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Legacies of invertebrate exclusion and tree secondary metabolites control fungal communities in dead wood

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Abstract

During decomposition of organic matter, microbial communities may follow different successional trajectories depending on the initial environment and colonizers. The timing and order of the species arrival (assembly history) can lead to divergent communities through priority effects. We explored how assembly history and resource quality affected fungal communities and decay rate of decomposing wood, 1.5 and 4.5 years after tree felling. Additionally, we investigated the effect of invertebrate exclusion during the first two summers. We measured initial resource quality of bark and wood of aspen (*Populus tremula*) logs and surveyed the fungal communities by DNA metabarcoding at different times during succession. We found that gradients in fungal community composition were related to resource quality and we discuss how this may reflect different fungal life history strategies. As with previous studies, the initial amount of bark tannins was negatively correlated with wood decomposition rate over 4.5 years. The initial fungal community explained variation in community composition after 1.5, but not 4.5, years of succession. Although the assembly history of initial colonizers may cause alternative trajectories in successional communities, our results indicate that the communities may converge with the arrival of secondary colonizers. We also identified a strong legacy of invertebrate exclusion on fungal communities, even after 4.5 years of succession, thereby adding crucial knowledge on the importance of invertebrates in affecting fungal community development. By measuring and manipulating aspects of assembly history and resource quality that have rarely been studied, we expand our understanding of the complexity of fungal community dynamics.

KEYWORDS
aspen, assembly history, dead wood, insects, latent fungi, secondary metabolites, wood decay fungi

1 | INTRODUCTION

Biological communities developing in newly emerged habitats depend on the interplay between the biotic and abiotic environment. The community assembly can lead to stable or alternative community compositions, that is, they either converge or diverge over time (Chase, 2010; Fukami, 2015; Young et al., 2001). This is inherently driven by deterministic and stochastic effects of the initial environment and is known as *historical contingency* (Ben-Menahem, 1997; Fukami, 2015). Perhaps the most well-known example of a stochastic effect is assembly history (i.e., the initial timing of species colonizations), which can determine the subsequent community structure in processes called *priority effects* (Chase, 2003; Drake, 1991; Fukami, 2015). An example of deterministic effects is when environmental conditions or biotic interactions exclude species from the community after they have arrived, although the two are not independent of one another (Cadotte & Tucker, 2017; Kraft et al., 2015; Vellend, 2010). Understanding the processes that govern community assembly is important for detecting drivers of biological communities which ultimately link to ecosystem functions.

Dead wood provides diverse habitats which are important for global biodiversity and carbon sequestration (Goodale et al., 2002; Seibold et al., 2021; Stokland et al., 2012). The organisms that are dependent on dead wood comprise mostly fungi and invertebrates—particularly insects. Fungi are by far the most effective decomposers of dead wood through their lignocellulolytic abilities (Baldrian, 2006; Rayner & Boddy, 1988). Although wood decomposition is a process that sometimes lasts for decades (Stokland et al., 2012), most experimental studies that try to assess the importance of dead wood communities have focused only on the first few years of decomposition (e.g., Seibold et al., 2021; Ulyshen, 2016; Venugopal et al., 2017). However, given the central role that fungi have in nutrient cycling in forests, it is crucial to expand this successional perspective.

Fungal community development in dead wood is governed by environmental conditions (e.g., water content, temperature) and biotic interactions, particularly combative interactions between fungi (Boddy, 2000; Boddy & Heilmann-Clausen, 2008; Rayner & Boddy, 1988; Venugopal et al., 2017). The importance of environment is reflected by the different life history strategies that dead wood fungi have evolved: stress-tolerant, ruderal and competitive (Boddy, 2001; Boddy & Heilmann-Clausen, 2008; Treseder & Lennon, 2015). For instance, different tree species host distinct fungal communities (e.g., Boddy & Heilmann-Clausen, 2008; Norberg et al., 2019), probably due to inherent physicochemical qualities of the wood (Hoppe et al., 2016), including pH, and nutrient and secondary metabolite content, and the selective advantages of fungal life history strategies (Rayner & Boddy, 1988).

The amount and type of inhibitory secondary metabolites which the tree produces give a competitive advantage to fungal species that tolerate this stress (Valette et al., 2017). Among the most common secondary metabolites are phenolic compounds, such as tannins and flavonoids, which inhibit growth of many fungi (Flores & Hubbes, 1979; Hammerbacher et al., 2018) and the rate of decomposition (Coq et al., 2010; Loranger et al., 2002; Valette et al., 2017). The concentration of phenolic compounds can differ markedly between and within individual trees of the same species (Birkemoe et al., 2021). However, few studies (Bailey et al., 2005; Birkemoe et al., 2021) have investigated whether such intraspecific variation in secondary metabolite concentration can govern community assembly of fungi in wood.

The initial fungal colonizers of wood arrive rapidly as spores or are latently present in functional sapwood (Boddy & Hiscox, 2016; Boddy et al., 2017). A wide diversity of fungi, including wood-decaying species, are present as latent propagules, but relatively few of them establish overtly when the sapwood begins to dry out (Chapela & Boddy, 1988b; Cline et al., 2018; Hiscox, Savoury, Müller, et al., 2015; Parfitt et al., 2010). These initial colonizers of wood are, however, soon replaced by more combative fungi arriving as spores or mycelial cords (Chapela & Boddy, 1988a; Hiscox et al., 2018). The outcome of such competitive interactions can be altered by invertebrate grazing (A'Bear et al., 2014; Crowther et al., 2011). Furthermore, invertebrates can affect fungi in other ways (Birkemoe et al., 2018), especially by aiding in dispersal (e.g., bark beetles: Harrington et al., 1981; Webber & Gibbs, 1989).

There is accumulating evidence that the developing community in dead wood can be shaped by altered assembly history (e.g., Dickie et al., 2012; Fukami et al., 2010). For example, on the forest floor, precolonization of wood by *Stereum hirsutum* led to distinct fungal communities compared to when seven other species were introduced first (Hiscox, Savoury, Müller, et al., 2015). There are also indications that the presence of invertebrates can lead to priority effects in fungal communities of dead wood (Jacobsen et al., 2018; Leopold et al., 2017). However, the few long-term studies that exist have only traced a limited number of fungal species (Jacobsen et al., 2015; Weslien et al., 2011), rather than looking at the effect on the whole community. Given that wood decomposition is a slow process and that such effects might attenuate over time (Dickie et al., 2012; Fukami, 2015; Norberg et al., 2019), there is a need for long-term studies investigating how assembly history affects fungal community composition and wood decomposition.

In this study, we explore how resource quality and assembly history shape fungal community development in dead wood of aspen (*Populus tremula*). In particular, we quantify the initial secondary metabolites, and carbon and nitrogen content, and identify the initial fungal community composition of the dead wood habitat. Furthermore, we manipulate assembly history by excluding invertebrates for two summers in an in-field experiment. To uncouple the effects of deterministic and stochastic processes caused by spatial variation on fungal communities, we transferred aspen logs to 30 different locations in two landscapes. To cover temporal variation in the fungal communities, the logs were sampled twice—after 1.5 and 4.5 years (termed year 2 and 5). By applying DNA metabarcoding of the ITS2 region, we ask: how does (1) resource quality (initial secondary metabolite, carbon and nitrogen content), and (2) assembly history (initial fungal community and invertebrate exclusion) affect fungal community composition, operational taxonomic unit (OTU)

richness and wood decomposition? Answering these questions will reveal important drivers of biological communities and dead wood decomposition.

2 | MATERIALS AND METHODS

2.1 | Study area

Seventeen aspen (*Populus tremula* L.) trees were felled from the same stand in Ås municipality in Norway (59°66′N, 10°79′E, 92 m a.s.l.) during March 2014. The trees were cut into 1-m-long logs (diameter [*D*] = 20.5–36.4 cm, mean = 27.6 cm), which were transported to 15 sites in each of two boreal forest landscapes in southeast Norway: Losby forest holdings in Østmarka (59°87′N, 10°97′E, 250–300 m a.s.l.) and Løvenskiold-Vækerø in Nordmarka (60°08′N, 10°58′E, 300–500 m a.s.l.). Both forests are dominated by spruce (*Picea abies* (L.) H. Karst.), with pine (*Pinus sylvestris* L.), birch (*Betula pubescens* Ehrh.) and aspen as subdominants. All sites were in mature, closed-canopy managed forest, with a mean distance between sites of 120 m in Østmarka and 276 m in Nordmarka.

2.2 | Wood and bark samples for analysis of the initial fungal community and resource quality

After felling, 53 discs were cut from the fresh wood between every two or three logs (Figure S1). The discs were used for DNA analysis and chemical measurements to identify the initial fungal community and determine resource quality, respectively. Aseptically (ethanol-and-flame-sterilization), bark was removed with a knife, then wood samples for DNA analysis were taken by drilling 10 cm into the wood with a 12-mm drill bit. Each sample consisted of woodchips from drilling at two different positions along the circumference of the section. Wood samples were stored at −80°C prior to DNA extraction. Wood samples for analysis of resource quality were taken next to the drilling holes where samples for DNA analysis had been taken. Samples were taken by drilling, using the same method as above, but followed by drying the samples at 30°C overnight and subsequently storing them at −20°C. Bark samples for analysis of resource quality were taken 3 months after felling, from logs stemming from the same 17 aspen trees and distributed in the same two forest landscapes. Bark samples were taken with a knife that was sterilized between each sample, subsequently dried at 30°C and stored at −20°C. Birkemoe et al. (2021) describes the sampling of these logs in detail.

2.3 | Invertebrate exclusion experiment

Four logs were placed at each of the 30 sites and subjected to the following treatments from April 2014 to November 2015. (i) Invertebrate exclusion ("cage") where logs were enclosed in a fine polyester plastic mesh (1 × 1 mm) net suspended from a frame. A mesh size of 1 mm effectively excludes macro- and larger meso-invertebrates, which we expect to have greatest impacts on fungi (Crowther, Boddy, et al., 2011; Crowther et al., 2011). Colonization from below was prevented by a polyethylene plastic sheet beneath the logs. Since the plastic sheets would prevent not only invertebrates but also fungi from colonizing from the soil, these were included in all treatments. (ii) "Cage control" logs placed within cages identical to those in (i), except with four large holes (*D* = 20 cm) allowing invertebrate access. This treatment was to control for microclimatic effects of the cage itself. (iii) Uncaged logs ("control") placed on a plastic sheet. (iv) Uncaged "positive control" logs baited with an ethanol bottle (1 L, 96% ethanol) with small holes for evaporation to attract wood-inhabiting invertebrates. In November 2015, cages and plastic sheets were removed, but all logs were kept in the same positions on the ground. More information on the experimental set-up can be found in Jacobsen et al. (2018).

2.4 | Wood samples for analysis of the successional fungal communities

Wood samples for DNA analysis were taken from the logs at the end of the exclusion period in November 2015 (termed year 2), and again in November 2018 (termed year 5). The same methods as for fresh wood samples were used, but with drilling at three different positions along the circumference (top and both sides) for each sample. In year 2, each log was sampled 25 cm (end section) and 50 cm (mid section) from the end with the least damage to the bark, resulting in 240 samples. In year 5, samples were taken 20 cm (end section) and 45 cm (mid section) from the same log end, but one log had disappeared, thereby resulting in 238 samples.

2.5 | Wood samples for analysis of wood decomposition

Wood samples for density analysis were taken in November 2015 (year 2) and in May–June 2018 (year 5), next to the drilling holes for DNA analysis samples in the respective years. Unfortunately, wood density and woodchips for DNA were not sampled in parallel during 2018 as the risk of forest fire prevented flame sterilization during summer. Samples for density analysis were taken with a core sample drill (length = 10 cm, *D* = 12 mm) in two replicates—top and one side—for each section and pooled together for analysis. Only the outer 5 cm (without bark) of the core sample was used for analyses because the inner region was often too decayed to be sampled in year 5 (Tangnæs & Andelic, 2019). Wood density was calculated as dry mass divided by fresh volume. Fresh volume was first measured by water displacement, and the samples were then oven-dried at 103°C overnight before measuring dry mass.

2.6 | Chemical analyses

The analyses of carbon, nitrogen and phenolic compounds are described in detail in Birkemoe et al. (2021). Briefly, dried wood and bark samples were grounded to powder with a mixer mill (Retsch MM400) from which subsamples for different analyses were taken. Carbon and nitrogen concentrations were analysed on a Micro Cube elemental analyser (Elementer Analysen). Low molecular weight phenolic compounds were extracted in methanol and subsequently analysed by high-performance liquid chromatography (HPLC) (Agilent Series 1200; Agilent Technologies). Compound identification was based on retention times, online UV spectra and cochromatography of commercial standards (Nybakken et al., 2012). Individual phenolic compounds were assigned to chemical groups (phenolic acids, salicylates and flavonoids; Birkemoe et al., 2021). Two fractions of condensed tannins, methanol-soluble and methanol-insoluble, were quantified by the acid butanol assay for proanthocyanidins (Hagerman, 2002) from the liquid HPLC-extract and from the extraction residue, respectively.

2.7 | DNA isolation, amplification and sequencing

Samples in year 2 and immediately after felling (fresh wood; initial fungal community) were analysed for DNA as described in Jacobsen et al. (2018). Samples in year 5 were analysed for DNA in the same way, except for minor differences in centrifugation speed (always 14,000 *g* in year 2 and fresh wood) and primer use (see below). To 50-ml Falcon tubes, 15 ml woodchips and 15 ml CTAB lysis buffer (pH 8.0; 100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA, 2% w/v CTAB) were added along with seven stainless steel beads (5 mm). The tubes were homogenized in a mixer mill (Retsch MM400) at 30 Hz for 90 s, placed at −80°C for 30 min and then incubated at 54°C overnight. Next, samples were inverted, left to cool and vortexed with 15 ml chloroform. They were centrifuged for 25 min in a Rotina 46 (Hettich Zentrifugen) at 1,780 *g* until the phases had separated. In a clean tube, 5 ml of the upper phase was transferred along with 5 ml cold isopropanol. To precipitate the DNA, the tubes were inverted and placed at −20°C for 30 min. The tubes were centrifuged at 13,000 *g* for 10 min in an Eppendorf 5804R (Eppendorf) and the supernatant was discarded. The remaining pellet was washed with 1,000 µl cold 70% ethanol, vortexed, centrifuged for 3 min at 13,000 *g* and discarded. Excess ethanol was decanted by incubation at 60°C and dried on laboratory tissue paper. Finally, the DNA was resuspended with 60 µl milli-Q H_2O. The extracts were cleaned using an E.Z.N.A. Soil DNA kit (Omega Bio-tek) as recommended by the manufacturer, then washed again with 500 µl DNA Wash Buffer and eluted twice with 20 µl Elution Buffer, resulting in 40 µl suspended DNA.

Amplicon libraries were constructed from 238 DNA extracts, 18 technical replicates and three polymerase chain reaction (PCR) negatives. They were prepared on three 96 PCR plates with uniquely tagged ITS2 primers in 20-µl volume samples: 6.2 µl milli-Q H_2O, 4 µl 10×-dilution DNA template, 4 µl Phusion HF Buffer, 2 µl dNTPs (10 mM), 1 µl bovine serum albumin (BSA; 10 µg μl^{-1}), 0.6 µl dimethyl sulfoxide (DMSO), 0.2 µl Phusion Hot Start II High-Fidelity DNA Polymerase (2 U μl^{-1}) and 1 µl each of forward and reverse primers (10 µM, but 5 µM in 2016). The forward primer, gITS7 (GTGARTCATCGARTCTTTG) differed with one nucleotide code (*R* → *A*) from the one used for year 2 and fresh wood samples (fITS7) (Ihrmark et al., 2012). The reverse primer for both years was ITS4 (TCCTCCGCTTATTGATATGC; White et al., 1990). PCR was run on an GeneAmp PCR System 2700 (Life Technologies, Applied Biosystems) with the following settings: initial denaturation at 98°C for 30 s, then 30 cycles of 98°C/10 s denaturation, 56°C/30 s annealing and 72°C/15 s elongation, ending with 10 min elongation at 72°C. Amplifications were cleaned with Wizard SV Gel and PCR Clean-Up System (Promega) as recommended by the manufacturers but with a longer centrifugation step of Membrane Wash Solution to remove excess ethanol.

A qualitative assessment was made of the cleaned PCR products with electrophoresis on a 1% agarose gel, ordering each sample in one of three categories based on band strength. The average amount of DNA for each category was estimated from a random selection and quantified with a Qubit dsDNA HS Assay Kit on a Qubit 2.0 Fluorometer (Life Technologies, Invitrogen). All samples were then normalized according to these categories before pooling them in three libraries. The libraries were purified with Agencourt AMPure XP magnetic beads (Beckman Coulter). Quality control was also done with Qubit. The libraries were barcoded with Illumina adapters, indexed with 30% PhiX and sequenced in an Illumina Miseq (Illumina) lane with 2 × 300-bp paired-end reads at StarSEQ.

2.8 | Bioinformatic analyses

The sequencing runs resulted in two data sets: I, two libraries from year 2 and fresh wood samples that consisted of 60,822,032 sequence reads; and II, three libraries from year 5 samples that consisted of 28,249,006 reads. The two data sets were demultiplexed in separate steps using CUTADAPT version 2.7 (Martin, 2011). Tags, and forward and reverse primers were cut simultaneously and reads less than 100 bp were discarded (data set I: 6,294,571 reads, data set II: 3,293,456 reads). Ten samples with low read numbers were removed (I: two samples, II: 15 samples). The reads were denoised, dereplicated, checked for chimeras and merged with DADA2 version 1.12 (Callahan et al., 2016) in R version 3.5.1 (R Core Team, 2021). Before denoising, low-quality samples were filtered with "filterAndTrim" (see Supp. Material 2 for details), but with optimized parameters for data set I to retain more sequences without changing the read length characteristics (Figure S2). The reads were merged while allowing overhangs with "mergePairs." Putative chimeric sequences were checked and removed with "removeBimeraDenovo," specifying a more relaxed abundance distance threshold from parental sequences for data set II. After combining the two data sets, the resulting table contained 26,395,685 reads and 7,626 amplicon sequence variants (ASVs) from 560 samples. The ITSx algorithm (version 1.1b1; Bengtsson-Palme et al., 2013) was used to remove

conserved regions adjacent to ITS2 and nonfungal ASVs. Then, with VSEARCH version 2.9.1 (Rognes et al., 2016), the reads were dereplicated and clustered with 97% similarity using a distance-based greedy clustering, resulting in 1,668 OTUs. These were then curated with the LULU algorithm (Frøslev et al., 2017) to avoid potential oversplitting, ending up with 1,287 OTUs. Taxonomy was assigned to 1,126 OTUs (88.13%) with a BLAST+version 2.8.1 search (evalue 1×10^{-4}, max_target_seqs 1) against the UNITE+INSD version 8.2 database of fungal sequences (Abarenkov et al., 2020). Information on ecological guilds was annotated to 758 OTUs (58.81%) using FUNGUILD (Nguyen et al., 2016) as of February 2020.

2.9 | Statistical analyses

All analyses and figure preparation were carried out in R version 4.1.1 (R Core Team, 2021) and the package sjPlot (Lüdecke, 2021). Rarefaction and richness estimations were performed with the package PHYLOSEQ (McMurdie & Holmes, 2013) and ordinations with the package VEGAN version 2.5-7 (Oksanen et al., 2013).

Invertebrate exclusion, landscape and log section were used as categorical explanatory variables, while wood and bark resource quality and initial fungal community were used as continuous explanatory variables to explain successional fungal community composition, OTU richness and wood density (Table S3). Bark variables were used as average values per tree ($n = 17$), while wood variables stemmed from the closest wood disc that had been sampled between logs at each tree (Figure S1). All continuous variables were transformed to zero skewness (Økland et al., 2001) to approximate normality of errors, that is log-transformation of right-skewed and exponential transformation of left-skewed variables, then standardized to a 0–1 scale (Table S3). The initial fungal community was identified by DNA analysis and described by ordination—specifically, the two first axes of a detrended correspondence analysis (DCA1 and DCA2) (see Supporting Material 3.1). Wood and bark secondary metabolites from fresh wood were used as the total sum of all phenolic groups (phenolic acids, salicylates and flavonoids), as identified by Birkemoe et al. (2021). Additional variables of resource quality were: bark methanol-soluble and methanol-insoluble condensed tannins; and wood carbon and nitrogen.

Negative and technical replicates were checked and are described in Supporting Material 3.2. For each pair of technical replicates, the sample with most reads was retained. All analyses were performed on a filtered (OTUs >10 reads) and rarefied data set of samples from year 2 and 5 (Supporting Material 3.3). Twenty-five samples were removed from year 2 and 10 samples from year 5 because they were either below the subsampling depth (14,407 reads) or not present in both years. This resulted in a data set of 773 fungal OTUs from 426 samples originating from 213 aspen logs.

Fungal community composition of year 2 and 5, and their relationship with the explanatory variables, were explored with (i) indirect and (ii) direct gradient analysis. (i) A four-dimensional global nonmetric multidimensional scaling (gNMDS; Kruskal, 1964a, 1964b) was generated on a Bray–Curtis dissimilarity matrix of samples from year 2 and 5. The choice of dimensionality followed Liu et al. (2008), and detailed evaluation and settings of the ordination are described in Supporting Material 4.1. The samples were balanced between the two years to consider displacement of the logs in ordination space. The axes were sorted by most variation explained and scaled in half-change units of compositional turnover (Faith et al., 1987). The ordination was interpreted by fitting linear mixed models (LMMs) to each gNMDS axis (Dargie, 1984; Økland, 2007), using the package LME4 (Bates et al., 2014) and with site as a random effect. Models were chosen after forward selection of explanatory variables with second-order Akaike information criterion (AICc) Burnham and Anderson (2002); package MuMIn: Barton (2020). Degrees of freedom and p values were calculated with Satterwaithe's approximation from the LMERTEST package (Kuznetsova et al., 2017) and α was set to 0.05. Additionally, we used Tukey's honest significant difference (HSD) test to see if the invertebrate exclusion treatment levels were different along the gNMDS axes (Tukey, 1949).

(ii) Direct gradient analysis was applied using hypothesis testing with partial constrained ordination. Essentially, canonical correspondence analysis (CCA; ter Braak, 1986) was used with forward selection of explanatory variables and a Monte Carlo permutation test (perm. = 999; Legendre et al., 2011). The p value was used as a selection criterion, or F value if p values were equal between competing models. Model selection was done on the data set from year 2, year 5 and both data sets combined. In the final models, inertia units were used to compare the variation explained by each variable. As the final models contained at least seven variables and the shared variation between variables was negligible, variation explained was presented by each variable independent of all others. Variables that were congruent with both indirect and direct gradient analysis were fitted to the gNMDS with the "envfit" function from VEGAN. This is based on factor averages and vectors (for continuous variables) and the significance of the variables fit was tested (perm. = 999).

To test whether wood density (g cm^{-3}) or fungal OTU richness in year 2 and 5 were related to initial resource quality, assembly history, log section or landscape, an LMM was applied as described above. Model assumptions were deemed satisfactory except a very severe violation of linearity of fixed effect residuals (Figure S5.1). This was because the bark and fresh wood values were derived from fewer samples than the wood density and fungal OTU samples. To provide more certainty to these results, OTU richness and wood density of year 2 and 5 in linear regressions were tested in aggregated data sets (i.e., $n = 17$ for bark samples and $n = 50$ for wood samples; Supporting Material 5).

3 | RESULTS

3.1 | Taxonomic and functional groups of OTUs

There were 773 OTUs, of which 537 occurred in year 2 and 422 in year 5. Most OTUs were Ascomycota (61.1%) and 27.6% were Basidiomycota. Helotiales was the most common order (116 OTUs), followed by Agaricales (36) and Polyporales (34). Around half of the

reads from year 2 (52%) and a third (32%) from year 5 were from OTUs annotated as "Wood saprotrophs." The functional distribution of OTUs across both years were: 411 saprotrophs (of which 118 were wood saprotrophs), 156 symbiotrophs (e.g., mycorrhiza), 153 pathotrophs and 243 that were not assigned to guilds. Note that many OTUs are assigned to more than one guild, for example pathotroph-saprotroph. The initial fungal community from fresh wood (Figure S3.1a, S3.2) consisted of 375 OTUs of which 68.8% were Ascomycota and 18.9% Basidiomycota. The functional distribution was: 171 saprotrophs (49 wood saprotrophs), 87 symbiotrophs, 83 pathotrophs and 129 that were not assigned to guilds. Note that while it is possible that this may include airborne fungal spores that arrived during sampling, it still reflects the initial fungal community. The initial community was closer to year 2 than year 5 in ordination space (Figure S3.3).

3.2 | Fungal community composition

We identified four compositional gradients of the fungal community from year 2 to 5 that were interpretable in terms of temporal variation, spatial variation, initial resource quality and assembly history. Specifically, the main compositional gradient (gNMDS1) separated the fungal communities from year 2 to year 5, while also being related to spatial variation and wood phenolic acids (Figure 1; Table S4.2). gNMDS3 was related to the initial fungal community (DCA1) and wood carbon (Figure S4.3, Table S4.2, S4.5). The second and fourth axes formed gradients related to the initial fungal community (DCA2) and resource quality: from higher amounts of wood carbon to bark flavonoids in gNMDS2 (Figure 1; Table S4.2, S4.4), and from higher amounts of wood nitrogen to bark methanol-soluble condensed tannins in gNMDS4 (Figure S4.4, Table S4.2, S4.6). The second axis showed an effect of the invertebrate exclusion, with a gradient from caged to positive control logs (Figure 1). Although the variable was not included in the LMM explaining variation in gNMDS2, a Tukey HSD test showed significant dispersion in plot scores between caged and positive control logs along this axis ($p = .047$; Figure S4.5).

From the model selection with constrained ordination of both years, temporal variation accounted for 37.3% of the explained variation, followed by invertebrate exclusion (11.4%) and variation between landscapes (8.7%) (Table S4.3). Invertebrate exclusion was the most important initial condition explaining the fungal community composition in year 2 and year 5 (Tables 1 and 2). Combined, the secondary metabolites contributed to around half of the explained variation in year 2 and 5 (Figure S4.6). All initial conditions were deemed important in explaining fungal community composition in year 2, while invertebrate exclusion, flavonoids, phenolic acids and nitrogen were important in year 5 (Tables 1 and 2).

3.3 | Fungal OTU richness

Wood carbon was negatively correlated with fungal OTU richness in year 2 (Figure 2; Figure S5.2, Table S6). Also, richness

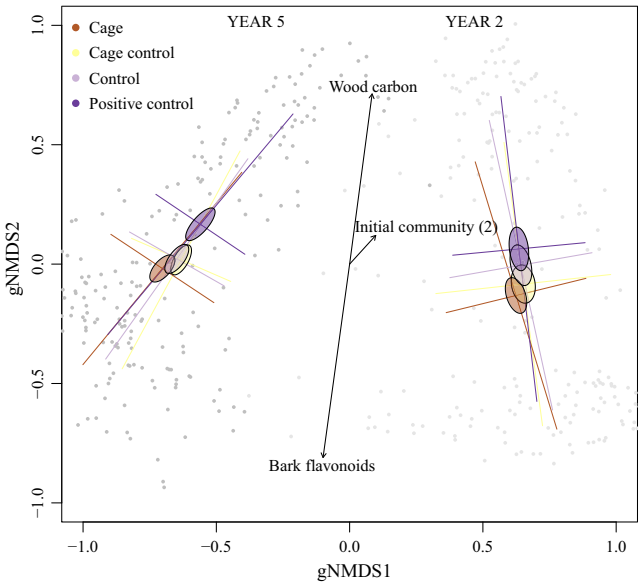

FIGURE 1 Ordination biplot of the first and second compositional gradients of fungal OTUs from aspen logs ($n = 424$) in year 2 and 5 after tree felling (gNMDS of Bray–Curtis dissimilarities; stress = 0.14). Coloured ellipses display the standard error of treatment levels, while error bars display the standard deviation. Vectors are fitted with the "envfit" function in the package VEGAN and are based on a forward selection of linear mixed models of each gNMDS axis. Light grey dots represent plot scores of year 2 and dark grey of year 5

was estimated to be higher in mid, rather than end, log sections ($p = .019$) and in Østmarka, rather than Nordmarka landscape ($p = .039$). Bark flavonoids positively affected fungal OTU richness in both years, but the effect was not significant ($p = .07$). Wood phenolic acids positively affected fungal OTU richness in year 5 ($p = .013$; Table S6; Figure S5.3).

3.4 | Wood density

Bark phenolic acids and methanol-insoluble condensed tannins, as the only included variables after model selection, had a positive effect on wood density (Table 3). The relative contribution of bark tannins was stronger in year 5 than in year 2. Phenolic acids only explained wood density in year 2, but this effect was not consistent in models that took into account the linearity of fixed effect residuals (Table S5.1, S5.2, Figure S5.4).

4 | DISCUSSION

By combining DNA metabarcoding with chemical analyses in an experimental field study of dead wood of aspen, we have shown that exclusion of invertebrates had conditional effects on the fungal community for at least 3 years after exclusion ceased. However, the initial fungal community present in fresh wood was only important

TABLE 1 Variation partitioning with constrained ordination (CCA) of fungal community composition in aspen logs in year 2 after tree felling ($n = 213$). Variables are based on a forward model selection with p as the selection criterion. Inertia of constrained variables is modelled alone without conditioning variables to show variation explained independent of other variables

Variable	Inertia	Proportion explained
Landscape	0.1335	0.0200
Invertebrate exclusion	0.1142	0.0171
Bark flavonoids	0.0879	0.0131
Wood phenolic acids	0.0849	0.0127
Bark soluble tannins	0.0805	0.0120
Wood salicylates	0.0788	0.0118
Wood flavonoids	0.0750	0.0112
Wood carbon	0.0702	0.0105
Initial fungal community (1)	0.0679	0.0101
Bark phenolic acids	0.0651	0.0097
Initial fungal community (2)	0.0641	0.0096
Bark insoluble tannins	0.0640	0.0096
Log section	0.0586	0.0088
Wood nitrogen	0.0504	0.0075
Unconstrained	5.7361	0.8574
Total	6.6900	–[a]

[a]Inertia of constrained and unconstrained axes does not add up to the total inertia because 2.11% of the variation is shared between one or several variables.

TABLE 2 Variation partitioning with constrained ordination (CCA) of fungal community composition in aspen logs in year 5 after tree felling ($n = 213$). Variables are based on a forward model selection with p as the selection criterion. Inertia of constrained variables is modelled alone without conditioning variables to show variation explained independent of other variables

Variable	Inertia	Proportion explained
Invertebrate exclusion	0.3071	0.0173
Landscape	0.2049	0.0116
Bark phenolic acids	0.1681	0.0095
Bark flavonoids	0.1395	0.0079
Wood phenolic acids	0.1314	0.0074
Wood flavonoids	0.1272	0.0072
Wood nitrogen	0.1027	0.0058
Unconstrained	16.5813	0.9352
Total	17.7301	–[a]

[a]Inertia of constrained and unconstrained axes does not add up to the total inertia because 0.18% of the variation is shared between one or several variables.

after 1.5 years of succession. Moreover, intraspecific variation in initial bark and wood secondary metabolites, carbon and nitrogen affected not only fungal community composition but also wood decomposition rates.

4.1 | Secondary metabolites, carbon and nitrogen structure fungal communities

After 1.5 and 4.5 years of succession, we identified compositional gradients of fungal communities in dead wood that were related to both the initial community and resource quality. The gradient spanned from higher amounts of carbon or nitrogen at one end to higher amounts of secondary metabolites at the other. Specifically, the concentration of flavonoids and phenolic acids from bark and wood affected fungal community composition and diversity.

Fungi in dead wood have evolved different life history strategies based on the amount of stress, disturbance and competition they face within their environment (Boddy, 2001). Phenolic compounds, including flavonoids, from wood can cause intracellular stress by directly disrupting the degradative capacities of fungi in dead wood, and even destroying the fungal cell (Valette et al., 2017). Indeed, phenolics isolated from bark and wood of American aspen (*Populus tremuloides*) inhibited growth and spore germination of a tree canker-causing fungus, *Entoleuca mammata* (Flores & Hubbes, 1979, 1980; Hubbes, 1962, 1969). The gradients we identify that structure fungal communities in relation to resource quality may reflect strategies to tolerate stress and competition for territory (i.e., combat). For instance, in resources with higher concentrations of secondary metabolites, a combative species might have lower net energy intakes or use more energy on maintenance, thereby reducing its combative abilities relative to that of a stress-tolerant species (Crowther et al., 2014; Schoener, 1973). Because of the harsh environment that latent fungal communities face when the tree is alive, many of these species are stress-tolerant (Boddy & Heilmann-Clausen, 2008; Rayner & Boddy, 1988). Indeed, the trade-off between combative ability and stress tolerance in dead wood fungi has been strongly asserted (Boddy & Heilmann-Clausen, 2008; Crowther et al., 2014; Maynard et al., 2019; Treseder & Lennon, 2015). Environmental gradients in resource quality can increase the number of ecological niches that are available in the wood and allow more fungal species to coexist. This is suggested by the positive effect that wood phenolic acids and bark flavonoids had on fungal OTU richness in our study. Even though we have not measured the combative ability of fungal species, nor classified the fungal community according to life history strategies, our results show that gradients of resource quality structure successional fungal communities in dead wood.

4.2 | Initial fungal community has transient effects on fungal community development

The initial fungal community was an important driver of community composition after 1.5 years, but not after 4.5 years, of succession. Song et al. (2017) found an effect of the initial fungal community on later fungal colonizers in isolated mesocosms. However, their study ended after only 6 months of succession and they excluded natural colonizers. Field studies agree with our findings, in that initial fungal

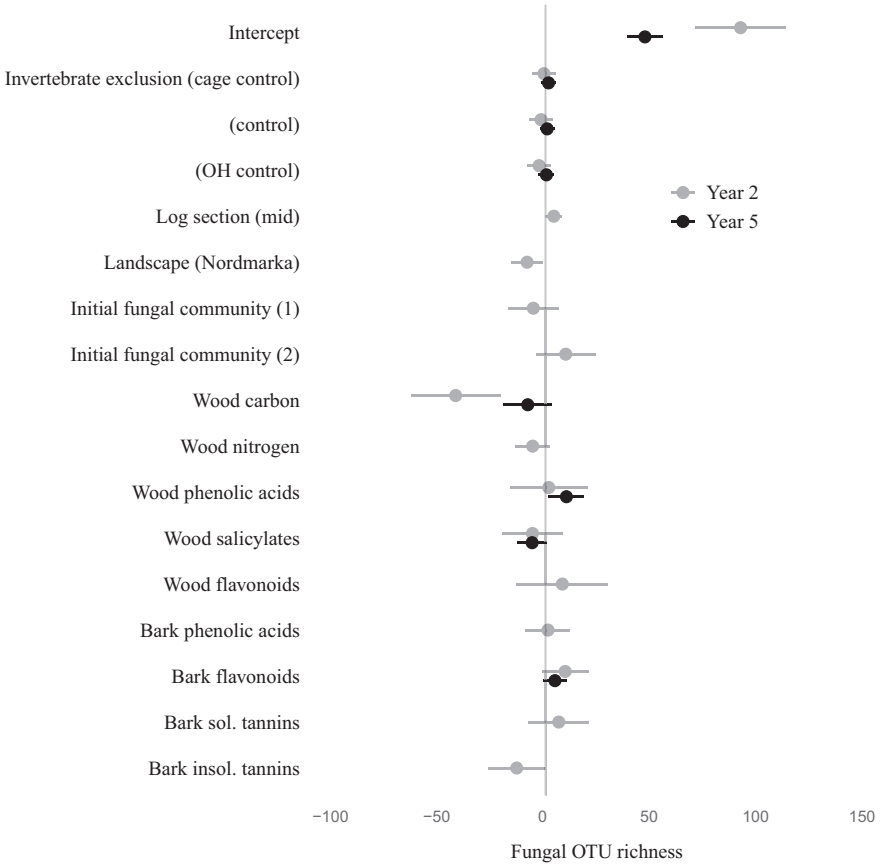

FIGURE 2 Linear mixed model effect sizes of initial conditions explaining fungal OTU richness in aspen logs in year 2 and 5 after tree felling, based on a forward model selection with Site as a random effect. Effect sizes that do not overlap with zero are significant ($\alpha = 0.05$). Reference levels in the intercept are: Invertebrate exclusion (cage), Log section (end) and Landscape (Østmarka)

TABLE 3 Linear mixed models of initial conditions explaining variation in aspen wood density in year 2 and 5 after tree felling. The models are based on a forward model selection (AICc) with Site as a random effect

	Wood density, year 2			Wood density, year 5		
Predictors	**Effect size (95% CI)**	**Statistic**	***p* value**	**Effect size (95% CI)**	**Statistic**	***p* value**
Intercept	0.36 (0.35–0.37)			0.33 (0.31–0.34)		
Bark phenolic acids	0.02 (0.01–0.04)	3.45	**.001**			
Bark methanol-insoluble tannins	0.04 (0.02–0.05)	5.12	**<.001**	0.06 (0.04–0.08)	6.07	**<.001**

Abbreviation: CI, confidence interval.

Significant *p* values (<.05) in bold.

communities have short-term priority effects in early stages of succession, but that these effects attenuate over time (Chapela & Boddy, 1988a; Cline et al., 2018; van der Wal et al., 2016). This can be because the initial fungal colonizers are replaced over time by secondary colonizers with highly combative abilities (Chapela et al., 1988; van der Wal et al., 2016) and it highlights the importance of long-term observations in studies on dead wood communities. Secondary colonizers arrive in the dead wood via airborne or animal-vectored spores, or from the soil as cords or rhizomorphs (Boddy & Heilmann-Clausen, 2008). As we placed all logs on plastic sheets to exclude soil invertebrates from the logs during the first two summers, this would have also excluded fungi that colonize from the soil with mycelial cords. Cord-forming fungi are strong competitors (Hiscox et al., 2015, 2018; Rayner & Boddy, 1988) and this could explain why the effect we found of the initial fungal community was only transient; when the plastic sheets were removed after 1.5 years, secondary colonizers, such as cord-forming fungi, may have replaced the initial community via competitive exclusion.

4.3 | Legacies of invertebrate exclusion on fungal communities after 4.5 years

We detected a strong effect of invertebrate exclusion on fungal community composition, even after 4.5 years of succession, although the experimental set-up excluding invertebrates was removed 3 years previously. Many invertebrates help fungi to establish in dead wood, for example bark beetles that vector ascomycete mutualists (Klepzig & Six, 2004) and other rot fungi (Harrington et al., 1981; Persson et al., 2011). Dispersal of fungi by nonmutualistic beetles to dead

wood may also be common, although the importance for fungal colonization and population dynamics remains unknown (Jacobsen et al., 2017; Seibold et al., 2019). On the other hand, invertebrates can reduce fungal growth (A'Bear, Jones, et al., 2014) or alter the outcomes of competitive interactions between fungi (Crowther, Boddy, et al., 2011; Rotheray et al., 2011), as shown in soil microcosms by mycelial grazers. However, as indicated by studies on the woodlouse (Isopoda) *Oniscus asellus*, the effects of fungivory may be weaker in the field than in laboratory experiments (A'Bear et al., 2014; Crowther & A'Bear, 2012; Crowther, Boddy, et al., 2011). This makes our results even more intriguing. Although we cannot know the underlying causes behind the effects of invertebrate exclusion demonstrated in our study, long-term studies show that beetles can have variable, but significant, effects on later fungal colonizers (Jacobsen et al., 2015; Weslien et al., 2011). By providing evidence of such effects at the community scale, we add crucial knowledge to the importance of invertebrates in affecting fungal community development through altered assembly history.

4.4 | Bark secondary metabolites impede wood decomposition

High concentrations of bark phenolic acids and methanol-soluble condensed tannins impaired wood decomposition rates, but only tannins had an effect 4.5 years after tree felling. Simple molecular compounds, such as phenolic acids, are targeted during early stages of litter decomposition (Chomel et al., 2016; Moorhead & Sinsabaugh, 2006). Tannins, on the other hand, are polymers of repeating phenolic units and are therefore more difficult to break down. Furthermore, condensed tannins complex with proteins and even with fungal chitin (Adamczyk et al., 2019), thus altering the enzymatic activity of microbes and as such may impede decomposition considerably (Adamczyk et al., 2018; Kraus et al., 2003; Smolander et al., 2012). In a study on four tropical tree species, Loranger et al. (2002) found that simple phenolic compounds controlled early stages of leaf litter decomposition, while later stages were correlated with tannin concentrations. Although we only have initial measurements of secondary metabolites, we suspect that the concentrations of phenolic acids in aspen logs decreased more than tannins during the study period, explaining the lingering effect of tannins after 4.5 years.

In a study using the same samples as here, Jacobsen et al. (2018) found, in contrast to us, a significant difference in decomposition between logs where invertebrates had been excluded and control logs. The essential difference between our two models was that we did not fit the tree identity nor the log's position on the trunk as random effects, because we aimed to use initial secondary metabolite, carbon and nitrogen content to explain this variation. The diverging results between our studies indicate that there was considerable variation between or within trees that we did not manage to account for. Furthermore, there was a large amount of "unexplained variation" in our models on fungal community composition. Although this variation can partly be attributed to inherent problems with the model when fitting the data (Økland, 1999), many other tree qualities may govern wood decomposition or fungal community development. These could be, for example, bark anatomy (Dossa et al., 2018), wood water content (Rayner & Boddy, 1988; Venugopal et al., 2017), or the identity of fungal (Rayner & Boddy, 1988; Venugopal et al., 2017), invertebrate (Seibold et al., 2021; Ulyshen, 2016) or even bacterial (Johnston et al., 2016) communities.

The altered effect of several variables from 1.5 to 4.5 years in our study underlines the importance of long-term observations in dead wood systems. Many previous studies that have linked priority effects to wood mass loss only lasted for 1 year (Dickie et al., 2012; Leopold et al., 2017; Song et al., 2017), although wood decomposition might take decades (Stokland et al., 2012). It appears that the link between community development and functioning is far from obvious. Over 4.5 years, we found that resource quality, but not assembly history, were drivers of wood decomposition rates. However, altered assembly history through invertebrate exclusion controlled fungal community development, even 3 years after exclusion had ceased.

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CONFLICT OF INTEREST

We declare that we have no competing interests.

AUTHOR CONTRIBUTIONS

L.F.L. performed laboratory work, bioinformatic and statistical analyses, and led the writing of the manuscript; T.B. conceived, designed and coordinated the project and performed sampling; R.M.J. conceived and designed the project, performed sampling and laboratory work, and contributed to statistical analyses; A.S.T. and H.K. designed the project; L.N. contributed to chemical analyses. All authors contributed to interpretation and writing of the manuscript, and gave final approval for publication.

OPEN RESEARCH BADGES

This article has earned an Open Data Badge for making publicly available the digitally-shareable data necessary to reproduce the reported results. The data is available at https://doi.org/10.5061/dryad.zcrjdfndj.

DATA AVAILABILITY STATEMENT

The raw sequence reads are deposited in NCBI SRA SUB10694654. The OTU table, metadata, mapfiles and R script for reproducing

figures and analyses in this paper are deposited in Dryad (https://doi.org/10.5061/dryad.zcrjdfndj).

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