extracellular enzymes (van der Wal et al. 2007; Hoppe et al. 2016). In addition to basidiomycetes, certain ascomycetes are capable of decomposing plant materials, including celluloses and hemicelluloses (Bani et al. 2018). The presence of ascomycetes may influence decomposition rates through interactions or competition with basidiomycetes, particularly in the early stages of decomposition (Hoppe et al. 2016; Gómez-Brandón et al. 2020). While fungal lignin degradation has been extensively studied, microbial lignin breakdown outside of fungi is less explored. However, some bacteria, mainly from the classes Actinomycetes, α-Proteobacteria, and γ-Proteobacteria, have been reported to degrade lignin (Janusz et al. 2017). Despite these findings, our understanding of the interactions between and within fungal and bacterial communities that regulate lignin degradation and influence SOM formation and accumulation remains limited. Further research is needed to clarify how nitrogen availability affects these microbial associations.

2. Research objectives

This thesis primarily investigated the initial stages of lignin degradation dynamics of plant material in boreal forest soils. Through a combination of laboratory incubations and field experiments (papers I-III), this research aimed to elucidate the breakdown of lignin monomers and linkages using different genotypes of Aspen wood (*Populus tremula*) with differing lignin content and composition as a model substrate. The application of 1H–13C Hetero nuclear Single Quantum Coherence Nuclear magnetic resonance (2D HSQC NMR) aids in the analyses of the complex lignin structure by allowing for the identification of both lignin subunits and their associated linkages in their true form (Kim & Ralph 2014). The 2D HSQC NMR approach surpasses the typical extraction protocols and results in highly increased resolution to identify organic carbon moieties relative to other common methods such as 13C Cross-Polarization Magic-Angle Spinning (CP-MAS) NMR and (Fourier Transform InfraRed) FTIR-spectroscopy. This thesis is structured into three sections, with the objectives of each section outlined below.

I. Examine lignin degradation dynamics in field and laboratory experimental settings (paper I).
II. Investigate the impact of lignin content and nitrogen addition on decomposition, and elucidate lignin degradation dynamics and associated microbial communities under laboratory conditions (paper II).
III. Evaluate the impact of lignin content and long-term effects of in situ nitrogen additions on decomposition and assess lignin degradation dynamics across Pine and Spruce forest stands (paper III).

3. Materials and methods

3.1 Field description

For Papers I and II, organic (O) horizon spodzol soil (0-15 cm) was collected from the Kulbäcksliden experimental forest, near Vindeln in Västerbotten County, northern Sweden (64°11′N, 19°33′E). The elevation is 258 meters above sea level. Given that coniferous forest is the dominant biome in the boreal region, the selected forest comprises a mixed stand of spruce (*Picea abies)* and pine (*Pinus sylvestris*), with a vegetation dominated by *Vaccinium vitis-idaea*, *Vaccinium myrtillus*, and *Pleurozium schreberi.* The mean annual air temperature and the average annual rainfall at the site is 2.96 °C and 646 mm respectively.

For Paper III, two distinct tree stand types were chosen as experimental sites: the Flakaliden spruce forest stand and the Norrliden pine forest stand. In Flakaliden, Vindeln municipality, Sweden (at latitude 64°07′N and longitude 19°24′E), forest dominated by Norway spruce (*Picea abies*) the tree species exists at an elevation of 310-320 meters above sea level. The average yearly temperature is 2.4°C, and the area receives 647 mm of precipitation annually, with a third of that precipitation falling as snow (Bergh et al. 1999; Rütting et al. 2021). The nitrogen deposition in the area is moderate, at 4.6 kg ha^{-1} yr^{-1} (Hedwall et al. 2013). At Flakaliden the soil is defined as a podzol on sandy till, with a 3-6 cm organic layer. The stand was established in 1963 by planting locally grown seedlings of Norway spruce. The fertilization experiment began in 1986, with four replicates arranged in a randomized block design using 50 x 50 m plots. The nutrient mixture's composition and quantity were adjusted annually based on foliar analysis and soil water nutrient concentrations (Linder 1995). The field layer

in the fertilized plots was sparse, while in the control plots, it was dominated by dwarf shrubs such as *V. myrtillus* and *V. vitis-idaea* (Strengbom et al. 2011).

The Scots pine (*Pinus sylvestris*) forest stand is located in Norrliden, Sweden (64°23′ N, 19°45′ E), at an elevation of 260 meters. This site is dominated by *Vaccinium vitis-idaea* and *Vaccinium myrtillus* as the understory species. The sandy soil, formed by glacial till, is classified as a Haplic Podzol (FAO) or Typic Haplocryod (US system). The average annual temperature is 1.2°C, with moderate precipitation of 595 mm. Snow covers the ground for roughly half the year, from late October to early May. A randomized block design experiment with three replicates (n=3) was established in 1971. Prior to the current Scots pine stand, the area was dominated by mature spruce trees. Clearcutting took place in 1951, followed by a prescribed burn in 1952, which was a common site preparation practice at the time. Two-year-old Scots pine seedlings were planted in 1953. Further site and experiment details are available in Tamm et al. (1999). The nitrogen addition experiment began in 1971, testing four levels of nitrogen (N0, N1, N2, N3) applied as ammonium nitrate (NH_4NO_3). The experiment followed a randomized block design with three replicate plots (30 × 30 m) for each treatment. Nitrogen fertilizer in the form of NH_4NO_3 pellets was applied annually to the forest floor in June. The plots used in this study (N1) had received a total of 1620 kg N ha^{-1} prior to the start of the study and were subsequently supplemented with approximately 32 kg N ha^{-1} yr^{-1}. Meanwhile, the N0 plot served as a control, receiving only the background nitrogen deposition of around 3 kg N ha^{-1} yr^{-1} (Högberg et al. 2024).

3.2 Pre-processing of samples

The wood of *Populus tremula* clones was selected as the model substrate for both laboratory incubation and field experiments (Papers I-III). *Populus tremula* is one of the most common tree species in the boreal region and is characterized by substantial genetic variation, particularly due to nucleotide polymorphisms that influence its wood's chemical composition (Jalas & Suominen 1989; Escamez et al. 2023). These clones were obtianed from the Skogforsk research station in Sävar (63.4°N, Umeå district), as part of the SwAsp collection (Luquez et al. 2008), which represents trees from 12 different locations across Sweden. For this study (Papers I-III), four clones—

(clones#: 35, 114, 91, and 52) were selected based on their natural variation in lignin content and composition. Lignin and carbohydrate contents were determined using Pyrolysis Gas Chromatography Mass Spectrometry (Py-GC-MS) (see section 3.5). Clones 35 and 114 were classified as low lignin (LL) substrates; with lignin contents of 32 ± 0.6% (mean ± SE) and carbohydrate contents of 67 ± 0.6% (mean ± SE). In contrast, Clones 91 and 52 were categorized as high lignin (HL) substrates, having lignin contents of 41 ± 0.9% (mean ± SE) and carbohydrate contents of 58 ± 0.9% (mean ± SE). Prior to processing, the bark was completely removed from the branches of each clone. The wood was then cut into small pieces and milled using a Tube Mill Control (IKA, Germany), followed by lyophilization for 72 hours. The milled wood was stored at room temperature until the experiments began. To assess organic matter (OM) and organic carbon (OC), replicate subsamples (n = 4) of the milled wood were analyzed. OM was determined as a loss on ignition (LOI; 4 hours at 550°C), while OC was quantified using an Elemental Analyzer (Flash EA 2000, Thermo Fisher Scientific, Bremen, Germany) (Werner et al. 1999). The OM of the wood was found to be 99.4 ± 0.1%, with negligible mineral content. Across all wood substrates, the OC content was 49.7 ± 0.2%, and the nitrogen content was 0.10 ± 0.006% (mean ± SE).

The O horizon spodzol soil collected for Papers I and II (refer Section 3.1) was transported to the lab and stored at 4 °C overnight. The next day, the soil was homogenized by passing it through a cutting sieve (6 x 3.5 mm) while still field moist. During this process, needles, cones, visible roots, and other debris were removed. The homogenized soil was then stored at -20 °C until the start of the experiment. Replicate subsamples (n = 4) of the homogenized soil were taken to measure organic matter content (OM), organic carbon (OC), and pH. The OM content of the soil was found to be 74.6 ± 0.44% (mean ± SE), and the OC content was 45.1 ± 0.04% (mean ± SE) organic nitrogen content was 1.38 ± 0.13 % (mean ± SE). The soil pH was determined using soil-water suspension method with a soil to water ratio of 1:2, resulted in a pH of 4.5 ± 0.003 (mean ± SE).

3.3 Lab and field experimental setup

In papers I and II, respirometer (A. Nordgren Innovations AB, Sweden) was used to measure biogenic production of carbon dioxide (CO_2). The

production of CO_2 was tracked every hour throughout the incubation period. Each incubation jar contained a small container with 10 mL of 0.5M potassium hydroxide (KOH) solution and two platinum electrodes. As CO_2 formed in the jar, it was absorbed by the KOH solution. By measuring the change in electrical conductivity of the solution, the amount of CO_2 produced is determined.

Briefly, 2.55 g of dry soil was placed into each 250 mL incubation jar. The moisture level of the homogenized soil was adjusted to -25 kPa, representing the optimum moisture content for microbial activity (Ilstedt et al. 2000). For jars with nitrogen amendment, 35 mg of ammonium sulfate was added as a solution (0.06M) to the freeze-dried wood. Jars without nitrogen amendment were only amended with an equal amount of Milli-Q water. With or without nitrogen amendments, 1.5 grams of milled wood was placed inside a 160-micron nylon mesh bag (4cm x 4cm) and incubated with the soil in each jar. All samples were incubated for 56 weeks in an insulated water bath set at 15°C.

For paper III, Six replicate plots (3 control and 3 fertilized) were used at Norrliden and Flakaliden sites. A single plot (non fertilized) was selected at Kulbäcksliden site for comparison with the lab incubation experiment (Section 4.1). Within each plot, six replicates of four different clonal wood types were deployed at three time points (3 months, 1 year, and 2 years) as milled wood samples inside a mesh bag. This resulted in 936 meshbags deployed across all sites. Each mesh bag consisted of approximately 2 grams of dry-weight wood material enclosed in a 160-micron nylon mesh bag measuring 4 cm x 4 cm. The size of the nylon mesh was chosen to be above 10 µm to ensure it does not restrict the movement of microbial communities in and out of the bag.

3.4 Post- processing of samples

For Papers I and II, 32 mesh bags were retrieved at four designated sampling time points (12, 24, 36, and 56 weeks following the experiment's initiation) from the incubation jars for further analysis. Each set of 32 bags included 16 bags from substrates without nitrogen amendment and 16 bags from substrates with nitrogen amendment. Within these sets, there were eight replicates each for both LL and HL substrates.

For Paper III, mesh bags were retrieved from the field at 3 months, 1 year, and 2 years. At each collection point, 312 bags were collected, comprising 144 bags each from Norrliden and Flakaliden, and 24 bags from Kulbäcksliden. All the mesh bags collected for Paper III were stored at 4°C and processed within 8 days of field retrieval.

The wood material extracted from the mesh bags was homogenized and sub-sampled for various analyses. These analyses included determining the mass loss (dry weight; 48 hours at 80°C) in Papers I-III, enzyme activities and microbial composition in Papers I and II, and molecular characterization utilizing 2D HSQC NMR in Papers I-III. Additionally, the initial and changes in the chemical composition of clones were assessed using pyrolysis Gas Chromatography-Mass Spectrometry (Py-GC/MS) in Paper I.

3.5 Pyrolysis GC/MS

For Papers I and II, approximately 75 µg of the freeze dried sample was transferred to auto sampler containers (Eco-cup SF, Frontier Laboratories, Japan). The Py-GC/MS setup consisted of an oven pyrolyzer with an auto sampler (PY-2020iD and AS-1020E, FrontierLabs, Japan) connected to a GC/MS system (Agilent, 7890A-5975C, Agilent Technologies AB, Sweden). The setup of pyrolysis GC/MS conditions was similar to (Gerber et al. 2012). The pyrolyzing temperature was set at 450 °C. The temperatures of the pyrolysis GC interface and GC injector were at 340 °C and 320 °C respectively. Helium was used as the carrier gas with a split ratio of 16:1. The gas saver mode was activated with a flow rate of 3 mL min^{-1} after one minute to vent away the pyrolysate remaining in the pyrolyzer oven. DB-5MS capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness; J&W, Agilent Technologies AB, Sweden) was used to separate the pyrolysate. The GC temperature was increased from 40 °C to 320 °C with a rate of 10 °C min^{-1}. The GC/MS interface was maintained at 300 °C. The mass spectrometer operated at unit mass resolution with a quadrupole type analyzer and the mass range was scanned from m/z 30 to 500 at 3.1 scan s^{-1}. For ionization, 70 eV electron bombardment was used (Tolu et al. 2015).

The raw data from Agilent Chemstation Data Analysis (Version E.02.00.493) were exported to NetCDF format and processed in R (version 2.15.2, 64-bit) following the method described by Jonsson et al. (2005) and Tolu et al. (2015). The data were processed using alternate regression (MCR-

AR), which includes chromatogram smoothing and alignment, background correction, and multivariate curve resolution (Karjalainen 1989). For each sample, a data table containing peak areas and a text file with mass spectra for each peak were generated. The mass spectra were then imported into the NIST MS Search 2.0 software, where peaks were annotated using the NIST/EPA/NIH 2011 library. A total of 55 peaks, excluding CO_2, were annotated and categorized as either carbohydrates or lignin (guaiacyl, syringyl, and phenols) (refer to paper I). The relative proportions of carbohydrates and lignin were determined by normalizing the peak areas to the total sum for each sample.

3.6 NMR spectroscopy

For Papers I-III, the wood substrates were prepared according to the protocol described in Soucémarianadin et al. (2017). Briefly, 200 mg of freeze-dried wood substrates were ground in a 50 mL ZrO_2 jar with ten 10 mm ZrO_2 ball bearings using a Retsch PM100 planetary ball mill (Fritsch, Idar-Oberstein, Germany) for 5 cycles of 10 minutes at 500 rpm, with 10-minute breaks in between. After grinding, 50 mg of the ball-milled wood sample was transferred to an NMR tube and dissolved in 600 µL of deuterated dimethyl sulfoxide (DMSO-d6). The sample was thoroughly mixed and left to stand overnight before NMR analysis.

^{1}H–^{13}C heteronuclear single quantum coherence (HSQC) correlates ^{1}H atoms with their directly bonded ^{13}C atoms through one-bond J coupling, resulting in a cross peak for each C-H group at their respective ^{1}H and ^{13}C chemical shifts (in parts per million, ppm). Spectra were acquired on an 850 MHz Bruker Avance III HD spectrometer equipped with a 5mm cryoprobe (TCI HCN) and an automatic sample changer (SampleXpress), employing the hsqcetgpsisp2.2 pulse sequence from the Bruker pulse sequence library. Spectra were collected at room temperature (298 K) with 40 scans. A recycle delay of 1 s was used and 128 points were collected in the indirect dimension. Each 2D experiment was approximately 2 hours and the raw data were zero-filled to a 2048 × 1024 spectrum size. Spectral widths of 10 ppm (8504 Hz) in the ^{1}H and 165 ppm (35274 Hz) in the ^{13}C dimensions were employed. The spectra were manually phase- and baseline-corrected and referenced to the residual DMSO peak at 40.0/2.50 ppm. Data processing was performed using Topspin 3.2 (Bruker BioSpin Corporation, Billerica, USA).

To monitor the compositional changes in lignin structure, ten peaks in the 2D HSQC spectra were assigned to different lignin moieties and integrated (Table 1). Assignments were based on previously published data (Kim et al. 2008; Del Río et al. 2012). The HSQC spectrum is divided into the several regions: aromatic, di-O-alkyl, O-alkyl, and alkyl. Peaks corresponding to lignin subunits and linkages were identified and integrated in the aromatic and O-alkyl regions (Fig. 2). In Poplar lignin, H-lignin content is very low and was not detected. Instead, the *p*-hydroxybenzoate groups are present, linked to the γ-position of either syringyl or guaiacyl units (Smith 1955; Goacher et al. 2021).

The relative abundance of each moiety was determined by calculating the total integrated cross-peaks within specific groups (lignin subunit and lignin linkages) at each sampling time point. By doing so, the sum of lignin subunits and linkages was 100% individually, enabling us to observe the change in abundance of the moieties over time within the groups separately. A decline in the decomposition pattern for a particular moiety over sampling time indicates a greater degree of degradation compared to other moieties within the same group. Conversely, an increase suggested lesser degradation relative to other moieties within the group. In instances where multiple peaks could be utilized to track the same sub-structure, the best-resolved peak was selected (Table 1).

Table 1: Assignments of HSQC signals to the lignin linkages and lignin subunit moieties. Each cross peak in the spectrum resulted from the correlation between 1H–and 13C, separated by one bond. The 1H and 13C chemical shifts of moieties are shown in parts per million (ppm).

Assignments	**δ ^{1}H (ppm)**	**δ ^{13}C (ppm)**	**Relative %**
Lignin subunits			
$C_{2,6}$-$H_{2,6}$ in syringyl units	6.65	103.7	53
$C_{2,6}$-$H_{2,6}$ in C_{α}-oxidized syringyl units	7.19	106.2	9
C_{2}-H_{2} in guaiacyl units	6.92	110.7	32
$C_{2,6}$-$H_{2,6}$ in *p*-hydroxybenzoates	7.64	131.2	6
Lignin linkages			
C_{β}-H_{β} in resinol	3.03	53.5	16
C_{β}-H_{β} in spirodienone	2.75	59.5	4
C_{β}-H_{β} in β-O-4' ethers linked to guaiacyl	4.26	83.5	18

C_β-H_β in β-O-4' ethers linked to syringyl	4.08	85.8	50
C_α-H_α in phenylcoumaran	5.43	86.9	4
C_β-H_β in cinnamyl alcohol	6.20	127.9	8

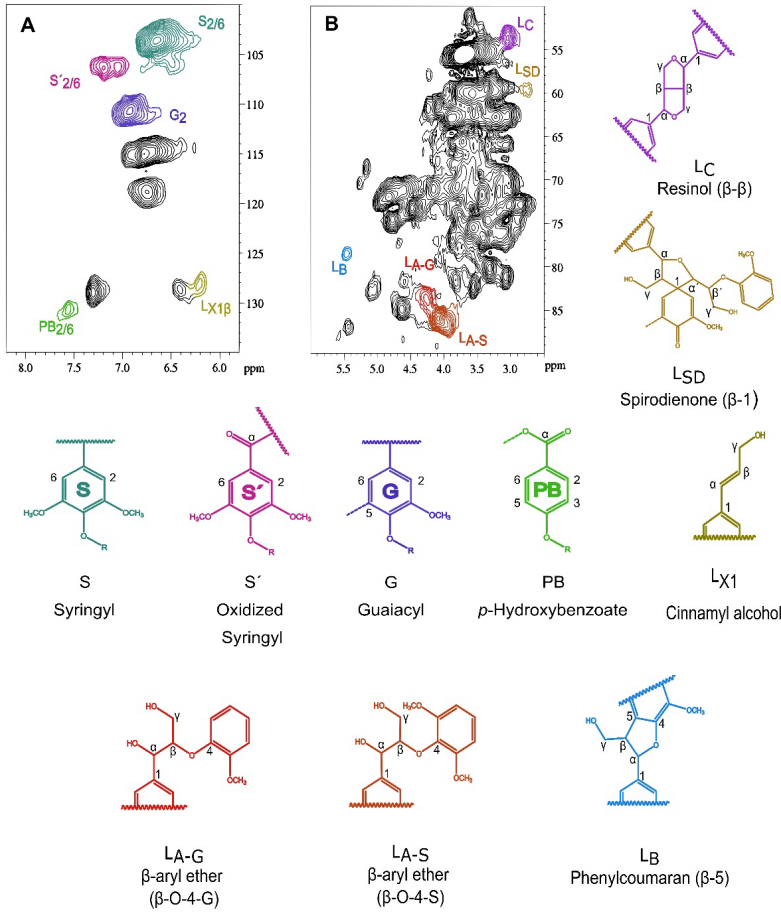


Figure 2: Peak assignments in the aromatic (A) and O-alkyl (B) regions of the HSQC spectrum of the undecomposed Aspen wood DMSO-extract. Color-coding represents, the chosen lignin linkages and lignin subunit moieties for this study and their corresponding chemical structure shown using respective colors and labeling.

3.7 16S rRNA Gene Amplicon Sequencing

For paper II, Aliquots of 200 mg of milled wood were collected from each incubation bag at each sampling time point. Four replicates were prepared for each of the four wood types, both with and without nitrogen addition. DNA extraction was carried out using the DNeasy PowerSoil kit (Qiagen, Valencia, CA, USA). DNA concentrations in the extracts were measured using the Qubit 1X dsDNA Broad Range (BR) Assay kit and a Qubit 3.0 fluorometer (Invitrogen, Waltham, MA, USA).

To construct bacterial 16S rRNA gene libraries, the V3–V4 hypervariable region was amplified via polymerase chain reaction (PCR) using primers 341F (CCTACGGGNGGCWGCAG) and 805NR (GACTACNVGGGTATCTAATCC) (Herlemann et al. 2011). The PCR and library preparation protocols were adapted from Sinclair et al. (2015), with modifications to fit the experimental setup. The first PCR was performed with 1 µl of a 10-fold diluted DNA template from each sample (average concentration 25.4 ng/µl, ranging from 8.4 to 36.2 ng/µl) in a 20 µl reaction mixture containing 0.2 µl Q5 High-Fidelity DNA Polymerase (2 U/µl; New England Biolabs), 2 µl of 2 mM dNTPs, 0.5 µl each of 5 µM forward and reverse primers, and 15.8 µl of nuclease-free water. Thermal cycling included an initial denaturation at 98°C for 3 minutes, followed by 20 cycles of 98°C for 10 seconds, 48°C for 30 seconds, and 72°C for 30 seconds, with a final extension at 72°C for 2 minutes and a hold at 6°C. PCR product quality and size were verified by agarose gel electrophoresis, and products were purified using Agencourt AMPure XP magnetic beads (Beckman Coulter, Brea, CA, USA).

For the secondary PCR (15 cycles), Illumina sequencing adapters and unique index primers were attached to the amplicons. This step used 4 µl of DNA template from the first PCR in a 20 µl reaction containing 0.2 µl Q5 High-Fidelity DNA Polymerase, 2 µl of 2 mM dNTPs, 1 µl each of forward and reverse index primers (5 µM), and 11.8 µl of nuclease-free water. The thermal protocol included an initial denaturation at 98°C for 30 seconds, followed by 15 cycles of 98°C for 10 seconds, 66°C for 30 seconds, and 72°C for 30 seconds, with a final extension at 72°C for 2 minutes and a hold at 6°C. Final product sizes were verified by gel electrophoresis and purified with the same magnetic bead protocol. DNA concentrations were quantified with the Qubit 3.0 fluorometer, and equal amounts of DNA (20 ng per sample) were pooled to create the sequencing library.

Sequencing was performed using the Illumina MiSeq system with the Reagent Kit v3. Data analysis followed the DADA2 pipeline (version 1.16) in R (version 4.0.2), as described by Callahan et al. (2016). Forward and reverse primers were removed, and sequences were trimmed to 282 and 240 bp, respectively. Quality control thresholds were set at maxEE = (2, 5) and truncQ = 2, based on FIGARO predictions (Weinstein et al. 2019). Taxonomic assignments were made using the SILVA rRNA database (release 138.1) (Quast et al. 2013).

For fungal community analysis, the ITS2 region of the ribosomal operon was amplified in a single PCR step using the primers gITS7 (Ihrmark et al. 2012) and ITS4 (Innis et al. 2012), with sample-specific tags. Reactions included 25 µl of 10-fold diluted DNA template, 0.25 µl DreamTaq polymerase (5 U/µl; Thermo Fisher Scientific, MA, USA), 5 µl of 10x buffer, 5 µl of 2 mM dNTPs, 1.5 µl of 25 mM MgCl2, 5 µl of primer mix (5/3 µM), and 8.25 µl of nuclease-free water in a total volume of 50 µl. The thermal program included an initial step of 5 minutes at 94°C, followed by 22 cycles of 30 seconds at 94°C, 30 seconds at 56°C, and 30 seconds at 72°C, with a final extension of 7 minutes at 72°C. Sequencing adaptors were added via ligation, and the library was sequenced on a PacBio Sequel II system at SciLifeLab (Uppsala, Sweden).

Fungal sequence processing and clustering were conducted using the SCATA pipeline (Ihrmark et al. 2012). Quality control included trimming primers and tags, removing single-occurrence sequences, and collapsing homopolymers to three bases. Sequences with less than 1.5% divergence were clustered using pairwise alignment and single-linkage clustering with USEARCH. Taxonomic assignments were made using the UNITE database (Nilsson et al. 2019), with non-fungal sequences removed. Representative sequences were assigned to species hypotheses (Kõljalg et al. 2013). Raw data are available in the NCBI database under BioSample accession numbers SAMN41557728 (bacteria) and SAMN41598886 (fungi), within BioProject PRJNA1117022.

3.8 Statistical analysis

In Paper I, the relative percentages of lignin subunits and linkages, as determined by HSQC NMR, were analyzed using linear mixed-effects (LME) models. Time was treated as a random factor, while the fixed

factors—site (lab vs. field) and substrate type (high vs. low lignin)—were tested for significance using F-tests with Type II ANOVA and Kenward-Roger degrees of freedom. The analyses were conducted with the "car" and "lmerTest" packages in R (Kenward & Roger 1997; Kuznetsova et al. 2017).

In Paper II, a two-way ANOVA was used to assess the difference in total net mass loss between high-lignin (HL) and low-lignin (LL) substrates, both with (+N) and without (-N) nitrogen, by the end of the incubation period. Assumptions of normality and homogeneity of variance were confirmed. Repeated measures ANOVAs were conducted to evaluate the impact of initial lignin content and nitrogen amendments on accumulated CO_2-C and the relative abundance of lignin subunits and linkages, as determined by 2D HSQC NMR. Assumptions regarding outliers, normality, and variance homogeneity were checked, and if violated, robust ANOVA tests were performed using the WRS2 package (Mair & Wilcox 2020).

PERMANOVA (via the "vegan" R package, version 2.6-4) was used to examine the effects of nitrogen addition, lignin content, and time on bacterial and fungal community compositions. To compare microbial community composition at the genus level between nitrogen amendments, the "Phyloseq" (version 1.44.0) and "MicrobiotaProcess" (version 1.12.3) packages in R (McMurdie & Holmes 2013; Xu et al. 2023) was utilized. ANOVA was performed using Phyloseq to test for significant effects of nitrogen amendment on microbial community composition at the genus level. Alpha diversity indices, including Chao1 (estimated richness), observed Amplicon Sequence Variant (ASV) richness, Simpson diversity (diversity and dominance), and Shannon diversity (evenness), were calculated using MicrobiotaProcess. ANOVA was then applied to compare these diversity indices between nitrogen amendments. Bacterial ASVs and fungal OTUs were rarefied to the lowest read counts (4437 and 9093, respectively) before conducting the above mentioned multivariate analyses.

To explore the core bacterial and fungal groups involved in lignin degradation and their potential functional interactions, co-occurrence network analysis was performed. This analysis was based on the relative abundance dynamics of 16S and ITS2 markers for samples with and without nitrogen addition. ASVs and OTUs representing over 1% of total sequence reads were selected for the analysis, which was conducted using the RCy3 and igraph packages in R. Spearman’s rank correlations were calculated for each ASV pair, with p-values adjusted using the Benjamini-Hochberg

method to control the false discovery rate (Jones et al. 2008). Only significant correlations ($p < 0.001$, $r > 0.5$) were included in the network. Key indices like degree, betweenness, and closeness centrality were calculated to identify potential keystone microorganisms, following Berry & Widder (2014). Cytoscape (version 3.7.2) was used for network visualization.

In Paper III, net litterbag mass loss was analyzed using Type II ANOVA to examine the main effects and interactions of substrate type (high vs. low lignin), nitrogen amendment (fertilized vs. non-fertilized), site (pine vs. spruce), and time (weeks 12, 52, and 104). Assumptions of normality and homogeneity of variance were satisfied. The relative percentages of molecular moieties were further analyzed using linear mixed-effects (LME) models, with substrate type (high vs. low lignin), nitrogen amendment (fertilized vs. non-fertilized), site (pine vs. spruce), and time (weeks 12, 52, and 104) specified as fixed effects. To account for potential variation among individual clones, the initial relative percentage of each lignin moiety was included as a random effect. The significance of random effects (clonal variation) was assessed with the maximum likelihood ratio test (Zuur et al. 2009), and p-values were adjusted for multiple comparisons using the Tukey method. All statistical analyses were conducted in R software version 4.0.1 (R Core Team 2021)."

4. Summary of results and discussion

4.1 Comparison of lignin degradation dynamics of model substrates: Field vs. Laboratory experiments

Significant variations in lignin degradation often occur among studies (Thevenot et al. 2010; Polman et al. 2021) these variations can be attributed to differences in subunits that make up lignin and the distribution of linkages between them(Lourenço & Pereira 2018). The lignin in our model, distributed among S, G, and H-type subunits, reflects the functional groups found in a range of boreal plant species provides an effective conceptual proxy across diverse plant materials, offering a reliable framework for studying lignin degradation. Paper I investigated the impact of lignin content on decomposition and elucidated lignin degradation dynamics. Additionally, model substrates were incubated at the same field site from which the soil for the laboratory incubation experiment was taken. The lignin degradation dynamics observed in Paper I were then compared with changes in lignin chemistry observed at the field site.

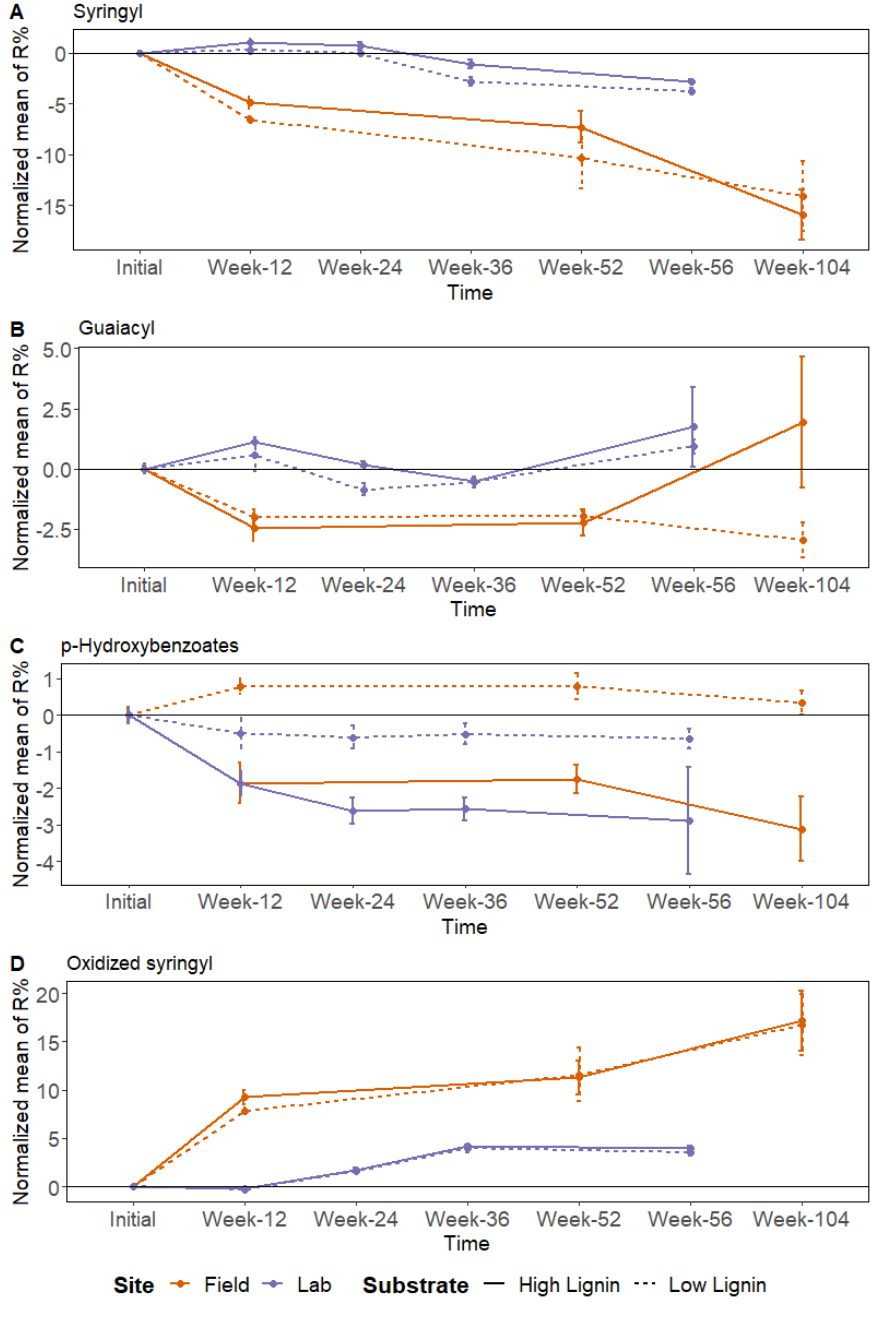
In our model substrates, except for *p*-hydroxybenzoate, the overall changes in the moieties of lignin subunits between HL and LL substrates were similar throughout the lab incubation experiment. The relative abundance of *p*-hydroxybenzoate decreased more rapidly in HL substrates than in LL substrates (Fig. 3C). Similar degradation pattern of p-hydroxybenzoate were observed with the field experiments (Fig. 3C). This suggests that, similar to *p*-hydroxyphenyl (H-lignin) (Goñi et al. 1993; Otto & Simpson 2006; Soucémarianadin et al. 2017; vandenEnden et al. 2018; Campo et al. 2019), *p*-hydroxybenzoate belongs to the faster-decomposing

lignin fraction, alongside syringyl, as compared to guaiacyl. However, the greater degradation of guaiacyl in field experiments compared to lab incubations (Fig. 3B) underscores the potential role of soil physical properties such as soil structure, texture, porosity and water content in the degradation of lignin subunits.

A small proportion of syringyl was present in its oxidized form (Cα-oxidation) in our model substrates. Interestingly, oxidized syringyl was more resistant to degradation than other lignin subunits and this fraction increased significantly during decomposition (Fig. 3D). Previous studies have shown that Cα-oxidation deactivates the aromatic ring, preventing it from undergoing electrophilic substitution and affecting subsequent reactions after enzyme oxidation (Kirk et al. 1986; Kawai et al. 2002). This suggests that Cα-oxidation inhibits further lignin degradation (Van Erven et al. 2019; Chen et al. 2021). However, during the lab incubation experiment, the increase in Cα-oxidation observed in our substrates was primarily due to a relative decrease in the abundance of other subunits, with no clear evidence of additional oxidized syringyl (Cα-oxidation) being formed from lignin depolymerization. In contrast, the field experiment provided clear evidence that degraded syringyl accumulated as oxidized syringyl (Fig. 3D), providing further insights into the influence of soil's physical properties on the secondary lignin degradation products.

The differences in lignin subunit degradation can be attributed to the varying stability of the linkages connecting the subunits (Talbot et al. 2012). β–O–4 linkages are more commonly associated with syringyl than guaiacyl (Kim & Ralph 2010), and they are considered more easily degradable compared to other lignin linkages. Additionally, guaiacyl is more resistant to degradation as compared to syringyl, due to the availability of the C5 position, which allows for the formation of more stable linkages, such as β–5, 5–5, and 5–O–4 ((Boerjan et al. 2003a). Our findings align with this, as the abundance of β–O–4 linkages associated with syringyl decreased more than guaiacyl's phenylcoumaran (β–5) linkages (Fig. 3E & 3G). Furthermore, β–O–4 linkages attached to guaiacyl were more resistant to microbial decomposition than those linked to syringyl (Fig. 3F). However, the degradation of β–O–4 linked to guaiacyl increased, compared to syringyl, during the first year of the field experiment (up to week 52), then becoming resistant to degradation in the second year (week 52- 104) (Fig. 3A & 3B). This suggests that the degradability of β–O–4 linkages might potentially be

influenced by other factors such as climatic conditions and microbial activity.



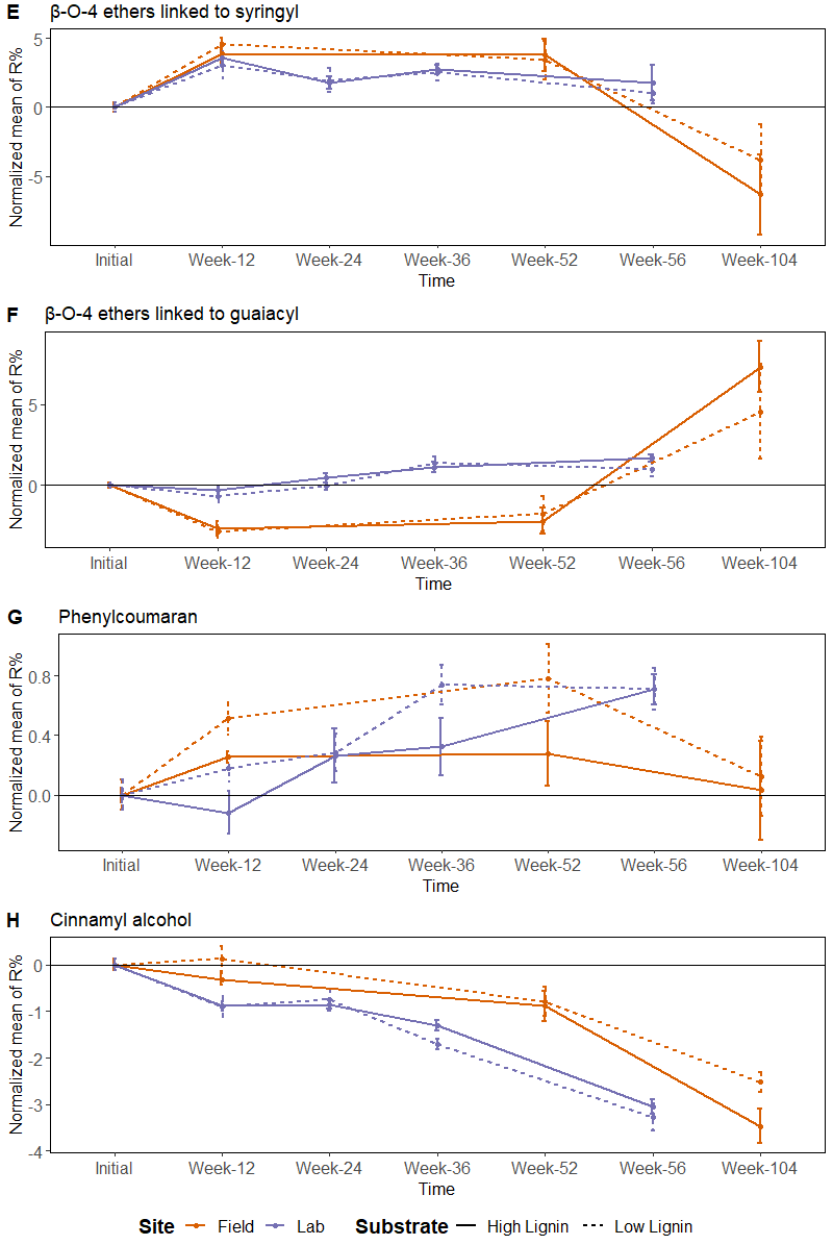


Figure 3. The temporal dynamics of the average relative percentage of lignin moieties (n=8) in high and low lignin substrates between field and lab incubations. Values are normalized to the initial relative abundances. Error bars indicate the standard error (±SE)

4.2 Impact of lignin content and nitrogen addition on plant material decomposition: Insights into lignin degradation dynamics and microbial communities under lab conditions

Paper II examined the effect of lignin content and nitrogen addition on the decomposition of our substrates (HL & LL). There were no significant differences in CO_2-C from the substrates or net mass loss of the substrates due to variations in lignin properties (Fig. 4). This similarity emphasis the absence of significant physical constraints induced by the lignin on the microbial access to the cell wall components of e.g. polysaccharides. Our results agree with Talbot et al. (2012) that physical protection of cell wall polysaccharides by the lignin appeared to play a smaller role than the chemical protection in the decay of the plant material.

The cumulative CO_2-C from the incubation vessels and net mass loss of the substrates were significantly lower in nitrogen amended substrates than in non-nitrogen amended substrates ($p<0.001$) (Fig. 4). The total net mass loss for nitrogen amended and non-nitrogen amended substrates was 37 ± 2 % (mean ± SE) and 54 ± 4 % (mean ± SE). This aligns with the common understanding that nitrogen additions often reduce respiration rates and impede decomposition (Berg & Matzner 1997; Janssens et al. 2010; Xing et al. 2022). However, there was no suppressive effect of nitrogen (N) on the lignin-rich HL substrates compared to LL substrates. This contradicts the common understanding that high N availability reduces the lignin degradation and thereby increases the physical constraints induced by the lignin on the microbial access to the polysaccharides (Keyser et al. 1978; Carreiro et al. 2000; Craine et al. 2007; Talbot & Treseder 2012).

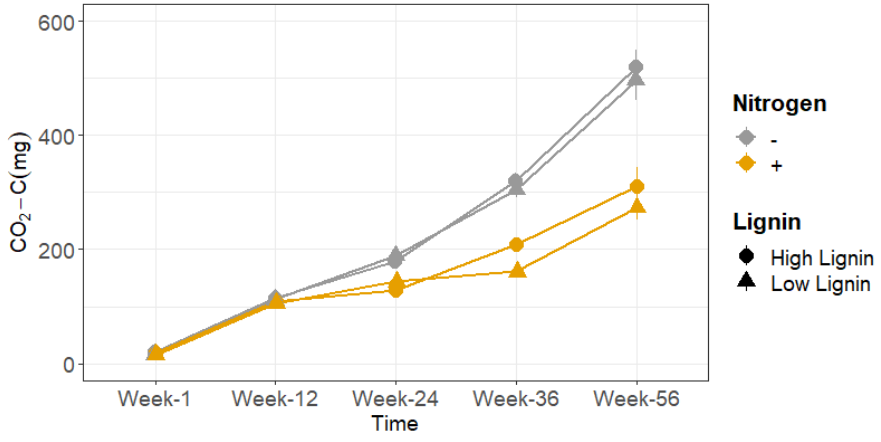


Figure 4. The average CO_2-C accumulation (n=8) from soil and wood over the 394-day incubation period. Coloured lines represent different substrates with (+) and without (-) nitrogen amendment. Error bars indicate the standard error (±SE).

In Paper II, I also investigated the microbial community dynamics during the incubations that showed nitrogen addition elicited contrasting responses in the bacterial and fungal diversity. Nitrogen addition decreased the richness (Chao1 and Observed richness) and evenness (Shannon and Pielou) of bacterial communities, thereby reducing the overall diversity. Conversely, nitrogen addition increased the evenness and diversity of fungal communities (Fig. 5). Species like *Penicillium longicatenatum*, *Mucor abundans*, and *Mucor silvaticus* were predominant and their abundance remained similar (p=0.06, R=0.1) throughout the incubation period in nitrogen-treated substrates (Fig. 6). However, in the non-nitrogen amended substrates. *Tausonia pullulans* (homotypic synonym: *Trichosporon pullulans*) and *Renatobasidium notabile* emerged as the predominant species. As incubation progressed, *Renatobasidium notabile* gradually replaced *Tausonia pullulans* as dominant in the fungal community (Fig. 6) and led to an uneven community with low diversity.

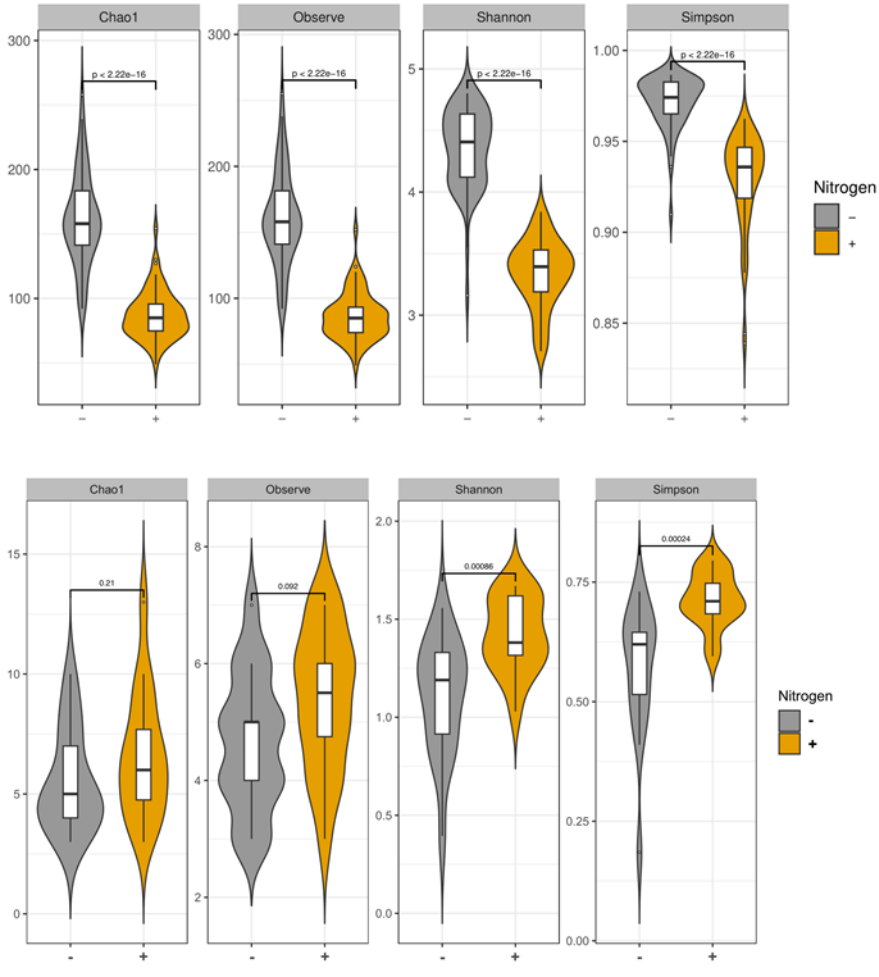


Figure 5. Bacterial (Top) and fungal (Bottom) diversity indices are represented as violin plots for all samples with (+) and without (-) N amendment (Chao1: estimated ASV richness; Observe: observed ASV richness; Simpson: amplicon sequence variants (ASVs) diversity, and Shannon: ASVs evenness). The significance (p-values) was presented between two groups of samples for each diversity index.

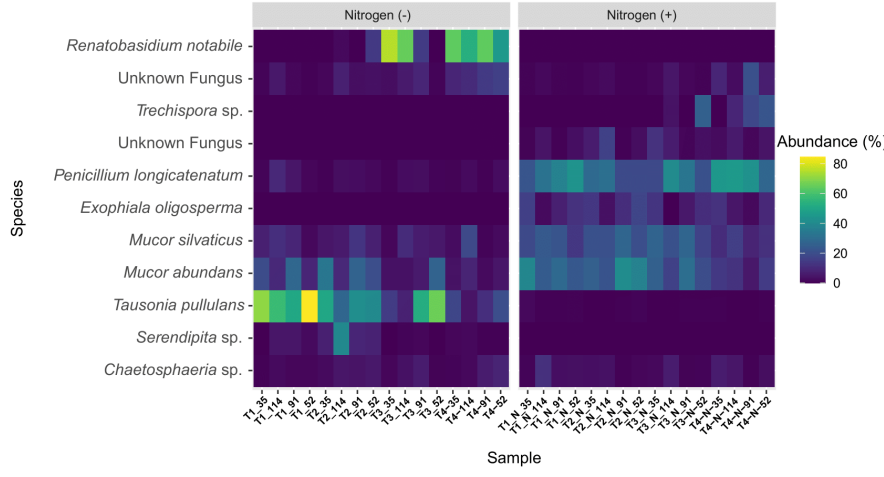


Figure 6. Relative abundance of the fungal community based on the reads of ITS genes from samples grouped according to the nitrogen amendment. T1, T2, T3 and T4 represent weeks 12, 24, 36, and 56.

This is particularly evident from the network analysis (Fig .7). At the beginning of incubation, microbial clusters with many positive correlations constituted by several genera belonging to *Proteobacteria*, such as *Methyloferula, Bradyrhizobium, Luteibacter, Pseudomonas* and family *Magnetospirillaceae*, as well as the fungi *Serendipita sp*., *Mucor abundans*, and *Tausonia pullulans* exits in non-nitrogen amended substrates. However these clusters diminished over time with the onset of *Renatobasidium notabile.* The dominance of *Renatobasidium notabile* was suppressed by nitrogen addition, potentially increasing the diversity of other fungi in the nitrogen amended substrates. The results diverge from the field studies that have generally reported decreased fungal diversity following nitrogen amendment (Allison et al. 2007; Yan et al. 2017; Entwistle et al. 2018). This discrepancy could be attributed largely to the absence of colonization of wood by airborne spores and typical wood-decaying fungi from the Basidiomycetes class, particularly those from the *Polyporaceae*, *Thelephoraceae*, and *Agaricaceae* families, in our study(Dickinson 2012).

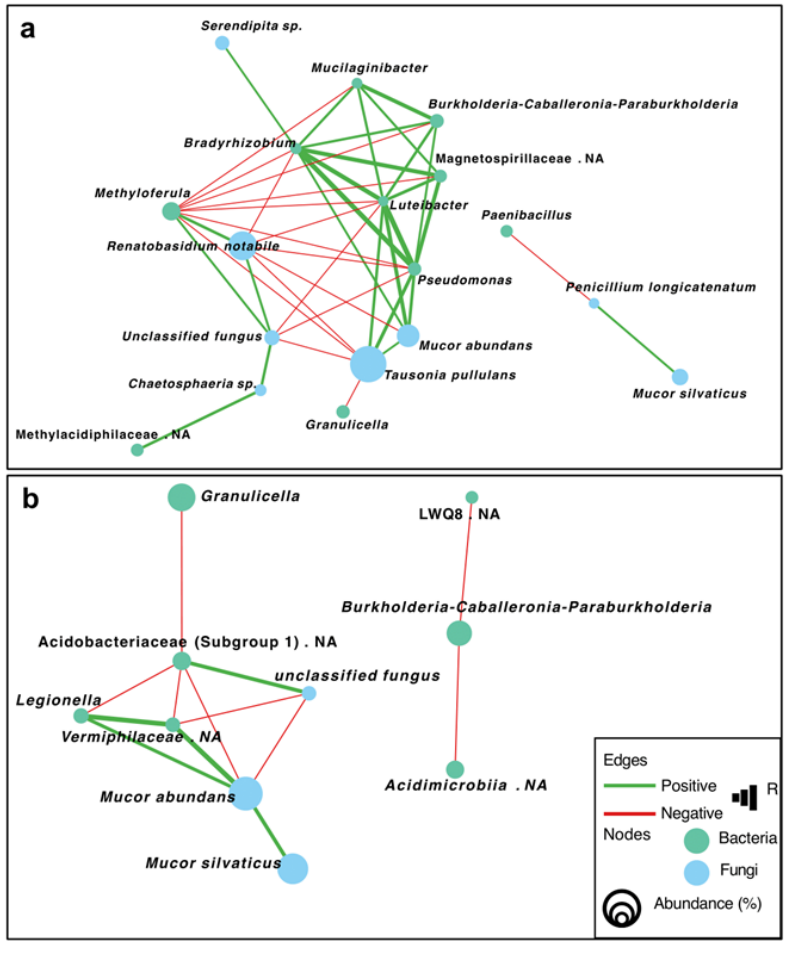


Figure 7. Co-occurrence network analysis based on the correlation of the relative abundance of amplicon sequence variant (ASV) reads for microbial profiles at the genus level or the nearest classified taxonomic level (relative abundance >1%) for samples (a, top) without nitrogen amendment and (b, bottom) with nitrogen amendment. Nodes represent bacterial (green) and fungal (blue) groups. Each edge represents significant correlations between node pairs ($p < 0.05$), with positive correlations shown in green and negative correlations in red. The thickness of each edge corresponds to the R-value of the correlation ($R > 0.5$).

Renatobasidium notabile belongs to the order *Auriculariales* (jelly fungi), which are well-recognized white-rotters with a high capacity for oxidative ligninolysis (Nagy et al. 2016). The presence of bacterial communities like

Methyloferula and *Methylacidiphilaceae* suggest a shift in local conditions, as these bacterial communities are known to thrive in acidic environments (Radajewski et al. 2002; Op den Camp et al. 2009). These changes in conditions can be beneficial for lignin degradation. For instance, acidic environment like in this study (pH=4.5) favours the non-enzymatic degradation of lignin via Fenton's chemistry (Kapich et al. 1999; Arantes et al. 2009; de Boer et al. 2010; Daniel 2016). Moreover, these communities were positively associated with fungi, *Renatobasidium notabile.* Therefore, it is likely that *Tausonia pullulans* fed primarily on carbohydrates and later, with an increased abundance of *Renatobasidium notabile,* a greater amount of syringyl units was degraded in non-nitrogen amended substrates (Fig. 6 & 8A). Interestingly, in nitrogen amended substrates during the later stages of incubation*, Trechispora sp* became abundant, coinciding with the rise of *Renatobasidium* in non-nitrogen amended substrates (Fig. 6). *Trechispora sp*., a wood inhabiting fungus, is known for its enzymatic capacity to degrade lignin (Zhao & Zhao 2021; Kalntremtziou et al. 2023). This could explain the enhanced degradation of guaiacyl related moieties in the nitrogen amended substrates (Fig. 8B & 8F), suggesting that different lignin components can degrade depending on the microbial communities present. Furthermore, with nitrogen addition, microbes showed preference for degrading guaiacyl-related linkages, including β–O–4 bonds associated with guaiacyl and phenylcoumaran along with cinnamyl alcohol linkages, all of which decreased in abundance (Fig. 8F, 8G & 8H). These results contrast with studies that observed increased syringyl degradation following nitrogen amendments (Pisani et al. 2015; vandenEnden et al. 2018), yet are consistent with findings that suggest higher nitrogen levels can inhibit syringyl degradation (Chen et al. 2019; Berg & McClaugherty 2020).

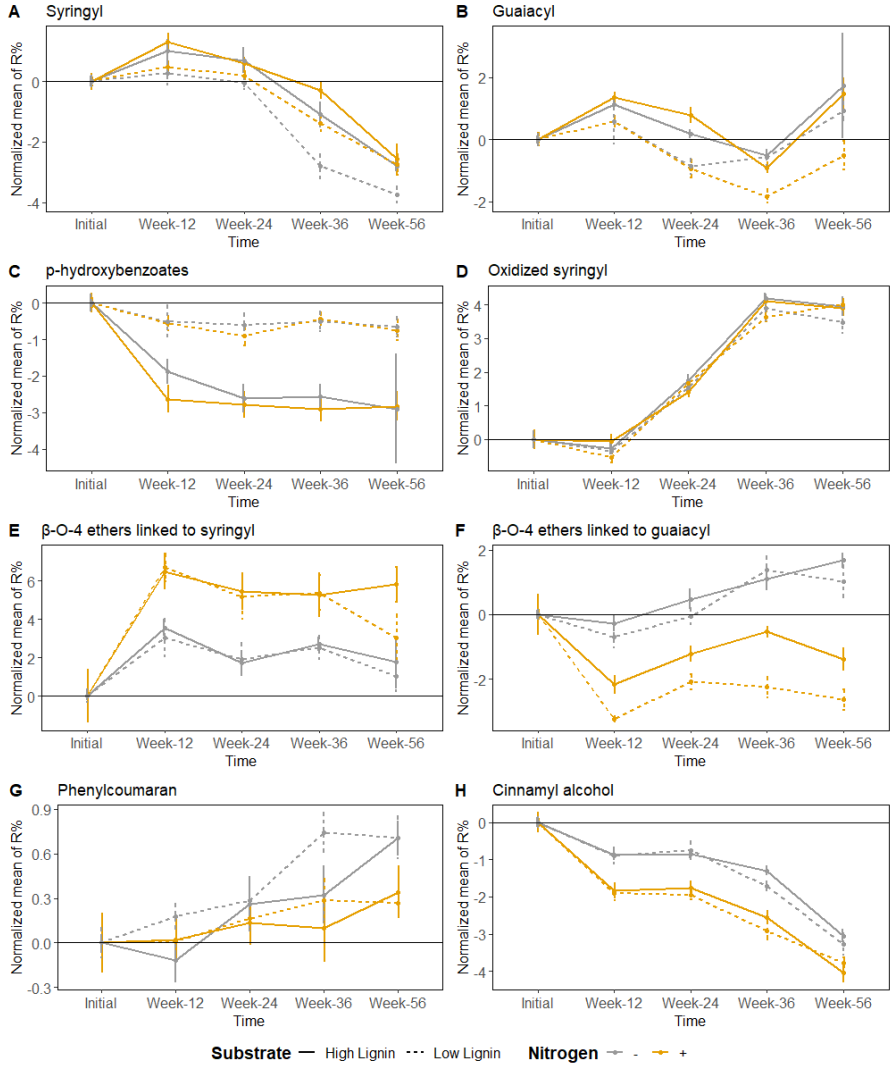


Figure 8. The temporal dynamics of the average relative percentage of lignin moieties (n=8) in high and low lignin substrates with (+) and without (-) nitrogen amendments. Values are normalized to the initial relative abundances. Error bars indicate the standard error (±SE).

4.3 Effect of lignin content and long-term effects of *in situ* nitrogen additions on decomposition and lignin degradation dynamics across Pine and Spruce forest stands

Paper III evaluated the effect of long-term nitrogen fertilization on the decomposition of model substrates, with a focus on differences in lignin degradation dynamics between pine and spruce forest stands. Both spruce and pine forest stands exhibited a greater overall net mass loss of substrates over two years in fertilized plots (56 ± 2.1%, mean ± SE) compared to non-fertilized plots (46 ± 2.7%, mean ± SE) (Fig. 9 & Table 2). These findings contrast with previous studies, which generally report a decrease in plant organic matter decomposition following long-term nitrogen addition (Maaroufi et al. 2017, 2019; Bowden et al. 2019; Forsmark et al. 2021). One potential explanation is that plant organic matter in fertilized plots typically contains higher nitrogen levels, which can slow degradation. However, in this study, the model substrates that had lower nitrogen content were used across all stands and nitrogen treatments, likely contributing to the observed acceleration in overall decomposition rates.

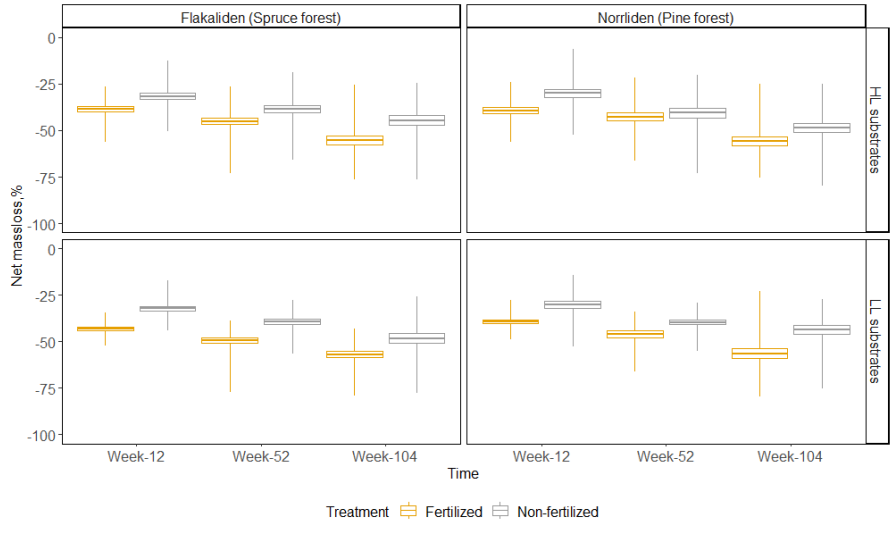


Figure 9. The average net mass loss (n=36) of high lignin (HL) and low lignin (LL) substrates across fertilized and non-fertilized plots at Flakaliden and Norrliden. Week 12, week 52, and week 104 represent the duration litterbags remained in the field for decomposition.

Table 2. ANOVA results for net mass loss of high and low lignin substrates over time in control and treatment plots. Asterisks (*) indicate statistically significant differences.

Variables	Df	Sum Sq	F value	*p*-value
Substrates	1	283	2.22	0.136
Treatment	1	13879	109.08	<2e-16 ***
Time	2	33257	130.69	<2e-16 ***
Site	1	155	1.21	0.269

Lignin content, commonly regarded as a key factor influencing decomposition rates (Knorr et al. 2005; Talbot & Treseder 2012; Frey et al. 2014; Yue et al. 2016) , did not affect substrate decomposition. Net mass loss between substrates with high and low lignin content remained similar across both fertilized and non-fertilized plots (Fig. 9 & Table 2). These findings suggest that lignin content may have a limited role in regulating decomposition during the early stages of litter breakdown. Although the net mass loss and degradation of lignin subunits were similar across different forest stands, the degradation of lignin linkages varied between pine and spruce forests. Specifically, the breakdown of β-O-4 linkages associated with syringyl and cinnamyl alcohol differed between spruce and pine sites (Fig. 10A & 10D). This indicates that while the overall rates of plant material decomposition may appear similar, the pathways and mechanisms underlying lignin degradation differ between spruce and pine stands. Previous studies have also reported similar decomposition rates between these forest types (Herrmann & Prescott 2008; Kriiska et al. 2021), but our results emphasize the need to consider forest-specific mechanisms of lignin breakdown. These differences in lignin degradation pathways across forest types highlight the challenges of generalizing findings about lignin decomposition across ecosystems.

Across all sites, the model substrates decomposed gradually, with syringyl subunits and their associated β-O-4 linkages exhibiting greater degradation compared to other lignin subunits and linkages (Fig. 10A & 10C). This aligns with the conclusions of paper I, as well as previous research (Boerjan et al. 2003b; Talbot et al. 2012; Rinkes et al. 2016), which highlights the higher susceptibility of syringyl subunits and their linkages to degradation. These findings underscore the importance of lignin composition, rather than lignin content alone, in driving the decomposition

of plant material. Notably, the preferential degradation of syringyl-related moieties was more pronounced in fertilized plots than in non-fertilized ones, a pattern consistently observed in both spruce and pine forest stands (Fig. 10A & 10C).

However, oxidized syringyl increased significantly during decomposition (Fig. 10B) suggesting that oxidized syringyl is more resistant to degradation than other lignin subunits. The observed correlation between the relative decrease in syringyl and the rise in its oxidized form implies that degraded syringyl tends to accumulate as it becomes oxidized. Previous studies have shown that oxidized syringyl is considerably more difficult to degrade and tends to persist in the environment (Van Erven et al. 2019; Chen et al. 2021). With nitrogen fertilization, the relative abundance of oxidized syringyl increased, particularly in low-lignin substrates. This finding aligns with earlier research indicating that lignin oxidation is often enhanced under nitrogen fertilization, likely due to reduced microbial activity (Feng et al. 2010; Frey et al. 2014; Pisani et al. 2015; Bonner et al. 2019). These results suggest that nitrogen addition may promote the accumulation of more recalcitrant, oxidized lignin forms.

Previous studies have reported diverging effects of nitrogen on syringyl degradation, ranging from decreases to increases or no effect, depending on the context. For example, Man et al. (2021) found that the increase in lignin degradation diminishes above nitrogen application rates of 145 kg $ha^{-1}yr^{-1}$, while Sjöberg et al. (2004) observed enhanced syringyl degradation at a site receiving ammonium sulfate at a rate of 100 kg $ha^{-1}yr^{-1}$. Conversely, Stoica et al. (2023) reported no significant effect on syringyl degradation in organic soil with nitrogen treatment. These varied outcomes may be linked to differences in nitrogen application rates. In this study, a relatively low nitrogen fertilization rate was applied (<35 kg $ha^{-1}yr^{-1}$), which may explain the increase in syringyl degradation and its associated linkages. This finding suggests that low nitrogen input creates more optimal conditions for lignin-degrading microbes, by potentially avoiding the inhibitory effects on lignin-degrading enzymes, such as phenol oxidases and peroxidases, which have been observed under higher nitrogen inputs.

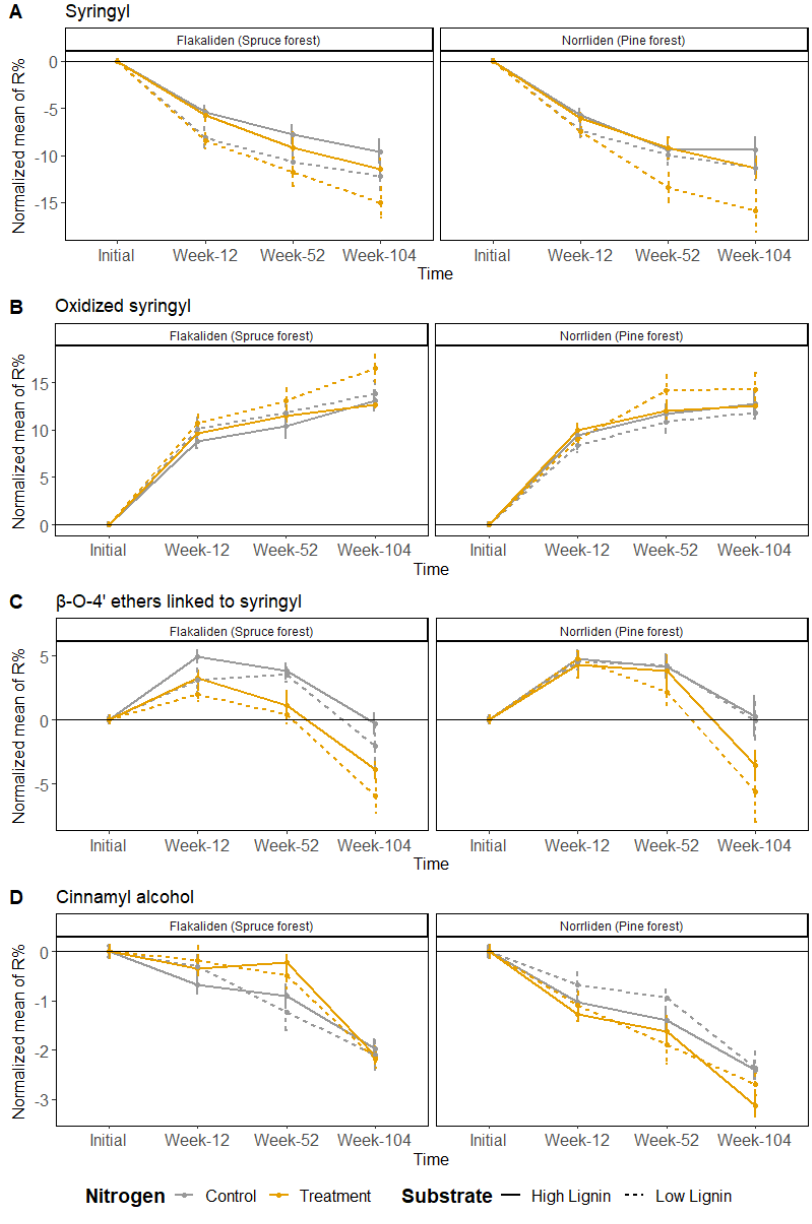


Figure 10. A-D depict the temporal dynamics of the average relative percentage of lignin linkages (n=12; initially n=8) in high and low lignin substrates across both fertilized and non-fertilized treatments at different forest stands (Flakaliden and Norrliden). Values are normalized by initial relative abundance. Week 12, week 52, and week 104 represent the duration that litterbags remained in the field for decomposition. Error bars indicate the standard error (±SE).

Pine and Spruce forest, Kulbäcksliden, Sweden

5. Conclusions

This study concludes that lignin content *per se* is not a critical factor in controlling the initial stages of plant material decomposition. Both laboratory and field incubation experiments demonstrated no significant differences in the decomposition of model substrates that differed in lignin content. This finding challenges the conventional view that lignin is highly resistant to decomposition during the early stages of plant material decomposition. Instead, this research underscores the importance of lignin composition, including the types of subunits and their linkages, as key determinants of lignin stability.

This study supports emerging paradigms suggesting an energetically coupled decomposition of lignin and carbohydrates. Both laboratory and field studies revealed contrasting effects of nitrogen addition over different timescales: short-term nitrogen inputs slowed overall decomposition, whereas long-term additions accelerated it. These results highlight the critical role of nitrogen addition in terms of both quantity and duration. Furthermore, laboratory study showed that short-term nitrogen addition preferentially increased the degradation of guaiacyl units and their associated linkages compared to syringyl units. This effect was attributed to structural shifts in microbial communities and their interactions, indicating that nitrogen's influence on plant material decomposition is mediated through changes in microbial composition and the functional roles different communities represents.

Meanwhile, field study demonstrated that while the degradation of lignin subunits was consistent across pine and spruce forest stands, the degradation of lignin linkages varied significantly. This suggests that although lignin degradation remains similar between forest types, the underlying mechanisms may differ. Investigating lignin chemistry on the molecular

level is crucial for understanding its decomposition dynamics, but is also very challenging. Overall, HSQC NMR provides a powerful tool for comprehensively investigating lignin degradation. It enables the identification of specific regions within the lignin polymer that are more susceptible to enzymatic degradation, distinguishing them from regions contributing to the stability and accumulation of SOM.

6. Future perspectives

This study, along with growing evidence in the emerging literature, strongly challenges the long-held notion that lignin is entirely resistant to degradation. Increasingly robust findings indicate that, under suitable conditions, appropriately adapted decomposer organisms can degrade lignin more efficiently than previously thought (Klotzbücher et al. 2011; Lehmann & Kleber 2015; Andlar et al. 2018; Ferrari et al. 2021; Atiwesh et al. 2022; Grgas et al. 2023). A key factor influencing lignin degradation is the chemical nature of its phenolic and non-phenolic structures, which are integral components of its complex polymeric framework. Microorganisms generally depolymerize phenolic lignin more readily, while the degradation of non-phenolic lignin requires small molecular weight agents known as mediators (Longe et al. 2018; Chan et al. 2019; Chen et al. 2021). These mediators facilitate electron transfer, enabling ligninolytic enzymes to catalyze the oxidation of non-phenolic lignin subunits indirectly. Additionally, studies (Bao et al. 1994; Kapich et al. 1999) have demonstrated that unsaturated fatty acids can function as mediators in the oxidation of non-phenolic lignin structures. Another structural factor influencing lignin degradation is the arrangement of its constituent units. For example, Lu et al. (2004) found that in woody plant like poplar, lignin precursors such as sinapyl alcohols were modified by *p*-hydroxybenzoylation at Cγ position. When lignin linkages break, these *p*-hydroxybenzoates can also be released from the polymer, becoming accessible for microbial uptake. These findings underscore the diverse mechanisms by which lignin is degraded.

The findings of this thesis challenge the conventional classification of lignin as a recalcitrant compound and carbohydrates as entirely easily degradable. Instead, this research highlights that lignin contains units that are relatively easy to degrade, much like carbohydrates. On the other hand,

certain components of carbohydrates, such as the crystalline cellulose, exhibit high resistance to degradation. However, the degradation pathways of these crystalline regions of carbohydrates remain poorly understood.

The early-phase decomposition of plant material is often considered a carbohydrate-regulated process, and this phase shows significant variability among the litters of different tree species. This variability may be attributed to the properties of carbohydrate-dominated fractions, but further research is needed to enhance our understanding of how this translates into soil organic matter accumulation. The question of why a significantly larger portion of carbohydrate polymers (e.g., cellulose) persists in the surface moor layer of boreal forests remains unanswered. While this thesis discounts the role of lignin in physically protecting carbohydrates during the early stages of decomposition, the possibility of such protection occurring in later stages cannot be ruled out. Furthermore, the chemical protection of carbohydrates by lignin remains poorly understood. The 2D NMR techniques employed in this thesis offer a promising approach to addressing these unresolved questions. A recent study by Nishimura et al. (2018) has combined various NMR techniques such as HSQC (Heteronuclear Single Quantum Correlation), TCOSY (Total correlation) and HMBC (Heteronuclear Multiple Bond Correlation) to provide direct evidence of lignin-carbohydrate bonds, which could help address longstanding hypotheses regarding lignin's role in protecting carbohydrates.

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Norway Spruce forest, Flakaliden, Sweden

Popular science summary

Soil holds an enormous amount of carbon, second only to oceans. In boreal forests, carbon is largely stored in the uppermost layer, known as the "moor layer". The secret to this carbon storage lies in the microbial breakdown of plant materials that takes decades or even centuries, transforming plant material into soil organic matter (SOM). This layer is rich in carbohydrates, which is also the building blocks of plant life. However, these carbohydrates are often intertwined with lignin, a tough, complex compound that acts like a protective shield. Lignin has long been thought to slow down decomposition by shielding plant material from microbial attack, thereby contributing to the long-term storage of carbon in soils. However, this research seek to question that assumption. By using model plant substrates that represent lignin from various boreal species this study aimed to explore whether lignin truly acts as a bottleneck in the early stages of decomposition. The substrates, with varying lignin content and composition, were incubated under controlled laboratory conditions and in the field. State-of-the-art technique like two-dimensional NMR spectroscopy was applied to investigate how lignin breaks down in boreal forest soils. The study found that the amount of lignin in plant material may not be the most crucial factor in determining how quickly it decomposes. Instead, the type of lignin, its specific structure and the way its subunits are linked together, play a more significant role.

In Paper I, model substrates were incubated under controlled laboratory conditions, revealing that lignin degraded at rates similar to carbohydrates. This finding challenges the conventional view that lignin is highly resistant to decomposition. Papers II and III explored the effects of nitrogen availability on decomposition and uncovered complex, time-dependent impacts. In the short term, increased nitrogen availability slowed overall

decomposition. However, decomposition accelerated with a long-term and continuous nitrogen input. Notably, different lignin components degraded depending on nitrogen availability, as nitrogen influenced microbial communities by altering the balance of fungi and bacteria. This shift affected how these microbes interacted with lignin and other plant material components. In Paper III, lignin decomposition patterns were consistent across pine and spruce forest stands, but the underlying mechanisms differed, underscoring the influence of forest ecosystem characteristics on decomposition processes. These findings reshape our understanding of how plant materials break down into SOM, highlighting the critical roles of lignin's molecular structure and microbial activity. This work also demonstrates how long-term environmental changes, such as increased nitrogen deposition, could significantly impact soil carbon storage.

Populärvetenskaplig sammanfattning

Mark innehåller en enorm mängd kol och det är bara världshaven som utgör ett större enskilt förråd. I boreala skogar lagras kolet till stor del i det översta marklagret. Hemligheten bakom denna kollagring ligger i den mikrobiella nedbrytningen av växtmaterial, som tar decennier eller till och med århundraden, och som omvandlar växtmaterial till markorganiskt material. Växtmaterial är rikt på kolhydrater, som också är byggstenarna i växters biomassa. Dessa kolhydrater är dock ofta sammanflätade med lignin, en komplex förening som kan fungera som en skyddande sköld. Nedbrytning av lignin har länge ansetts gå sakta och skyddar på så sätt växtmaterial från mikrobiella angrepp och bidrar därigenom till den långsiktiga lagringen av kol i marken. Forskningen som presenteras i denna avhandling syftar till att utmana detta antagande. Genom att använda växtsubstrat med varierande ligninninnehåll och sammansättning, inkuberade under kontrollerade laboratorieförhållanden och i fält, syftade studien till att klargöra om lignin verkligen fungerar som flaskhals i de tidiga stadierna av nedbrytningen. Avancerade tekniker som tvådimensionell NMR-spektroskopi användes för att undersöka hur lignin bryts ned i jordar i boreala skogar. Studien visade att mängden lignin i växtmaterial inte är den mest avgörande faktorn för att avgöra hur snabbt det bryts ned. Istället spelar typen av lignin, dess specifika struktur och hur dess olika kemiska enheter är sammanlänkade en viktigare roll.

I artikel I inkuberades modellsubstrat under kontrollerade laboratorieförhållanden, vilket visade att lignin bryts ned i samma takt som kolhydrater. Detta resultat utmanar den traditionella uppfattningen att lignin är mycket motståndskraftigt mot nedbrytning. I artikel II och III undersöktes effekterna av kvävetillgång på nedbrytningen och man fann komplexa, tidsberoende effekter. På kort sikt bromsade ökad kvävetillgång den totala

nedbrytningen. Men med långvarig, kontinuerlig kvävetillförsel accelererade nedbrytningen. Det var framför allt olika ligninkomponenter som bröts ned beroende på kvävetillgången, eftersom kvävet påverkade de mikrobiella samhällena genom att förändra balansen mellan svampar och bakterier. Detta skifte påverkade hur dessa mikrober interagerade med lignin och andra komponenter växtmaterialet. I artikel III var mönstren för nedbrytning av lignin desamma i tall- och granskogsbestånd, men de underliggande mekanismerna skilde sig åt, vilket understryker hur skogsekosystemets egenskaper påverkar nedbrytningsprocesserna. Dessa resultat omformar vår förståelse för hur växtmaterial bryts ned till SOM och hur kol lagras i marken. Resultaten belyser rollen av ligninets molekylära struktur och mikrobiell aktivitet i marken samtidigt som de visar hur långsiktiga miljöförändringar, såsom ökat kvävenedfall, kan påverka markens kollagring avsevärt.

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I

Plant Soil
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RESEARCH ARTICLE

Unraveling the dynamics of lignin chemistry on decomposition to understand its contribution to soil organic matter accumulation

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Abstract

Aims Plant inputs are the primary organic carbon source that transforms into soil organic matter (SOM) through microbial processing. One prevailing view is that lignin plays a major role in the accumulation of SOM. This study investigated lignin decomposition using wood from different genotypes of *Populus tremula* as the model substrate. The genotypes naturally varied in lignin content and composition, resulting in high and low lignin substrates.

Methods The wood was inoculated with fresh soil and decomposition was interpreted through mass loss and CO_2 produced during a 12-month lab incubation. Detailed information on the decomposition patterns of lignin was obtained by Two-dimensional Nuclear magnetic resonance (2D NMR) spectroscopy on four occasions during the incubations.

Results The lignin content per se did not affect the overall decomposition and ~60% of the mass was lost in both substrates. In addition, no differences in oxidative enzyme activity could be observed, and the rate of lignin decomposition was similar to that of the carbohydrates. The 2D NMR analysis showed the oxidized syringyl present in the initial samples was the most resistant to degradation among lignin subunits as it followed the order *p*-hydroxybenzoates > syringyl > guaiacyl > oxidized syringyl. Furthermore, the degradability of β–O–4 linkages in the lignin varied depending on the subunit (syringyl or guaiacyl) it is attached to.

Conclusions Our study demonstrates that lignin contains fractions that are easily degradable and can break down alongside carbohydrates. Thus, the initial differences in lignin content per se do not necessarily affect magnitude of SOM accumulation.

Keywords Soil organic matter · Decomposition · Lignin · 2D NMR

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Introduction

Soil organic matter (SOM) contains the largest pool of organic carbon in the biosphere second only to

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that found in the ocean. Approximately 2400 Gt of organic carbon is estimated to be stored in the top 3 m of soil with about 828 Gt of organic carbon stored in the top 30 cm (Beillouin et al. 2023; Friedlingstein et al. 2020; Lin et al. 2023; Stockmann et al. 2013). Plant biomass is the primary organic input to soils and subsequently transforms into SOM through microbial processing. Besides this, soil physical properties—such as porosity, permeability, and surface area—along with chemical properties, including nutrient availability, pH, cation exchange capacity, and organic matter content, also influence decomposition rates and the soil's capacity for carbon sequestration (Berg and McClaugherty 2020). The current view about SOM is that it is continuous in nature and consists of plant organic fragments of all molecular sizes at various stages of decomposition with a significant contribution from microbial biomass and necromass (Cotrufo et al. 2015; Lehmann and Kleber 2015; Liang et al. 2019). Therefore, understanding the decomposition and transformation of plant inputs is important for long term carbon storage (Prescott 2010; Cotrufo et al. 2015). However, the biogeochemical mechanisms that govern the long-term storage of carbon in soil remain unclear.

Plant biomass is composed of approximately 20–40% lignin, which has been considered challenging to degrade due to its intricate chemical structure (Zhu et al. 2017; Zoghlami and Paës 2019). Lignin in plants forms mainly through the polymerization of three monomers (also known as monolignols) that differ in their degree of methoxylation. After incorporation into lignin, these are referred to as syringyl (S), guaiacyl (G), and *p*-hydroxyphenyl/*p*-hydroxybenzoate (H) respectively (Adler 1977; Boerjan et al. 2003; Vanholme et al. 2010). During the lignification process, the monomers are connected through ether (C–O–C) or carbon-carbon (C–C) linkages. Among them, the most frequent linkage is the β–O–4 linkage while linkages such as β–5, β – β, 5–5, 5–O–4, and β – 1 exist to a lesser extent (Fig. 1) (Ralph et al. 2004; Brown and Chang 2014). To depolymerize such complex lignin structure, microbes use a broad range of extracellular enzymes like peroxidase and phenol oxidase, each employing distinct mechanisms. For instance, phenol oxidase catalyzes non-specific reactions, including the oxidation of Mn^{2+} and Fe^{2+}, which can lead to the depolymerization or transformation of lignin. In contrast, peroxidase can directly oxidize the carbon-carbon (C-C) bonds between lignin monomers, facilitating lignin breakdown through a more targeted approach (Sinsabaugh 2010; Janusz et al. 2017).

The traditional rationale offered for lignin being difficult to decompose is that the microorganisms tend

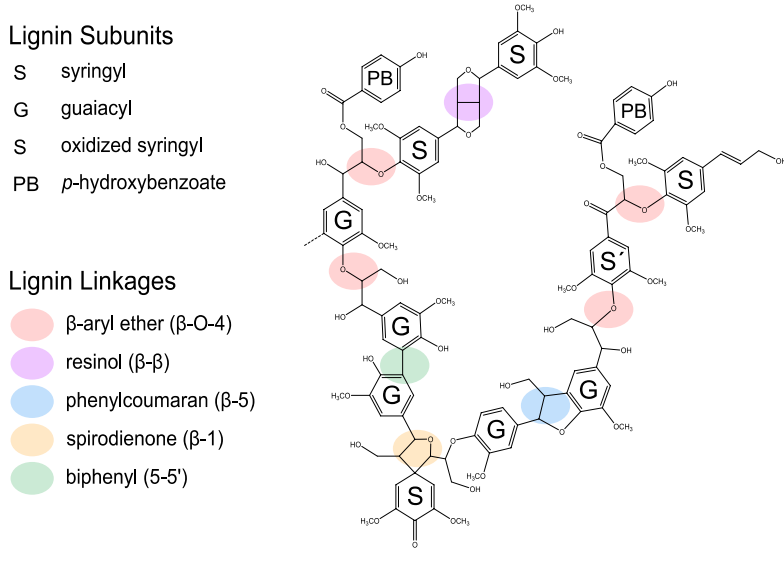

Fig. 1 An example of a lignin polymer. Different possible lignin subunits and lignin linkages are shown. The colors represent different lignin linkages that connect subunits. Note that the structure does not accurately represent the relative abundances of subunits and linkages

to receive a lesser amount of energy in comparison to the energy spent in producing the enzymes for lignin degradation (Schimel and Weintraub 2003; Moorhead and Sinsabaugh 2006; Herman et al. 2008). However, recent studies suggest that lignin degradation is triggered during the early stage of decomposition when the input of an easily decomposable fraction, carbohydrates is abundant. In this way, microbes will have sufficient energy to produce lignin-degrading enzymes (Klotzbücher et al. 2011; Lehmann and Kleber 2015; Angst et al. 2021). Lignin also acts as a binding material within the lignocellulose biomass of plants by being involved in the cross-linking of hemicellulose and cellulose to provide rigidity and stiffness to the cell wall (Boer et al. 2005; Datta et al. 2017). As a result, lignin could physically protect the more labile cell wall polysaccharides from microbial attack or the presence of covalent bonds between lignin and the labile cell wall polysaccharides could potentially protect hemicellulose and cellulose chemically from hydrolysis during the decomposition (Boerjan et al. 2003; Cadisch et al. 1997; Talbot et al. 2012). Thus, lignin could be the important control over the decomposition and transformation of plant inputs. However, the biogeochemical mechanisms by which saprotrophic decay of lignin occurs are still poorly understood.

One major reason for the lack of mechanistic understanding of lignin decomposition is the difficulties in analyzing lignin at adequate resolution. Pyrolysis – Gas Chromatography Mass Spectrometry (Py-GC/MS) is a technique used widely in characterizing different wood types in terms of polysaccharides and lignin-derived products. However, it gives information mainly on lignin derived monomers, leaving out lignin linkages that can vary in their degradation (Lv et al. 2022). Thus, the composition of linkages can be important for decomposition. For instance, syringyl is mostly associated with β-O-4 linkages that are more easily broken by oxidation than the phenylcoumaran (β- 5) and aryl-aryl linkages (5–5') that, in addition to β-O-4 linkages, polymerize guaiacyl (Fig. 1) (Kim and Ralph 2010). Other common methods such as ^{13}C Cross-Polarization Magic-Angle Spinning (CP-MAS) NMR and FTIR-spectroscopy used for OM characterization, lacks in resolution as they can detect only a few broad functional groups (Mao et al. 2017; Ong et al. 2020). However, the two dimensional ^{1}H-^{13}C Heteronuclear Single Quantum Coherence Nuclear magnetic resonance (2D HSQC NMR) is a state–of–art technique that has been increasingly applied in wood and lignin analysis. It has the advantage of analyzing complex organic matrices without extractions or chemical modifications (Kim and Ralph 2014) and results in much higher resolution by allowing for the identification of both lignin subunits and their associated linkages.

The aim of this study is to investigate the degradation of lignin during its initial decomposition stages. To achieve this we conducted a lab incubation experiment using four clones of *Populus tremula* (aspen wood) as model substrates. These clones were chosen as they naturally vary in both lignin content and composition. We hypothesized that the degradation of lignin should be lower than the carbohydrates and the high lignin substrates (HL) will provide more protection to labile plant polymers than in low lignin substrates (LL), thereby reducing the mass loss and carbon respired from the HL substrates. We also hypothesized that differences in lignin composition among the substrates influence its degradation. Therefore, the wood substrates with a higher abundance of syringyl and *p*-hydroxybenzoate units, would decompose faster than substrates relatively higher in guaiacyl.

Materials and methods

Sample properties

Populus tremula clones were used as model substrates for this decomposition study, as it is one of the most common tree species in boreal forests and is naturally rich in genetic variations due to nucleotide polymorphism. This variation significantly impacts the chemical composition of wood (Jalas and Suominen 1989; Escamez et al. 2023). The clones were obtained from the Skogforsk research station in Sävar (63.4°N, Umeå district) and are part of the SwAsp collection, which includes trees from 12 different localities in Sweden (Luquez et al. 2008). Four clones (Clone #: 35, 114, 91, 52) from the SwAsp collection, were chosen for their natural variation in lignin content and composition (Table S2). The wood material was taken from the branches of these clones, with the bark removed completely before further processing. The lignin: carbohydrate ratio was determined

by Py-GC-MS (refer Sect. 2.5). Clones 35 and 114 were categorized as low lignin substrates (LL) with a lignin content of 32 ± 0.6% (mean ± SE) and a carbohydrate content of 67 ± 0.6% (mean ± SE) and clones 91 and 52 were categorized as high lignin substrates (HL), with a lignin content of 41 ± 0.9% (mean ± SE) and a carbohydrate content of 58 ± 0.9% (mean ± SE).

The wood was then cut into small pieces and milled using a Tube mill control (IKA, Germany). The wood was milled to increase surface area, thereby enhancing the availability of sites for enzymatic attack. Although this pretreatment differs from in situ conditions and may affect decomposition rates, our substrate, serves as a conceptual proxy for lignin decomposability on a molecular level, even if it does not fully capture the diversity of lignin morphologies present in natural environments. The milled wood was lyophilized for 72 h and stored at room temperature until the start of the experiment.

Incubation experiment

The soil used in this study originated from the surface O-horizon (0–15 cm) of a boreal spodozol collected from the Kulbäcksliden experimental forest, near Vindeln in the county of Västerbotten, northern Sweden (64°11′N, 19°33′E). Spodzol cover large areas of the northern hemisphere and is thus representative for the boreal forest landscape. The site is a a mixed spruce (*Picea abies*) and pine (*Pinus sylvestris*) stand with *Vaccinium vitis idaea*, *Vaccinium myrtillus*, and *Pleurozium schreberi* as ground vegetation. The soil was transported to the lab and stored at 4 °C overnight after which the soil was homogenized by passing it through a cutting sieve (6×3.5 mm) in its field moist state. During the homogenizing process, needles, cones, visible roots, and other debris were removed. The soil was stored at -20 °C until the start of the experiment.

Organic matter (OM) and carbon (C) contents were determined on replicate subsamples ($n = 4$) of milled wood and homogenized soil. OM was determined by loss on ignition (LOI; 4 h at 550 °C), and organic C was determined using an Elemental Analyzer (Flash EA 2000, Thermo Fisher Scientific, Bremen, Germany). The OM content of the wood was 99.4 ± 0.1% (mean ± SE) with a negligible amount of minerals. The organic carbon and nitrogen contents were 49.7 ± 0.2% and 0.10 ± 0.006% (mean ± SE), respectively, for all wood substrates. For the soil, the OM content was 74.6 ± 0.44% (mean ± SE), with an organic C content of 45.1 ± 0.04% (mean ± SE) (Fig.S1) and organic nitrogen content of 1.38 ± 0.13% (mean ± SE). The soil pH was determined using soil-water suspension method with a soil to water ratio of 1:2, resulted in a pH of 4.5 ± 0.003 (mean ± SE).

The soil incubation experiment was conducted using a respirometer (A. Nordgren Innovations AB, Sweden) to measure CO_2 production hourly. Each incubation jar was equipped with a small vessel containing 10 mL of 0.5 M potassium hydroxide (KOH) and two platinum electrodes. The CO_2 produced during the incubation was trapped in the KOH solution and its production was measured by the induced change in electrical conductivity (Nordgren 1988). For the experiment, 2.55 g dry weight of soil was placed in each 250 mL incubation jar. The moisture content of the homogenized soil was set to -25 kPa, representing optimum moisture content for microbial activity (Ilstedt et al. 2000). Based on the previous litter mass loss studies (Berg et al. 1984; Berg and Ekbohm 1991; Wardle et al. 2003) and in-house incubation studies with boreal forest soils (Nordgren 1988, 1992; Nordgren et al. 1988), approximately 1.5 g dry weight of milled wood in a 160-micron nylon mesh bag (4 cm × 4 cm) was placed inside each jar along with the soil. The size of the nylon mesh bag was chosen to be above 10 µm to ensure unrestricted movement of microbial communities. The samples were incubated in an insulated water bath at 15 °C for 56 weeks, reflecting a typical summer temperature at the site from which the soil was taken. At each of the four sampling time points (12, 24, 36 & 56 weeks after the start of the experiment) 16 sample bags were removed from the incubation jars for analysis, with eight replicates each for both LL and HL substrates. Upon removal, the samples were taken out of the mesh bags and homogenized. Subsamples were then taken for the determination of mass loss (dry weight; 48 h at 80 °C), enzyme activities, and chemical characterization using 2D HSQC NMR and Py-GC/MS.

Enzyme assay

In addition to CO_2 production rates, we used enzyme assays to evaluate differences in the microbial

activity between HL and LL substrates. Specifically, we tracked oxidative enzymes phenol oxidase and peroxidase that are known to be associated with lignin oxidation and broadly reflecting microbial activity. Oxidative enzyme activity was estimated using the protocol from Bonner et al. (2019) that was modified from Saiya-Cork et al. (2002). Approximately 1 g (wet weight) of the decomposed wood substrate was added into two 15 mL falcon tubes. 4 mL of milli-Q water and 1 mL of 5mM L-DOPA (L-3,4-dihydroxyphenylalanine) were added. To one of the two tubes, 0.2 mL of 0.3% hydrogen peroxide was added. The tubes were manually shaken to homogenize and incubated in the dark at room temperature for 2 h. After incubation, the tubes were centrifuged at 3000 rpm for 2 min and the absorbance of each supernatant sample was determined at 460 nm using a spectrophotometer. The increase in L-DOPA oxidation induced by hydrogen peroxide addition was used to calculate peroxidase activity. The absorbance measured from the tube without the addition of hydrogen peroxide was used to calculate phenol oxidase enzyme activity. A concentration gradient of 180 µl of 5mM L-DOPA with 20 µl $NaNO_2$ and 10 µl of 1 M HCl was incubated overnight and a standard curve was plotted by reading the absorbance at 460 nm.

The measured absorbance was subtracted for blank (without L-DOPA) and negative controls (without wood material) and the concentration (mM) for each measured absorbance was determined from the standard curve. Furthermore, the concentration was multiplied by the assay volume (milli-Q water + L-DOPA + hydrogen peroxide) of 5.2 mL. The enzyme activity was normalized to the dry weight of wood used and incubation time, resulting in nmol $g^{-1}min^{-1}$.

NMR spectroscopy

Two dimensional ^{1}H-^{13} C Heteronuclear Single Quantum Coherence Nuclear magnetic resonance (2D HSQC NMR) was used to determine the changes in the lignin composition over time. This technique enabled the detailed analysis of lignin subunits and their associated linkages. Sample preparation was done according to the protocol described in Soucémarianadin et al. (2017). Briefly, 200 mg of freeze-dried wood material were ground in a 50 mL ZrO_2 jar with ten 10 mm ZrO_2 ball bearings using a Retsch PM100 planetary ball mill (Fritsch, Idar-Oberstein, Germany) for 5 × 10 min at 500 rpm with 10 min breaks. 50 mg of ball-milled wood sample was transferred to the NMR tube and dissolved in 600 µl of deuterated dimethyl sulfoxide (DMSO-d6). The sample was mixed thoroughly and left to stand overnight before NMR analysis.

HSQC correlates ^{1}H atoms with their directly bonded ^{13}C atoms through their one-bond J coupling, so that each C-H group gives rise to a cross peak (Fig. 2) at the respective ^{1}H and ^{13}C chemical shifts (in parts per million, ppm). Spectra were acquired on an 850 MHz Bruker Avance III HD spectrometer equipped with a 5 mm cryoprobe (TCI HCN) and automatic sample changer (SampleXpress), using the hsqcetgpsisp2.2 pulse sequence from the Bruker pulse sequence library. Spectra were collected at room temperature (298 K) using 40 scans. A recycle delay of 1 s was used and 128 points were collected in the indirect dimension. Each 2D experiment was approximately 2 h and the raw data were zero-filled to a 2048 × 1024 spectrum size. Spectral widths of 10 ppm (8504 Hz) and 165 ppm (35274 Hz) were used in the ^{1}H and ^{13}C dimensions respectively. Spectra were manually phase- and baseline-corrected and referenced using the residual DMSO peak at 40.0/2.50 ppm. Data were processed with Topspin 3.2 (Bruker BioSpin Corporation, Billerica, USA).

Identification of lignin moieties

The HSQC spectrum was divided into the following regions: aromatic, di-O-alkyl, O-alkyl, and alkyl. To follow the changes in lignin structure, ten peaks in the 2D HSQC spectra were assigned to different lignin moieties and quantified (Table 1). Assignments were based on previously published data (Kim et al. 2008; Del Río et al. 2012). The peaks related to lignin subunits and linkages were identified and integrated into the aromatic and O-alkyl regions (Fig. 2). In Poplar lignin, *p*-hydroxyphenyl content is very low and not detected. Instead, the *p*-hydroxybenzoate groups are present and it is linked to the γ-position of either S or G units (Smith 1955; Goacher et al. 2021).

The relative abundance of each moiety was determined from the specific grouped sum of cross-peak integrals for each sampling time point. Therefore, the sum of subunits was 100% and the sum of linkages

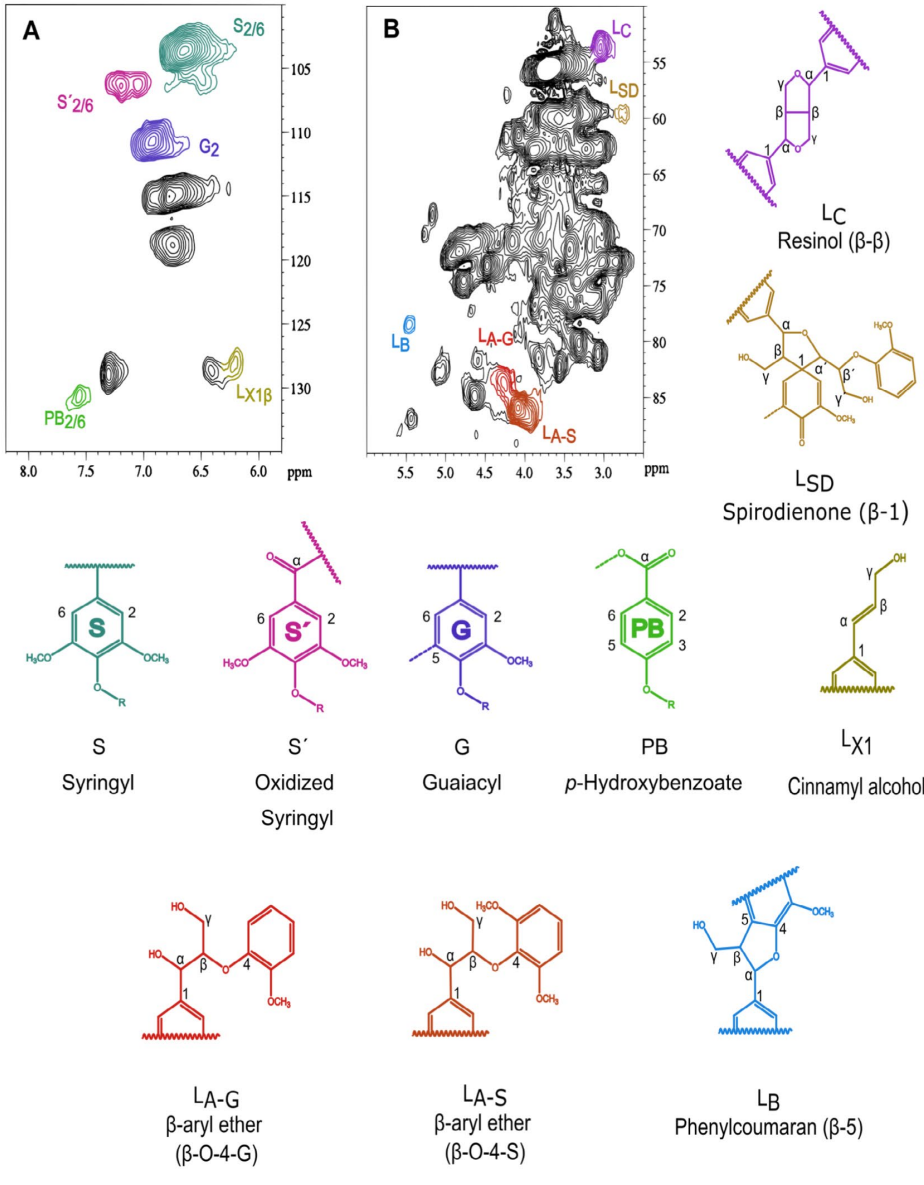

Fig. 2 Peak assignments in the aromatic (**A**) and O-alkyl (**B**) regions of the HSQC spectrum of the undecomposed aspen wood DMSO-extract. Color-coding represents, the chosen lignin linkages and lignin subunit moieties for this study and their corresponding chemical structure shown using respective colors and labeling

Table 1 Assignments of HSQC signals to the lignin linkages and lignin subunit moieties. Each cross peak in the spectrum resulted from the correlation between ^{1}H and ^{13}C, separated by one bond. The ^{1}H and ^{13}C chemical shifts of moieties are shown in parts per million (ppm)

Assignments	δ ^{1}H (ppm)	δ ^{13}C (ppm)
Lignin subunits		
$C_{2,6}$-$H_{2,6}$ in syringyl units	6.65	103.7
$C_{2,6}$-$H_{2,6}$ in C_{α}-oxidized syringyl units	7.19	106.2
C_2-H_2 in guaiacyl units	6.92	110.7
$C_{2,6}$-$H_{2,6}$ in *p*-hydroxybenzoates	7.64	131.2
Lignin linkages		
C_{β}-H_{β} in resinol	3.03	53.5
C_{β}-H_{β} in spirodienone	2.75	59.5
C_{β}-H_{β} in β-O-4' ethers linked to guaiacyl	4.26	83.5
C_{β}-H_{β} in β-O-4' ethers linked to syringyl	4.08	85.8
C_{α}-H_{α} in phenylcoumaran	5.43	86.9
C_{β}-H_{β} in cinnamyl alcohol	6.20	127.9

was 100%. In this way, we see the abundance of these moieties over time. A decreasing trend of the relative abundance of a certain moiety as a function of sampling time meant that it degraded to a larger extent than other moieties included within the same group. Likewise, an increasing trend implies less degradation relative to other moieties within the same group. In cases where several peaks could be used to track the same sub-structure, the best-resolved peak was chosen (Table 1).

Pyrolysis gas-chromatography mass spectrometry (Py-GC/MS)

To determine the differences in the lignin contents of the model substrates, milled wood from the four clones (see above) was analyzed by Py-GC/MS for their lignin: carbohydrate ratios. This was repeated at the end of the experiment to evaluate the extent to which the polymeric groups had decomposed in relation to each other. Approximately 75 µg of freeze-dried sample was transferred to auto sampler containers (Eco-cup SF, Frontier Laboratories, Japan). The Py-GC/MS setup consisted of an oven pyrolyzer with an auto sampler (PY-2020iD and AS-1020E, FrontierLabs, Japan) connected to a GC/MS system (Agilent, 7890 A-5975 C, Agilent Technologies AB, Sweden). The setup of pyrolysis GC/MS conditions was similar to (Gerber et al. 2012). The pyrolyzing temperature was set at 450 °C. The temperatures of the pyrolysis GC interface and GC injector were at 340 °C and 320 °C respectively. Helium was used as the carrier gas with a split ratio of 16:1. The gas saver mode was activated with a flow rate of 3 mL min^{-1} after one minute to vent away the pyrolysate remaining in the pyrolyzer oven. DB-5MS capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness; J&W, Agilent Technologies AB, Sweden) was used to separate the pyrolysate. The GC temperature was increased from 40 °C to 320 °C with a rate of 10 °C min^{-1}. The GC/MS interface was maintained at 300 °C. The mass spectrometer operated at unit mass resolution with a quadrupole type analyzer and the mass range was scanned from m/z 30 to 500 at 3.1 scan s^{-1}. For ionization, 70 eV electron bombardment was used (Tolu et al. 2015).

From the Agilent Chemstation Data Analysis (Version E.02.00.493), the raw data were exported to NetCDF and proceeded in R (version 2.15.2, 64 bits) similar to the processing method specified in (Jonsson et al. 2005; Tolu et al. 2015). Alternate regression (MCR-AR) is used to process the data that involves chromatogram smoothing and alignment, background correction, and multivariate curve resolution (Karjalainen 1989). Then for each sample, a data table with peak areas and a data text file with mass spectra for each peak were obtained. The latter was imported into the mass spectra library software 'NIST MS Search 2.0' to annotate the peaks based on spectra from the library 'NIST/EPA/NIH 2011'. In total, 55 peaks were annotated, excluding CO_2. The annotated peaks were categorized either carbohydrates or lignin (guaiacyl, syringyl and phenols) (Supplementary Table S1). The relative percentage of carbohydrates and lignin were then determined by normalizing to the total sum of peak areas for each sample.

Statistical analysis

The difference in mass loss between the HL and LL substrates after 56 weeks and the initial difference in the lignin: carbohydrates ratio between HL and LL substrates were determined using the unpaired two-sample t-test. Both the assumptions of normality and homogeneity of variance were satisfied. To

investigate if the difference in lignin abundance of the substrates significantly affected the increase in total accumulated CO_2-C over time and lignin: carbohydrates ratio determined from pyrolysis products, we conducted two way repeated measure Analysis of Variance (ANOVA). The normality assumption of data was satisfied but the variance of the differences between groups was not equal (assumption of sphericity). To solve this, the p-values were adjusted using Greenhouse-Geisser and Huynh-Feldt corrections (Girden 1992). The difference in the enzyme activities for the HL and LL substrates and the difference between the phenol oxidase and peroxidase enzyme activity among substrates were determined using three-way repeated measure ANOVA. The assumption of normality was satisfied and the p-values were adjusted for violation of the assumption of sphericity.

The relative percentage of each molecular moiety was analyzed for the difference in decomposition between the HL and LL substrates by using Linear mixed effects (LME) models. Using the lme4 package in R (Bates et al. 2015), LME models were performed by considering the different starting values of four clones individually as the random factor. *p*-values of the fixed factors (HL & LL substrates) were approximated by F-test using Type II ANOVA tests with Kenward-Roger Degrees of Freedom using the "car" and "lmerTest" packages in R (Kuznetsova et al. 2017; Kenward and Roger 1997). The significance of random effects was tested using the maximum likelihood ratio test (Zuur et al. 2009). If the random effects were not significant then two way repeated measure ANOVA was performed. The post hoc test of pairwise comparison was performed using emmeans packages in R. The *p*-values were adjusted using the Tukey method (Searle et al. 1980). All statistical analysis was performed with R environment software v.4.0.1(R Core Team 2021).

Results

Overall decomposition of model substrates

The overall decomposition rate of our substrates was interpreted through mass loss and the total CO_2-C produced during the incubations. The total CO_2-C generated from the soil in the absence of substrates over 56 weeks was 53 ± 0.7 mg (mean $\pm$ SE). After accounting for this, the decomposition of HL and LL substrates resulted in 466 ± 29 mg and 443 ± 34 mg of CO_2-C, respectively, representing $62 \pm 4\%$ and $59 \pm 4\%$ of carbon lost from the substrates, with no significant difference between them ($p = 0.55$)

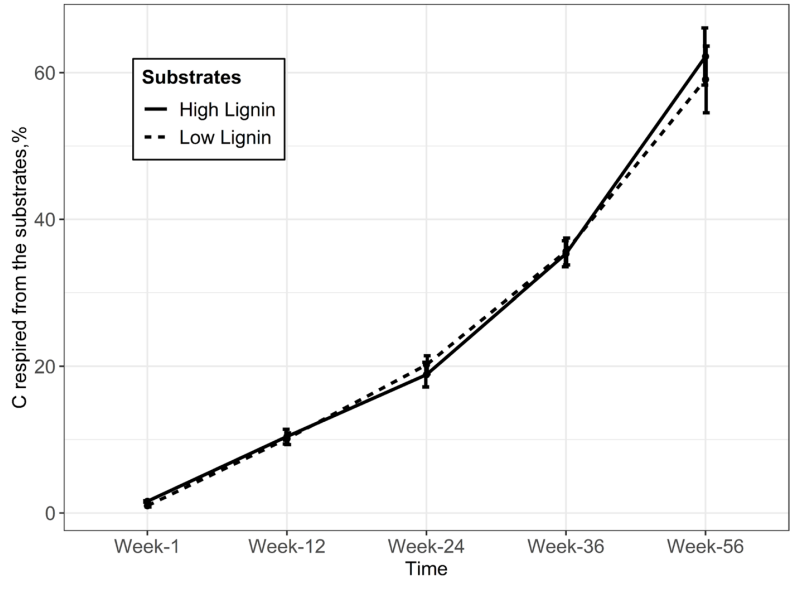

Fig. 3 Average percentage (relative to C content of added substrate, $n = 8$) of carbon respired from high and low lignin substrates as function of incubation time. The error bars represent standard error ($\pm$SE)

(Fig. 3). Similarly, the total mass loss for HL and LL substrates was 59 ± 5% and 50 ± 5%, respectively, also showing no significant difference ($p = 0.23$). Furthermore, carbon loss through mass reduction and CO_2-C respiration remained statistically similar for both the substrates ($p = 0.24$). The enzyme activity did not differ significantly neither between peroxidase and phenol oxidase activity ($p = 0.28$) nor between HL and LL substrates ($p = 0.41$). By the end of week 56, the peroxidase activity for HL and LL substrates was 69 ± 10 nmol g^{-1} min^{-1} (mean ± SE) and 100 ± 10 nmol g^{-1} min^{-1} (mean ± SE) respectively. During the same period, the phenol oxidase activity for HL and LL substrates was 57 ± 14 nmol g^{-1} min^{-1} (mean ± SE) and 77 ± 15 nmol g^{-1} min^{-1} (mean ± SE) respectively (Fig. 4).

The difference in the lignin: carbohydrates ratio (determined by Py-GC/MS) among our substrates at the start of the incubation was statistically significant ($p < 0.001$). The mean lignin: carbohydrates ratio of HL substrates was 0.72 ± 0.02 (mean ± SE) and 0.48 ± 0.02 (mean ± SE) for the LL substrates. By the end of week 56, the mean lignin: carbohydrates ratio of HL substrates was 0.57 ± 0.01 (mean ± SE) and 0.58 ± 0.05 (mean ± SE) for the LL substrates (Fig. 5). The lignin: carbohydrates ratio had a significant interactive effect between substrates and time ($p < 0.05$). Relatively more lignin was decomposed compared to carbohydrates in the HL substrates, while lignin and carbohydrates decomposed at equal rates in the LL substrates (Fig, S2, S3 and S4).

Decomposition of lignin subunits and linkages

The changes in the relative abundance of lignin subunits and lignin linkages across different time points were statistically significant (Figs. 6 and 7; $p < 0.0001$). Within the lignin subunits, the relative abundance of syringyl decreased significantly ($p < 0.0001$ for HL and LL substrates) during the period from 24 − 56 weeks (Fig. 6a). However, the relative abundance of oxidized syringyl increased significantly ($p < 0.0001$ for HL and LL substrates) during the weeks 12–36 (Fig. 6d). For the guaiacyl, the relative abundance did not change significantly until the week 36 ($p = 0.07$ for HL and LL substrates) (Fig. 6b).

Within the lignin linkages, the relative abundance of β-O-4 linked to guaiacyl increased significantly ($p < 0.0001$ for HL substrates; $p < 0.001$ for LL substrates) during the weeks 12–56 (Fig. 7b). In contrast, the relative abundance of β-O-4 linked to syringyl did not change significantly ($p = 0.15$ for HL substrates; $p = 0.09$ for LL substrates) during this time

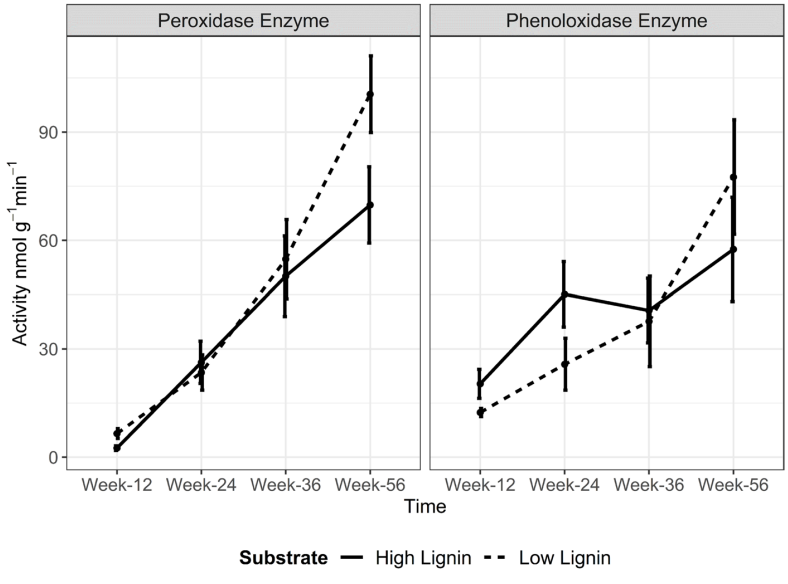

Fig. 4 The average ($n = 8$) peroxidase and phenol oxidase activities for the high and low lignin substrates. The error bars represent standard error (± SE)

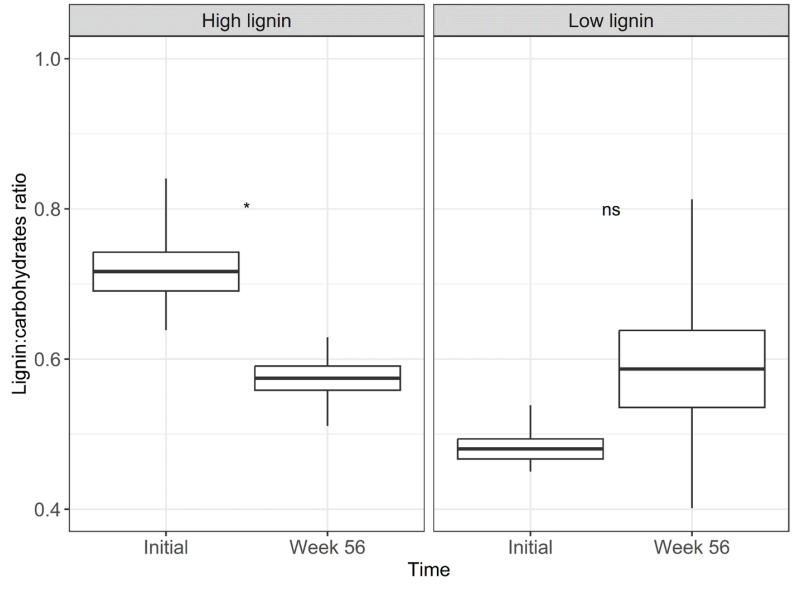

Fig. 5 Lignin: Carbohydrates ratio of pyrolysis products from high and low lignin substrates for the initial, undecomposed, substrates and after week 56 of incubation. The boxplot shows means ($n=8$) and standard errors (±SE). The sign (*) indicates p-value < 0.05 and (ns) indicates statistical non-significance

period (Fig. 7a). For the first 12 weeks, the relative abundance of β-O-4 linked to syringyl increased significantly ($p<0.001$ for HL substrates; $p<0.01$ for LL substrates) (Fig. 7a). During the same time, the relative abundance of resinol linkages decreased significantly ($p<0.01$) for HL substrates but not for LL substrates ($p=0.29$) (Fig. 7c). For the cinnamyl alcohol linkages, the relative abundance decreased significantly for LL substrate ($p<0.05$) but not for HL substrates ($p=0.43$) during the weeks 12–36 (Fig. 7d). In addition, the relative abundance of spirodienone linkages decreased ($p<0.0001$ for HL and LL substrates) significantly during the weeks 12–36 (Fig. 7f). Finally, for the phenylcoumaran linkages, the relative abundance increased significantly until week 56 ($p<0.001$ for HL and LL substrates) (Fig. 7d).

The random intercept LME model was employed to address the initial variations in the relative abundance of lignin linkages and subunits among the substrates. After controlling for the initial randomness, the decomposition patterns of β-O-4 linked to syringyl ($p=0.15$), β-O-4 linked to guaiacyl ($p=0.39$), and phenylcoumaran ($p=0.10$) linkages showed no significant differences between HL and LL substrates (Fig. 7a, b & d). The decomposition patterns of resinol ($p<0.05$) and cinnamyl alcohol linkages ($p<0.05$) were significantly different between HL and LL substrates (Fig. 7c & e) but not for the spirodienone linkages ($p=0.06$) (Fig. 7f). Similarly, for the lignin subunits, there was no significant difference between HL and LL substrates in the decomposition pattern of syringyl ($p=0.09$), guaiacyl ($p=0.39$), *p*-hydroxybenzoate ($p=0.83$), and oxidized syringyl ($p=0.12$) units (Fig. 6). However, there was a significant interaction effect between the substrate type and time on the abundance of syringyl ($p<0.05$) and *p*-hydroxybenzoate ($p<0.05$). The relative abundance of syringyl decreased for HL substrates between weeks 36–56 ($p<0.001$). However, for the LL substrates, the relative abundance of syringyl did not significantly change during the same period (Fig. 6a). In the same way, the relative abundance of *p*-hydroxybenzoate decreased significantly for HL substrates ($p<0.0001$) from initial until week 56, but for the LL substrates, the relative abundance of *p*-hydroxybenzoate did not change significantly ($p=0.77$) during the same period (Fig. 6c).

Discussion

This study investigated lignin degradation during its initial decomposition phase using different high lignin (HL) and low lignin (LL) genotypes aspen wood as a model substrate. Our results suggest that the

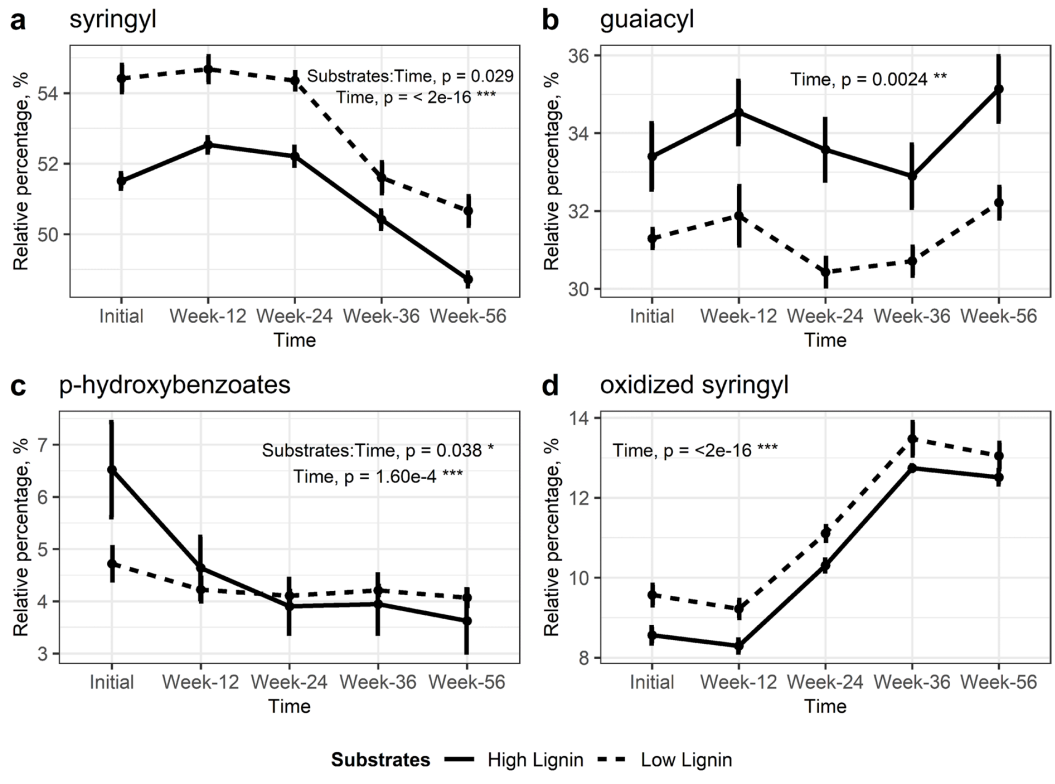

Fig. 6 Temporal dynamics of average ($n = 8$) relative percentage of lignin subunits in the high and low lignin substrates (**a**-**d**). The error bars represent standard error (± SE). The sign (*) indicates statistical significance

difference in lignin content and composition did not impact the decomposition of model substrates as CO_2 production, mass loss, and oxidative enzyme activity between our model substrates were similar (Figs. 3 and 4 & S5). Furthermore, the lignin degraded relatively more than carbohydrates for the HL substrates and was on par with the degradation of carbohydrates for the LL substrates (Fig. 5). This suggests that the potential for lignin degradation during the decomposition is high, Similar results of active degradation of lignin during the early decomposition phase were shown by He et al. (2019) in a 180 days laboratory incubation experiment using leaf litter from two plant species *Fagus lucida* and *Schima parviflora.* In addition, there is an increasing amount of laboratory and field studies suggesting that lignin is not particularly resistant to microbial decomposition during its initial stages of the decomposition (Huang et al. 2023; Kalbitz et al. 2006; Klotzbücher et al. 2011; Miltner and Zech 1998; Yue et al. 2016).

The observation that CO_2 production and mass loss are similar suggests the absence of significant physical constraints induced by the lignin on the microbial access to the cell wall components of e.g. polysaccharides. This is also supported by Talbot et al. (2012) who concluded that physical protection of cell wall polysaccharides by lignin appeared to play a smaller role than the chemical protection of the decay of the plant material. They used litter from *Arabidopsis thaliana* that was genetically modified to differ in guaiacyl content, as compared to the wild type. Litter from the high guaiacyl plant lost polysaccharides at a similar rate as compared to the wild type, which led to their similar decay rates. Likewise, in our study, we observed lignin degradation to be more similar to that of carbohydrates for the model substrates. Thus, we

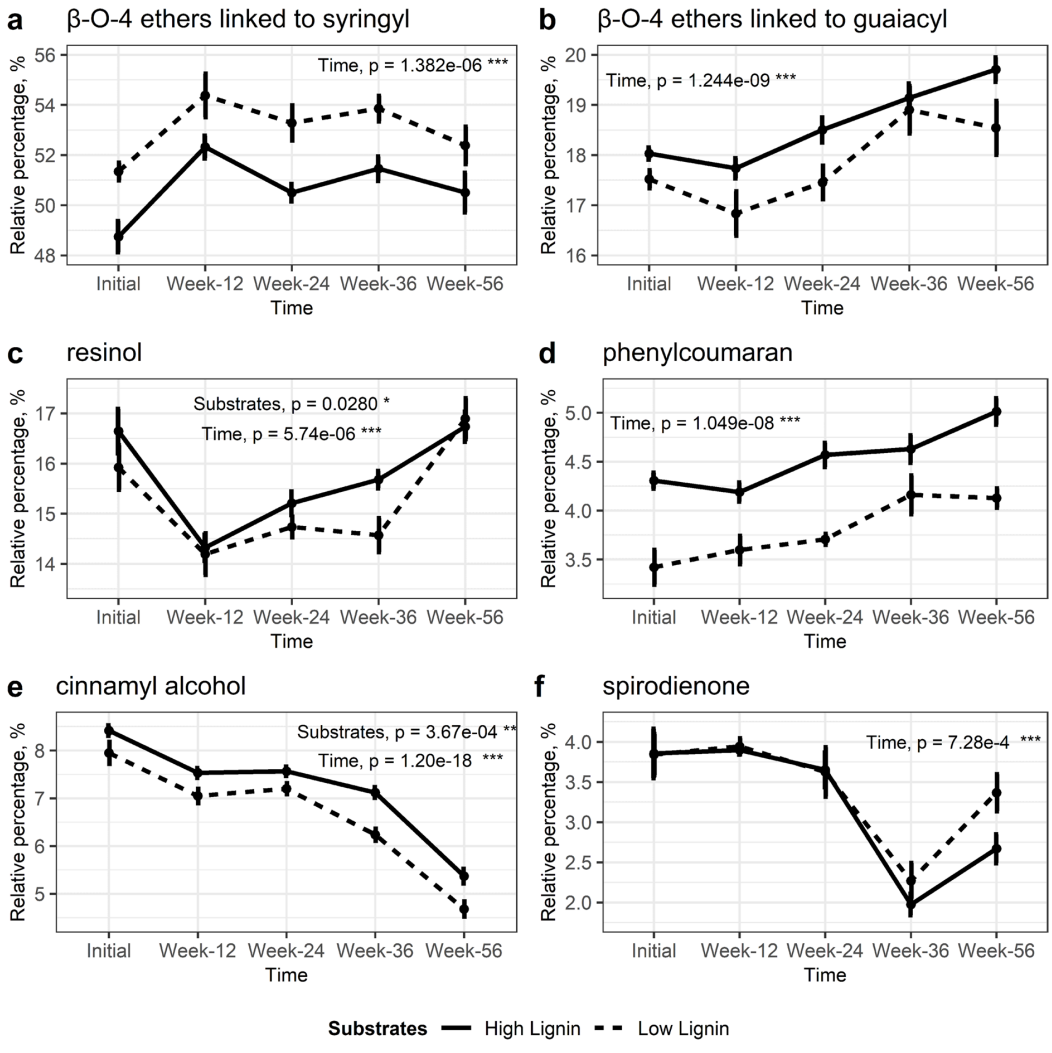

Fig. 7 Temporal dynamics of average ($n = 8$) relative percentage of lignin linkages in the high and low lignin substrates (**a**-**f**). The error bars represent standard error (±SE). The sign (*) indicates statistical significance

can reject our hypothesis that lignin degradation proceeds slower than carbohydrates.

During decomposition, the reduction in the abundance of syringyl was greater than that of guaiacyl (Fig. 6a & b). This is consistent with several studies that have reported a decrease in syringyl relative to guaiacyl during the decomposition of various plant litters (Otto and Simpson 2006; Pisani et al. 2015; Strukelj et al. 2012; Thevenot et al. 2010; vandenEnden et al. 2018). Because the HL substrates contained more lignin initially, we hypothesized HL substrates to retain more lignin during the incubation. This was partially true as the changes in the lignin chemistry over the course of the experiment, between HL and LL substrates were similar except for the *p*-hydroxybenzoate. The relative abundance of *p*-hydroxybenzoate decreased faster in HL substrates compared to LL substrates (Fig. 6c). This implies that

similar to the *p*-hydroxyphenyl (H-lignin) (Campo et al. 2019; Goñi et al. 1993; Otto and Simpson 2006; Soucémarianadin et al. 2017; vandenEnden et al. 2018), *p*-hydroxybenzoate also degrades faster and belong to faster-decomposing lignin fraction, alongside syringyl, as compared to guaiacyl.

Interestingly, a small proportion of syringyl was found to be in its oxidized form (Cα-oxidation) in our model substrates and its fraction increased significantly during decomposition (Fig. 6d). This indicates that oxidized syringyl was more resistant to degradation compared to any of the other lignin sub-units. Studies have shown that Cα-oxidation deactivates the aromatic ring from undergoing electrophilic substitution that determines their subsequent reactions after enzyme oxidation (Kirk et al. 1986; Kawai et al. 2002). Therefore, Cα-oxidation would suppress the degradation of lignin further (Van Erven et al. 2019; Chen et al. 2021). Moreover, microbes depolymerize lignin using various peroxidases and phenol oxidase enzymes that lead to Cα-Cβ cleavage, Cα-oxidation, β-ether cleavage, or aromatic ring cleavage reactions. However, the increase in Cα-oxidation fraction was mainly due to the relative decrease in the abundance of other subunits and there was no clear evidence of additional formation of oxidized syringyl (Cα-oxidation) resulting from the de-polymerization of lignin.

The differences in the degradation of lignin subunits can be attributed to the different stability of various linkages that these units form (Talbot et al. 2012). The β–O–4 linkages are more associated with syringyl than with guaiacyl (Kim and Ralph 2010) and they are considered more easily degradable than other lignin linkages. Moreover, because of the availability of the C5 position for the formation of linkages such as β–5, 5–5, and 5–O–4, guaiacyl was more resistant to degradation (Boerjan et al. 2003). This is in line with our results as we found β–O–4 linked to syringyl linkages decreased in abundance as compared to guaiacyl's phenylcoumaran (β–5) linkages (Fig. 7a & d). In addition, the linkage β–O–4 linked to guaiacyl was more resistant to microbial decomposition than β–O–4 linked to syringyl (Fig. 7a & b). Thus, the degradability of β–O–4 linkages depends on the degradability of the respective subunits to which they are attached. Along with β–O–4 linkages, a decrease in the abundance of other minor linkages such as resinol, cinnamyl alcohol, and spirodienone was also evident in our samples (Fig. 7c, e & f). During the initial phase of decomposition (< 12 weeks), the abundance of resinol (β– β) linkages, which is more resistant than β–O–4 linkages decreased faster compared to that of β–O–4 linked to syringyl linkages (Fig. 7a & c). Thus, our results suggests other unknown factors also regulates lignin linkage stability.

A common challenge in lignin degradation studies is the difficulty in generalizing results, as significant variations often occur among studies (Thevenot et al. 2010; Polman et al. 2021). These variations can be attributed to differences in e.g. the source and type of plant material and soil properties. Different plant species exhibit distinct lignin content and composition, particularly in the subunits that make up lignin and the distribution of linkages between them (Lourenço and Pereira 2018). For instance, in gymnosperms like pine and spruce, lignin is predominantly composed of guaiacyl (G-units), with minor amounts of *p*-hydroxyphenyl (H-units). In contrast, angiosperm dicots have lignin that mainly consists of guaiacyl (G-units) and syringyl (S-units). Softwood tends to have a higher abundance of H-units, with slightly elevated levels also found in grasses (Vanholme et al. 2010). Moreover, different parts of plants, such as internodes, leaves, and roots, exhibit variations in the presence of condensed lignin structures (where lignin units are attached at the C2, C3, C5, or C6 positions of adjacent units) and uncondensed structures (where these connections are absent) (Bertrand et al. 2006; Machinet et al. 2009). Various biotic and abiotic factors further impact lignin polymerization in plants (Moura et al. 2010; Liu et al. 2018; Renström et al. 2024), which in turn can be reflected in the overall rate of lignin degradation.

In our model substrates, the degradation patterns of lignin moieties were consistent between the HL and LL substrates. The composition of our model lignin in terms of S, G, and H-type subunits, is representative of the lignin found in a range of boreal plant species, though the relative distribution of these subunits may vary among species. On a molecular level, this distribution provides an effective conceptual proxy across diverse plant materials, offering a reliable framework for studying lignin degradation. Additionally, under low pH conditions like in our study, lignin can undergo non-enzymatic oxidation by hydroxyl radicals produced via the Fenton reaction, which oxidizes Fe^{2+} to Fe^{3+} (Jung et al. 2009; Shah et al. 2015; Op De Beeck

et al. 2018). It is also possible that other soil types with varying mineral compositions could exhibit different behaviors due to stronger interactions between mineral constituents and organic matter, potentially exerting greater control over decomposition (Cotrufo et al. 2013, 2015; Huang et al. 2019). However, irrespective of the specific degradation mechanisms or the structural variations within lignin, HSQC NMR can be applied to investigate the resistance of different lignin subunits and linkages that determine the lignin degradation rate.

Conclusions

HSQC NMR offers the unique possibility to investigate the degradation of lignin comprehensively, involving not just the various subunits of the lignin polymers but also the different polymerizing linkages. This technique also allows for the identification of sections within the lignin polymer that are more susceptible to enzymatic degradation, distinguishing them from those that contribute significantly to the stability and accumulation of SOM. Our results contradict the more traditional view on lignin degradation as it occurred at rates similar to that of carbohydrates. Instead, it lends support to the recent paradigms of energetically coupled decomposition of lignin and carbohydrates. Our findings emphasize that the type of subunits and their linkages determine lignin stability. Furthermore, our study reveals that lignin contains fractions that are easily degradable and can break down alongside carbohydrates, suggesting that initial differences in lignin content alone do not necessarily contribute to the magnitude of SOM accumulation.

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Data availability Data will be made available on request.

Declarations

Competing interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supporting information

Article title: Unraveling the dynamics of lignin chemistry on decomposition to understand its contribution to soil organic matter accumulation.

Table S1 A list of pyrolysates with their corresponding retention time for the model substrates.

S.no	Compound	Retention time (Sec)	Category
1	Propanal-2-one	60.1	Carbohydrates
2	Acetic acid	70.7	Carbohydrates
3	2-Butenal, (E)-	92.9	Carbohydrates
4	2-Propanone, 1-hydroxy-	96.7	Carbohydrates
5	2,3-Butanedione	130.4	Carbohydrates
6	2-Propenoic acid methyl ester	138.5	Carbohydrates
7	2-Methylfuran	140.4	Carbohydrates
8	3-Butenal-2-one	142.6	Carbohydrates
9	(3H)-Furan-2-one	151.6	Carbohydrates
10	Propanoic acid, 2-oxo-, methyl ester	159.9	Carbohydrates
11	(2H)-Furan-3-one	169.2	Carbohydrates
12	3-Furaldehyde	180.2	Carbohydrates
13	2(5H)-Furanone, 3-methyl-	184.9	Carbohydrates
14	Furfural	193.9	Carbohydrates
15	2-Furanmethanol	210	Carbohydrates
16	Dihydro-methyl-furanone	215.8	Carbohydrates
17	4-Cyclopentene-1,3-dione	235	Carbohydrates
18	gamma-Lactone derivative	242.7	Carbohydrates
19	2(5H)-Furanone	258.2	Carbohydrates
20	1,2-Cyclopentanedione	270.4	Carbohydrates
21	2(5H)-Furanone, 5-methyl-	282.2	Carbohydrates
22	Isomer of 4-Hydroxy-5,6-dihydro-(2H)-pyran-2-one	307.3	Carbohydrates

S.no	Compound	Retention time (Sec)	Category
23	2H-Pyran-2,6(3H)-dione	333.6	Carbohydrates
24	3-Methylcyclopentane-1,2-dione	363.4	Carbohydrates
25	2-Furoic acid methyl ester	417.9	Carbohydrates
26	2,5-Dimethyl-4-hydroxy-3(2H)-furanone	421.2	Carbohydrates
27	2H-Pyran-2-one, 5,6-dihydro-	436.9	Carbohydrates
28	Maltol	444.3	Carbohydrates
29	2(3H)-Furanone, dihydro-4-hydroxy-	485	Carbohydrates
30	4H-Pyran-4-one, 3,5-dihydroxy-2-methyl-	508.5	Carbohydrates
31	1,4:3,6-Dianhydro-α-d-glucopyranose	537.9	Carbohydrates
32	5-Hydroxymethylfurfural	546.9	Carbohydrates
33	Levoglucosan	765.2	Carbohydrates
34	Phenol, 2-methoxy-	422.5	Lignin
35	3-methoxy-catechol	574.9	Lignin
36	Phenol, 4-ethyl-2-methoxy-	589.9	Lignin
37	2-Methoxy-4-vinylphenol	621.1	Lignin
38	Eugenol	655.1	Lignin
39	Vanillin	691.7	Lignin
40	Phenol, 2-methoxy-4-(1-propenyl)-	730.9	Lignin
41	Phenol, 2-methoxy-4-propyl-	735.7	Lignin
42	6-Methoxy-3-methylbenzofuran	755.6	Lignin
43	Guaiacylacetone	788.6	Lignin
44	4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol	936.6	Lignin
45	Phenol	320.4	Lignin
46	p-Cresol	409.6	Lignin
47	Creosol	516.2	Lignin
48	Phenol, 2,6-dimethoxy-	649.7	Lignin
49	3,5-Dimethoxy-4-hydroxytoluene	724.5	Lignin
50	Phenol, 2,6-dimethoxy-4-(2-propenyl)-	838.4	Lignin
51	Benzaldehyde, 4-hydroxy-3,5-dimethoxy-	880.7	Lignin
52	(E)-2,6-Dimethoxy-4-(prop-1-en-1-yl)phenol	909.6	Lignin

S.no	Compound	Retention time (Sec)	Category
53	Acetosyringone	929.5	Lignin
54	Sinapyl alcohol (trans)	1044.4	Lignin
55	trans-Sinapaldehyde	1086.4	Lignin

Table S*2* Information about each of the parental trees of the clones: place of origin, longitude and latitude, altitude of the place where they grow and estimated age.

Clone number	Lignin content	Place	Lattitude	Longitude	Altitude (m)	Estimated Age (years)
35	Low	Ydre	57,8	15,3	180	15
114	Low	Luleå	65,6	22,4	10	70
91	High	Umeå	63,9	20,6	45	60
52	High	Uppsala	59,8	17,8	10	50

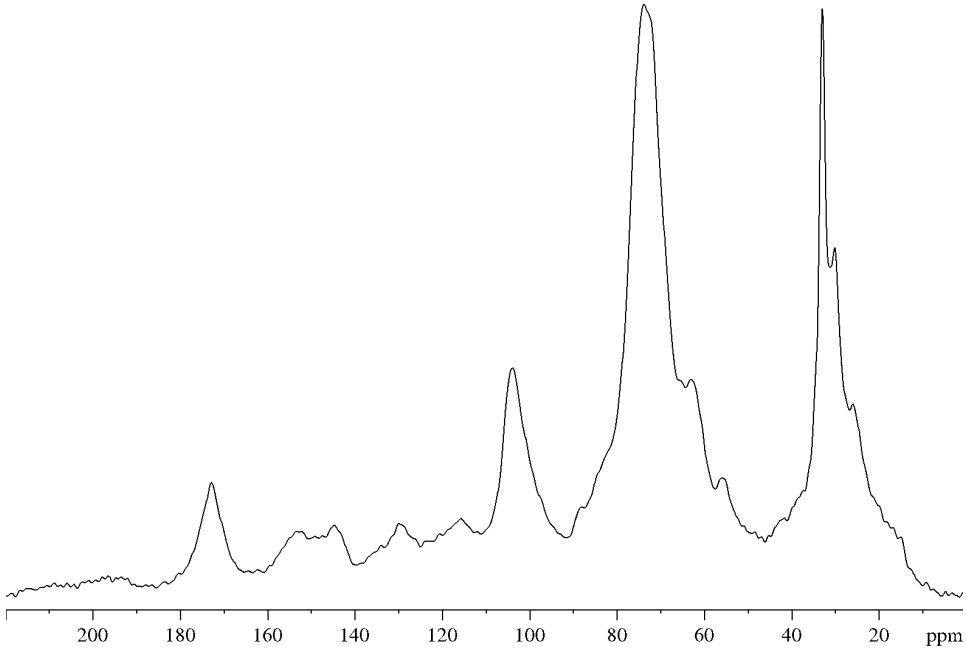

Fig. S1 Solid state CPMAS ^{13}C NMR spectra of the soil used in the incubation experiment. 10—50 ppm: alkyl C, 50-90 ppm: non anomeric carbohydrate C, 90-110 ppm: anomeric carbohydrate C, 110-150 ppm: aromatic C, 150-200 ppm: carboxyl C.

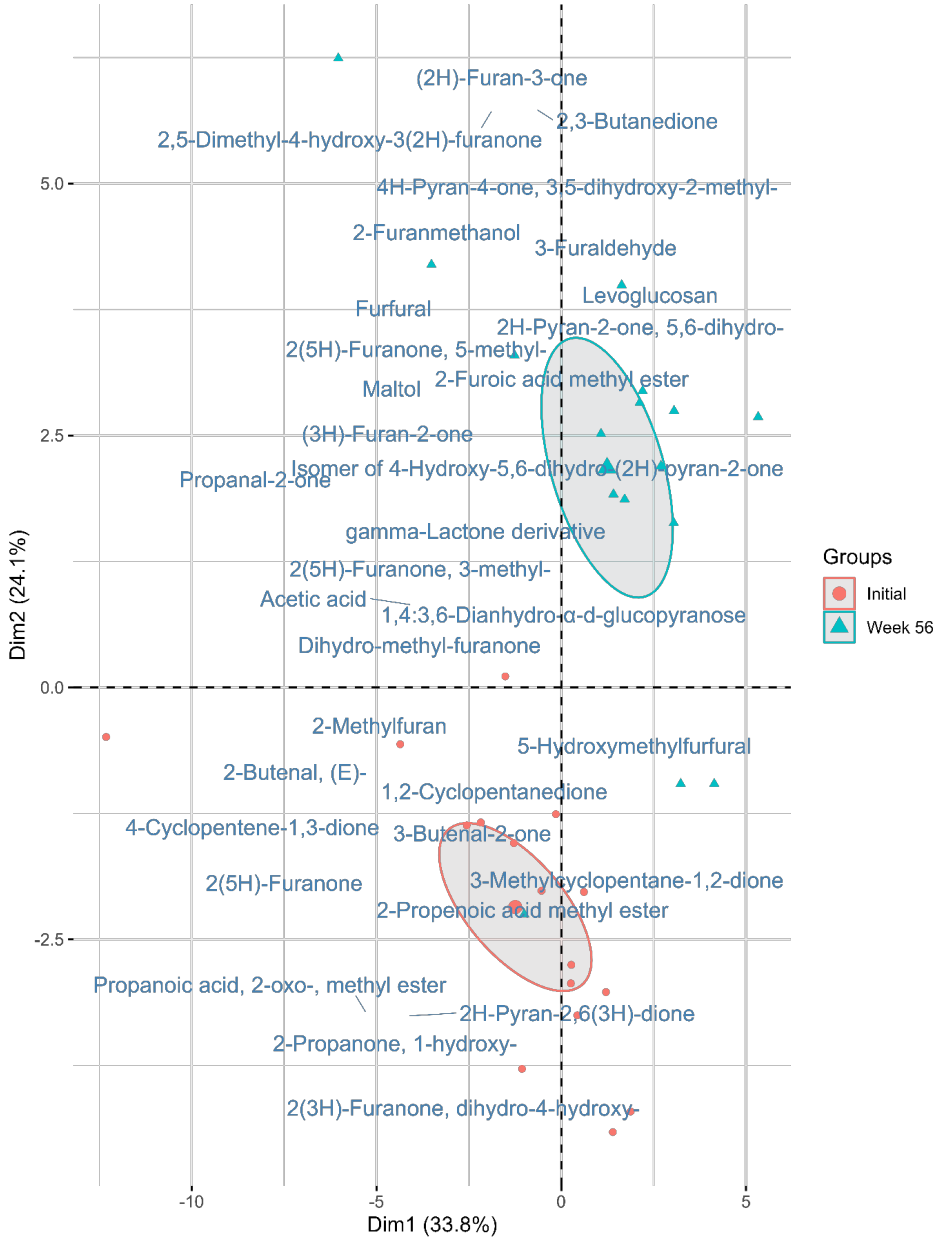

Fig. S2 Principal component analysis (PCA) of carbohydrate pyrolysates at the start and the end the incubation period (R^2= 0.58). The colored individual points represent temporal dynamics of decomposition, with "initial" denoting undecomposed substrates (n=16), and week 56 representing decomposed substrates at the end of the experiment (n=16). The ellipse with a centroid represents the group mean with a confidence interval of 95%.

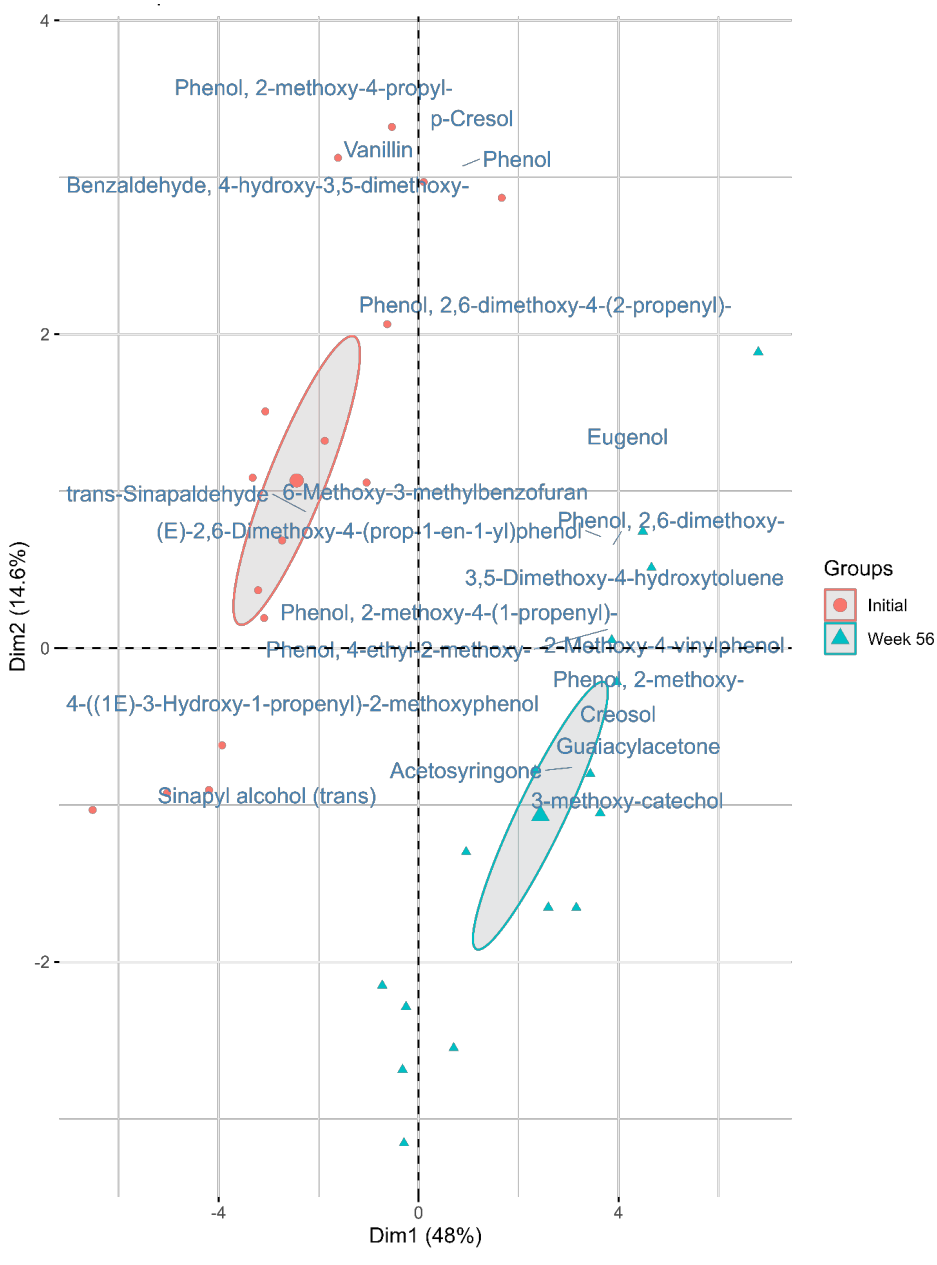

Fig. S3 Principal component analysis (PCA) of lignin pyrolysates at initial and week 56 of the incubation period (R^2= 0.63). The colored individual points represent temporal dynamics of decomposition, with "initial" denoting undecomposed substrates (n=16), and week 56 representing decomposed substrates at the end of the experiment (n=16). The ellipse with a centroid represents the group mean with a confidence interval of 95%.

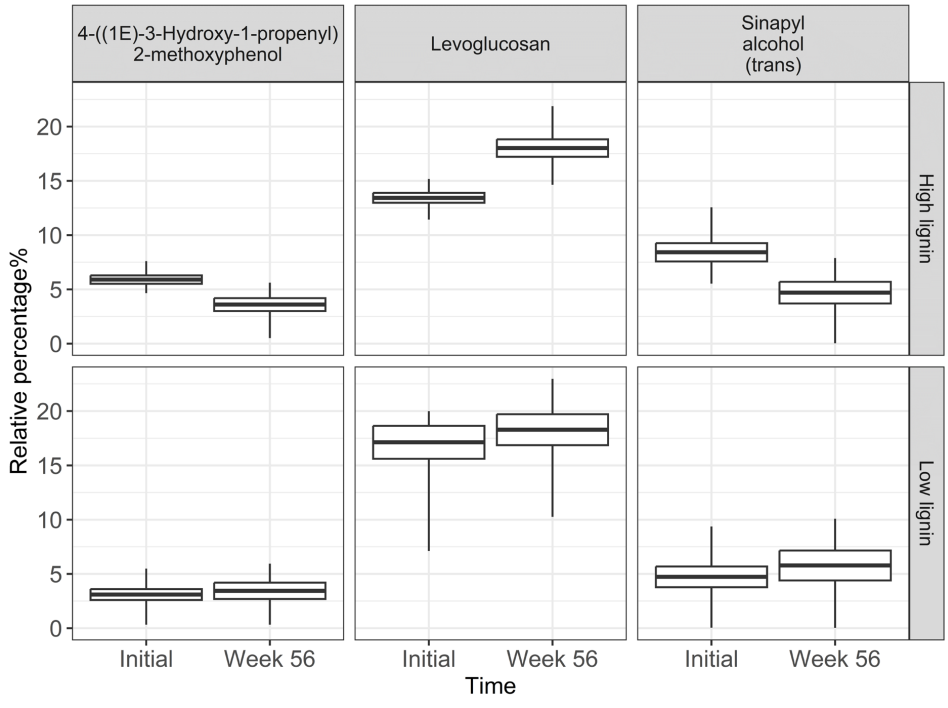

Fig. S4 Changes in the relative abundance of pyrolysates over time for high and low lignin substrates. 4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol, Levoglucosan and Sinapyl alcohol represents the dominant peak among guaiacyl, carbohydrates and syringyl pyrolysates respectively.

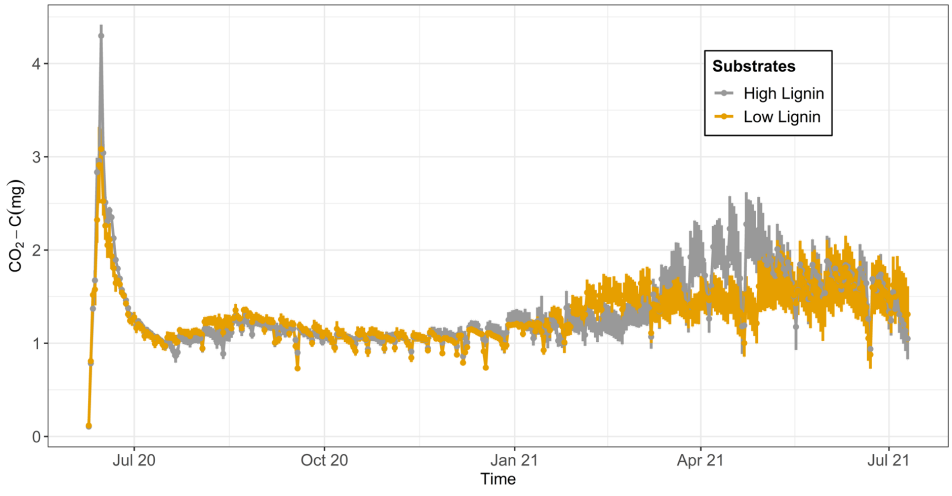

Fig. S5 The average (n=8) CO_2-C per hour from the decomposition of high and low lignin substrates over 56 weeks. The error bars represent standard error (±SE).

Acta Universitatis Agriculturae Sueciae

Doctoral Thesis No. 2025:3

Soil organic matter (SOM) is crucial for the global carbon cycle, with lignin considered a key contributor due to its recalcitrance favouring SOM accumulation. This thesis investigated lignin's role in the plant material decomposition using aspen genotypes with varying lignin traits. Findings reveal that decomposition is regulated by lignin composition and microbial community dynamics, rather than lignin content. These results underscore the importance of microbial interactions and chemical composition in advancing our understanding of SOM dynamics and carbon cycling processes.

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