MEASURING WARFARE IN WOOD: LINKING COMPETITION AMONG WOOD-DEGRADING FUNGI OF NORTHERN FORESTS TO ITS ECOLOGICAL CONSEQUENCES

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Dedication

For Ella and Ming

Abstract

Competition between distinctive groups of fungi determines the pattern of wood decomposition in forests, but the outcome of these battles may shift in a changing climate. With more than 70% of Earth's biotic carbon stored in woody tissues, understanding the processes that unlock this carbon and release the greenhouse gas CO_2 is critical. For my thesis research, I am addressing several key questions about how fungi colonize and dominate wood on the forest floor. Quantitative PCR was developed to measure biomass of specific fungi from a community in Chapter one. This technique was coupled with ergosterol, dilute alkali solubility, pH and carbon component analysis to measure biotic and abiotic dynamic during wood decomposition. With these comprehensive tools, factors that may influence fungal competition and decomposition outcomes were studied in the following chapters. In Chapter two, wood type was shown not to influence the competition between a brown rot fungus and a white rot fungus. It is contrary to the observations on wood preferences in nature, but reflected different foraging strategies by fungi. This led to the study of Chapter three on priority effect. By increasing the inoculum potential either inside or outside wood substrate, I have shown evidence that a weak competitor fungus can outcompete its more aggressive opponents, thus achieving co-existence. Another two factors, temperature and endophytes, along with priority effect were studied in Chapter four. Endophytes showed a much larger effect in influencing wood decomposition than temperature, mostly through antagonisms against soil fungi. Studies on these factors reveals potential for a more comprehensive model for wood decomposition. Emphasis on the role of microbial components, especially the often neglected endophytic communities,

is possible to explain the variability in wood decomposition that can not be explained by abiotic factors, alone.

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INTRODUCTION

Wood decomposition plays an important part in carbon and nutrient cycling, and also fulfills diverse ecosystem functions (Harmon *et al.* 1986). Decomposition of wood is an important component of the global carbon budget (Weedon *et al.* 2009; Freschet *et al.* 2012). Carbon emissions are balanced with uptake by plants, and this balance determines how much carbon is held in an ecosystem. The ability to hold carbon gives natural ecosystems critical potential to offset human-induced greenhouse gas emissions. Likewise, an imbalance that provisionally favors carbon release will do the opposite, adding more CO_2 and warming potential to the atmosphere. Globally, around 8% of carbon (73 ± 23 Pg) in forest stocks is in dead wood (Pan *et al.* 2011). Another 42% (363 ± 28 Pg) carbon is in live biomass, most of which belongs to wood, is also a substrate for decomposition (Pan *et al.* 2011). Estimating and predicting wood decomposition thus are crucial for an accurate estimation of the global carbon budget, an effect not just scientifically important but also politically (IPCC 2013). Nutrients that lock in dead wood represent critical long-term nutrient pools (Laiho & Prescott 2004). Some limited nutrients can be accumulated in wood and can facilitate seed germination of other plants (Zielonka 2006; Lonsdale *et al.* 2008). Formation of dead wood is often accompanied by new forest gaps, again benefiting the regeneration of forests (Franklin *et al.* 2002). Rotten wood also supports a high diversity of organisms using wood as habitats (Müller & Bütler 2010).

The ecological role of dead wood, or coarse woody debris, was realized decades ago (Harmon *et al.* 1986). Starting in the last century (1900s), numerous studies have been done to estimate the stock and turnover of the woody debris component in most types of forests (*e.g.* 36 studies summarized by Weedon *et al.* 2009). Researchers also tried to find

models that can predict the decomposition rate of dead wood. In most of the studies, abiotic factors were included as the key parameters (Chambers *et al.* 2001; Weedon *et al.* 2009; Tuomi *et al.* 2011; Freschet *et al.* 2012). However, it is not uncommon that abiotic factors resulted in a poor performance or over-simplified models (Bradford *et al.* 2014). Lacking a good predictive mechanism for wood decomposition, a very simplified process is simulated for wood decomposition in most of current earth system models (Dai *et al.* 2003). In most cases, wood was considered a recalcitrant/slow carbon pool, and its turnover rate was determined by C/N ratio or Q_{10} (rate of change as a consequence of increasing the temperature by 10°C, Dai *et al.* 2003). Incorporating biotic factors into current model thus is among top priorities in the study of wood decomposition.

In boreal and temperate forests, major wood decomposers are constrained in two distinct but evolutionarily related functional groups of Basidiomycetes – the white rot and brown rot fungi (Gilbertson 1980). Lignin, a recalcitrant carbon matrix, can be degraded by white rot fungi, often simultaneously with carbohydrates (cellulose and hemicellulose). In contrast, the majority of lignin remains behind in wood residues after carbohydrates are removed by brown rot fungi. These rot types may represent more of a gradient than previously assumed (Riley *et al.* 2014), but collectively create wood residues with distinctive physiochemical properties that can influence soil processes and the cycling of other soil organic matter (Rypáček & Rypáčková 1975; Gilbertson, 1980; Jurgensen *et al.* 1997; Filley *et al.* 2002, Song *et al.* 2012). Brown rot involves production of small size oxidative radicals (in most cases hydroxyl radicals) that can penetrate lignin and attack cellulose (Daniel *et al.* 2007). Cellulose can be cut into small fragments by these radicals and diffuse back to the hyphae of brown rot fungi. This is different than white rot fungi, in

which two sets of enzymes are produced to attack lignin and cellulose (Worrall *et al.* 1997). Thus, although white rot fungi can degrade both wood components, their efficiency against cellulose is often lower than brown rot fungi (Song *et al.* 2012). Current studies show that brown rot fungi evolved multiple times (at least seven) from different lineages of white rot fungi in history (Floudas *et al.* 2012). The evolution may be under the pressure of cold environment and the presence of coniferous trees, since brown rot fungi mostly colonizes coniferous trees in high latitude forests, while tropical forests are dominated by white rot fungi (Fukami *et al.* 2010). Since the ability to degrade wood is mostly constrained in these two groups, understanding the factors that determine the rotting types, *i.e.* pattern of fungal colonization in wood, are likely to be very useful toward a new approach for modelling wood decomposition.

My thesis focuses on linking the biotic components to abiotic factors and finally the process of wood decomposition. Chapters are broken down by factors that could influence the dynamic of fungal community, which in turn determines the pathway of wood decomposition. Underlying these trials is the development of a molecular means to determine who 'wins' when wood-degrading fungi compete inside the wood.

In Chapter 1, I introduce a method using quantitative PCR to measure the competition of fungi in wood. The purpose is to find a tool that can extract fungal DNA and measure fungal biomass quantitatively, and not just the presence/absence of a species. Issues related to fungal DNA extraction and fungal biomass measurement are discussed in detail. This chapter has been published in *Fungal Ecology* (Song *et al.* 2014).

In Chapter 2, two fungi were used to test the effect of wood type on fungal competition. Considering the observation of substrate preference of brown rot and white

rot fungi, it is reasonable to think that substrate imposes strong influence on fungal competition and niche separation. No significant effect, however, of wood type was discovered in the microcosm studies, with results instead suggesting the importance of inoculum potential and priority effect in successful competition in wood. This chapter was published in *FEMS Microbiology Ecology* (Song *et al.* 2012).

In Chapter 3, I tested whether the sequence of species colonizing wood can determine the outcome of competition. Several studies have shown that the structure of fungal community can be determined by looking at which species arrives first. Early colonists may modify the wood environment and antagonize or facilitate late arrivals, thus become a controlling factor for competition and decay rate. The same pair of fungi from Chapter 2 were used to test this 'priority effect'. This chapter has been submitted to peer review as an invited paper (invited by Dr. Jennifer Talbot, Stanford University) in a special issue 'The Forest Microbiome' in *Microbial Ecology*.

In Chapter 4, two new factors, temperature and endophytes, were studied. By examining the effect of temperature, I tried to link the dynamics of the fungal community in wood with aspects related to potential changes in temperature. The role of endophytic communities in wood decomposition was rarely studied. Our lab and others have observed potential saprotrophic fungi living as endophytes in wood. The potential of endophytic communities as early colonizers and potential wood decomposers were studied in this chapter. If wood-degrading fungi are present in wood as endophytes, this will be taken into consideration for all the models of wood decomposition. My study revealed a more interesting and important role of endophytes in saprotrophy, relative in this case to any effects of temperature. Part of the results in this chapter were presented in an invited

symposium talk in 2014 at the Mycological Society of America annual meeting, and it will be submitted as a manuscript in 2014.

Chapter One: Probing wood-degrading fungi and their competition in wood

Chapter Summary

The biomass of brown and white rot fungi were estimated using ergosterol and quantitative PCR (qPCR). Each biomass estimate was compared with biomass measured gravimetrically from liquid cultures, as well as from three wood substrates at two decay stages. Fungal morphological changes in two different substrates, agar and pine, were measured using a chitin-specific fluorophore and confocal microscopy. In liquid culture, the two fungal isolates had significantly different biomass conversion factors for both methods. In wood colonized for 3 wk, qPCR yielded a lower estimate than did ergosterol, while at week 8 it yielded a higher estimate. Changes in average fungal cell dimensions partially explained the differences between the two methods. Overall, our results suggest that a constant conversion factor cannot be assumed as default for either method. Instead, it demonstrates the importance of standardizing wood species, decay class, fungus-specific conversion factor, and DNA extraction protocol in order to properly estimate fungal biomass in woody substrates. The issues of designing qPCR primers for specific fungal species, and extracting fungal DNA from wood matrix were also discussed. A modified protocol of DNA extraction was developed in order to improve the accuracy of qPCR.

1.1 Background

Wood-degrading fungi are ecologically and economically important decomposers in forests and wooden structures. These fungi can cause structural failure in trees and lumber (Franklin *et al.* 1987), and some pathogenic species, such as the root rot fungus *Armillaria mellea*, cause tree death (Coetzee *et al.* 2000). Dead wood, which represents the largest

detritus pool in forests and Earth's largest biotic carbon pool, is also predominantly cycled by wood-degrading fungi in temperate and boreal forests (Freschet *et al.* 2012). Connecting the ecosystem functions of dead wood to those fungi responsible for decomposition is difficult yet essential, and it demands a capacity for accurate quantification of key fungi within complex microbial communities.

There are two major difficulties in quantifying the biomass of plant-decomposing fungi in situ. First, it is not practical to separate fungal hyphae from wood to determine fungal biomass, and unfortunately both change mass as the fungus colonizes and the plant material is mineralized. Second, species diversity of microbial communities on plant materials adds complexity on measuring the biomass of a specific wood-degrading fungus (Kubartová *et al.* 2012).

The first issue can be approached by using indirect measurements of fungus-specific biomarkers, (Jones & Worrall 1995; Newell *et al.* 1988). But most of these methods for assessing wood-degrading fungi in situ provide semi-quantify or qualify result on fungi. Morphological identification requires expertise, and isolating and culturing fungi from wood typically reveals a small fraction of the fungi present (Tringe *et al.*, 2005). Active and total fungal biomass in wood can be calculated using ergosterol (Newell *et al.*, 1988) and chitin assays (Ekblad & Näsholm, 1996), but these techniques are not specific to fungal taxa. Molecular approaches can better resolve which fungi are present, but, at present, have difficulty weighting the presence of specific taxa relative to others present. Phospholipid fatty acid (PLFA) analysis (Frostegard *et al.*, 1993), denaturing gradient gel electrophoresis (DGGE) (Muyzer *et al.*, 1993), randomly amplified polymorphic DNA (RAPD) (Groppe *et al.*, 1995; Marzorati *et al.*, 2008), and carbon source utilization

profiling (Spears *et al.*, 2003) can "fingerprint" communities, but richness measures provide little insight into functional diversity. All these methods lack the ability to measure the abundance of a single fungus within a community with up to thousands of other species.

By designing a specific primer set, quantitative PCR (qPCR) can be a versatile tool targeting different species of fungus at various accuracy. In most cases, internal transcribed spacer (ITS) DNA regions (Schoch *et al.* 2012) was used as the target. Like ergosterol, fungal biomass is calculated from the abundance of the biomarker by applying a conversion factor, i.e. a ratio between biomarker and biomass, but these adjustments assume a static biomarker-to-biomass ratio that is constant across all treatments. Variability in the conversion factors for ergosterol has been discussed in a range of fungal species (Klamer & Bååth 2004; Baldrian *et al.* 2013) and environmental conditions (Newell *et al.* 1987; Li *et al.* 2009). In addition, decomposer communities vary by environments (Pasanen *et al.* 1999; Raviraja *et al.* 2004; Klamer & Bååth 2004). It is logical that these issues concerning the use of traditional biomarkers would persist when relating biomass or other abundance measures to DNA sequence copy numbers, and this will affect studies targeting specific fungi as well as those extrapolating biodiversity estimates when using high-throughput sequencing. Therefore, a better understanding of factors modulating this ratio when using DNA copy number, along with a targeted set of methodological guidelines to reduce variability, are needed to accurately measure fungal biomass *in planta*.

Quantitative PCR offers the ability to determine gene copy number of specific fungi, but it similarly bears assumptions as do other indirect biomarkers such as ergosterol. Ergosterol in different treatments can be compared directly (Meier *et al.* 2010) or converted to fungal biomass using universal conversion factors. For terrestrial environments, a value

of 5 μg mg^{-1} is often used for all conversions (Ruzicka *et al.* 2000; Bååth 2001). These studies, however, cover a limited number of fungal species, and a wide range of ergosterol-to-biomass ratios have been reported (Pasanen *et al.* 1999; Klamer & Bååth 2004). The influence of environmental factors, such as growth medium (Newell *et al.* 1987) and temperature (Li *et al.* 2009) have also been shown to affect ergosterol content in fungi and, indirectly, their conversion factors. A similar condition applies to the assumption when using qPCR. The copy number of a target DNA sequence is measured during qPCR by monitoring the exponential amplification in PCR reaction (Eikenes *et al.* 2005). Whole fungal groups (Graaff *et al.* 2010), such as Basidiomycetes (Fierer *et al.* 2005), or a specific fungal species (Horisawa *et al.* 2009) can be quantified in the same wood sample by using different primers targeting the ITS region, a universal DNA barcode marker for fungi (Schoch *et al.* 2012). Similar to chitin and ergosterol, this DNA-based approach assumes a constant ratio between copy number and biomass, and it is known that ITS copy number can vary among fungal species (Maleszka 1993) and strains (Herrera *et al.* 2009). For example, a conversion factor of 1.88×106 copies mg^{-1} (copy number per fungal biomass) for Trichoderma harzianum (López-Mondéjar *et al.* 2010) has been proposed, while Piloderma croceum has a conversion factor of 1.27×109 copies mg^{-1} (Raidl *et al.* 2005). This exemplifies the potential problems of using a universal conversion factor. While the influence of the environment on conversion factors has not been currently addressed, fungal hyphae are known to be morphologically diverse (Klein & Paschke 2004) and may respond to environmental changes *in planta*. In the case of using ITS copy number for fungal biomass, by comparing with ergosterol and fatty acid based methods, Baldrian *et al.* 2013 showed that DNA based methods suffered a higher level of variation and

underestimated fungal biomass in litter and soil. Defining the underlying factors introducing this variability can help guide experimental design to limit these issues and improve the use of a method being proposed and utilized with increasing frequency.

Current protocol of extracting fungal DNA from wood still has space for improvement. The most effective protocol used CTAB as the major reagent to digest the wood matrix, while several commercial kits failed in a previous selection trial. One widely used protocol was chosen as the extraction method in Chapter 2 (Jasalavich *et al.* 2000; Song *et al.* 2012). However, this protocol is relatively long (3 hr of water bath, 2 - 3 hr for other procedures), and still not effectively eliminate PCR inhibitor for some wood substrates (*e.g.* Level of extractives is high in oak wood). A shorter protocol is needed to accommodate high throughput sampling without compromising on the efficiency of extraction. PCR inhibitors and small size DNA fragments (<100 bp) should also be removed from extracted DNA as much as possible. This will increase the quality of PCR amplifiable DNA.

In this chapter, species specific primers were designed for four fungal species, two white rot fungi and two brown rot fungi. The first pair, *Gloeophyllum trabeum* (brown rot) and *Irpex lacteus* (white rot), were cultured on three types of wood (oak, birch and pine) to compare the effects of fungal species, substrate type, and decay stage on biomass measurements determined *via* ergosterol or qPCR approaches. Previous studies indicated that these isolates differed significantly in their respective ergosterol:biomass ratios (Song *et al.* 2012), making a useful side-by-side comparison for a molecular approaches. Our goal was to make this comparison over a time course of decomposition and on distinct substrates in order to inform both molecular analyses and experimental design, and more importantly

to reduce the number of assumptions used when using qPCR to measure biomass of fungal decomposers *in planta*. A second pair of fungi was used to test qPCR assays targeting specific fungus from a complex community. Pooled samples from the experiments of Chapter 4 were used to test various protocols of DNA extractions and estimate the optimum sample size for qPCR assays. The protocol was modified to require less handling time, and also with a filter column to reduce the level of inhibitor. In addition, the new protocol was applied on wood samples with a wide range of weight, in order to test if the extracted amount of DNA and qPCR signal scale proportionally with sample amount.

1.2 Methods

1.2.1 Quantitative PCR assay for wood-degrading fungi

Study Isolates

The brown rot fungus, *Gloeophyllum trabeum* (Persoon: Fries) Karsten strain M617 (ATCC 11539), and the white rot fungus, *Irpex lacteus* (Fries:Fries) Fries strain M517 (ATCC 11245) were used in experiments in Chapter 1, 2 and 3. Both fungi are native to, and commonly found in, Minnesota (USA). Both were isolated from a single forest plot in Cloquet, MN recently.

Another pair of fungi, *Piptoporus betulinus* (brown rot) and *Fomes fomentarius* (white rot) were used for experiments in Chapter 1 and 4. These two fungi were common species on birch and were isolated from a same standing dead birch at the Cloquet Experimental Forest of University of Minnesota.

Primer design

Species specific primers were designed for these four species of wood-degrading fungi. Primers for the pair *G. trabeum* and *I. lacteus* were designed for microcosms that contain

only the two species. Primers for *P. betulinus* and *F. fomentarius* were designed to work in the presence of other species, in order to use in the experiments in chapter 4 with endophytes community.

All fungi were sequenced for their ITS region and aligned in pairs to search for proper primers. Four sets of primers were designed to only amplify the ITS region of the target fungi for quantitative PCR assay. The primer sets were summarized in Table 1.1. During the designing process, sequences were loaded into Primer Blast (Ye *et al.* 2012) for an initial picking. *P. betulinus* and *F. fomentarius* were also aligned with their close relatives, *Fomitopsis pinicola* and *Ganoderma spp*., respectively for primer picking. Candidate primer sets were then tested by Gradient PCR ranging from 55 to 65°C to optimize annealing temperature. In most of the case, 60°C resulted in highest Ct, good PCR efficiency and single peak in the melting curve.

Fungal DNA was also extracted from 2% malt extract agar culture stock using a standard CTAB method (Jasalavich *et al.* 2000) and sequenced for primer verifications.

1.2.2 DNA extraction method for wood-degrading fungi

Fungal DNA in wood samples was extracted by the protocol of Jasalavich *et al.* 2000 in the experiment in Chapter 1, 2 and 3. Two modifications to the original protocol were tested for Chapter 4 to accommodate large number of samples (~400). The first modification shortened the total length of water bath (65°C) from 3 hr to 1 hr. The second modification replaced ethanol wash of extracted DNA with a spin column filtering. The DNA solution after the second chloroform extraction (250 µL) was mixed with 1250 µL buffer PB (binding buffer, 5M Guanidine-HCl, 0.9 M potassium acetate, pH 4.8). The

mixture was loaded to a spin column (Epoch Life Science, Sugar Land, TX, USA) in three potions and centrifuged for 1 min at 14,000 g. The filter was washed with 750 µL buffer PE (washing buffer, 10 mM Tris-HCl pH 7.5, 80% ethanol) by centrifuging for 1 min at 14,000 g. The column was centrifuged again for 1 min to get rid of residue ethanol. The column was then laced on a clean 1.7 ml microcentrifuge tube and 100 µL buffer EB (Qiagen, Valencia, CA, USA) was then added to the center of the white filter and incubate at RT for 2 min. Purified DNA was washed down to the tube by centrifuging for 1 min at 14,000 g. Amounts of extracted DNA were compared among the three protocols. Water bath was also removed from the protocol to test its effect in separating fungal DNA from wood matrix. Pooled samples containing hyphae of *P. betulinus* and *F. fomentarius* from experiments of Chapter 4 were used in this test.

The optimized protocol was used to extract ten pooled wood with their wet weight ranged from 20 to 140m. This gave an evaluation of the stability of this protocol on the amount of samples, which are often varied in real experiment. The amount of input wood samples were correlated with the amount of extract DNA and Ct value of both fungi in their qPCR assays.

1.2.3 Compare qPCR and ergosterol in measuring fungal biomass

Soil-block microcosms

Soil-block microcosms (ASTM D 1413, 2007) containing 1:1:1 mixture of peat, vermiculite, and fertilizer-free soil were used for fungal cultivation as described by Song *et al.* 2012. Round agar plugs (38 mm2, 7 mm dia.) were placed on 2 x 5 cm birch feeders (cut from tongue depressors). Two birch feeders, each with two agar plugs of one fungus,

were placed onto the surface of the soil mixture, with a 5-mm gap between the feeders. The inoculated fungi were cultured for 7 days at 25°C before three wood dowels of white birch (Betula papyrifera), red oak (Quercus rubra), or pine (Pinus sp., southern yellow pine) were added to each microcosms. For each type of wood, five microcosms containing three autoclaved dowels were assembled and inoculated using a randomized complete block experimental design.

The dowels were harvested after 3 and 8 wk. For each replicate, one dowel was ground fresh for ergosterol measurement. A second dowel was flash frozen in liquid nitrogen and ground with a Midas Rex bone mill (Medtronic Inc., Minneapolis, MN), modified by sharpening blades as discussed in Song *et al.* 2012. Blocks were ground whole to allow expression of fungal biomarkers relative to a volume of wood, not mass, and to eliminate issues of declining wood density during wood decay. The third dowel was oven-dried and used to measure mass loss in order to gauge the extent of decay. The three dowels were separated for different methods instead of mixing powder extracted from a single dowel in order to prevent degradation of ergosterol and DNA by oven drying and flash freezing.

Conversion factors

Both fungi were grown in 100 ml of 2% malt extract solution (2 g malt extract dissolved in 100 ml distilled water and sterilized at 121°C for 20 min) in three 250-mL flasks. The flasks were incubated at 25°C for 3 wk as static cultures. Fungal tissue was collected by vacuum filtration through a filter paper and ground using the modified bone mill after flash freezing in liquid nitrogen. Ten replicates were weighed; five of them were added to 5 mL

cold methanol for ergosterol extraction and stored at 4°C. The other five samples were added to 6 mL 2X CTAB buffer, and DNA was extracted immediately.

The water content of the fungal tissue was obtained by oven drying five pre-weighed tissue samples at 103°C for 48 hr. Total ITS copy numbers and ergosterol were calculated after adjusting for extraction efficiency (Song *et al.* 2012), and dividing the dry fungal biomass to obtain the conversion factors for qPCR (ITS copies mg-1 dry fungus) and ergosterol (µg mg-1 dry fungus).

Ergosterol

Fresh ground materials were extracted in methanol as described by Newell *et al.* 1988. Cholesterol (9.6 ng) was added in each extraction reaction as an internal standard. Extracted samples were analyzed for ergosterol and cholesterol by using high-performance liquid chromatography (HPLC) as described by Schilling & Jellison 2005.

Quantitative PCR

Fungal DNA from fresh mycelium and ground wood dowels was extracted as described by Jasalavich *et al.* 2000 using 2X and 1X CTAB buffer for mycelium and wood, respectively. This extraction protocol yielded higher amounts of DNA relative to several kits tested. The DNA was precipitated with isopropanol overnight, washed 2 times in 70% ethanol, and re-suspended in 100 µl of nuclease-free water (Ambion, Austin, TX, USA). DNA concentration was determined with a Qubit™ fluorometer (Invitrogen, Carlsbad, CA, USA), and diluted to 1.5 ng µl-1.

Primers were designed to amplify a specific ITS region of the two fungi as described by Song *et al.* 2012. The primers: GT94-F (5'-TCA GGC TGT CCT TCC TAT GTC-3') and GT521-R (5'-GTC AAA TTG TCC GAA GAC G-3') were developed to

amplify a 427-bp DNA fragment from *G. trabeum*, whereas primers IL54-F (5'-ATC GAG TTT TGA ACG GGT TG-3') and IL633-R (5'-AAA TGA TTG TCT CGG CAA GG-3') were used to amplify a 579-bp DNA fragment from I. lacteus. The ITS fragments were cloned into the StrataClone PCR cloning vector, pSC-A-amp/kan (Stratagene, La Jolla, CA, USA). The resulting plasmids were used to create standard curves representing 101 to 106 copies. Quantitative PCR was performed using the ABI Prism™ 7000 sequence detection system. Each reaction contained plasmid template DNAs or 4.5 ng of sample DNA, 400nM of each primers, and 20uL of 2X iTaq™ SYBR® Green Supermix with ROX (Bio-Rad, Hercules, CA, USA) in a total volume of 40 μL. All measurements were performed in triplicate. The thermal cycling conditions for DNA amplification from *I. lacteus* were an initial denaturation at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, 60 °C for 30 s and 72 °C for 1 min. A similar thermal cycling protocol was used for *G. trabeum* detection, except the annealing temperature was reduced to 58 °C.

Staining of hyphae and confocal microscopy

To better understand the influence of substrate on biomarker yields, fungal morphological characters relevant to cell dimensions and nuclei-per-cell counts were quantified from *in planta* images of fungi. The *G. trabeum* and *I. lacteus* were cultured for 10 d on southern yellow pine wafers (30 mm × 27 mm × 3 mm) in soil-block microcosms (ASTM 2007) or in 2% malt extract agar. For agar culturing, an ultrathin layer of agar was made by streaking a drop of hot agar solution onto a sterile microscopic slide using another slide as a spreader, as described by Raidl 2005. After inoculating the fungus on one corner of the agar layer, the cultures were kept in a glass petri dishes containing 5 ml sterile distilled water. All cultures were incubated for 10 d, in the dark, at 25°C. At the time of harvest, a small piece

of wafer (5 mm × 5 mm) was cut from one end with a razor blade, soaked in 25% TFMTM tissue freezing medium (Triangle Biomedical Sciences, Inc., Hatfield, PA, USA) by vacuum infiltration, and thin sectioned (30-40 µm) using the OM 2488 MinotomeR microtome-crystat at at -20°C (International Equipment Company, Needham Heights, MA, USA). Pine was the only wood substrate used in confocal microscopy for comparing the morphological difference between agar and wood substrates.

Fungal cell walls were stained with wheat germ agglutinin-tetramethylrhodamine (WGA-TMR, Invitrogen, Carlsbad, CA). Samples were incubated at room temperature in the dark for 15 min in 1X phosphate buffered saline (PBS, pH 7.4) containing 10 µg ml-1 WGA-TMR before rinsing with 1X PBS (pH 7.4). Confocal fluorescence images were captured using a Nikon A1 spectral confocal microscope (Nikon, Japan), in most cases by using Z-stacking. Fluorescence of WGA-TMR was excited at 543nm and detected at 560-630nm, and was distinguished against wood autofluorescence using a separate excitation at 488nm and detection at 505-540nm. The width and length of individual hyphae were measured and the surface area of each hyphal filament was calculated assuming the cells were cylinders. For *G. trabeum*, 179 and 121 cells were measured in agar and wood, respectively. For *I. lacteus*, 126 cells were measured in agar. Since *I. lacteus* lacks clamp connections, only 55 cells in wood were found to be intact and measured for length, while 172 cells were recorded for width.

Statistical analyses

Data are expressed as means ± standard error (SE). The calculated biomass based on qPCR and ergosterol were compared using two-sample t-tests ($\alpha = 0.05$) within each wood type

and fungal isolates. ANOVA was applied with Fisher's LSD multiple comparison (α = 0.05) on log transformed copy number data to ergosterol ratios of each fungus. The hyphal width, length, and surface area in agar and wood were tested for significant differences by using a two-sample t-test (α = 0.05).

1.3 Result and Discussion

1.3.1 Validate the specificity of qPCR assays

Primers sets that only amplify the target fungus were developed and listed in Table 1.1. Although *G. trabeum* has an optimum temperature at 58°C, later test found out that qPCR efficiency was within the good range (90 – 110%) at 60°C. It was not surprising that in most of the latest qPCR kits, 60°C was recommended as the default annealing temperature. For fungi with amplicons less than 500 bp, such as *P. betulinus* and *F. fomentarius*, a single 60 °C step was enough for the detecting stage.

Primer sets picked by Primer Blast in many cases amplified the close relative of *P. betulinus* and *F. fomentarius*, or produced multiple bands. The final primers were found by manually checking their alignment (Supplementary document S1). It is also worth noticing that *F. fomentarius* has two phenotypes that differ their ITS region by around 15 nucleotides (Judova *et al.* 2012). Primers were designed to amplify both phenotypes although only one were isolated from on birch at Minnesota.

1.3.2 Evaluate the modified protocol of fungal DNA extraction from wood

The original protocol (Jasalavich *et al.* 2000) required twice water bath (2 hr and 1 hr). Similar amount of DNA were extracted (Fig 1.1) after reducing water bath to once and 1 hr.

The amount of DNA recovered by spin column filtering was about half of that by other methods (Fig 1.1). But since only small size fragment (<100bp) were filter out, the amplifiable DNA should be similar in all three protocols. It is also worth notice that using spin column may reduce the levle of PCR inhibitors, since less number of samples required to be diluted before they can be amplified by PCR.

The effect (extracting power) that needed to extract DNA from different fungal species also varied. For the two *Basidiomycota* tested, 1 hr water bath at 65°C is required to extract the DNA of *P. betulinus* (Figure 1.1). On the other hand, DNA of *F. fomentarius* can be extracted by simply vortex sample with the extraction buffer. It is possible that the attachment forces of hyphae to wood matrix differ by species. Although not common in reports on fungal DNA, an additional sonication step was necessary to extract the DNA of some Streptomyces (Schlatter *et al.* 2010). Considering the huge diversity of fungal species, water bath is thus recommended for the CTAB related protocol. An additional step of water bath should also be considered for commercial kits.

For a wide range of input sample size (20 to 140 mg), the modified protocol kept a good linear relationship with the outcomes. Amount of extracted DNA increased linearly with the amount of wood samples (Figure 1.2). The pooled sample tested was abundance in *P. betulinus* and rare in *F. fomentarius* as shown in their Ct values (Figure 1.2). Ct of both species also linearly related to the sample amount (after Log_{10} transformation). The sampling size of the modified protocol is larger than that of PowerSoil and PowerPlant DNA kits (50 mg, MOBIO, Carlsbad, CA, USA), and can be easily scaled up for larger samples.

In general, the modified protocol with shorter water bath and spin column provide a solution for high throughput sample processing. Roughly, if samples were weighted the day before extraction, up to 96 samples can be processed within one day and ready for downstream analysis. The sample size of this protocol (up to 150mg) were generally larger than some commercial kits (~50mg) with a much lower cost (~$1 per sample). We also showed that downstream analysis was not influenced by the amount of input sample, *i.e.* the extraction ability of this protocol did not saturate at a wide range. Although we only test this protocol for qPCR, it should be valuable for other applications that require high quality of DNA, such as next generation sequencing.

1.3.3 Conversion factors for qPCR and ergosterol

The conversion factors for both ergosterol and ITS copy number varied significantly (up to 4-fold) between the fungi tested, suggesting that applying a universal conversion factor with either approach will involve assumptions. For *G. trabeum*, $2.58 \times 10^6 \pm 2.11 \times 10^5$ ITS copies per mg dry mass and 7.25 ± 0.92 μg ergosterol per mg dry mass were obtained from liquid cultures, compared to $10.00 \times 10^6 \pm 1.38 \times 10^6$ ITS copies per mg dry mass and 3.70 ± 0.64 μg mg^{-1} dry mass for *I. lacteus*. The variations of ergosterol and ITS copy number among species were also reported in other studies. For ergosterol, a 1 to 24 μg mg^{-1} range was found in 11 common fungi (Klamer & Bååth 2004), and a 0.017 to 68 μg mg^{-1} range was found in fungi that commonly inhabit building materials (Pasanen *et al.* 1999). Baldrian *et al.* 2013 reported wide ranges of ergosterol content (2.2 to 7.2 μg mg^{-1}) as well as ITS copy number (67×10^6 to 910×10^6 ITS copies mg^{-1}) in fruiting bodies of 12 species, with greater variation in ITS content. Taken together, these data suggested that ergosterol

and ITS copy number variation are intrinsic to fungi, and that measurement should be accompanied with species composition data.

In environmental samples, the assumption is often that species richness can compensate for species variations. In plant tissues, however, this is problematic when priority effects during colonization and competition for space result in low species richness and single-species dominance, as often is the case for wood-degrading (Fukami *et al.* 2010) and mycorrhizal fungi (Kennedy *et al.* 2007).

1.3.4 Influences of wood types and decay stages on qPCR and ergosterol

Wood type (as tree species) was a major factor influencing biomass estimates, as reflected in significantly different biomass calculated from ITS copy number and ergosterol measurements (Fig 1.3). In oak, using qPCR to estimate fungal biomass yielded lower estimates of biomass than when using ergosterol, while in pine, the opposite was generally true. Extraction efficiency was previously shown to be different among these wood types (Song *et al.* 2012) when mixing non-degraded wood with known amounts of fungal biomass. This is likely due to different substrate properties, such as the content of phenolic extracts. While biomass can be compared statistically in a field trial between samples taken from residues of different plant species, the comparison is best if the effect of substrate is insignificant. This was not true in our case.

The stage of decay was also a compounding factor influencing biomass estimates using either biomarker. With birch as the clearest example (Fig 1.3), the use of qPCR yielded lower biomass estimates than did ergosterol in week 3 residues, but qPCR generally yielded a higher estimate at week 8. The copy number:ergosterol ratio increased

significantly from weeks 3 to 8 for *I. lacteus*, but not for *G. trabeum* (Fig 1.4). This ratio may reflect changing hyphal length, which is longer at 3 wk, as the two fungi changed strategies from resource occupation to utilization (Boddy *et al.* 2000). This is in accordance to the rate of mass loss which was minor after the first 3 wk period, but increased to 20% - 50% after 8 wks (Fig 1.3). It is also possible that extraction efficiency was affected by the substrate, which could be different as cellulose and lignin are modified or removed over 8 wk of wood degradation. Similar to comparisons among wood types, comparing fungal biomass among decay stages is also likely affected by hyphal morphology, as well as other factors.

1.3.5 Morphological changes on agar and wood

In planta confocal microscopic analyses indicated that hyphal morphology changes were likely a major reason for changes in qPCR conversion factors (Fig 1.5 & 1.6). Fungal hyphae morphology, including branching (Harris 2008), cytoplasm movement (Klein & Paschke 2004) and diameter (Singh *et al.* 2008) is known to be influenced by environmental factors. Such morphological changes may result in different biomass estimates between two biomarkers if they target different organelles, in this case cell membrane versus nuclei. While such differences were not observed in ergosterol and phospholipid-derived fatty acids (18:2ω6.9) measurements, both compounds in the cell membrane (Klamer & Bååth 2004), it is difficult to ascertain how this might be affected within a wood matrix. By directly observing individual hyphae, we showed that both fungi had different morphology, in the terms of diameter, length and surface area, when grown in agar versus wood (Fig 3 & 4). If such observations were true for all the wood types, then

qPCR analyses were likely to result in lower biomass estimation for *G. trabeum* than for *I. lacteus*. This, however, was not in accordance, in some cases, with the pattern of biomass estimates in wood (Fig 1.3). For example, *G. trabeum* biomass after 8 wks was greater as measured by qPCR than ergosterol in birch and pine (Fig 1.3). It should be notable that confocal microscopy analyses may not reveal the real morphological characteristics of the fungus nor be readily used to extrapolate overall biomass due to the relative small proportion of wood sample examined. In addition, the chitin-bind fluorescent dye also assumes the same caveats as any chitin-based biomarker. Furthermore, observations based on 10 d culture may also be different than that of 3 and 8 wk cultures, and extraction may change with decay stage. Although hyphal morphologies were only measured in pine, morphological changes occurring among different types of wood can be implied from the changing ratios of ITS copy number to ergosterol content (Fig 1.4) and inconsistent patterns of biomass using the two biomarkers (Fig 1). Nevertheless, both converted biomass (Fig 1.3) and confocal measurements (Fig 1.5) indicated that changing fungal morphologies between agar and wood, and potentially among different wood types, may vary the efficacy of conversion factors developed from pure cultures in growth media.

1.3.6 Suggestions for minimizing the variation of different methods

These results underscore the complexities of extrapolating biomass estimates from copy number and ergosterol measurements when sampling fungal tissue from wood, but also offer clear guidance for analytical and experimental design. Species composition and substrate types are two major sources of variability, and should be quantified and used as weighting factors. When comparing multiple fungi, biomarker content should be estimated

from fresh tissue for each individual species. Comparisons among different wood species or decay stages should also be avoided and this is plausible in field studies, especially where early or moderate stages of decay are used for extractions. In addition to tree species, using a system of decay classes is common practice in wood biogeochemical studies (Hale and Pastor 1998) and can guide standardization. Sampling techniques based on volume, instead of weight, should be used to compensate for mass loss during decomposition. This can be done in field using drill bits or other approaches to excise precise volumes of plant tissue rather than extracting per mass, and perhaps using volume compensation strategies for more degraded material, as outlined by Fraver *et al.* 2013. When taking these factors into account, it would also be complementary to measure properties of substrate such as wood density, dilute alkali solubility, and pH to report as a reference of the status on the substrate and the outcomes driven by the dominant colonizers. To further lower the variability from substrate, fresh tissue can be mixed with the substrate to ascertain extraction efficiency, as was done in this study. In addition to variation due to species and substrate, the accuracy of qPCR can also benefit from using an internal DNA standard, such as *Escherichia coli* used by Park & Crowley 2005. Collectively, these additional considerations can inform experimental design prior to sampling, ensure a more integrated and true interpretation of fungal activity, and provide more power when making statistical comparisons.

Chapter Two: Linking wood decomposition to fungal competition: substrate preference

Chapter Summary

Many wood-degrading fungi colonize specific types of forest trees, but often lack wood specificity in pure culture. This suggests that wood type affects competition among fungi and indirectly influences the soil residues generated. While assessing wood residues is an established science, linking this information to dominant fungal colonizers has proven to be difficult. In the studies presented here we used isolate-specific quantitative PCR to quantify competitive success between two distinct fungi, *Gloeophyllum trabeum* and *Irpex lacteus*, brown and white rot fungi, respectively, colonizing three wood types (birch, pine, oak). Ergosterol (fungal biomass), fungal species-specific DNA copy numbers, mass loss, pH, carbon fractions, and alkali solubility were determined 3 and 8 weeks post-inoculation from replicate wood sections. Quantitative PCR analyses indicated that *I. lacteus* consistently outcompeted *G. trabeum,* by several orders of magnitude, on all wood types. Consequently, wood residues exhibited distinct characteristics of white rot. Our results show that competitive interactions between fungal species can influence colonization success, and that this can have significant consequences on the outcomes of wood decomposition.

2.1 Background

Colonizing wood by fungi are determine by many factors. These include wood type, fungal germination success, synergism, and antagonism likely play roles in fungal colonization

and the overall rate of wood decomposition, in addition to temperature and moisture (Boddy & Heilmann-Clausen, 2008). In laboratory trials, wood-degrading fungi often exhibit metabolic flexibility, degrading non-host woods and non-woody plant tissues as efficiently as their normal wood substrates (e.g., Valášková & Baldrian, 2006). In forests, however, wood-degrading fungi are often associated with specific wood types, and this is reflected in many species names (e.g., Piptoporus betulinus is found primarily on Betula papyrifera) (Gilbertson, 1981). There are also large-scale associations between brown rot fungi and conifers, and between white rot fungi and angiosperms (Gilbertson, 1980; Hibbett & Donoghue, 2001). While these fungus-wood correlations are far from absolute, the predictability of the presence of specific sporophores on individual wood species suggests that wood type has a major influence on the competitive success of individual fungi among a diversity of wood-colonizing fungi.

In this chapter, isolate-specific quantitative PCR (qPCR) was used to examine fungal colonization success as a function of wood type. Physiochemical analyses of the resulting residues generated from decay were used to qualify fungus-specific outcomes and as a benchmark for confirming qPCR results, most notably using dilute alkali solubility (DAS). Three wood species were examined and at least one treatment per fungus (brown and white rot isolates) included a normal 'host' wood (a common natural association, for example Gloeophyllum trabeum on pine).

2.2 Methods

2.2.1 Fungal isolates

The brown rot fungus, Gloeophyllum trabeum (Persoon: Fries) Karsten strain M617 (ATCC 11539), and the white rot fungus, Irpex lacteus (Fries:Fries) Fries strain M517 (ATCC 11245), were used in this study. Both fungi are native to, and commonly found in, Minnesota (USA). *I. lacteus* is often found fruiting on angiosperm woods, such as alder and birch, whereas *G. trabeum* can be isolated from angiosperm woods or conifers, such as spruce or pine, or on softwood lumber (Gilbertson & Ryvarden, 1986). Both fungi were isolated from a single forest plot in Cloquet, MN during this experiment. Fungi were maintained in a culture collection at the University of Minnesota, using agar slants with pine or birch wafers for periodic culturing and maintenance of lignolytic activity. For this study, isolates were maintained in the dark at 25°C on 20 ml malt extract agar. Soil-block microcosms were inoculated with round agar plugs (38mm2 with a diameter of 7mm) of equal agar thicknesses, obtained from within the hyphal margin, from 14-d-old cultures.

2.2.2 Soil-block microcosms

Modified soil-block microcosms (ASTM D 1413, 2007) were used to study wood colonization and competitive interactions among fungal species (Figure 2.1). Soil-block jars (7 cm I.D. x 12 cm height) contained a 1:1:1 wetted mixture of peat (Conrad Fafard Inc., MA, USA), vermiculite (Good Earth Horticulture Inc., NY, USA), and fertilizer-free soil mix (Gertens, Inver Grove Heights, MN, USA). Birch feeder strips (2 x 5 cm) were placed onto the surface of the soil mixture, autoclaved twice for 1 hr with a 48 hr interval, and inoculated with plugs of fungi. A single fungus species (two plugs per strip – four total

plugs) or both fungal species in mixed fungus competitive treatments (two plugs of a specie on the same strip – two strips for two fungi). A 5 mm gap was left between parallel birch feeder strips and a small trench (~5 mm deep) was excavated in the soil between the feeder strips. Fungi were grown for 7 d at 25 oC, in the dark, to the edge of the feeder strips without meeting each other in the soil trench. By later adding wooden dowels to span the trench, isolates colonized wood simultaneously on opposite sides of dowels before encountering each other.

Three matched wooden dowel sections (2 cm long x 1.5 cm dia) per wood species were added along the groove between the feeder strips. A space was left between each dowel section to allow fungal access to cross-sectional faces. Sapwood dowels of white birch (*Betula papyrifera*), red oak (*Quercus rubra*), and pine (*Pinus sp*. southern yellow pine, SYP), were the substrate treatments. Oak and birch dowels were purchased locally, while pine dowels were shaped from strips using a bullnose router bit. Pine was selected because it is a natural 'host' wood for *G. trabeum*, while *I. lacteus* is rarely found on southern pine but more commonly on hardwoods like birch and oak. Dowels were initially cut into 6 cm lengths and then into three matched 2 cm lengths in order to reduce wood chemistry variability among dowels within a jar. These sections were oven-dried (48 h, 100 oC), weighed, and autoclaved for 1 h prior to adding to jars.

Fungus treatments included feeder strips inoculated with pure cultures of each fungus alone or as mixed inocula containing both fungi. Each fungus treatment with each wood type was replicated ten times, giving five replicates (n=5) for each of two harvests at incubations times of 3 and 8 weeks post inoculation. Control microcosms, which lacked fungal inoculum treatments, provided baseline measurements used for wood

characterization and as negative controls for PCR reactions. Microcosms were incubated at 25 °C in the dark. At each harvest period, one dowel section from each replicate microcosm was ground fresh, without drying, to 40-mesh in a Wiley mill, and added to cold methanol (0.4 g wet weight wood per 5 ml) for ergosterol measurements. A second section was flash frozen in liquid nitrogen, ground for 30 s into a fine powder using a Midas Rex bone mill (Medtronic, Inc, Minneapolis, MN, USA), assessed for moisture content, and stored at -70 oC. The bone mill uses autoclavable teflon cups containing dual steel blades. Cups were modified by sharpening the blunt 90o leading edge of the lower blade to a 45o blade angle. Between each grinding, the teflon cup was washed with 95% ethanol and diluted liquid Luminox (Alconox, Jersey city, NJ, USA) to remove DNA residue without damaging milling cups. The third dowel section was oven-dried to calculate mass loss and to characterize residue chemistry.

2.2.3 Ergosterol measurements

Wood dowel material was extracted in methanol as described by Newell *et al.* (1988). Methanol extracted samples (5 ml per 15 ml tube) were refluxed, saponified with 4% KOH in ethanol, and extracted into pentane for precipitation and dissolution back into MeOH. Samples were extracted and analyzed for ergosterol by using high-performance liquid chromatography (HPLC) as described by Schilling & Jellison (2005).

2.2.4 DNA isolation

Fungal DNA was isolated from fresh mycelia and inoculated wood blocks (birch, oak, and pine) as previously described (Jasalavich *et al.*, 2000), with slight modifications. Wood

blocks colonized by fungi were ground to a fine powder using the Midas Rex bone mill. Aliquots (150-550 mg) of the resulting wood powder were added to 50 ml conical tubes, and resuspended in 6 ml of 1X CTAB buffer (Doyle & Doyle 1987). The DNA was precipitated overnight at -20 °C with 0.6 volume of isopropanol, washed with 70% ethanol, dried in vacuo, and resuspended in 100 µl of nuclease-free water (Ambion, Austin, TX, USA). DNA concentrations in samples was determined by using a Qubit™ fluorometer (Invitrogen, Carlsbad, CA, USA), and adjusted to final concentrations of 1.5 ng µl-1. DNA samples were stored at -20 °C until used.

2.2.5 Quantitative PCR assays

Primers for PCR were designed to specifically-amplify a portion of the ITS region from *G. trabeum* and *I. lacteus*. The following published nucleotide sequences were aligned by using MultAlin software (Corpet, 1988; http://multalin.toulouse.inra.fr/multalin/multalin.html): *G. trabeum* (Accession AF423117), *G. trabeum* strain 900.73 (AY673077), *G. trabeum* strain BAM Ebw.109 (EF524032), *G. trabeum* isolate 259 (AJ420950), *G. trabeum* isolate 183 (AJ420949), *I. lacteus* isolate XSD-2 (EU273517), and *I. lacteus* isolate MAFF 420244 (AB079267). Potential forward and reverse primer pairs were manually chosen based on variations between the *G. trabeum* and *I. lacteus* nucleotide sequences within the ITS region. Primer pairs were examined for compatible melting temperatures and decreased secondary structures using Net Primer software (PREMIER Biosoft Internationl, Palo Alto, CA, USA). Primers: GT94-F (5'-TCA GGC TGT CCT TCC TAT GTC-3') and GT521-R (5'-GTC AAA TTG TCC GAA GAC G-3') were developed to amplify a 427-bp DNA fragment from *G. trabeum*, whereas primers IL54-F

(5'-ATC GAG TTT TGA ACG GGT TG-3') and IL633-R (5'-AAA TGA TTG TCT CGG CAA GG-3') were constructed to amplify a 579-bp DNA fragment from I. lacteus. The primers were synthesized by Integrated DNA Technologies (Coralville, IA, USA).

Conventional PCR was used to detect cross amplification between each fungal species. The amplification reaction (25 µl) contained 50 µM (each) dNTPs, 0.25 µM of each primer, 1X Taq buffer, and 1 U Choice-Taq DNA polymerase (Denville Scientific Inc., Metuchen, NJ, USA). PCR was performed using a MyCycler thermocycler (Bio-Rad, Hercules, CA, USA) using the following reaction conditions to detect *G. trabeum*: 95 °C for 5 min, and 35 cycles of 95 °C for 30 sec, 58 °C for 30 sec, and 72 °C for 1 min, and a final extension at 72 °C for 5 min. The same thermocycler program was used to detect I. lacteus, except the annealing temperature was increased to 60 °C. PCR products were separated by electrophoresis for 1 hr at 100 V on 1% agarose gels in 0.5X TBE buffer containing 0.5 µg ml-1 ethidium bromide. A 1 Kb Plus DNA Ladder (Invitrogen) was used to determine sizes of the PCR products.

Template DNAs for making standard curves were PCR products of the ITS region of *G. trabeum* and *I. lacteus* produced using primers GT94-F and GT521-R and primers IL54-F and IL633-R, respectively. The fungal ITS PCR fragments were cloned into the StrataClone PCR cloning vector, pSC-A-amp/kan, using the StrataClone PCR Cloning Kit (Stratagene, La Jolla, CA, USA), resulting in constructs, pSGT and pSIL for the cloned PCR fragment amplified from *G. trabeum* and I. lacteus, respectively. Plasmids were isolated using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol and each contained a single copy of the inserted DNA fragments as determined by using conventional PCR with the amplification protocol

described above. The concentration of plasmids DNAs was determined using a Qubit™ fluorometer.

Real-time, quantitative PCR amplification of the standard templates pSGT and pSIL and sample DNAs were performed using the ABI Prism™ 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA). Quantitative amplification of plasmid template DNAs (ten-fold dilutions of 101 to 106 copies) or 4.5 ng of sample DNA were conducted in reaction wells containing a total volume of 40 µl. Triplicate measurements of each standard concentration were made. Reactions contained 400 nM of each forward and reverse primers, and 20 ul of 2X iTaq™ SYBR® Green Supermix with ROX (Bio-Rad, Hercules, CA, USA). All reactions were performed in triplicate, and each run contained replicate non-template controls. The thermal cycling conditions for DNA amplification from *I. lacteus* were an initial denaturation at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, 60 °C for 30 s and 72 °C for 1 min. A similar thermal cycling protocol was used for *G. trabeum* detection, except the annealing temperature was reduced to 58 oC. Dissociation curve analysis was performed post-PCR on all plates. All raw data were analyzed using 7000 system sequence detection software (Applied Biosystems, Foster City, CA, USA), version 1.2.3. The average slope for *G. trabeum* and *I. lacteus* standard curves were -3.32 (n=6) and -3.25 (n=4), respectively, with the r2 of all fits >0.993. The PCR efficiencies of the plates ranged between 97-109% for *I. lacteus* and 90-105% for *G. trabeum*. The DNA quantity, or copy number, obtained from the quanititative cycle (Cq) from the real-time PCR assays was normalized to total DNAs and dry wood weight. Differences in fluorescence emission due to variability in DNA fragment length were insignificant in this study and did not require standardization of signal. Assessment

of the potential for PCR inhibition in DNA extracts from colonized wood samples was performed in two random week 8 samples from each wood type cultured with either *G. trabeum* or I. lacteus. 4.5 ng sample DNAs were spiked with 105 copies of the standard DNA template of the same type of fungus (pSGT or pSIL). Triplicate qPCR runs for each fungus/wood combination demonstrated no significant effects on recovery of the DNA spike.

2.2.6 Extraction efficiency

Since wood particles and liquid nitrogen may influence ergosterol determinations and yields of fungal DNA, the extraction efficiency of each fungus from pure culture and from inoculated fungus-wood combinations was assessed, as well as extracting from non-inoculated control wood. Fungi were grown on basal salts medium (Highley, 1973), mixed with fresh wood to create samples with known fungal density, and samples were flash-frozen in liquid nitrogen and ground using the Midas Rex bone mill. Extracted DNA and ergosterol concentrations from these samples were compared with those obtained following extraction of pure cultures and extraction efficiencies were calculated as the ratio of DNA and ergosterol concentrations from pure fungus to those obtained from fungus-wood sample (Table 1). Although imperfect, mixing hyphae with wood as opposed to calculating extraction efficiency from colonized wood avoids assessments of ergosterol or chitin or measurements of mass change of colonized wood. These approaches either require their own extraction efficiency estimates, to infer biomass within an inoculated wood sample, or are complicated by mass loss in the wood along with fungal weight gain after colonization.

2.2.7 Wood characterization

Mass loss and change in wood specific gravity were calculated in single blocks by measuring oven-dry weight lost during decay. Samples were ground to 60-mesh in the Wiley mill after determining post-decay weight, and stored over CaSO4 desiccant. Carbon fractions (w/w) were determined for cellulose (glucan), for hemicellulose (arabinan, galactan, glucan, mannan, and xylan) carbohydrates, and for acid-soluble Klason lignin, as previously described (Schilling *et al.*, 2009). The pH and dilute alkali solubility (DAS) of decayed and non-decayed wood samples were determined following Shortle *et al.* (2010). The DAS is an indirect measure of decay extent and, at a similar mass loss, a proxy for decay type (DAS increases during brown rot, while staying similar to control wood during white rot), in both cases by gauging concentration of low molecular weight carbohydrates (hemicellulose and hydrolyzed cellulose). For DAS, wood powder was autoclaved in 0.2 M NaOH, filtered through a tared fritted glass crucible, and rinsed with distilled water and 0.1 M HNO3. The percent loss on extraction (LOE%) was calculated by weighing extracted materials. For pH determinations, wood powder was equilibrated in 5 mM CaCl2 at a ratio of 0.1 mg µl-1.

2.2.8 Statistical analyses

Data is expressed as means and standard error (S.E.). Analysis of variance (ANOVA) (α=0.05) was used for Tukey's means comparisons, using wood types as individual cases and after log-transforming data requiring normalization. Lignin, carbohydrate, ergosterol and fungal biomass were normalized both as percent-of-original content and as weight-to-original volume using weight loss and wood density. The ratio of copy number between *I.*

lacteus and *G. trabeum* in single and mixed treatments was calculated since the comparison of absolute ITS copy numbers between two fungal species fails to provide a meaningful comparison. The variances of copy number ratio of single treatments were calculated based on delta method according to Cox, 1990. The copy number ratios were tested for difference with two-sample t tests (α=0.05).

2.3 Result and Discussion

2.3.1 Mass loss

Mass loss of wood degraded by the tested fungal isolates for 8 weeks was greater for the brown rot fungus *G. trabeum* than for the white rot fungus I. lacteus, and was greatest (nearly 50%) for *G. trabeum* grown on pine (Figure 2.2). Mass loss was statistically different between the fungi when colonizing birch and pine ($p \leq 0.05$ for both wood types), but not when degrading oak ($p = 0.420$). Mass loss in wood colonized by both fungi (mixed), in degraded birch and pine, was similar to, but not statistically different from, mass loss in wood degraded by the single *I. lacteus* inoculum.

2.3.2 Wood residue characterizations

Similar to mass loss data, each of the wood residue characteristics measured from the mixed-fungus inoculations resembled *I. lacteus* values more than they resembled those measured in *G. trabeum* residues. This was true for lignin and carbohydrate contents (% original) (Table 2.1), lignin removal in oak, carbohydrate removal in all wood types (per wood volume) (Figure 2b & c), wood acidification, the alkali solubility of the residues (Figure 2d & e), and wood moisture contents at the time of harvest. White rot residues,

generated in all cases in the mixed-fungus treatments, are known to impart unique humic chemistries on the forest floor and different CO2 emission rates, relative to brown rot residues (Gilbertson, 1980; Harmon *et al.*, 1986).

2.3.3 Ergosterol content

The ergosterol contents of week 3 and 8 samples did not show an obvious pattern of biomass accumulation (Figure 2.3), after compensating for mass loss (per wood volume) and applying extraction coefficiency to degraded wood samples. Because ergosterol includes a large fraction of active biomass, this is not surprising at these decay stages when colonizing fungi are likely recycling their own biomass toward a unified decay front (Klein & Paschke, 2004). Ergosterol levels matched those of *I. lacteus* in each case with the exception of oak, where ergosterol levels of the mixed-fungus treatment matched *G. trabeum* levels and not those of *I. lacteus*. The ergosterol to biomass ratio also differed two-fold in *G. trabeum* and *I. lacteus* (Table 2.1), indicating that caution is needed when measuring ergosterol in samples with mixed fungal species.

2.3.4 Quantitative PCR on fungal competition outcomes

Quantitative PCR analyses of wood blocks inoculated with both fungi revealed that *I. lacteus* was the dominant fungus by week 3 on all wood types tested (Figure2. 4). *G. trabeum*, however, remained detectable throughout the incubation period. All of the *I. lacteus*-to-*G. trabeum* copy number ratios in mixed treatments were significantly greater ($p \leq 0.05$) than their single treatment counterparts, except for the week 8 oak sample. This was likely due to high variability among replicates as the average ratio of mixed oak

treatment was 300 times that of the single treatment. However, the dominance of white rot in week 8 oak was confirmed by wood characterization analyses described above. This indicates that the wood characterization method provides complementary data for molecular analyses. The ITS copy number ratio method used in this study eliminated problems due to difference of DNA extraction efficiency between wood types and ITS copy number variation in the genomes of the two fungal species. The copy number ratio method was previously used to compare the biomass of fungi and bacteria (Fierer *et al.* 2005; Graaff *et al.* 2010).

2.3.5 Linking fungal competition to residue characterizations

These results link the dominant biomass component of *I. lacteus* to certain outcomes of white rot in residues over a relatively large volume of wood. Typically, PCR-based approaches have been used to detect pest or pathogenic decay fungi in wood, using sub-sampling with drill bits or pulverization of very small wood fragments (Kim *et al.*, 1999; Jasalavich *et al.*, 2000; Guglielmo *et al.*, 2008, 2010). Minimizing destructive sampling is important when sampling lumber or living trees. In our case, however, we needed to assess the contribution of individual fungi in a mixed-species environment occupying a larger wood volume than a drill bit, and thus required whole-block milling of fresh samples and without DNA carryover issues and with a reliable extraction protocol. By thoroughly characterizing the relationships between DNA copy number, ergosterol, and mycelial biomass, along with extraction efficiencies from wood, and residue characters that distinguish brown from white rot, one can begin to make a connection between wood residues and the fungi responsible for producing them. Importantly, despite inclusion of a

range of wood species for each fungus, we were able to establish that there was only a small influence of wood type on fungal competitive success in the test microcosms inoculated with these two co-existing fungi.

Based on other studies and on field guides, one can argue that associations between wood-degrading fungi and woody substrates are generally not rigid. Substrate-specificity in a tropical sporophore study was weak among 32 wood-degrading fungi (only 9% with specificity) (Lindblad, 2000). Shaw (1973) created a host fungus index for specific tree types and found the number of different potential wood-rotting fungal species colonizing a given wood type ranged from 102 to 241. Similar examples can be seen in the Pacific Northwest Fungi Database (http://pnwfungi.wsu.edu).

One surprising result of this study was that *I. lacteus* so successfully colonized and degraded pine conifer wood in the mixed fungus treatment, despite knowing that it would rarely be isolated from pine in the field (Gilbertson & Ryvarden, 1986). One explanation is that *I. lacteus* simply colonized more of each wood dowel than *G. trabeum*, growing faster in this particularly stable, moist environment. Assembly history can affect fungal community structure and decay rate in wood (Fukami *et al.* 2010) and colonization by pathogenic *Trichoderma spp.* (Bruce & King, 1991). Other explanations for the lack of competitive success of *G. trabeum* include use of bark-free wood blocks, temperature, moisture and heartwood content, and a simplified microbial community structure. While the influence of bark was negligible in a white rot and brown rot fungal spore trial by Tsuneda & Kennedy (1980), the presence of bark (causing hydrophobic encapsulation) along with naturally fluctuating temperature and moisture may affect competition among fungi with distinct moisture and temperature optima. Heartwood also contains phenolics

and other water- or solvent-extractable fungitoxic compounds and tolerance to these wood extractives has long been known to vary among decay fungi (Cowling & Scheffer, 1966). It seems more likely, however, that controlled-inoculum microcosms may not best reflect how competition, nor microbial succession, would proceed in a naturally diverse microbial community, collectively influenced by environment. Even if priority effect is a strong determinant of the rate of wood decomposition, it remains important to explain common fungus-wood associations and their consequence over longer time frames on both rate and character of decay. Therefore, scaling this approach to field decomposition studies, similar to the PCR-based competitive trial among ectomycorrhizal fungi (Kennedy *et al.*, 2007), may lend important insight into long-term drivers of competition among fungi colonizing forest woody debris.

Chapter Three: Linking wood decomposition to fungal competition: priority effect

Chapter Summary

The relative amounts of hyphal inoculum in forest soils may determine the capacity for fungi to compete with and displace early colonizers of wood in ground contact. Our aim in this study was to test the flexibility of priority effects (colonization timing) by varying exogenous inoculum potential. We controlled these variables in soil-block microcosms using fungi with known competitive outcomes in similar conditions, tracking isolate-specific fungal biomass and residue physiochemistry over time. In the priority trial (Experiment I), a brown rot fungus *Gloeophyllum trabeum* was given 1, 3, or 5 weeks to precolonize wood blocks (oak, birch, pine, spruce) prior the introduction of a white rot fungus, *Irpex lacteus,* a more aggressive colonizer in this set-up. In the inoculum potential trial (Experiment II) the fungi were inoculated simultaneously, but relative hyphal inoculum amounts were varied. As expected, longer priority duration increased the chance for the less-competitive brown rot fungus to outcompete its white rot opponent. Increasing inoculum outside of the wood matrix, however, also significantly increased competitive success of the brown rot isolate on most but not all wood types tested. These temporal shifts in fungal dominance were detectable in a 'community snapshot' as isolate-specific quantitative PCR, but also as functionally-relevant consequences of wood rot type, including carbohydrate depolymerization and pH. These results from a controlled system reinforce fungal-fungal interaction results on soils, and suggest that relative inoculum availability *beyond* the wood matrix (i.e., soils) might regulate the duration of priority effects and shift the functional trajectory of wood decomposition.

3.1 Background

Wood decomposition is an important component of carbon and nutrient cycling in forest ecosystems (Harmon *et al.* 1986; Malhi 2002), but remains a major uncertainty in global carbon budgets (Weedon *et al.* 2009; Freschet *et al.* 2012). In most forests, decomposition of woody tissue is largely fulfilled by two distinct but evolutionarily related functional groups of Basidiomycetes – the white rot and brown rot fungi (Gilbertson 1980). Lignin, a recalcitrant carbon matrix, can be degraded by white rot fungi, often simultaneously with carbohydrates (cellulose and hemicellulose). In contrast, the majority of lignin remains behind in wood residues after carbohydrates are removed by brown rot fungi. These rot types may represent more of a gradient than previously assumed (Riley *et al.* 2014), but collectively create wood residues with distinctive physiochemical properties that can influence soil processes and the cycling of other soil organic matter (Rypáček & Rypáčková 1975; Gilbertson, 1980; Jurgensen *et al.* 1997; Filley *et al.* 2002, Song *et al.* 2012). Although the physiological functions of these fungal groups have been well documented (Blanchette 1991; Kerem *et al.* 1999; Leonowicz *et al.* 1999; Schwarze *et al.* 2007; Worrall *et al.* 1997), the factors that control their community dynamics in forests are still unclear and likely not predictable by temperature and moisture, alone (Bradford *et al.* 2014). In particular, the colonization events among competing wood-degrading fungi likely vary the long-term outcomes of wood decomposition, carbon release, and soil formation (Fukami *et al.* 2010; Song *et al.* 2012).

Although environmental factors were shown in many cases to influence fungal community structure (Chase 2010; Lebrija-Trejos *et al.* 2010; Garcia-Pichel *et al.* 2013; Fortunel *et al.* 2014), assembly history, *i.e.* the order of species arrival, is also considered

to be a key contributor to community structure and ecosystem functions (Hooper *et al.* 2005; Fukami *et al.* 2010; Kennedy *et al.* 2009; Huston *et al.* 2014). During occupation of a new resource, the early colonists gain advantages of resource utilization and often impose negative effects upon later arrivals, referred to as a 'priority effect'. Priority effects have been shown to determine the community structure of many microorganisms, including bacteria (Tan *et al.* 2012), mycorrhizal fungi (Kennedy *et al.* 2009) and wood-degrading fungi (Fukami *et al.* 2010; Dickie *et al.* 2012). Fukami *et al.* 2010 showed that priority effects played an important role in community assemblage and ecosystem functions in a white rot fungal decay consortia (ten fungi) on black beech (*Nothofagus solandri*). Priority effect is also predictable when combative, non-lignocellulolytic fungi such as *Trichoderma spp.,* are inoculated on wood as biological control agents against wood-degrading fungi (Schubert *et al.* 2008; Susi *et al.* 2011). Many wood-degrading fungal species, however, show apparent preference for specific substrates (*eg*, *Lenzites betulina* on *Betula* spp. wood, Gilbertson 1980), implying that the substrate exerts some control over priority and/or that priority is not the sole determinant of competitive success.

Variation in inoculum potential, a function of fungal propagules (hyphae, spores, and other morphological adaptations) and resource availability (Liu & Luo 1994), is an alternative force that could complement or potentially overcome early priority effects over longer terms in structuring wood decomposer communities. The effects of inoculum potential were shown to be critical for plant colonization by pathogenic fungi (Rao & Rao 1963) and mutualistic mycorrhizal fungi (Daniels *et al.* 1981). Incomplete colonization on plant roots was shown to result in replacement by late arrivals among competing mycorrhizal fungi (Kennedy *et al.* 2009). For wood-degrading fungi, Holmer & Stenlid

(1993) showed that inoculum availability within a volume of wood was highly correlated to the competitive success between paired wood-decay fungi. Beyond the confines of woody material on the forest floor, the distribution of wood-degrading fungal mycelia is often patchy with varying connectivity, as shown in a boreal old-growth pine forest (Jönsson *et al.* 2008). Changes in the wood substrate itself may also affect colonization dynamics over time, as wood physiochemical changes due to decomposers can benefit early arrivals or late colonists by releasing then depleting preferred resources. Collectively, this supports investigations into the deterministic role of priority effects over a gradient of inoculum potentials.

In this study, priority effects and inoculum potential were studied in two separate experiments, exposing four wood types to brown and white rot fungi that cohabitate in nature but that have distinct substrate associations (Gilbertson 1980). In experiment I, a brown rot fungus (*Gloeophyllum trabeum)* precolonized wood resources for various lengths of time, imposing priority effects over a white rot fungus (*Irpex lacteus*) that was previously shown to dominate when simultaneously inoculated (Song *et al.* 2012). In experiment II, the brown rot fungus was inoculated simultaneously, but with a larger amount of inoculum compared to the white rot fungus. In this simplified community structure, our goal was to connect relative colonization success, as determined by using quantitative PCR (qPCR), with wood physiochemical changes imparted by the 'winning' fungus in order to gauge the plasticity of priority effects and to demonstrate functional consequences of the interactions.

3.2 Methods

3.2.1 Fungi and wood substrates

The brown rot fungus *Gloeophyllum trabeum* (Persoon: Fries) Karsten strain M617 (ATCC 11539), and the white rot fungus *Irpex lacteus* (Fries:Fries) Fries strain M517 (ATCC 11245) were used in this study. Isolates were stored on 2% malt extract (MA) medium at room temperature during the period of experiment.

To study competition between the two fungi, soil-block microcosms were used to mimic competition on a single wood substrate by two fungi colonizing from soil. This followed a modified protocol (ASTM D 1413, 2007) described by Song *et al.* 2012[41]. Soil in this study was 1:1:1 mixture of peat (Conrad Fafard Inc., MA, USA), vermiculite (Good Earth Horticulture Inc., NY, USA), and fertilizer-free soil (Gertens, Inver Grove Heights, MN, USA). Four types of wood, white birch (*Betula papyrifera*), red oak (*Quercus rubra*), pine (*Pinus* sp., southern yellow pine), or white spruce (*Picea glauca*) were used. Wood blocks (19 mm cubes) were oven dried at 100°C for 48 hr and weighed for initial dry mass. All blocks were soaked in distilled water for 24 hr to regain moisture before wrapping in foil. Wood blocks were autoclaved twice, 1 hr each run with a 24 hr interval. Stock isolates were transferred to fresh 2% malt extract medium and cultured for 14 d. Agar plugs were cut from petri plates, and placed on two parallel birch feeders (15 mm × 50 mm) spaced 5 mm on the soil surface. Microcosms were incubated at room temperature in the dark for 1 wk before three wood blocks were placed connecting (straddling) the two feeders. The assembled microcosms continued incubation before wood

blocks were harvested for downstream analyses. Top view photos were taken for each microcosm before harvest (Fig S1 & S2).

3.2.2 Microcosms designs

Experiment I: Precolonization

To test the effects of precolonization by the slower-growing isolate, wood blocks were first incubated on agar for 1, 3 or 5 wk with *G. trabeum* before transfer to soil-block microcosms (n=7 per treatment). Glass jars were filled with 40 ml of 2% water agar and autoclaved for 20 min. A round agar plug (7 mm in diameter) with *G. trabeum* mycelium (14-day-old culture on 2% MA, cut from the edge of 10 cm petri dish) was placed on the center of the agar. The agar-block microcosms were cultured in the dark at room temperature for 2 wk. Four wood blocks were placed, with wood grain vertical, on a round plastic mesh to prevent direct contact with the agar and incubated for various treatment times.

After precolonization, wood blocks were harvested in a biosafety cabinet. For each microcosm, one of the four blocks was split in half along the grain. One half was ground immediately in a modified bone mill (Song *et al.* 2012) and weighed into 5 ml HPLC grade methanol for ergosterol determination. The other half was weighed fresh and oven dried to obtain water content and dry mass of the whole block. The other three blocks were transferred to a new soil-block microcosm. The soil-block microcosms contained two agar plugs of *G. trabeum* on one feeder strip and two plugs of *I. lacteus* on the other feeder strip, and were incubated for 1 wk. The three blocks were positioned carefully so that the upper portion of the block in the previous microcosm was faced toward *I. lacteus* in the new microcosm environment (n = 7 per treatment). After assembling, the soil-block

microcosms were incubated for 8 wk. At harvest, each block was split in half, one half was kept frozen at -80°C before grinding in the bone mill and the other half was weighed fresh, oven dried to obtain water content and dry mass, and ground in a Wiley mill for chemical analyses. This experimental design consisted of 4 wood types × 3 time intervals of precolonization × 7 replicates, yielding 84 microcosms plus non-inoculated controls.

Experiment II: Inoculum potential

To test the effect of mycelial inoculum potential, autoclaved wood blocks were placed into soil-block microcosms, without precolonization, onto feeders inoculated with different amounts of the test fungi. *G. trabeum* was inoculated as a large agar rectangle (17 mm × 38 mm, top face area = 646 mm^2), and *I. lacteus* was inoculated as two round agar plugs (7 mm in diameter, top face area = 77 mm^2). Agar thickness was standardized by controlling petri agar volume (25 ml per plate). The soil-block microcosms were incubated for 3 or 8 wk (n = 6 per treatment). Wood blocks were harvested as described above for experiment I. This experimental design consisted of 4 wood types x 2 incubation periods x 6 replicates, yielding 48 microcosms plus non-inoculated controls.

3.2.3 Fungal colonization assessment

Fungal colonization was also determined by using the biomarker ergosterol. Fresh ground materials were extracted in methanol as described by Newell *et al.* 1988. Cholesterol (9.6 ng) was added in each extraction reaction as an internal standard. Extracted samples were analyzed for ergosterol and cholesterol by using high-performance liquid chromatography (HPLC) as described by Schilling & Jellison 2005.

The extent of colonization by each fungus was determined by qPCR. Fungal DNA was extracted following the procedure of Jasalavich *et al.* 2000, using 1X CTAB buffer. The DNA was precipitated with isopropanol overnight, washed 2 times in 70% ethanol, and re-suspended in 100 µl of nuclease-free water (Ambion, Austin, TX, USA). DNA concentration was determined with a Qubit™ fluorometer (Invitrogen, Carlsbad, CA, USA), and diluted to 1.5 ng μl^{-1}.

Primers were designed to amplify a specific ITS region of the two fungi as described by Song *et al.* 2012[41]. The primers: GT94-F (5'-TCA GGC TGT CCT TCC TAT GTC-3') and GT521-R (5'-GTC AAA TTG TCC GAA GAC G-3') were developed to amplify a 427-bp DNA fragment from *G. trabeum*, whereas primers IL54-F (5'-ATC GAG TTT TGA ACG GGT TG-3') and IL633-R (5'-AAA TGA TTG TCT CGG CAA GG-3') were used to amplify a 579-bp DNA fragment from *I. lacteus*. The ITS fragments were cloned into the StrataClone PCR cloning vector, pSC-A-amp/kan (Stratagene, La Jolla, CA, USA). The resulting plasmids were used to create standard curves representing 10^1 to 10^6 copies. Quantitative PCR was performed using the ABI Prism™ 7000 sequence detection system. Each reaction contained plasmid template DNAs or 4.5 ng of sample DNA, 400nM of each primers, and 20uL of 2X iTaq™ SYBR® Green Supermix with ROX (Bio-Rad, Hercules, CA, USA) in total volume of 40 µL. All measurements were performed in triplicate. The thermal cycling conditions for DNA amplification from *I. lacteus* were an initial denaturation at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, 60°C for 30 s and 72°C for 1 min. A similar thermal cycling protocol was used for *G. trabeum* detection, except the annealing temperature was reduced to 58°C.

Once copy counts were determined per fungus, the competitive success in mixed cultures was expressed as a ratio each fungus to the other. The proportion of *G. trabeum* relative to *I. lacteus* in wood was measured using DNA as the isolate-specific biomarker in mixed cultures and in wood blocks colonized by a single fungal species and incubated for 8 wk. In all cases, copy counts were adjusted to copies cm^{-3} by using mass loss to adjust for wood density. The *I. lacteus*/*G. trabeum* copy number ratio in theoretical equal (by volume) colonization of a wood block was set at 50/50 relative abundance of each fungus using single culture copy count information. The proportion of *G. trabeum* was calculated based on the equation below:

$$G = \frac{R_s/R_m}{R_s/R_m + 1} \times 100$$

G is the proportion (%) of *G. trabeum* in samples, R_s is the copy number ratio estimate for equal colonization projected from single culture measures, and R_m is the true ratio measured in mixed cultures. The copy number ratios (R_s and R_m) are calculated with *I. lacteus* as the numerator as it has more ITS copies per genome than *G. trabeum* (Song *et al.* 2014). Variance in copy number relationship with fungal biomass due to substrate and competition is discussed in depth in Song *et al.* 2014.

3.2.4 Wood physiochemical characterization

Each wood block was weighed immediately after harvest. Half of the block (split vertically along the grain to minimize spatial variability) was used for the analyses above, and the other half was weighed and oven dried at 100°C for 48 hr to obtain dry mass. Water content was calculated from this block portion and used to calculate dry mass of the whole block and, subsequently, mass loss induced by the decay fungi over the course of each experiment. The dried block half was then ground to 40-mesh in a Wiley mill to produce

wood flour for chemical analyses. The pH and dilute alkali solubility (DAS) of wood samples were determined following Shortle *et al.* 2010 and used as an indicator of the carbohydrate depolymerization characteristic of brown rot[39]. The DAS is dependably higher in brown-rotted wood than after white rot or in sound wood, so competition leading to *G. trabeum* (brown rot isolate) dominance should result in higher DAS than if *I. lacteus* (white rot isolate) dominated a wood block.

3.2.5 Wood-free competitive assay

To gauge the direct competitive outcomes upon contact outside of a wood matrix *G. trabeum* and *I. lacteus* were placed on the surface of 15 cm petri dishes to measure the relative change of mycelium area. Agar plugs of equal volume (77 mm^2, round plug top face area) of 14-day-old mycelium of *G. trabeum* and *I. lacteus* were positioned on the farthest points of petri dishes on MA medium. The mycelial boundary between the two fungi was marked on a transparent film every 24 hrs. The films were scanned, and area was calculated using imageJ and converted to percentage of the total area of the petri dish per pixel count. After the two fungi encountered each other, only the area of *G. trabeum* was recorded.

3.2.6 Statistical analyses

Data are expressed as means ± standard error (SE). ANOVA was applied with Fisher's LSD multiple comparisons on ergosterol amounts in each wood type and wood residue characteristics in experiment I. Linear regression was used to correlate the proportion of

G. trabeum with wood pH. Pearson correlation was used to correlate the proportion of *G. trabeum* and water content, mass loss, DAS or pH. The α for all tests was set at 0.05.

3.3 Results and Discussion

3.3.1 Experiment I: Precolonization

The competitive success of the brown rot fungus *G. trabeum* was enhanced with longer precolonization time prior to competition with *I. lacteus*. Ergosterol content in all wood types degraded by *G. trabeum* increased significantly ($p \leq 0.05$) from week 1 to week 3, then stabilized (Fig 3.1). When precolonized wood was then incubated in dual-species competition microcosms, qPCR indicated that the average proportion of *G. trabeum* relative to *I. lacteus* was greater in wood precolonized for a longer time. In most treatments, competition could apparently track toward either outcome once established in a given treatment (Fig 3.2). For example, most microcosms containing oak precolonized by *G. trabeum* for 1 week were eventually dominated by *I. lacteus*, but in some of the replicate microcosms, 70-80% of the total fungal biomass was that of *G. trabeum* (Fig 3.2). Overall, however, there was clearly a relationship between priority period and dominance later in wood decay.

These priority effects and the resulting shifts in dominance, as measured with qPCR, were also evident as physiochemical 'signatures' in residues. Longer precolonization by the brown rot fungus increased the likelihood that residues had brown rot characteristics after 8 weeks in competition, although there was an effect of wood type. In addition to mass lost during the *G. trabeum* precolonization period, mass loss increased significantly ($p \leq 0.05$) during competitive decomposition in most, but not all, treatments

(Fig 3.3). In oak and pine, precolonization for 5 weeks by *G. trabeum* stalled further decomposition in the 8-week trial. Water content in all four wood types reached a plateau by week 3. For most treatments, water content after 8 weeks of competition remained unchanged, except for significant decreases ($p \leq 0.05$) by 3 wks for birch and pine. Typical of brown rot, DAS increased and pH decreased from the 1st to 5th week. In the competitive period, DAS remained unchanged in all oak and spruce treatments but increased significantly in birch and pine for all three precolonization treatments. Wood pH was similar in oak in precolonized and wood degraded for 8 weeks, but decreased significantly ($p \leq 0.05$) in all other three types of wood.

Among the variables identified as physiochemical consequences of functional rot type (brown versus white rot dominance), all had significant correlation ($p \leq 0.05$) with *G. trabeum* dominancy (Table 3.1). Among these, pH had the most significant signature from dominance of brown rot, shown in regressions of proportion of *G. trabeum* biomass with wood pH in 3 and 5 weeks (Fig 3.4), when *G. trabeum* began to dominate many competitive interactions.

3.3.2 Experiment II: Inoculum potential

When the two test fungi were inoculated simultaneously, but at higher inoculum loading for *G. trabeum, I. lacteus* still dominated microcosms through 3 weeks; however, *G. trabeum* became dominant in a proportion of microcosms in the oak, birch and pine treatment after 8 wks (Fig 3.5, Supplementary document S2). This capacity to overcome an initial biomass deficit was reflected in the residue qualities in the wood as it was decomposed (Fig 3.5), similar to those from the priority trial in Experiment 1 (Fig 3.3).

DAS and pH had less variation after 3 than after 8 weeks for oak, birch and pine. Moreover, all wood types reflected the increase in DAS and acidification that accompanies brown rot. In this experiment, all blocks continued to lose significant mass from weeks 3 to 8. Water content of wood increased from 3 to 8 weeks in oak and spruce, decreased in pine, and remained similar in birch.

3.3.3 Petri dish competition studies

Complementing the observations from the inoculum potential trail (Experiment II), when the two test fungi were grown on the same agar medium, *I. lacteus* was faster in claiming new territory (~75% at first confrontation after 15 days). Similar to the outcome in competition, *G. trabeum* started to replace *I. lacteus* after confrontation and occupied around ~25% new area after 50 days, and was continuing to gain territory when the study was concluded (Fig 3.6).

3.3.4 The effects of assembly history and inoculum potential

Community assembly history and inoculum potential both affected the outcome of competition between wood-degrading fungi in this simplified competitive system. The fungus *G. trabeum,* previously shown to lose in 'equal-footing' competition with *I. lacteus* on wood types it would normally dominate in nature (Song *et al.* 2012), was able to win in this case when given priority. This is in line with priority results from more complex wood-decomposer community studies (Fukami *et al.* 2010; Dickie *et al.* 2012). The strength of priority effects are further reinforced by the potential for fungi such as *Trichoderma* spp. with minimal lignocellulolytic efficacy to outcompete and limit colonization by wood-

degrading fungi (Schubert *et al.* 2008). Wood provides some easily-metabolized sugars (eg, starch in wood ray parenchyma) to early colonizers who gain priority (Zabel & Morrell 1992). In addition, the low surface area/volume ratio of wood provides a space within which to defend a perimeter, giving colonizers the opportunity to guard territory via physical exclusion (*eg*, pseudosclerotial plate formation) (Cease *et al.* 1989).

In our Experiment II, we could also manipulate the competitive outcome by increasing the amount of *G. trabeum* inoculum relative to *I. lacteus*, overriding initial priority effects. Inoculum size has been shown to affect hyphal extension patterns and the foraging ability of the wood-degrading fungus *Resinicium bicolor* in forest soils (Zakaria & Boddy 2002). Competitive ability has also been shown to positively correlate with the volume of an inoculated disc between paired wood-degrading fungi (Holmer & Stenlid 1993). In our case, the hyphal colonization potential into the wood was improved, not by replacing with a more vigorous *G. trabeum* strain but by simply increasing the size/amount of mycelium. This likely increased the potential inside the wood matrix for territory replacement during secondary resource capture, as evidenced in Fig 5 and shown in the wood-free agar plate trial. These results confirm in these isolates an outcome shown among other wood-degrading fungi in dual-species agar trials (Boddy 2000).

An interesting observation, given the nature of these two isolates and the likelihood (or lack thereof) of immediate combat in forest soils, is the interaction between wood type and inoculum potential. The ability for *G. trabeum* to recapture substrate depended on the substrate type. These two fungi co-occur in forests but were selected originally with the assumption that fungal wood substrate affinities would drive outcomes of competition to favor 'home field advantage' dominance. Instead, the previous trails showed no effect of

substrate on the outcome of competition (Song *et al.* 2012), yielding *I. lacteus* as a predictable 'winner' in equal-footing combat. Here, we show that this outcome is flexible given shifts in inoculum potential but that there is an effect of wood type on this flexibility. These effects are not straight-forward (eg, a lack of significance in spruce), but they do suggest that common substrate-fungus relationships in forests could be shaped by substrate influences on competitive dynamics.

As a consequence of competition, the outcomes of shifts in competitive success were assessable using qPCR and confirmed as residue physiochemical signatures of functional rot type. While ITS copy number (with qPCR and omics measures) provides a community 'snapshot,' residue physiochemistry represents a consequence accumulated over time, which can be decades when woody debris decomposes (Harmon *et al.* 1986). Dilute alkali solubility (DAS) of decomposed wood has been helpful in other studies for identifying rot type (Shortle *et al.* 2010), and it reflects a larger ecologically-relevant set of residue qualities that include the following: redox reactivity, permeability, sorption dynamics, metal-binding capacity, forest floor residence time, and a capacity to alter cycling rates in other organic matter pools (Rypáček & Rypáčková, 1975; Jurgensen *et al.*, 1977; Gilbertson 1980; Zabel & Morrell, 1992; Filley *et al.*, 2002). These locally-relevant qualities are complemented by distinct rates of CO_2 evolution from wood depending on rot type (Herman *et al.* 2008). There are caveats when using DAS or pH to assess historical community types, particularly if chemical changes are irreversible, but when these tools are coupled with molecular community assessments, there is a potential to better connect cause with consequence.

Overall, the influence of inoculum potential is interesting in that it implies an alternative ecological force, including soil mycelial potential, on the outcomes of fungal competition for wood on the forest floor. The soil-inhabiting mycelium of individual wood-degrading fungi can be on the scale of hectares and very old, such as well-documented cases among *Armillaria* spp. (Ferguson *et al.* 2003). The hyphae of these fungi are also efficient in foraging among dispersed 'patchy' wood substrates, using soil as the medium and adapting hyphal morphology via cord-formation to enable long-distance transport of elements (Boddy 1993). This hyphal connectivity between the internal wood matrix and exogenous substrates, including soil and non-woody substrates such as building materials, can either slow or hasten decay in wood (Schilling 2010; Liew & Schilling 2012). Taken collectively, it is possible that soil mycelial prevalence may override early priority effects over the long process of wood decomposition, or at least shorten the tenure of priority colonizers. This, together with priority effects, may help explain in part the poor predictability of temperature and moisture on wood decomposition and the consistency of some fungus-substrate associations in forests.

Chapter Four: Linking wood decomposition to fungal competition: temperature and endophytes

Chapter Summary

Carbon in dead wood is principally cycled by wood-degrading fungi in forests, but current models for this process struggle to account for variability observed in ecosystem-level studies. Wood-degrading fungi include several functional groups, and the fraction of decomposition among these groups may shift along with shifting climate. Potential wood decomposers may also be present in live plant as endophytes. Endophytes provide crucial functions to live plant, but their roles in decomposition are largely unknown yet likely influenced as well by climate. In this chapter, I compared the effect temperature and endophytes on competition of two wood-degrading fungi on birch containing natural endophyte communities. These endophytic communities imposed a strong priority effect upon fungi in soil, but they also contained species that can substantially degrade wood. Influence of increased temperature was smaller than that of endophytes. Rot type of wood-degrading fungi determined the pattern of decomposition when these fungi were dominant in wood. Furthermore, the abundance of *Basidiomycota* and bacteria were better indicators of decomposition rate within the endophytic communities.

4.1 Background

The current trend of global change imposes highly uncertain influence upon the earth system, a major focus of many studies to evaluate the responses of ecosystems to global change (Cramer *et al.* 2001; Tilman *et al.* 2001; Schröter *et al.* 2005; Bardgett *et al.* 2008;

Collins 2009; Barnosky *et al.* 2012; Wheeler & von Braun 2013). Compared to other decomposers microorganisms, the abilities/functions of wood decomposers are mostly constrained in several clades of *Basidiomycota* (Riley *et al.* 2014). The functional redundancy of wood decomposition thus is likely to be limited compared to other ecosystem functions/processes, and also more vulnerable to environmental disturbances (Bader *et al.* 1995; Jonsell *et al.* 1998; Stenlid & Gustafsson 2001; Heilmann-Clausen & Christensen 2003; Lonsdale *et al.* 2008). Differentiated responses to environmental factors may result in a shift of functions and extinction of functional groups, as shown in a case of two species of cyanobacteria (Garcia-Pichel *et al.* 2013). Observations on fungal fruiting bodies showed significant changes on the fruiting period, production, and host range in the past 60 years (Gange *et al.* 2007; Heilmann-Clausen & Læssøe 2012; Kauserud & Heegaard 2012). In vitro studies have well documented the physiological changes of fungi due to environmental parameters. With temperature as an example, metabolite rate can increase and peak at certain optimum temperatures, after which the rate decreases (Toljander *et al.* 2006; Rousk *et al.* 2011; Wei *et al.* 2014). Functional shifts may occur if the dominant fungi have different optimum temperatures. This may, in part, explain the geographic distribution of brown rot (mostly high latitude areas) and white rot (lower latitude areas) fungi (Gilbertson 1980), with some parallel to evolution relative to historical climate shifts (Floudas *et al.* 2013).

In addition to abiotic kinetic drivers such as temperature, biotic roles for endophytes (microorganisms within live plants) are rarely considered as a 'trait' determining the rate or function of decomposing wood in forests. Current studies have shown intensively mutual interactions between endophytes and their host plants (Waller *et al.* 2005; Márquez *et al.*

2007; Hoffman & Arnold 2010; Behie *et al.* 2012; Bulgarelli *et al.* 2012; Lundberg *et al.* 2012; Parfitt *et al.* 2012; Bruez *et al.* 2014). Endophytes can boost the fitness of their hosts by increasing tolerance to environmental stresses, such as disease, salt or drought (Arnold *et al.* 2003; Berg *et al.* 2005; Waller *et al.* 2005). On the other hand, saprotrophic and pathogenic fungi also take dormancy in live plants, and begin saprotrophy when plants are sick or wounded (Carroll 1988; Saikkonen 1998). For wood-degrading fungi, several studies reported early decay symptoms that were unlikely caused by external fungi, *i.e.* it is too fast/far for fungi from soil or air to colonize freshly dead wood (Boddy & Rayner 1982, 1983a & 1983b; Hendry *et al.* 1998). Wood decay fungi have been isolated from fresh sapwood in some cases (Chapela & Boddy 1988), and typical wood-degrading fungi can be amplified by PCR in live trees using species specific primers (Parfitt *et al.* 2010). The presence of wood-degrading fungi as endophytes could be an important decay rate determinant by imposing strong priority effects (Ottosson *et al.* 2014), shown to be critical for the structure of fungal community (Fukami *et al.* 2010). This could also help to explain the host preference and niche separation of brown rot and white rot fungi (Gilbertson 1980). In general, the potential role of endophytes as wood decomposers, and their interactions with external fungi should be evaluated experimentally in order to improve predictive capacity for decomposition of dead woody stocks in forests.

Beside priority effect, interactions of wood-degrading fungi with other groups of fungi or bacteria could also be important. Such interactions included antagonism, parasitism or mutualism. Co-culturing bacteria or yeast with wood-degrading fungi has been shown to accelerate wood decomposition (Blanchette & Shaw 1978). This could be due to degrading of border pit by bacteria for easier wood tissue access (Tsuneda *et al.*

1995). Nitrogen fixing bacteria was also found to be prevalent in dead wood, but their functions and relationship to wood-degrading fungi are still unclear (Spano *et al.* 1982; Matzek *et al.* 2003; Hervé *et al.* 2014). Some species of *Trichoderma* can be potential mycoparasite of wood-degrading fungi (Tsuneda *et al.* 1995). Labile carbon, which release by wood-degrading fungi can be consumed by other opportunists, as shown in the decomposition of soil organic matter (Graaff *et al.* 2010). These interactions may change the relative abundance of fungi and bacteria in decaying wood. Graaff *et al.* 2010 has shown that the Fungi/bacteria (F/B) ratio increased following addition of labile carbon to soil. Whether F/B ratio can be used to predict wood-decomposition is still unknown. In case of dominancy of one species of wood-degrading fungi, it is possible that the pattern of decomposition is determined majorly by the rot type, instead of abundance. In any case, studies on the abundance of all microbial component, and not just wood-degrading fungi, will provide comprehensive information in wood with complex communities.

In this study, I used two wood-degrading fungi and one wood substrate as a model system to test the effects of temperature and endophytes on the competition of two fungi. The two fungi (*Piptoporus betulinus* the brown rot fungus and *Fomes fomentarius* the white rot fungus) are both commonly found on birch in northern forests (Figure 4.1). Young birch seedling were cut from the Cloquet Experimental Forest of University of Minnesota. Microcosms were used to incubate birch rounds with fungal isolates in two incubators with different temperatures (25°C and 30°C). Two conditions of endophytic community (absence/presence), four scenarios of fungi colonizing from the soil (single *P. betulinus*, single *F. fomentarius*, both fungi and no fungus), and also the priority effect (one soil fungus colonized wood 3 wk earlier) were tested. Wood residues were fully characterized

for their mass, carbohydrates and lignin loss, dilute alkali solubility, pH and water content. Biomass of different microbial component (*P. betulinus*, *F. fomentarius*, bacteria, fungi and *Basidiomycota*) were measured by qPCR. Collectively, this trial was geared to understand how the biotic predisposition of a community in the healthy tree would influence its decomposition process in the dead wood, relative to the most common abiotic factor measured to reflect climate change potential - temperature.

4.2 Method

4.2.1 Fungal isolates

Brown rot fungus, *Piptoporus betulinus* (Cloq001), and white rot fungus *Fomes fomentarius* (Cloq002) were isolated from a single standing dead tree in the Cloquet Experimental Forest of University of Minnesota in 2010 (Fig 4.1). Fungal hyphae were isolated by culturing a sterilized cut piece of fruiting body on 2% water agar. The isolated fungi were kept on 2% malt extract agar (MEA) in 4°C. Both fungi grew to the edge of 10cm petri dish with 2% MEA in two to three weeks. Fungi were identified following similar DNA extraction and work-up as in Chapter 2, using BLAST to query and verify sequence matches.

4.2.2 Wood substrates

Ten young and seemingly healthy birch trees (no discoloration, wounding, etc.) were cut down at 1 m height at Cloquet Experimental Forest on Oct, 2012. The diameter of the ten logs ranged 50 to 90 mm. The logs were stored in a 4°C cold room prior to use in the experiment, and were used as soon as possible in the trial. No surface hyphae were visible at the time of deployment.

These small-diameter logs were cut into rounds with ~40 mm length using a table saw. Any knot and side branches were avoided. The rounds were assigned to each treatment so that each treatment contained one round from each log, thus minimizing the effect of chemical difference among the logs. The rounds were weighed fresh, and then ten rounds each from a log were oven-dried to obtain the dry weight and water content. All other rounds were wrapped with aluminum foil. Half of the rounds were autoclaved 1 hr twice with a 24 hr interval, while the other half were kept cold and non-sterilized. All rounds were kept in 4°C until microcosm assembling. The process, including inoculations (4.2.4) and other assembly aspects, was less than 3 weeks.

4.2.3 Optimum temperature

To assess a logical temperature treatment structure, growth rates of *P. betulinus* and *F. fomentarius* were measured on 10 cm Petri dishes containing 2% malt extract agar and at five temperatures (20, 25, 28, 30 and 33°C) (Fig 4.1). Petri dish cultures were arranged that all cultures were inoculated with 2 wk old colonies, in order to minimize the influence of fungal physiological changes. The diameter of the colonies was measured daily until they reached the edge of the petri dishes. 25°C and 30°C were chosen as the two temperatures that favored the growth of *P. betulinus* or *F. fomentarius*.

4.2.4 Soil microcosm

Table 4.1 summarized the treatments in this experiment. The major factors were temperature (25°C and 30°C) and endophytes (absence/presence). The two wood-degrading fungi were arranged in all possible combinations (single, both and none). Both

fungi were given the chance to colonize wood 3 wk earlier than their opponent as the treatment for priority effect.

Soil-block microcosms were produced following a modified protocol (ASTM D 1413, 2007) described by Song *et al.* 2012. Soil was mixed with 1:1:1 peat, vermiculite, and fertilizer-free soil. 300g soil mixture was filled into each 8oz glass jar (Ball^{TM}, Daleville, IN, USA) and pressed to fill half of the jar (Fig 2.1). The lids were flipped to provide better ventilation for microcosms. The assembled microcosms were set for a day and autoclaved 1 hr twice with a 24 hr interval. The microcosms were stored at room temperature.

Fungi were pre-cultured on feeder strips (sterilized) before inoculating into microcosms. Agar plugs were cut from the edge of 2 wk-old culture on 2% MEA agar. Two plugs were put on each feeder strip and set on the edge of a new petri dish with 2% water agar on top of a plastic mesh (sterilized) for 3 wks (Fig 2.1, 4.2). All inoculations were carried out under a clean hood with ethanol/flame sterilized tools.

Two hyphae coated feeder strips were transferred to each soil-block microcosms and kept in dark under 25°C for 3 wk. The feeder strips were added according to the experimental design in Table 4.1. Fungal hyphae covered the entire soil surface after 3 wk. In microcosms containing two fungi, a boundary line can be visualized (Fig 2.1). Birch rounds were put in the center of each microcosm and incubated under corresponding temperature for 140 days at the designated temperature in the dark (Fig 2.1).

For the priority effect treatments, birch rounds were pre-cultured in the 8oz glass jars containing corresponding fungi for 3 wk at 25°C. Only one feeder strip (3 wk fungal mycelium) was put in the jars in order to colonize one side of the rounds. The precolonized

rounds were transferred to soil-block microcosms with the colonized side facing the fungus of the same species.

4.2.5 Sample harvest

After 140 d incubation, rounds were bagged with clean pre-weighed sterile baggies and measured for fresh weight in a clean safety cabinet. The rounds were then split into subsamples for molecular and chemical analysis. For single soil fungus treatments, the rounds were split along the grain into four pieces. For treatments containing two soil fungi, the rounds were split in the middle and then into eight pieces (Fig 2.1). Half of the upper and bottom subsamples for each round were stored in -80°C for molecular analysis. The other subsamples were weighed separately and oven-dried at 102°C for 48 hr. Dry weights for each subsample were measured to determine their moisture content. The dry weight of each non-dried round was then calculated based on the average moisture content of the subsamples. Methods preventing contamination, such as flame sterilizing and ethanol wipes, were used between processing of each round under a clean hood.

4.2.6 Wood characterization

Mass loss rate of the rounds was calculated as the loss of dry weight between initial and decayed samples. The oven dried subsamples of each round were pooled and ground to 40-mesh in the Wiley mill. Carbon fractions (w/w) were determined for cellulose (glucan), for hemicellulose (arbinan, galactan, mannan and xylan), and for acid-soluble Klason lignin, as previously described (Schilling *et al.* 2009). The pH and dilute alkali solubility (DAS) were determined following Shortle *et al.* (2010). For pH, wood powder was equilibrated in

5 mM $CaCl_2$ at a ratio of 0.1 $mg \cdot \mu L^{-1}$. For DAS, wood powder was autoclaved in 0.2 M NaOH, filtered through a tared fritted glass crucible, and rinsed with distilled water and 0.1 M HNO_3. The percent loss on extraction was calculated by weighing extracted materials.

4.2.7 DNA isolation

Frozen subsamples were ground to fine powder, as discussed earlier, with a bone mill after snap-freezing with liquid nitrogen. Additional liquid nitrogen was added during the process of grinding, if necessary. Ground powder was stored in 50ml conical tubes at -20°C.

Microbial DNA was extracted with a modified method as previous described by Jasalavich *et al.* (2000) and also described in Chapter 1. Between 60 and 70 mg of wood powder was added to 2 ml microcentrifuge tubes and resuspended in 800µL 2X CTAB buffer with 2% PVP and 2% β-mercaptoethanol. The tubes were incubated at 65°C for 1 hr after horizontally vortexing for 2 min using a vortex adapter (MO BIO, Carlsbad, CA, USA). Tubes were mixed at 15 min intervals. After incubation, samples were extracted in 800 µL phenol:chloroform:isopropanol (vortex to mix and centrifuge at maximum speed for 10 min; same procedure for the other two extractions). 600 µL upper liquid phase was transferred and extracted a second time in 600 µL choloroform:isopropanol. 400µL upper liquid phase was transferred, extracted a third time with 400µL choloroform:isopropanol. 250 µL upper phase was transferred to a clean 1.7ml microcentrifuge tube. 1.25 ml binding buffer PB was added and the mixed solution was loaded into Epoch mini spin columns (Epoch Life Science, Suagr Land, TX, USA) and spun for 1 min, two times. To wash, 750 µL washing buffer PE was added onto the column and spun for 1 min. The eluent was discarded and centrifuged again to get rid of residue ethanol. DNA was resuspended by

adding 100 μL EB buffer onto the center of column filter and spun at maximum speed for 1 min.

Concentrations of DNA were measured with a Qubit HS dsDNA assay (Life Technologies, Carlsbad, CA, USA). A working solution was made as a 1:10 dilution of the original DNA solution with nucleases-free water and stored at -20°C. Original DNA solution was stored at 4°C as backup. Although the original undiluted sample can be detected in qPCR, some samples showed a moderate effect of inhibitors which could be eliminated with a 1:10 dilution.

4.2.7 Quantitative PCR

The abundances of *P. betulinus*, *F. fomentarius*, bacteria, fungi and *Basidiomycota* were measured using qPCR with corresponding standard curves. Bacteria were included given the ease of primer sets and the potential preliminary data available relative to fungi dominating the wood deconstruction process. Primer sets for *P. betulinus*, *F. fomentarius* were designed in this study. Primers sets and qPCR protocol for bacteria, fungi and Basidiomycota followed the method of Fierer *et al.* 2005.

Primers were designed to amplify a segment of the ITS region specifically from either *P. betulinus* or *F. fomentarius*. DNA of the two isolates were extracted and sequencing for their ITS region. Primers were selected by checking the alignment of three pairs of fungi (*P. betulinus* and *F. fomentarius*, *P. betulinus* and *Fomitopsis pinicola*, *F. fomentarius* and *Ganoderma australe*). *P. betulinus* and *F. pinicola* are closely-related brown rot fungi with a large portion of overlapped sequence on ITS region. A same case applied to *F. fomentarius* and the genus *Ganoderma*). *F. pinicola* was isolated from the

Cloquet forest. *G. austral* was obtained from ATCC (ATCC90302). Primers: PB121F and PB576R were developed to amplify a 456 bp DNA fragment from *P. betulinus*, whereas FF149Fand FF420R were developed to amplify a 272 bp DNA fragment from *F. fomentarius* (Refer to Table 1.1 for the details of primers).

Quantitative PCR was performed using an ABI 7900HT system (Applied Biosystems, Foster City, CA, USA) on a 384-well plate. Each reaction well contained 5µL of standard or sample DNA, 1 µL each primer (10 mM), and 10 µL 2X SSo advance SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) in a total volume of 20 µL. The amount of standard DNA was added in the series of 2500, 500, 250, 50, 25, 5, 2.5 and 5 pg. All reactions were performed in triplicate, included three non-template controls. The thermal cycling condition were 98°C for 3 min, followed by 40 cycles at 60°C for 1 min. Dissociation curve analysis was performed on all plates using the default setting. DNA of pure cultured *P. betulinus* and *F. fomentarius* was used as the standard.

Following Fierer *et al.* 2005, primer set EUB 338 and EUB 518 was used for bacteria, primer set ITS1f and 5.8s was used for general fungi, and primer set 5.8sr and ITS4b was used for Basidiomycota as a more specific 'higher fungi' tag. PCR conditions were modified to 98°C for 3 min, followed by 40 cycles at 98°C for 15 s, 55°C for 30 s and 72°C for 1 min. Dissociation curve analysis was performed on all plates using the default setting. DNA of cultured *Escherichia coli*, *Saccharomyces cerevisiae* (baker's yeast) and an equal mix of *P. betulinus* and *F. fomentarius* DNA were used as standard for bacteria, fungi and Basidiomycota respectively. All qPCR assays had efficiencies ranging from 87.2% to 95.1% in the five assays (Table 4.2).

A test of inhibitors was performed by checking the series 1:10 dilution of standard and samples starting from 10 ng (2.5 ng was used in trial reactions). No change of linearity was detected within the range of our sample and standard.

The abundance of each species was calculated as the amount of DNA in measured samples ($ng \cdot mg^{-1}$), and adjusted with the mass loss rate into volume based measures ($ng\ cm^{-3}$).

4.2.8 Statistical analyses

Data are expressed as means and standard error (SE). Analysis of variance (ANOVA) was used for Fisher's LSD comparison among treatments at the same temperature. Two sample T-tests were used to compare the corresponding sample with and without endophytes (eg. between PAL and PNL in Table 4.1). Two-way ANOVAs were performed within each treatment to measure the effect of temperature and endophytes on decomposition. Linear regression was applied to correlate wood characters with the microbial abundance, as well as to correlate wood characters with microbe ratios (Fungi/Bacteria, *Basidiomycota*/Bacteria, *Basidiomycota*/Fungi). Principle component analysis (PCA) was performed using wood characterization (mass loss, carbohydrates loss, lignin loss, pH, DAS, water content) and microbial abundance (*P. betulinus*, *F. fomentarius*, bacteria, fungi, Basidiomycota) as variables. Data were averaged within each treatment before PCA.

4.3 Result and Discussion

4.3.1 Characterization of decay residues

After 140 d of decomposition, the rates of decomposition were generally lower under higher temperature (30°C) and in the presence of endophytes (Fig 4.3). Although the two no-fungus controls were colonized by unknown airborne fungi, their rates of mass, carbohydrate and lignin loss were negligible (Fig 4.3, upper panel). In no-endophyte treatments, single *P. betulinus* and *F. fomentarius* had different influences on the wood substrates. *P. betulinus* consumed more carbohydrates at 25°C than 30°C, while the rates for *F. fomentarius* remained similar. This likely resulted in the lower mass loss rate of single *P. betulinus* from 25 to 30°C. This could also explain the rates of "same time" treatments (with both fungi colonizing at the same time). Lignin was degraded in treatments dominated by the white rot fungus (Fig 4.3 E & F). The presence of endophytes generally lowered the rate of decomposition, but endophytes-only treatment had similar decomposition rates compared to other treatments. In many cases, the difference between the two temperatures was statistically insignificant. This is also evident by comparing the general effect of temperature and endophytes, with endophytes effects about twice that of temperature for mass loss and carbohydrates loss (Fig 4.5).

Other residue characters also showed a larger effect from the presence endophytes than a change in temperature (Fig 4.4). In no-endophytes treatments, DAS generally distinguished treatments with brown rot fungi from that with white rot fungi. DAS of same-time treatments were mostly the average of the two corresponding treatments (Figure 4.4 upper panel). This was generally the same case for pH. It is interesting that, comparing the no fungus control, brown rot fungus significantly lowered the pH while white rot fungus

increased the pH slightly. Again, endophytes had a larger effect than temperature. The presence of endophytes was associated with lower DAS in most of the treatments except for the endophytes-only treatment. Residue pH was generally higher at 30°C for the endophytes treatments (Fig 4.4 lower panel). The effect of endophytes was also larger than temperature for DAS, pH and water content, but not statistically significant (Fig 4.5).

4.3.2 Biomass of microbial components

In no-endophytes treatments, there was no significant difference in biomass of *P. betulinus* and *F. fomentarius* between the two temperatures (Fig 4.6), but the variation of biomass was much larger at 25°C. In the presence of endophytes, most of the treatments contained only a trace amount of biomass of the either of the two soil fungi inoculated. Bacteria, fungi and Basidiomycota can be detected in all samples, and biomass of Basidiomycota were lower in the presences of endophytes, and also lower under 30°C. Biomass of bacteria and fungi were largely consistent across the treatments.

Certain amount of bacteria were found in no-endophyte treatments and are in comparable size to the endophyte treatments. These bacteria communities may be introduced as contaminant during microcosm preparation. But we also detected bacteria biomass with qPCR from our culture stocks of *P. betulinus* and *F. fomentarius*. It is possible that bacteria could also be co-isolated from fruiting bodies on the culture medium.

4.3.3 Relationship between decay residues and microorganisms

PCA revealed clustered endophytes treatments and not segregated clusters among the no-endophytes treatments (Fig 4.7). All no-endophytes treatments could be separated by the

types of colonizer fungi, with wood residue characters also correlating with the type of wood-degrading fungi in the microcosms. For example, DAS and degraded carbohydrates were in the same ordination as brown rot fungi related treatments. The same was obvious for the link between degradation of lignin and the colonizer white rot fungus. The two no-fungus controls were separate from all other treatments, as would be expected. Although less pronounced, treatments at higher temperature were all lower relative to their higher temperature correspondents.

In the absence of endophytic community, there was no significant correlation between the abundance of each microbial component (bacteria, fungi or Basidiomycota) and mass or carbohydrates loss rate (Table 4.3; Figure 4.8). Abundance of Basidiomycota was positive related with lignin loss. But it seems unlike to reflect a factor of abundance since *F. fomentarius* always have high abundance in wood than *P. betulinus*. Abundance of bacteria was negative correlated with lignin loss, and was also higher in treatments related with the white rot fungus. It is possible that some bacteria opened the border pit for white rot fungus to reach the cell wall.

In the presence of endophytic communities, abundance of fungi and Basidiomycota were positively related with the rates of mass and carbohydrates loss, but contrary with no-endophyte treatment, was not significantly related with lignin loss. (Table 4.3; Fig 4.8). Abundance of bacteria was negative related with carbohydrates loss, and positively related with wood pH.

4.3.4 Neglected role of endophytic community

These data indicate an important and often ignored function of endophytes in controlling the structure and function of fungal communities during wood decomposition. Their strong antagonism against fungi that colonize via spores or soil inoculum suggests that endophytes might succeed in gaining priority by penetrating beyond the plant host active barrier in a living tree, This is not a consideration in most trait-based assessments aiming to predict rates of decomposition in dead wood, where chemical parameters are most often used but where predictions have been challenging (Bradford *et al.* 2014). This is not entirely new and is in accordance with the observation of early formation of decay columns and boundary lines (Boddy & Rayner 1982, 1983a & 1983b; Hendry *et al.* 1998). By overcoming exogenous colonizing fungi, endophytes in this study clearly contained wood-degrading members within the community present, nearly matching the decay rates of those inoculated by wood-degrading fungi (Fig 4.3 & 4.4). Again, results showed that priority effects were important (Fig 4.6), but here indicated that the endophytic life-history strategy may have certain advantages in the initial stages of decomposition. *F. fomentarius* has been detected by PCR on birch and beech trees (Parfitt *et al.* 2012), and latent propogules of both test fungi are known to reside in healthy sapwood of trees (Boddy and Watkinson 1995). Our study suggests these wood-degraders might have mutual relationships with plants, as certain plant life cycles for *e.g.* seed germination can be related with nutrient accumulation by fungi in wood (Zielonka 2006; Vanha-Majamaa *et al.* 2007). Given the inclusion of a biotic driver of wood decomposition that is, in some ways, regulated by the active host defense in a living tree, the logical next step is to ask whether selection has

pressured trees to house endophytic saprophytic fungi that might enable success among the next generation of seedlings.

4.3.5 Role of connectivity on forest floor

Exogenous fungi may gain advantage in competition by connecting with other resources in natural ecosystems. It is possible that the competitive ability of fungi colonizing from soil/feeder strips was underestimated in this study as there was a lack of connection with other resources. Distribution of dead wood in forests is often patchy and connected by networks of fungal hyphae/cords (Boddy & Watkinson 1995; Connolly & Jellison 1995; Laiho & Prescott 2004; Jönsson *et al.* 2008). Nutrients and carbon can be relocated from an established colony to a newly-developing one (Boddy & Watkinson 1995; Lindahl *et al.* 1999). This can increase the competitive ability of exogenous colonizer fungi against endophytes, which rely solely on limited resource in wood. Co-existence with endophytes can also occur in the forests due to heterogeneity of dead wood, which can result in a gradient of nutrients, water and oxygen (Paim & Beckel 1963; Silvester & Sollins 1982; Fromm *et al.* 2001; Romero *et al.* 2005). Endophytes may also not be fast enough to colonize all resources in larger-diameter woody debris, not testable in this study with small diameter birch. Considering these caveats, my work supports endophytes as a successful strategy for saprophytes in wood, but the competitive ability of exogenous fungi should be considered in the context of hyphal networks.

4.3.6 Interactions between bacteria and fungi

The relative abundances of bacteria and fungi were a somewhat strong index of decomposition, especially in the presence of endophytes (Fig 4.8 & 4.9). When wood substrates were most dominated by the inoculated isolates of wood-decay fungi, the pattern of decomposition was largely determined by the rot type, with weaker correlation with bacteria, fungi or Basidiomycota. In the presence of endophytes, however, decomposition rate was positively correlated with the abundance of Basidiomycota, and negatively with that of bacteria. Bacteria may act as a consumer of a "common good" such as free glucose or other carbohydrates that were released but not yet metabolized by saprotrophic fungi. In this study, bacteria were also co-isolated from the fruiting bodies of the two fungi (*P. betulinus* and *F. fomentarius*) and detected by qPCR from petri dish cultures. This hinted at a potential relationship between bacteria and wood-degrading fungi which are prevalent in various ecosystems (Clausen 1996; Bonfante & Anca 2009; Güsewell & Gessner 2009). For example, bacteria isolated from birch were shown to either inhibit or promote the growth of the white rot fungus *Heterobasidion annosum* (Murray & Woodward 2003), and more importantly were playing a role underestimated in single-isolate microcosm studies. These interactions between fungi and bacteria in biogeochemical cycles of wood should be evaluated, in addition to simple fungal community structure analyses.

Associations of bacteria with wood-degrading fungi also differed by rot types. In our study, the abundance of bacteria was positively related with wood pH in both endophyte and no-endophyte treatments. In no-endophyte treatments, abundance of bacteria was also positive related with lignin loss and negatively with DAS. High pH, lignin loss and low DAS showed typical signs of a white rot (Song *et al.* 2012). This indicated a

possibility of different bacteria association with brown rot and white rot fungi. Considering bacteria as a component of wood decomposition should be one of the future direction.

4.3.7 Incorporating microbes in predictive models of wood decomposition

Our study highlighted the role of microbial communities as determinants of wood decomposition rates and character. Most current models include wood as a recalcitrant carbon pool with a low C/N ratio and slower turnover rate compared to fine litter (Dai *et al.* 2003). These physiochemicial factors and the climate indices commonly used to predict decomposition rates often fall short of explaining variability in decomposing wood (Bradford *et al.* 2014). Endophytes, as shown in this study, represent another critical variable that could be included in these models (van der Wal *et al.* 2014) as a biotic factor if broad dominance of certain functional types could be predicted from an existing endophytic community. Collectively, by improving predictive modeling, we could begin to estimate the flux of greenhouse gases from wood to expect as our global climate changes.

Table 1.1 Primers used for qPCR assays to target specific fungus

Target Fungus	Primer Name*	Primer Sequence (5' – 3')	Annealing Temperature (°C)	Amplicon Size (bp)
Gloeophyllum trabeum	GT94F	TCAGGCTGTCCTTCCTATGTC	58	427
	GT521R	GTCAAATTGTCCGAAGACG		
Irpex lacteus	IL54F	ATCGAGTTTTGAACGGGTTG	60	579
	IL633R	AAATGATTGTCTCGGCAAGG		
Piptoporus betulinus	PB121F	GTCGGCTTTTGATGCAAAGTAA	60	456
	PB576R	GATTAGAAGCCGAGCCCTTT		
Fomes fomentarius	FF149F	GGCCCACGTTTTCTTTACAA	60	272
	FF420R	TCCAACATCCAACGCTACAA		

*Suffix "F" denotes forward primer, "R" denotes reverse primer.

Table 2.1 DNA and ergosterol extraction efficiencies from different wood types

Fungus	Wood	DNA (ng mg^{-1} dry fungus)	Extraction efficiency of DNA	Ergosterol (μg mg^{-1} dry fungus)	Extraction efficiency of ergosterol
G. trabeum	n/a	432.1[a]	n/a	8.83[b]	n/a
	Oak	396.0[c]	1.09	4.88[c]	1.90
	Birch	682.6	0.63	6.05	1.67
	Pine	281.8	1.53	7.40	1.31
I. lacteus	n/a	41.1[a]	n/a	4.39 [b]	n/a
	Oak	221.2[c]	0.19	4.23[c]	1.20
	Birch	158.0	0.26	4.76	1.09
	Pine	222.7	0.18	4.62	1.03

[a] From liquid-cultured fungus, frozen in liquid nitrogen and ground.
[b] From liquid-cultured fungus, added fresh and extracted in MeOH.
[c] From mixture of liquid-cultured fungus and wood, frozen and ground.

Table 2.2 Lignin and carbohydrates content of wood after 8 weeks (percentage of initial weight, %)

Wood Types	Fungi Types	Lignin	Carbohydrates					
			Glucose	Xylose	Galactose	Arabinose	Mannose	Total Carbohydrates
Oak	Control	27.3	41.2	11.7	0.7	0.7	4.7	59.1
	G. trabeum	27.0	22.6	2.3	3.2	0.2	1.0	29.3
	I. Lacteus	21.6	32.1	4.7	0.7	1.9	1.3	40.8
	Mixed	19.9	27.3	6.4	0.3	1.8	2.1	38.0
Birch	Control	32.9	45.0	9.3	0.3	0.3	1.5	56.4
	G. trabeum	25.6	18.7	3.5	1.7	0.2	0.4	24.5
	I. Lacteus	24.7	40.4	6.5	0.4	0.4	0.8	48.6
	Mixed	24.5	38.8	7.3	0.5	0.4	1.3	48.4
Pine	Control	33.0	41.1	5.5	5.0	1.1	8.9	61.6
	G. trabeum	23.9	17.3	2.9	2.4	0.3	4.3	27.1
	I. Lacteus	22.9	31.3	3.4	0.4	0.4	7.3	42.8
	Mixed	24.6	37.8	3.7	0.6	0.3	7.2	49.5

Table 3.1 Pearson coefficients when correlating fungal dominance (as proportion of *G. trabeum*) with fungal-induced changes in wood physiochemistry in Experiment 1 (priority effects). Correlations shown per wood type and *G. trabeum* (*Gt*) precolonization (priority period) lengths.

Wood types	*Gt* priority period (wks)	Mass loss	Water content	DAS	pH
Oak	1	0.23	**0.65**	0.32	-0.40
	3	**0.47**	-0.32	**0.54**	**-0.87**
	5	0.31	**-0.46**	0.15	**-0.72**
Birch	1	-0.39	-0.29	-0.26	-0.023
	3	0.24	**0.65**	**0.86**	**-0.85**
	5	-0.19	0.38	0.54	**-0.68**
Pine	1	**0.80**	**0.72**	0.13	-0.54
	3	0.08	**0.59**	0.28	-0.50
	5	**0.80**	0.09	**0.70**	**-0.84**
Spruce	1	-0.27	-0.43	-0.01	0.26
	3	**0.69**	0.04	**0.73**	-0.46
	5	0.32	0.20	0.24	**-0.69**

Numbers in bold indicate significant correlation ($P \leq 0.05$).

Table 4.1 Experiment design of Chapter 4

Treatment	Sample Label	Soil Fungi	Endophytes	Temperature	Precolonized Fungi	Replicates
Single soil fungi	PAL	Pb	Autoclaved	25	None	5
	FAL	Ff	Autoclaved	25	None	5
	PNL	Pb	Non-autoclaved	25	None	10
	FNL	Ff	Non-autoclaved	25	None	10
	PAH	Pb	Autoclaved	30	None	5
	FAH	Ff	Autoclaved	30	None	5
	PNH	Pb	Non-autoclaved	30	None	10
	FNH	Ff	Non-autoclaved	30	None	10
Both soil fungi	TAL	Pb, Ff	Autoclaved	25	None	10
	TNL	Pb, Ff	Non-autoclaved	25	None	10
	TAH	Pb, Ff	Autoclaved	30	None	10
	TNH	Pb, Ff	Non-autoclaved	30	None	10
Pb precolonization	XAL	Pb, Ff	Autoclaved	25	Pb	10
	XNL	Pb, Ff	Non-autoclaved	25	Pb	10
	XAH	Pb, Ff	Autoclaved	30	Pb	10
	XNH	Pb, Ff	Non-autoclaved	30	Pb	10
Ff precolonization	YAL	Pb, Ff	Autoclaved	25	Ff	10
	YNL	Pb, Ff	Non-autoclaved	25	Ff	10
	YAH	Pb, Ff	Autoclaved	30	Ff	10
	YNH	Pb, Ff	Non-autoclaved	30	Ff	10
Control	CAL	None	Autoclaved	25	None	1
	CNL	None	Non-autoclaved	25	None	10
	CAH	None	Autoclaved	30	None	1
	CNH	None	Non-autoclaved	30	None	10

Table 4.2 Slopes and efficiencies of quantitative PCR assays

Parameters	*P. betulinus*	*F. fomentarius*	Bacteria	Fungi	*Basidiomycota*
Slope*	-3.58 (0.16)	-3.45 (0.11)	-3.56 (0.17)	-3.68 (0.13)	-3.58 (0.11)
Efficiency	90.6% (5.9%)	95.1% (4.2%)	91.2% (5.8%)	87.2% (4.2%)	90.5% (4.0%)

* r^2 of all fits > 0.99.
n = 6 for each assays
mean (SD)

Table 4.3 Linear regression between microbial indexes and wood characterization in treatments with or without endophytes.

Microbial index	Endophytes	Mass loss	Carbohydrates loss	Lignin loss	DAS	pH
Bacteria	Absence	-0.37 (0.0%)	-2.04 (17.8%)	**1.62 (66.5%)**	**-1.28 (66.5%)**	**0.027 (55.6%)**
	Presence	-0.14 (18.7%)	**-1.86 (32.2%)**	0.13 (0.0%)	-0.29 (24.4%)	**0.017 (55.7%)**
Fungi	Absence	-0.0049 (0.0%)	-0.26 (29.0%)	**0.21 (62.3%)**	**-0.14 (71.0%)**	**0.0032 (67.4%)**
	Presence	-0.0039 (0.0%)	-0.19 (0.0%)	-0.011 (0.0%)	-0.019 (0.0%)	0.004 (11/7%)
Basidiomycota	Absence	0.0058 (10.5%)	-0.014 (0.0%)	**0.049 (60.5%)**	-0.02 (16.7%)	**0.0007 (58.4%)**
	Presence	**0.021 (54.0%)**	**0.22 (45.7%)**	-0.019 (0.0%)	**0.041 (53.7%)**	-0.0011 (17.8%)
Fungi/Bacteria	Absence	-0.57 (0.0%)	-24.9 (25.1%)	**19.8 (53.4%)**	**-11.1 (36.7%)**	**0.25 (36.5%)**
	Presence	**4.61 (35.8%)**	**47.7 (29.1)**	-4.61 (0.0%)	**9.27 (37.8%)**	-0.28 (15.9%)
Basidiomycota/Bacteria	Absence	0.337 (11.2%)	-0.342 (0.0%)	**2.53 (45.0%)**	-0.74 (0.0%)	**0.034 (35.6%)**
	Presence	**1.02 (62.7%)**	**10.9 (55.4%)**	-0.99 (1/2%)	**2.21 (79.4%)**	**-0.077 (53.2%)**
Basidiomycota/Fungi	Absence	**3.65 (38.2%)**	10.1 (0.0%)	14.7 (23.1%)	-1.46 (0.0%)	0.2 (17.6%)
	Presence	**3.25 (60.6%)**	**34.4 (53.0%)**	-2.43 (0.0%)	**7.18 (80.8%)**	**-0.24 (55.6%)**

a. Bolded values indicated significant level $P \leq 0.05$.
b. Each cell contains Slope (r^2).

Figure 1.1 Effect of extraction protocol on general amount of DNA and specific DNA of two fungi – *P. betulinus* and *F. fomentarius*. The data are means and standard errors (n = 5). Letters indicated significant difference between treatments in one way ANOVA by Tukey's multi-comparison ($P \leq 0.05$).

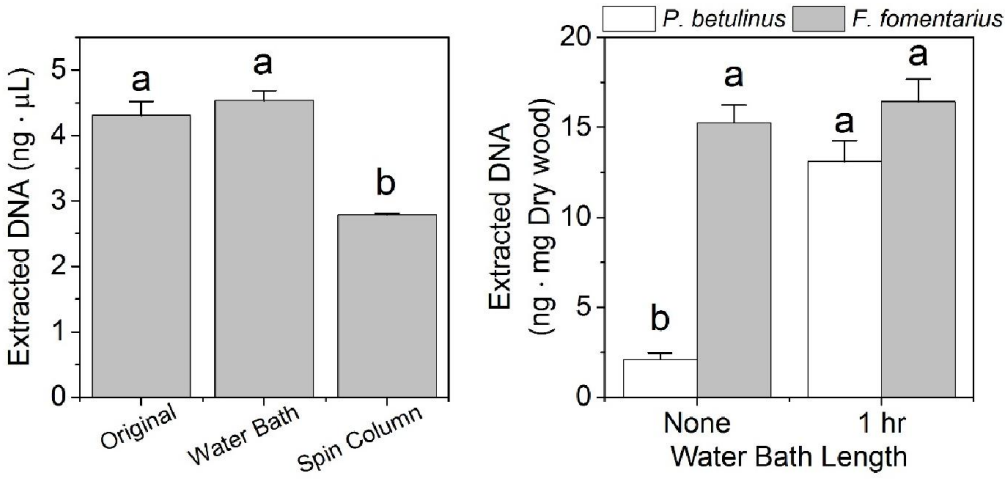


Figure 1.2 Extracted amount of DNA or Ct value from qPCR assays of *P. betulinus* and *F. fomentarius* as a function of the dry weight of input wood samples. * and solid lines indicated significant linear regression relation ($P \leq 0.05$).

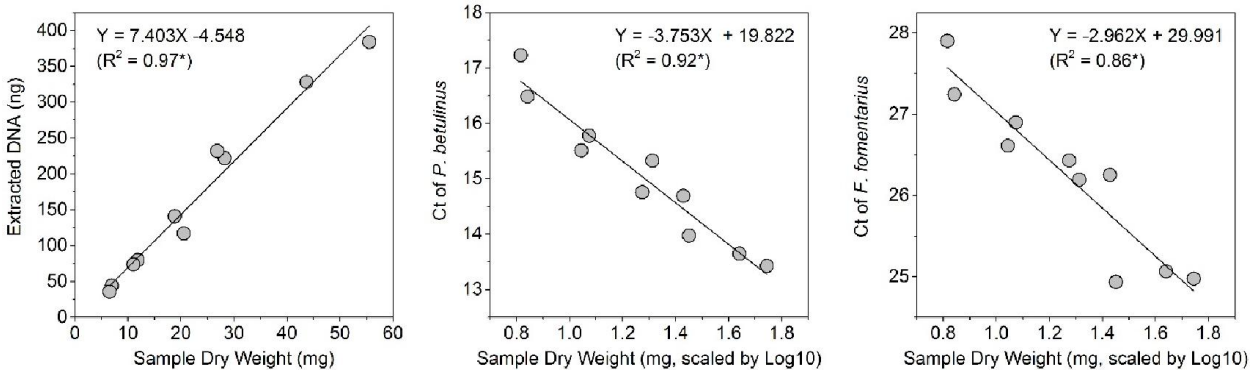


Figure 1.3 Fungal biomass per wood volume as measured using qPCR (open bars) or ergosterol (solid bars) and as a function of fungal species, wood type, and harvest time. The data are means and SEs (n = 5). Significant differences in ergosterol and qPCR methods, at $p \leq 0.05$, are indicated by an asterisk (*). Values above bars are average mass loss (n = 5) of the corresponding samples.

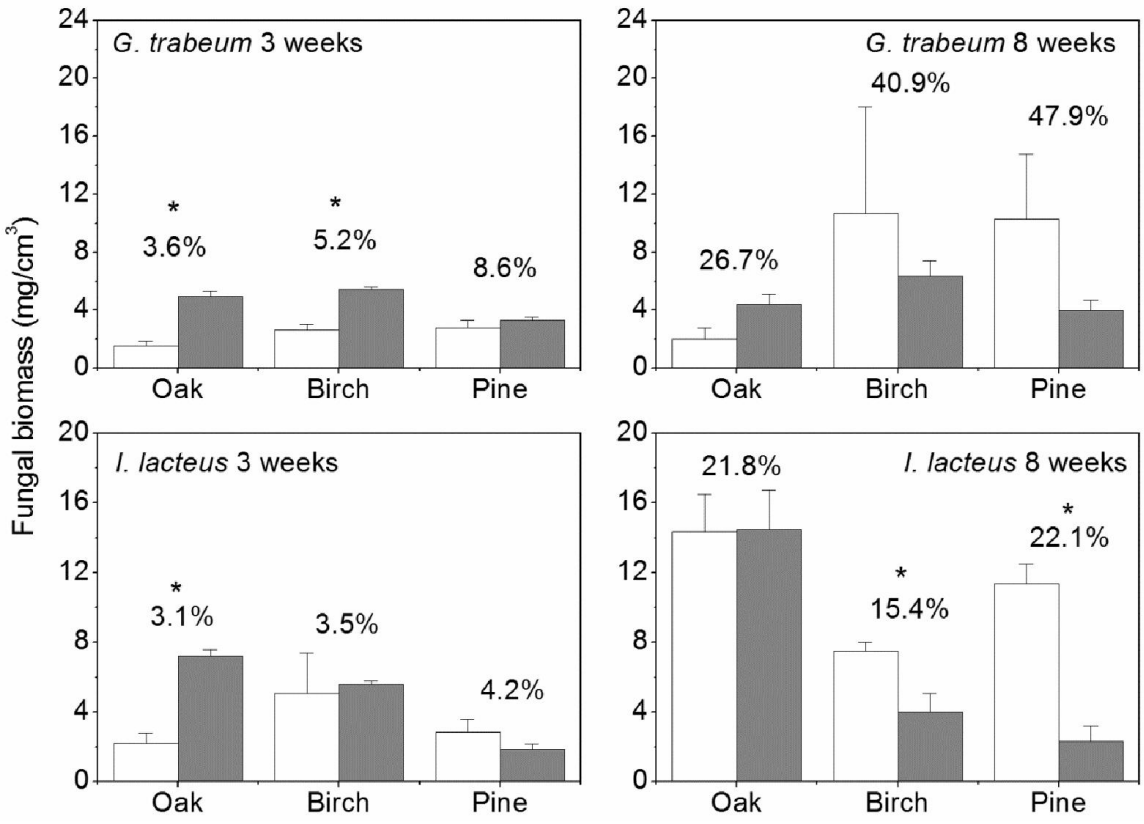


Figure 1.4 ITS copy number ratio with ergosterol (±se) for *G. trabeum* and I. lacteus, as a function of wood type at 3wks (open bars) and 8 wks (solid bars). Bars with different letters are significantly different ($p \leq 0.05$) within a fungal species.

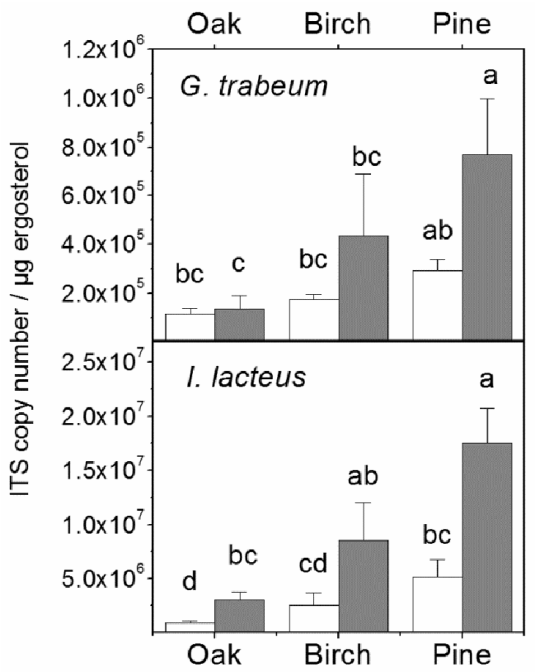


Figure 1.5 Measurement and distributions of hyphal dimensions for *G. trabeum* and *I. lacteus* growing either on agar (open bar) or in pine wood (solid bar) for 10 d. The data are means (±se). Significant differences in ergosterol and qPCR methods, at $p \leq 0.05$, are indicated by an asterisk (*).

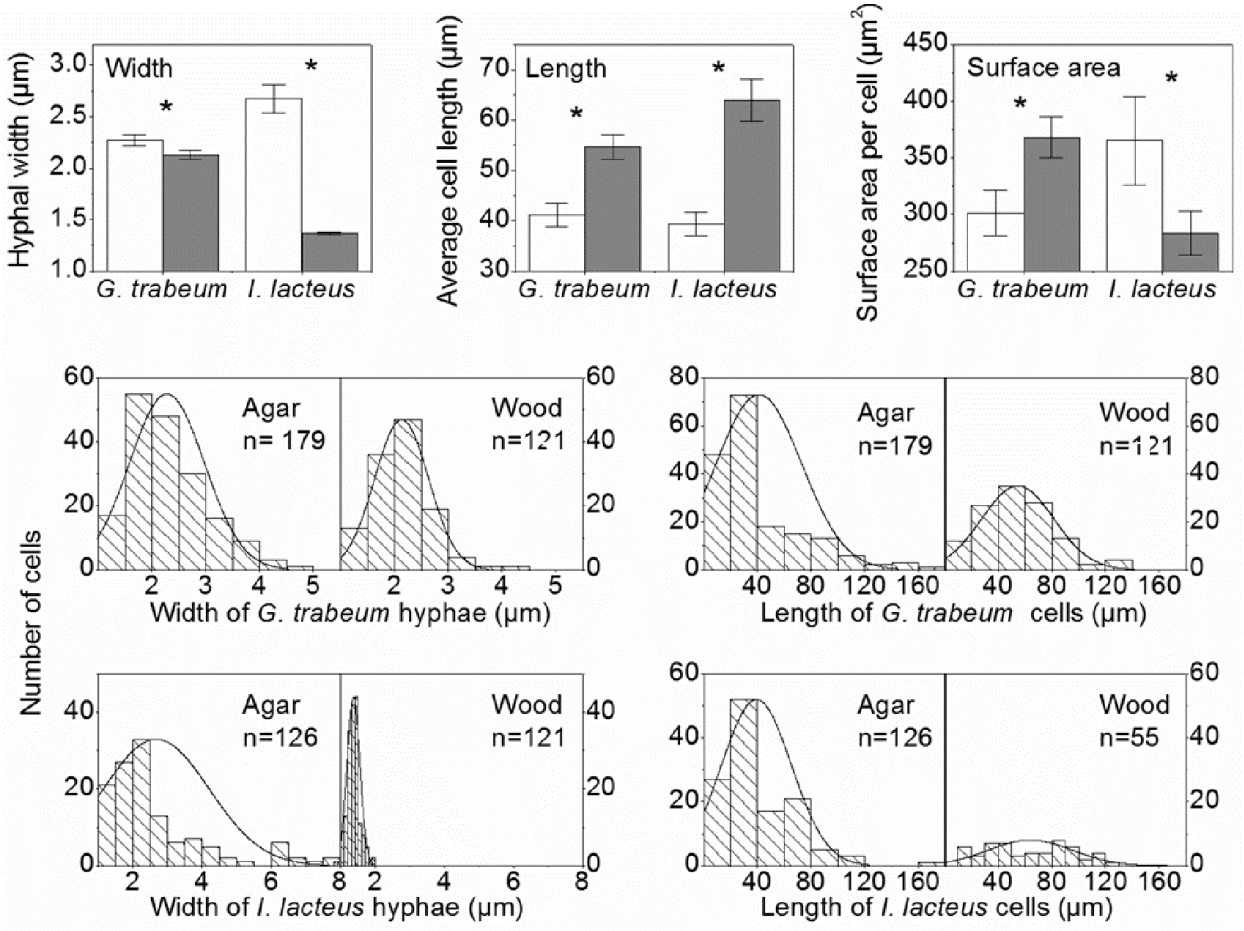


Figure 1.6 Confocal microscopic analyses of hyphae of *G. trabeum* and *I. lacteus*, grown for 10 d in agar and wood and stained with a chitin-specific fluorophore. (a). *G. trabeum* in agar, with arrows showing hyphae with and without clamp connection septa. (b). *G. trabeum* in pine wood, where hyphal cells were longer but only slightly thinner than in agar. (c). *I. lacteus* in agar, with arrows indicating thick and thin hyphae, and (d). *I. lacteus*

in pine wood, where hyphal cells were significantly longer and thinner than in agar. Scale bars = 20 µm.

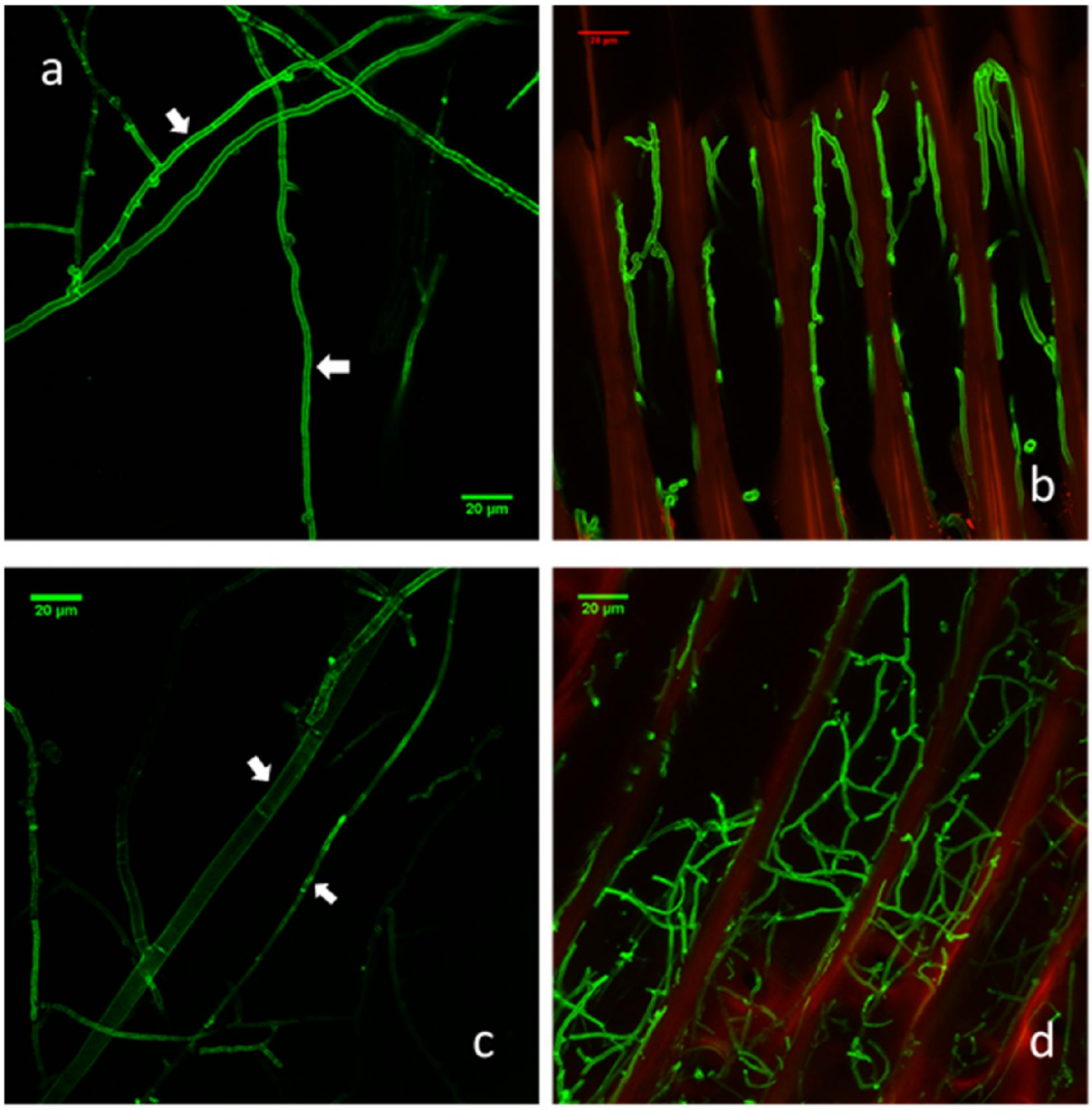


Figure 2.1 Soil-block microcosm used throughout this thesis. The upper panel described the general analysis on wood residues. The bottom panel included photos of microcosm preparation. Legend for the upper panel: **a.** 1:1:1 peat, vermiculite, fertilizer-free potting soil; **b.** Wood samples consisting of oak, pine, or birch; **c.** experimental fungus/fungi. **d.** Characterization included mass loss, carbon fractions, alkali solubility, and pH; **e.** Ergosterol content = fungal biomass. Legend for the bottom panel: **a.** The lid was flipped to avoid poor ventilation of the red rubber band. **b.** soil, vermiculate and peat moss. **c.** soil filled mason jar. d. Birch feeders with agar plugs were cultured on 2% MEA medium for 2 wks. **e.** Mycelium boundary between *P. betulinus* (right) and *F. fomentarius* (left) after 3 wks (used in Chapter 4). **f.** Fungal mycelium after 140 days incubation. Wood substrate were small size dowels or blocks in Chapter 1 to 3, and relative larger rounds in Chapter 4.

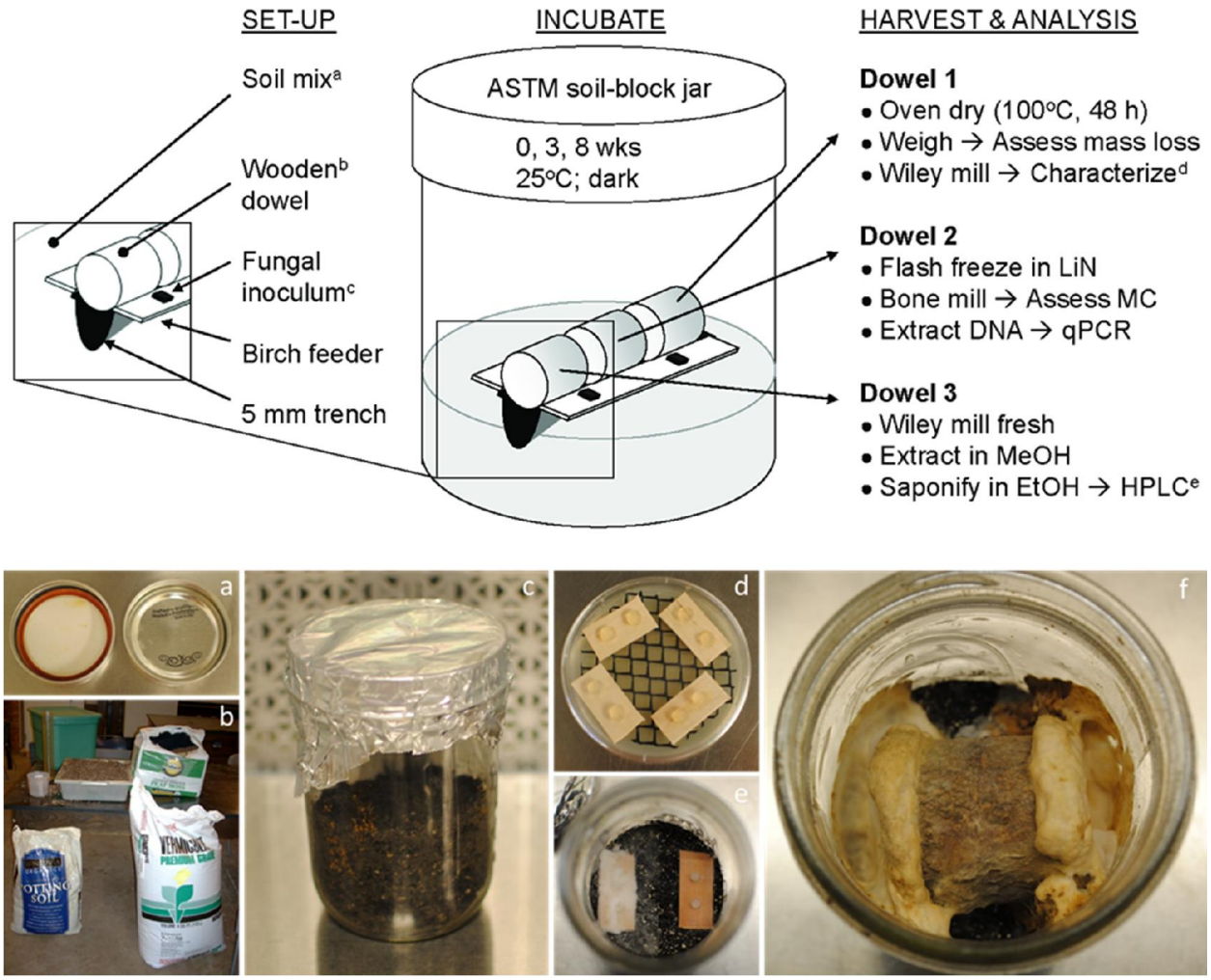

SET-UP
INCUBATE
HARVEST & ANALYSIS
Soil mix[a]
Wooden[b] dowel
Fungal inoculum[c]
Birch feeder
5 mm trench
ASTM soil-block jar
0, 3, 8 wks
25°C; dark
Dowel 1
• Oven dry (100°C, 48 h)
• Weigh → Assess mass loss
• Wiley mill → Characterize[d]
Dowel 2
• Flash freeze in LiN
• Bone mill → Assess MC
• Extract DNA → qPCR
Dowel 3
• Wiley mill fresh
• Extract in MeOH
• Saponify in EtOH → HPLC[e]
a
b
c
d
e
f

Figure 2.2 Characteristics of decay residues in three wood types after 8 weeks of decay by *G. trabeum*, *I. lacteus*, or a mixture of the two fungi. The data are means and standard errors (n = 5). **a.** mass loss (as % of original dry weight) after 3 and 8 weeks. The mass loss after 3 weeks was inside the bars of week 8 samples. The result of multiple comparison of week 3 samples was not shown. **b.** Lignin loss (as weight to original volume). **c.** Carbohydrate loss (as weight to original volume). **d.** Wood pH, error bars for pH are asymmetrical, calculated from $[H^+]$. **e.** Dilute alkali solubility (DAS).

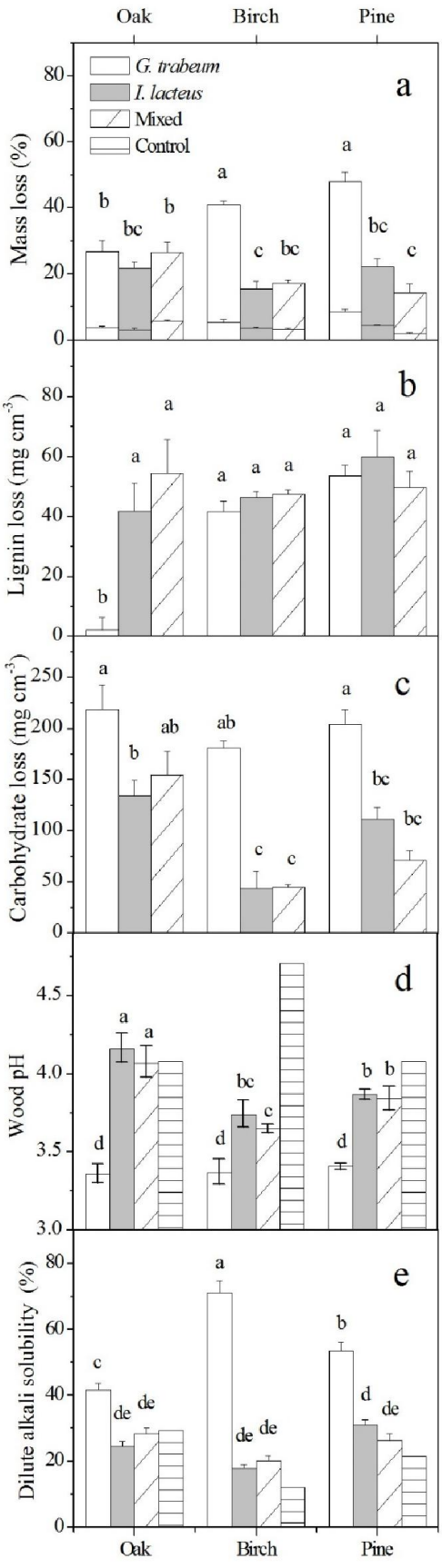


Figure 2.3 Ergosterol content (as weight to original volume) after 3 and 8 weeks of decay by *G. trabeum*, *I. lacteus*, or a mixture of the two fungi. The data are means and standard errors (n = 5). *Letters indicated significant difference between treatments in one way ANOVA by Tukey's multi-comparison (P ≤ 0.05).*

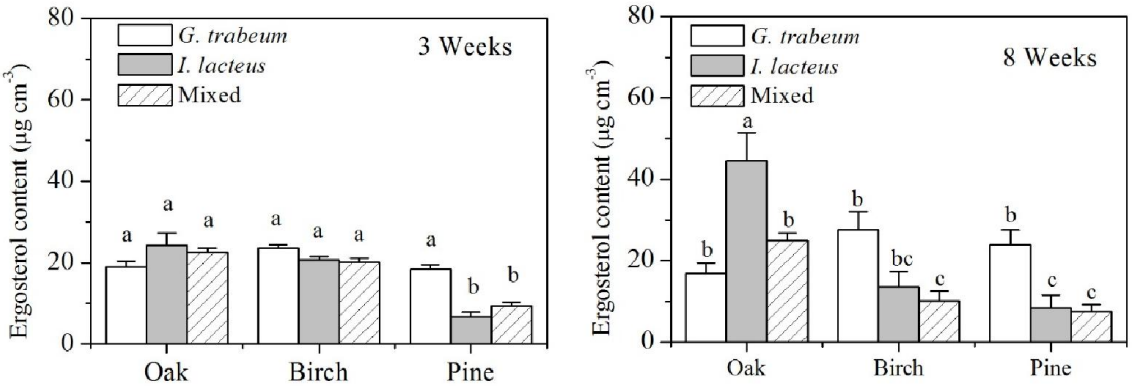


Figure 2.4 *I. lacteus*-to-*G. trabeum* ITS copy number ratio of single and mixed treatments after 3 and 8 weeks. The data are means and standard errors (n = 5). * indicated significant differences ($P \leq 0.05$) between the ratio of single and mixed treatments.

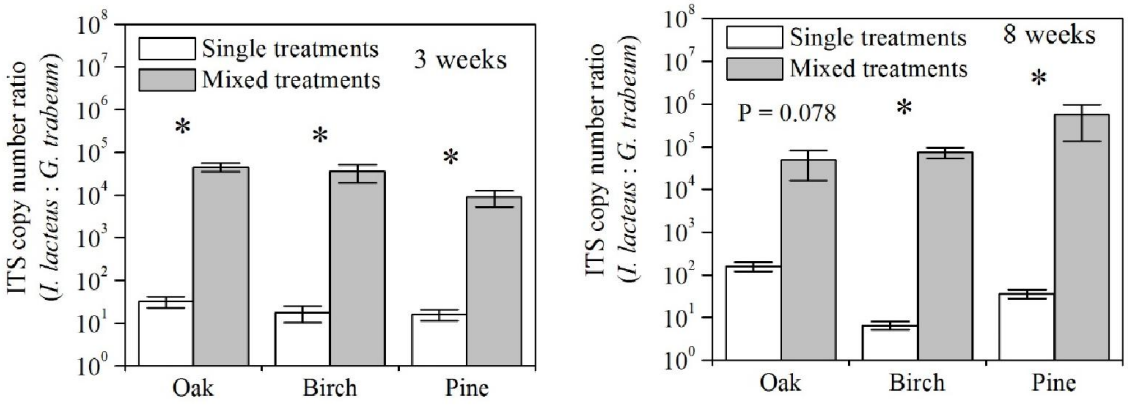


Figure 3.1 Ergosterol content (wt/vol) in wood after various lengths of precolonization (priority periods) by brown rot fungus *G. trabeum* but before addition to competition microcosms. Letters indicate significant difference in protected means comparisons (Fisher's LSD $P \leq 0.05$) within each wood type.

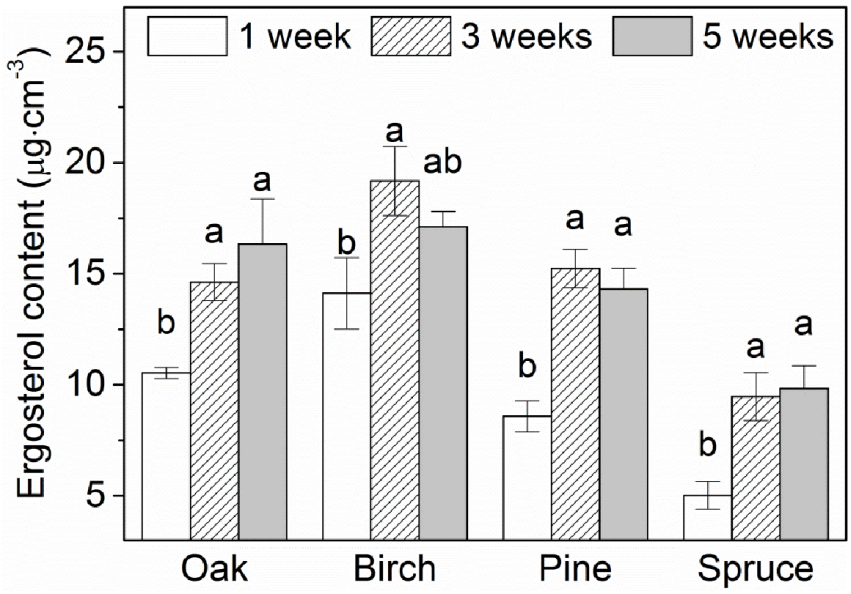


Figure 3.2 Proportion of *G. trabeum* (as a converted biomass percentage, G%) in wood samples after 8 weeks of competition with *I. lacteus* and in wood precolonized by *G. trabeum* prior to competition for various periods.

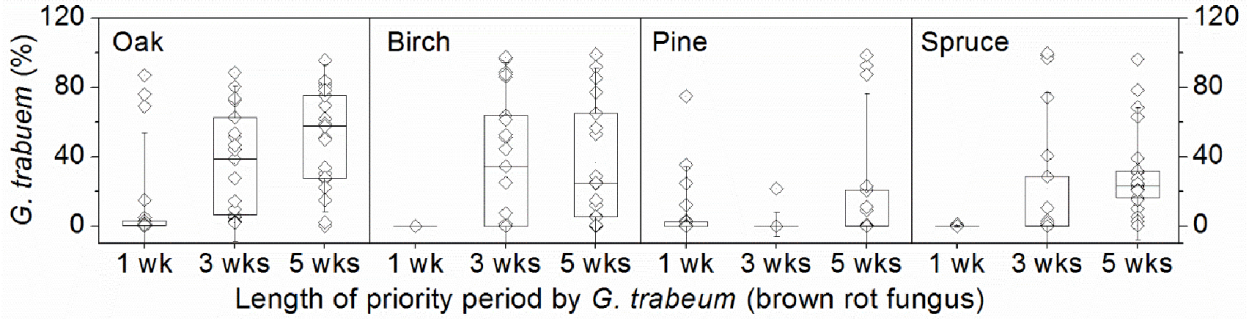


Figure 3.3 Physiochemistry of wood residues after precolonization by *G. trabeum* for various period lengths (open bars) and after an additional 8 weeks of decay in competition microcosms containing *G. trabeum* and *I. lacteus* (closed bars). Data are means and SEs (n = 7). Letters indicate significant difference in protected means comparisons (Fisher's LSD $P \leq 0.05$) within each wood type. Dashed lines (DAS, pH) indicate time zero values in non-degraded wood.

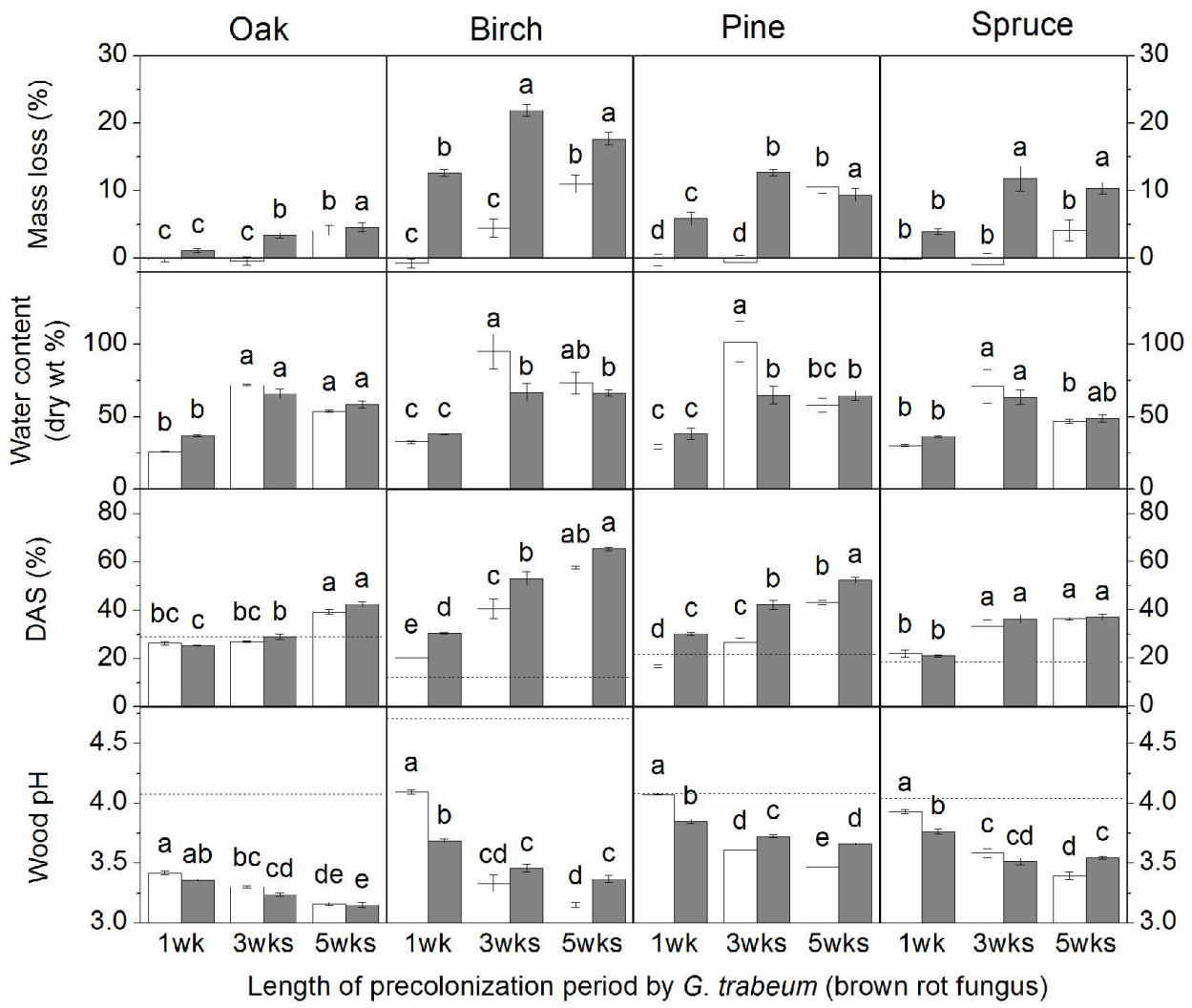


Figure 3.4 Linear regression of proportion of *G. trabeum* proportion and wood pH in samples precolonized by *G. trabeum* followed by 8 wks competitive trial. Bold number and asteroid (*) indicates significant level at $P \leq 0.05$.

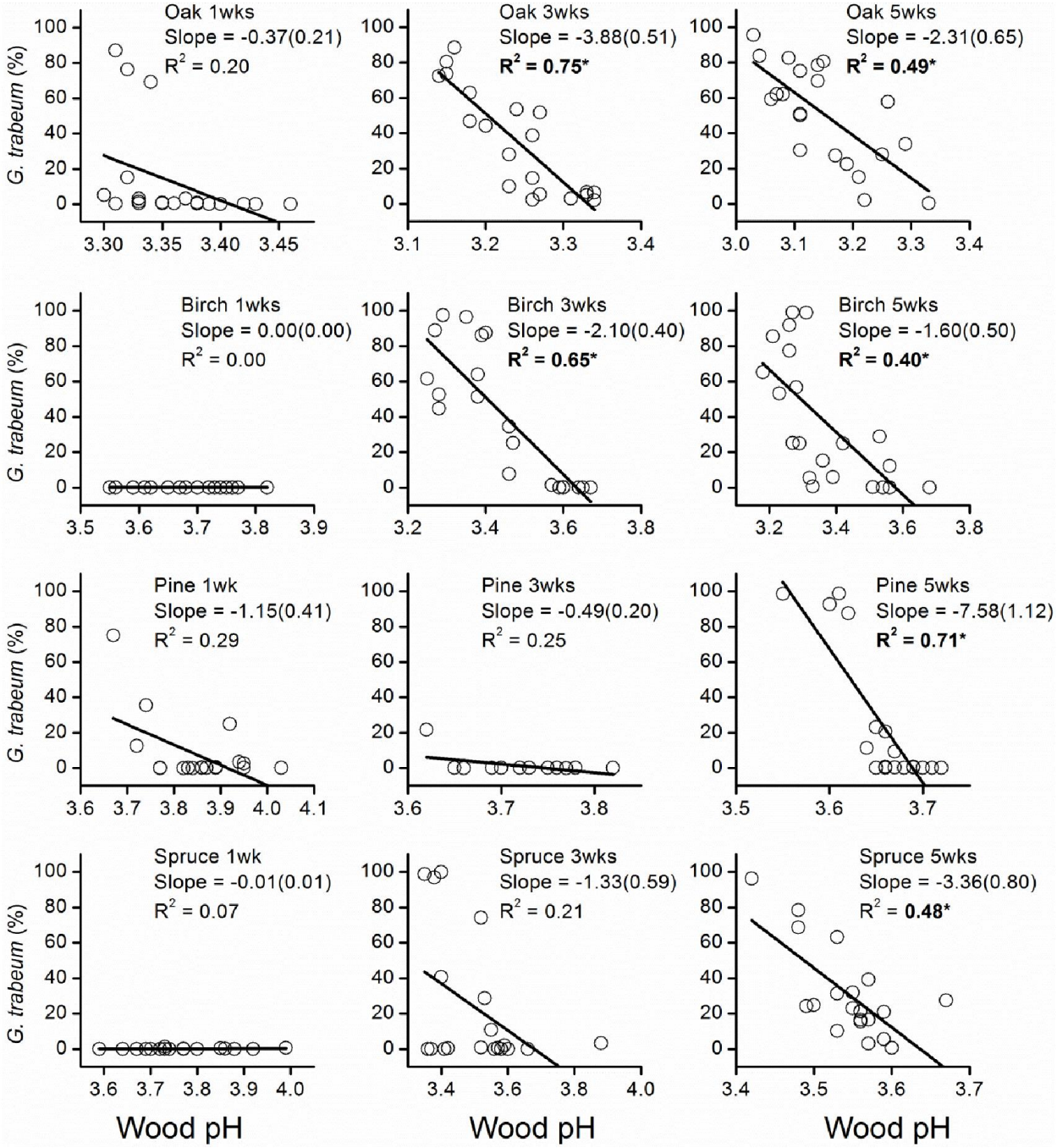


Figure 3.5 Proportion of *G. trabeum* in wood after 3 and 8 weeks competition with *I. lacteus* for various wood substrate types, and the consequences on residue characteristics. Inoculations were simultaneous but with 8x more *G. trabeum* than *I. lacteus* colonized agar volume.

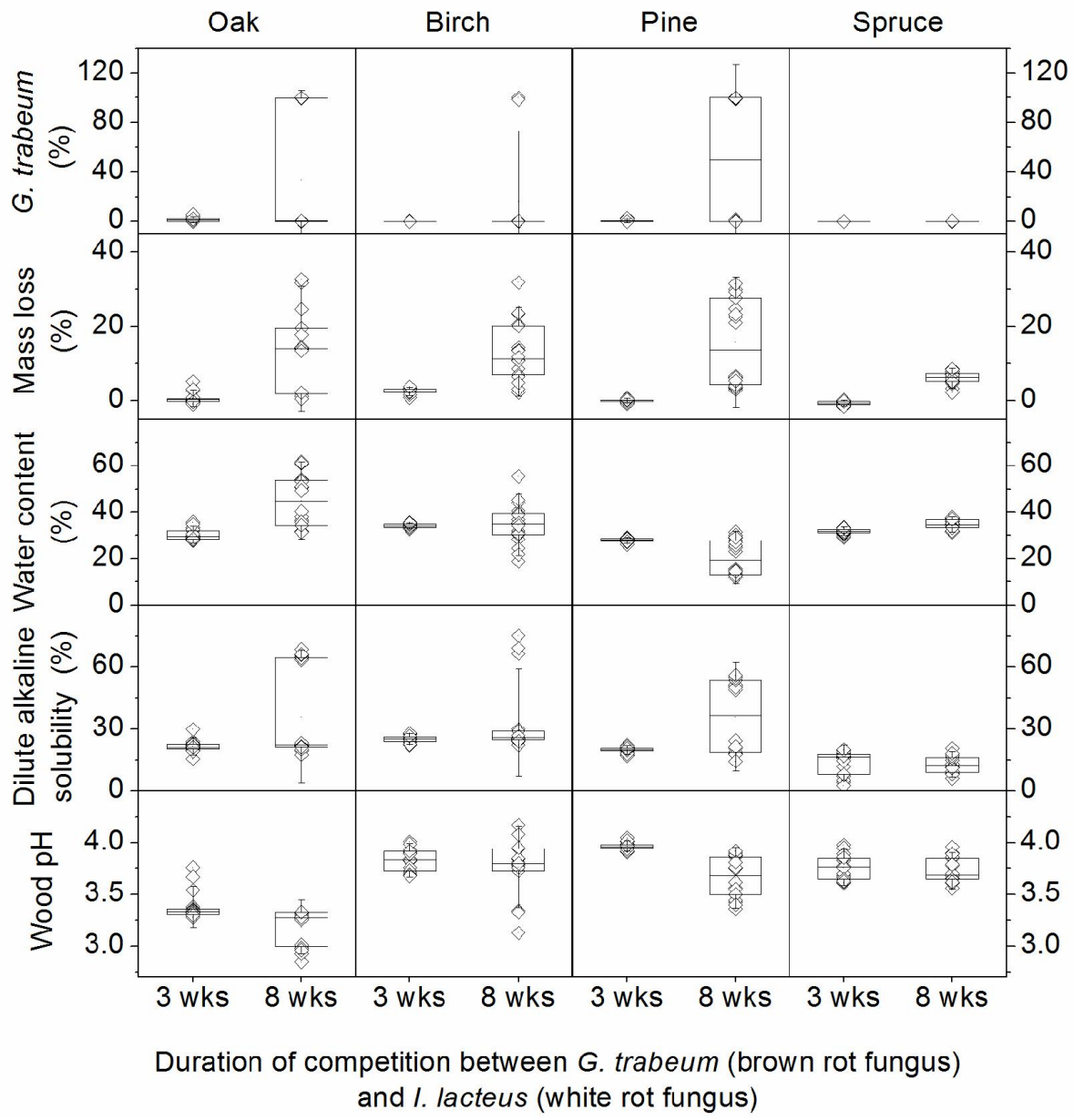


Figure 3.6 Petri dish competitive trial with *G. trabeum* and *I. lacteus* inoculated simultaneously with equal inoculum volumes. The two fungi confronted after 15 days, after which only *G. trabeum* is plotted.

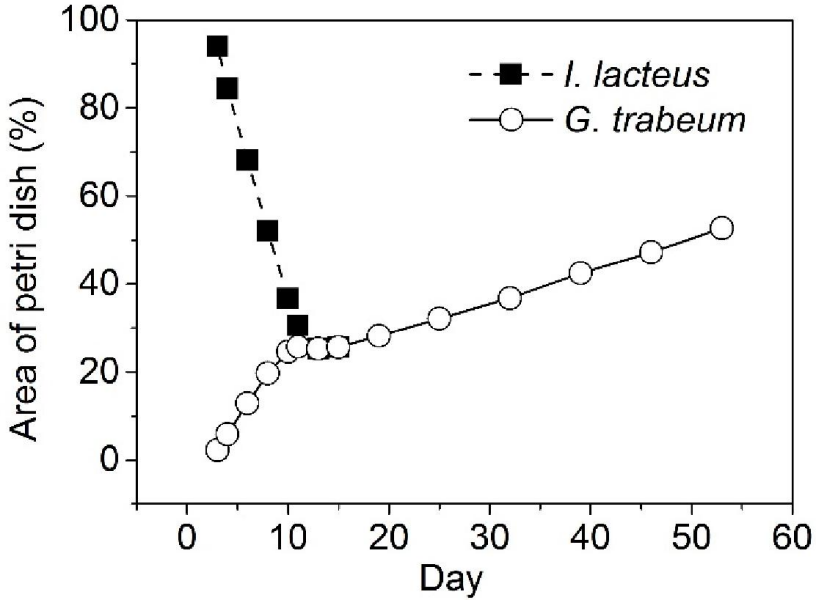


Figure 4.1 Fruiting bodies of *Piptoporus betulinus* (blue arrows) and *Fomes fomentarius* (red arrows) on a standing dead tree at Cloquet Experimental Forest of University of Minnesota (2010).

The bottom panel showed the growth rates of these two fungus under five different temperatures as measured by the diameter of their colonies on 10 cm petri dishes (2% MEA).

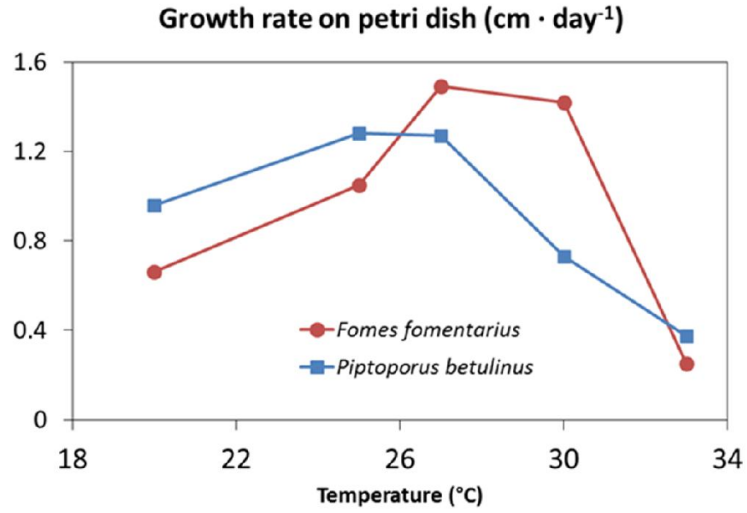


Figure 4.2 Microcosm assembly procedure. Feeders with fungal inoculums were cultured on 2% MEA agar before placed in the microcosms. Part of the birch rounds were not autoclaved and kept at 4°C to maintain an intact endophytic community. Another proportion of rounds were pre-inoculated with one types of fungus for 3 wk, in order the simulate priority effect. All rounds were divided into four (single fungus treatments) or eight (two fungi treatments, shown in this diagram) pieces for downstream analysis.

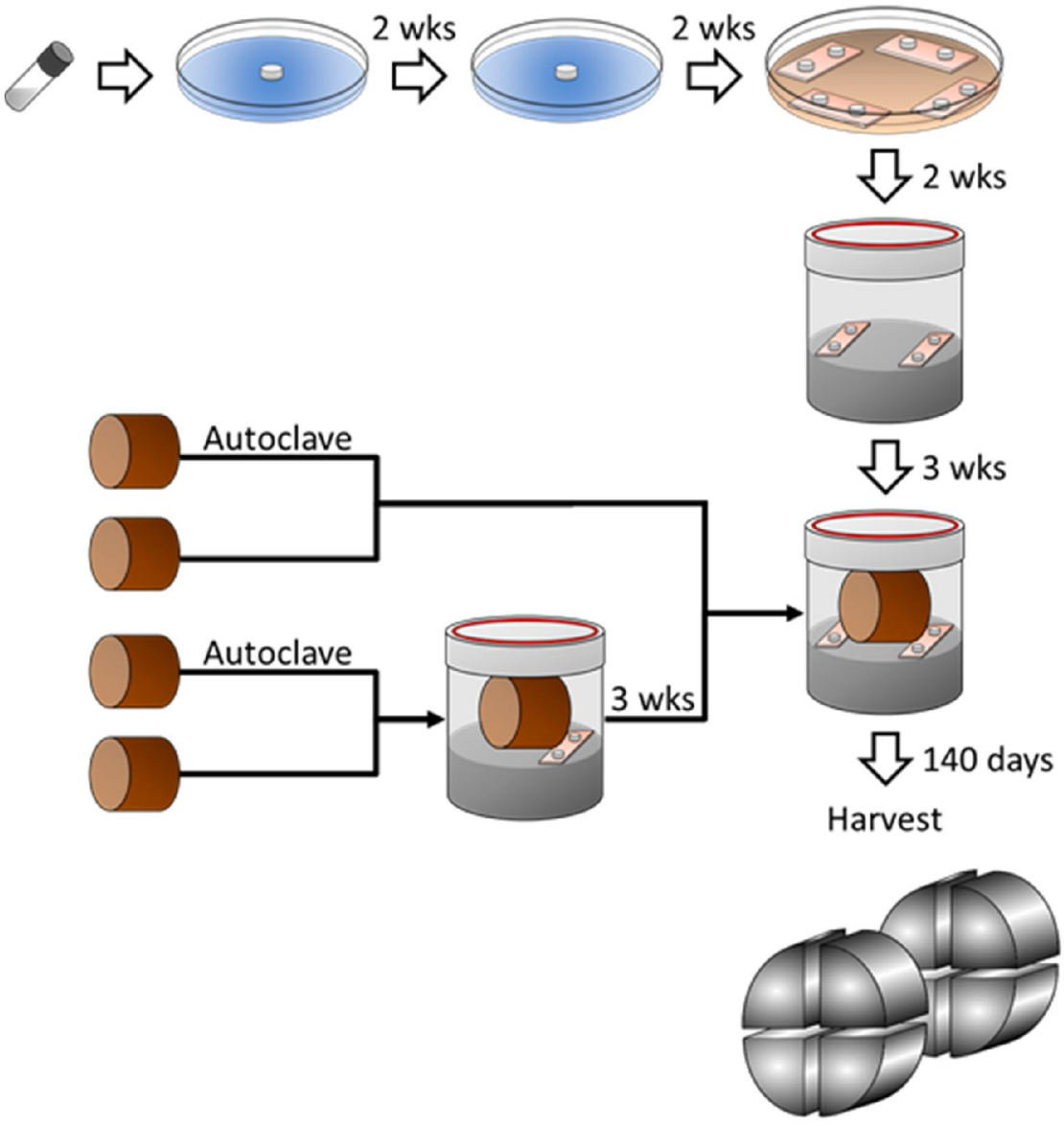


Figure 4.3 Loss rate of dry weight, carbohydrates and lignin of different treatments after 140 days decay under 25°C (open bars) and 30°C (closed bars), and without (upper panels) and with (bottom panels) endophytes. Mass, carbohydrates and lignin loss of treatments without endophytes were shown in **A**, **C** and **E**. Mass, carbohydrates and lignin loss of treatments with the presence of endophytes were shown in **B**, **D** and **F**. Within each plot, letters indicated significant difference among treatments in one way ANOVA by Fisher's LSD multi-comparison ($P \leq 0.05$). Arrows in lower panel indicated significant different between the correspondent treatment with/without endophytes. An up arrow means that no endophytes treatment has larger values, while a down arrow indicate that endophytes presence treatment has larger value. Letters on the X-axis indicated the treatment (types of fungi on soil in microcosms): **Pb**, single *P. betulinus*; **Ff**, single *F. fomentarius*; **ST**, both fungi at the same time; **PP**, *P. betulinus* prior for 3 wk, **FP**, *F. fomentarius* prior for 3 wk; **CT**, no fungus on soil.

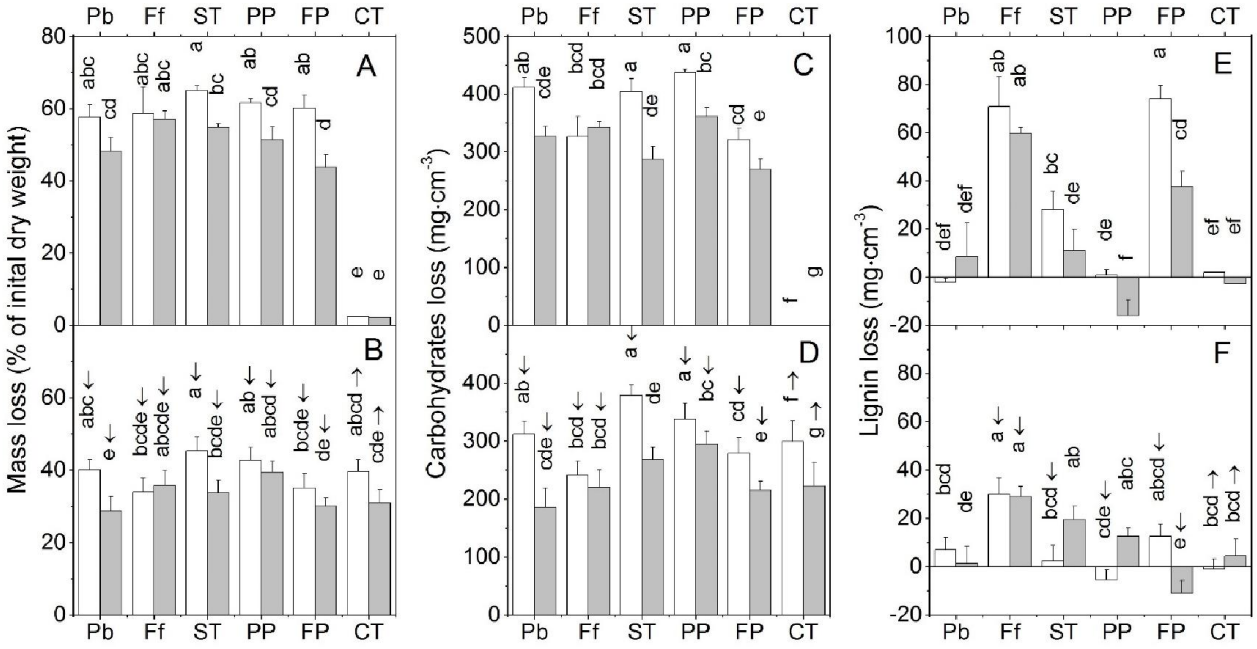


Figure 4.4 Dilute alkaline solubility (DAS) and pH of different treatments after 140 days decay under 25°C (open bars) and 30°C (closed bars), and without (upper panels) and with (bottom panels) endophytes. DAS and pH of treatments without endophytes were shown in **A** and **C**. DAS and pH of treatments with the presence of endophytes were shown in **B** and **D**. Within each plot, letters indicated significant difference among treatments in one way ANOVA by Fisher's LSD multi-comparison ($P \leq 0.05$). Arrows in lower panel indicated significant different between the correspondent treatment with/without endophytes. An up arrow means that no endophytes treatment has larger values, while a down arrow indicate that endophytes presence treatment has larger value. Letters on the X-axis indicated the treatment (types of fungi on soil in microcosms): **Pb**, single *P. betulinus*; **Ff**, single *F. fomentarius*; **ST**, both fungi at the same time; **PP**, *P. betulinus* prior for 3 wk, **FP**, *F. fomentarius* prior for 3 wk; **CT**, no fungus on soil.

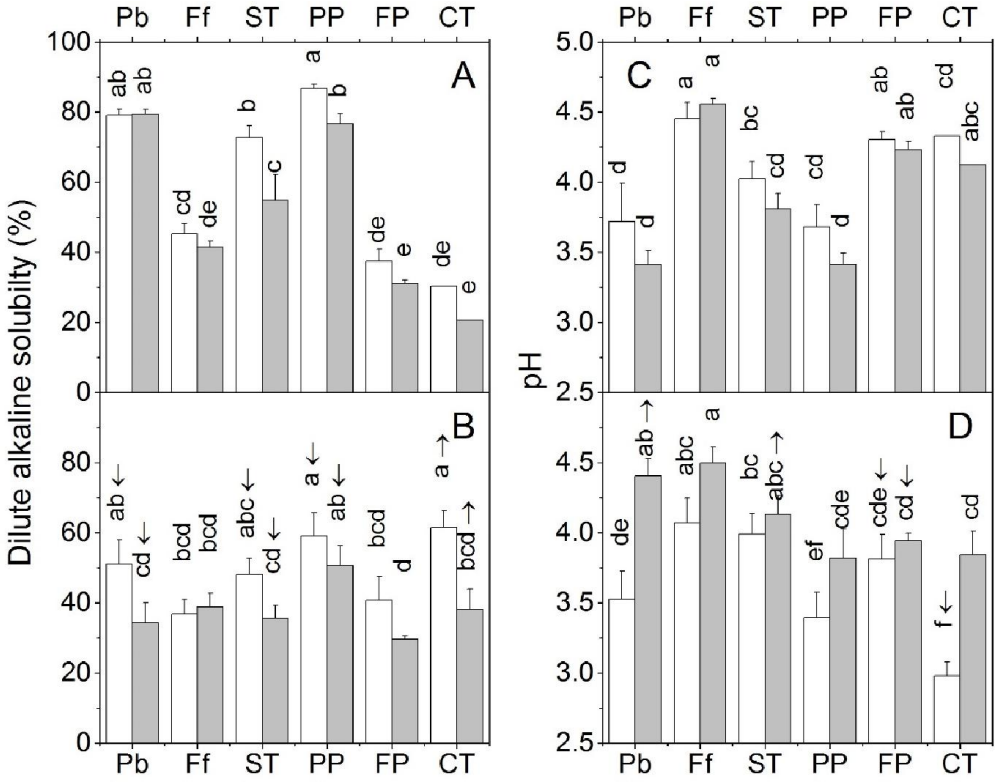


Figure 4.5 General effect of the two experimental factors: temperature and endophytes. The height of bars indicated from 25°C to 30°C (open bars), or from no endophytes to endophytes treatments (closed bars). * indicated significant different between the effect of temperature and endophytes (Two sample T test, $P \leq 0.05$, $n = 10$). Control treatments were not included in these calculations.

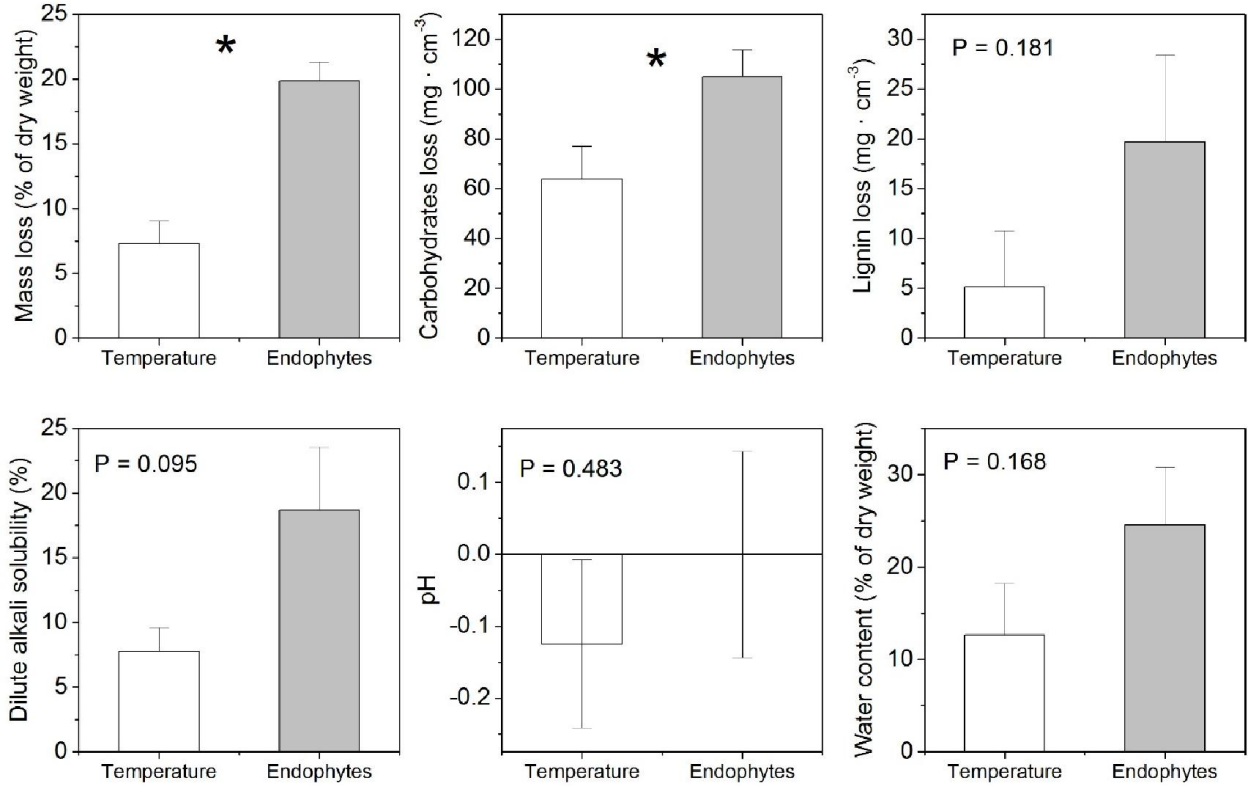


Figure 4.6 Abundance of different microbial groups in the amount of DNA in wood. Open: 25°C autoclaved, Gray: 25°C non-autoclaved, Slashed: 30°C autoclaved, Slashed and Grayed: 30°C non-autoclaved. Lower case letters within each plot indicated significant difference among treatments in one way ANOVA by Fisher's LSD multi-comparison ($P \leq 0.05$). Upper case letters in each plot indicated the level that is significant in two way ANOVA (T: temperature, E: endophytes, I: interaction).

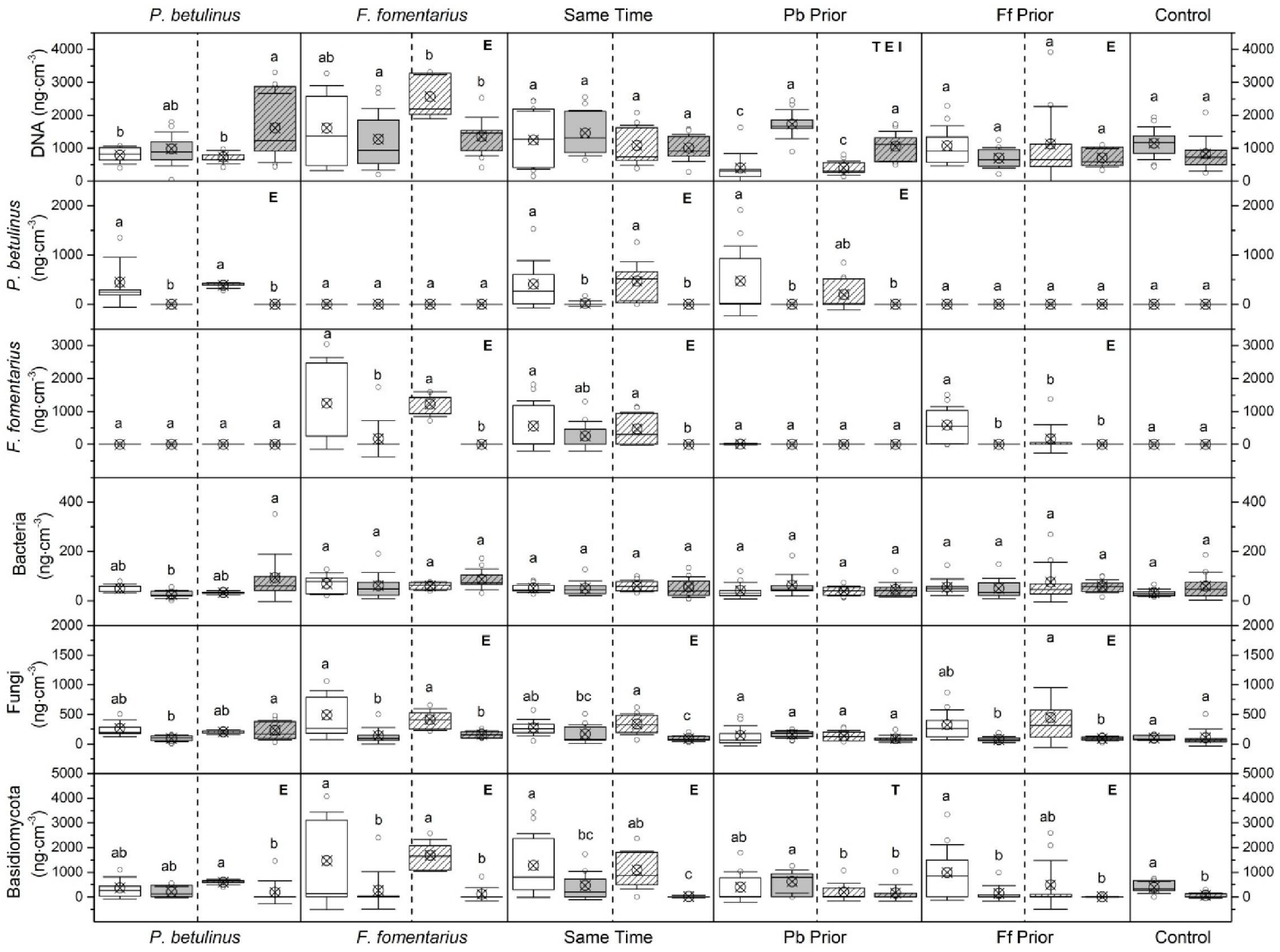

P. betulinus
F. fomentarius
Same Time
Pb Prior
Ff Prior
Control
DNA (ng·cm-3)
P. betulinus (ng·cm-3)
F. fomentarius (ng·cm-3)
Bacteria (ng·cm-3)
Fungi (ng·cm-3)
Basidiomycota (ng·cm-3)

Figure 4.7 Principle component analysis. Wood characterization and microbial abundance were used as variables for each treatment. Letters on the left panel annotated types of soil fungi, P: *P. betulinus* only. F: F.fomentarius only. T: Both fungi. X: *P. betulinus* precolonized for 3 wks. Y: *F. fomentarius* precolonized for 3 wks. C: No soil fungi.

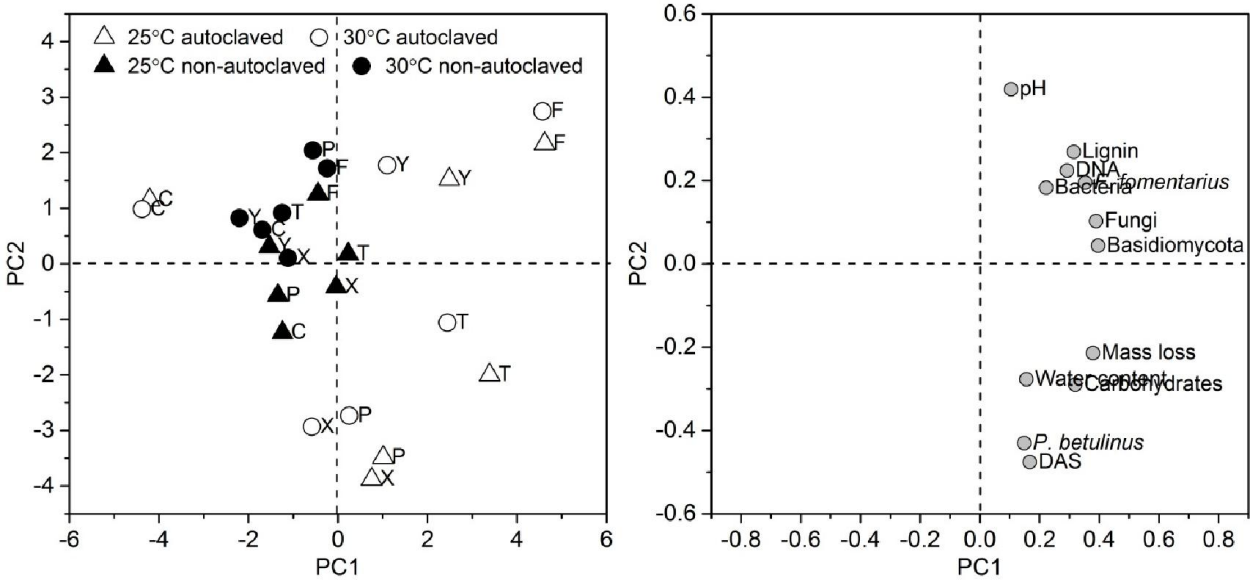


Figure 4.8 Linear regression between wood characterizations and abundance of different microbial groups. Open symbol: no endophytes, closed symbols: endophytes. Triangles: 25°C. Cycles: 30°C. Solid line indicated significant level at $P \leq 0.05$.

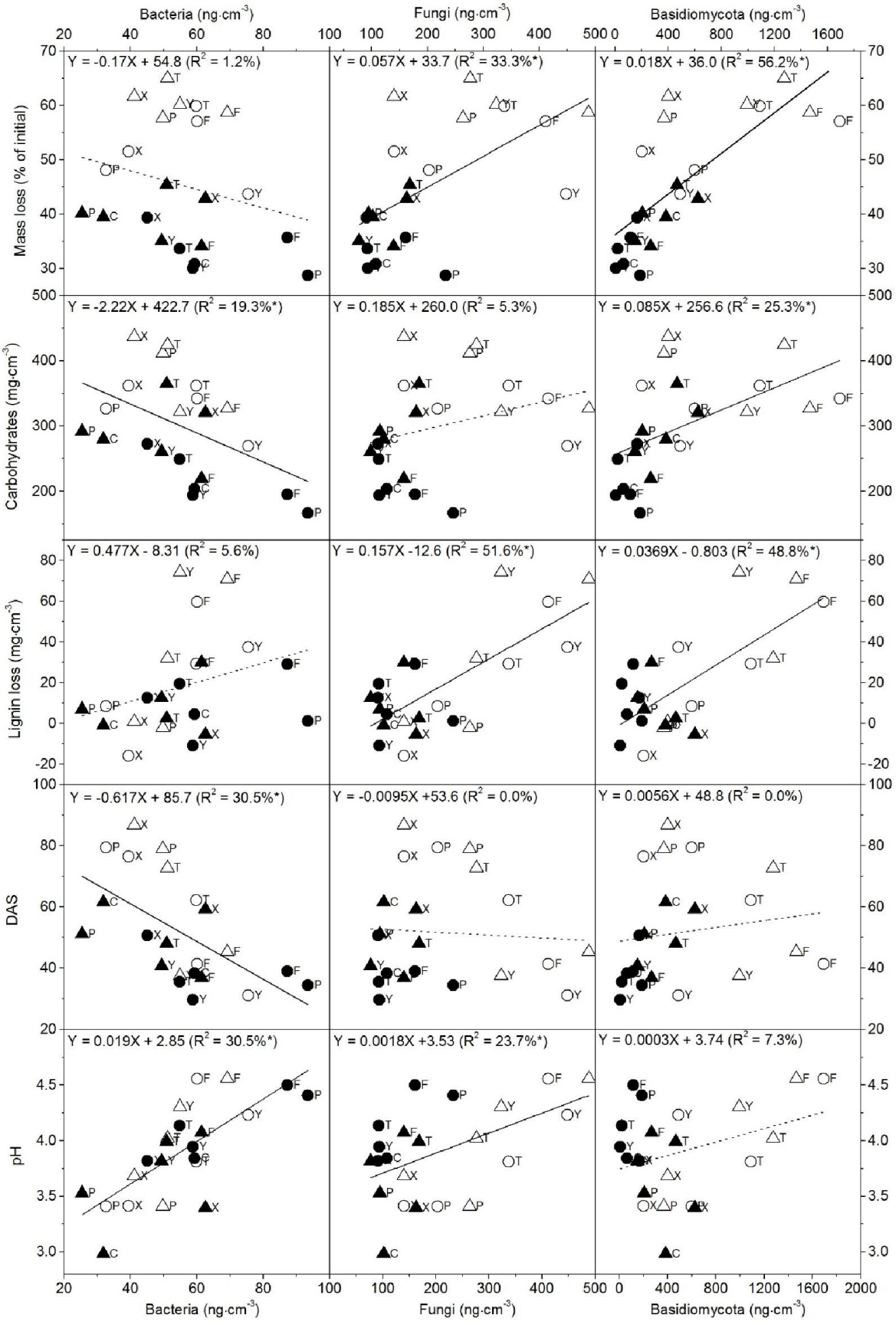
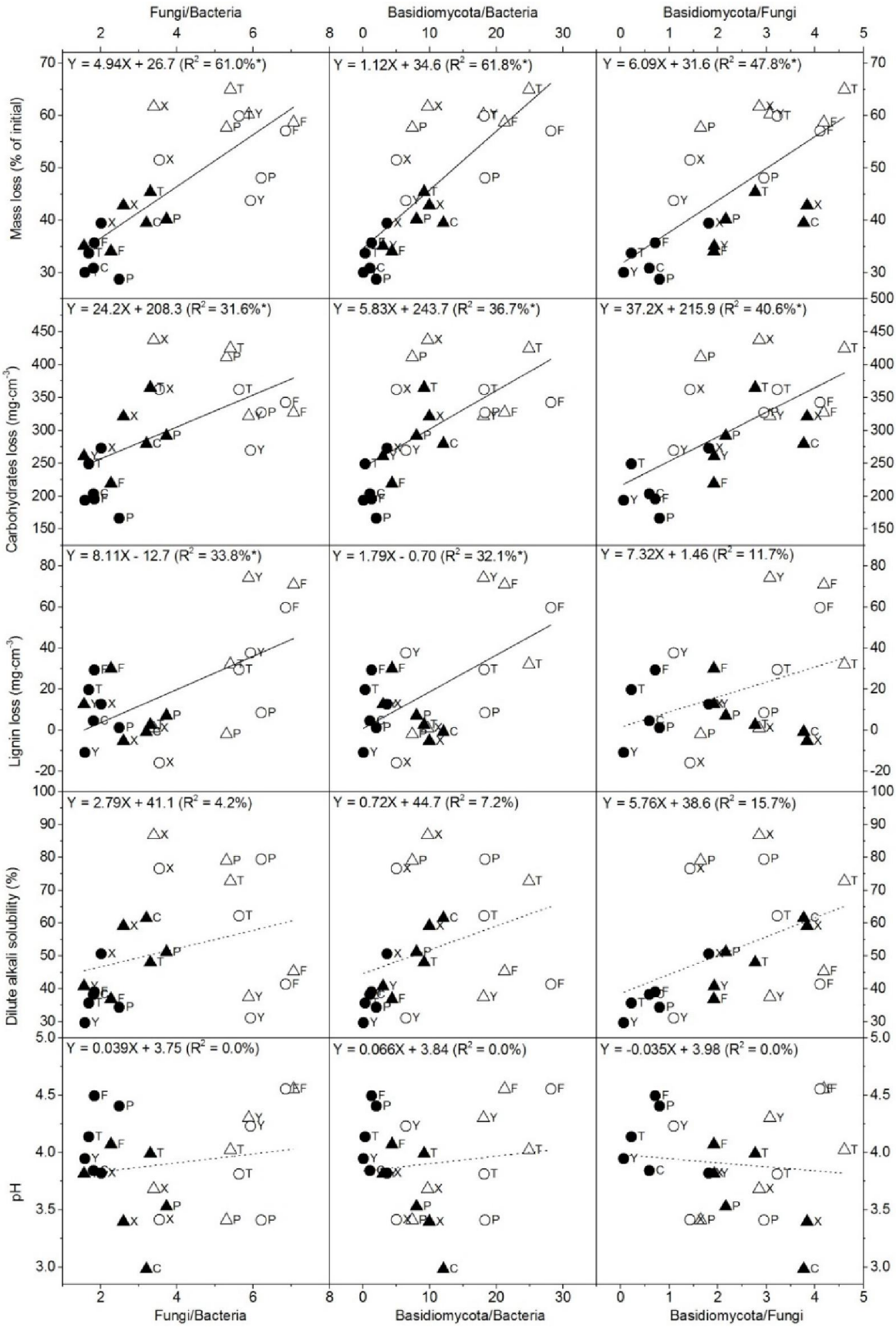

Bacteria (ng·cm-3)
Fungi (ng·cm-3)
Basidiomycota (ng·cm-3)
Mass loss (% of initial)
Carbohydrates (mg·cm-3)
Lignin loss (mg·cm-3)
DAS
pH
Y = -0.17X + 54.8 (R2 = 1.2%)
Y = 0.057X + 33.7 (R2 = 33.3%*)
Y = 0.018X + 36.0 (R2 = 56.2%*)
Y = -2.22X + 422.7 (R2 = 19.3%*)
Y = 0.185X + 260.0 (R2 = 5.3%)
Y = 0.085X + 256.6 (R2 = 25.3%*)
Y = 0.477X - 8.31 (R2 = 5.6%)
Y = 0.157X -12.6 (R2 = 51.6%*)
Y = 0.0369X - 0.803 (R2 = 48.8%*)
Y = -0.617X + 85.7 (R2 = 30.5%*)
Y = -0.0095X +53.6 (R2 = 0.0%)
Y = 0.0056X + 48.8 (R2 = 0.0%)
Y = 0.019X + 2.85 (R2 = 30.5%*)
Y = 0.0018X +3.53 (R2 = 23.7%*)
Y = 0.0003X + 3.74 (R2 = 7.3%)

Figure 4.9 Linear regression between wood characterizations and abundance ratio between microbial groups. Open symbol: no endophytes, closed symbols: endophytes. Triangles: 25°C. Cycles: 30°C. Solid line indicated significant level at $P \leq 0.05$.


Fungi/Bacteria
Basidiomycota/Bacteria
Basidiomycota/Fungi
Y = 4.94X + 26.7 (R² = 61.0%*)
Y = 1.12X + 34.6 (R² = 61.8%*)
Y = 6.09X + 31.6 (R² = 47.8%*)
Mass loss (% of initial)
Y = 24.2X + 208.3 (R² = 31.6%*)
Y = 5.83X + 243.7 (R² = 36.7%*)
Y = 37.2X + 215.9 (R² = 40.6%*)
Carbohydrates loss (mg·cm⁻³)
Y = 8.11X - 12.7 (R² = 33.8%*)
Y = 1.79X - 0.70 (R² = 32.1%*)
Y = 7.32X + 1.46 (R² = 11.7%)
Lignin loss (mg·cm⁻³)
Y = 2.79X + 41.1 (R² = 4.2%)
Y = 0.72X + 44.7 (R² = 7.2%)
Y = 5.76X + 38.6 (R² = 15.7%)
Dilute alkali solubility (%)
Y = 0.039X + 3.75 (R² = 0.0%)
Y = 0.066X + 3.84 (R² = 0.0%)
Y = -0.035X + 3.98 (R² = 0.0%)
pH
Fungi/Bacteria
Basidiomycota/Bacteria
Basidiomycota/Fungi

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