Gry Alfredsen*, Annica Pilgård and Carl Gunnar Fossdal

Characterisation of *Postia placenta* colonisation during 36 weeks in acetylated southern yellow pine sapwood at three acetylation levels including genomic DNA and gene expression quantification of the fungus

DOI 10.1515/hf-2016-0009
Received January 12, 2016; accepted April 4, 2016; previously published online May 9, 2016

Abstract: One way to protect timber in service against basidiomycete deterioration is by means of acetylation via reaction with acetic anhydride. The reason why acetylated wood (W_{Ac}) is resistant against decay fungi is still not exactly understood. The aim of this study was to contribute to this field of science, and *Postia placenta* colonisation after 4, 12, 20, 28 and 36 weeks was observed at three acetylation levels of Pinus spp. sapwood. Mass loss (ML) and wood moisture content (MC) data reflected the acetylation levels. The initial equilibrium MC (EMC) proved to be a good indicator of subsequent ML. Genomic DNA quantification showed *P. placenta* colonisation in all samples, also in samples where no ML were detectable. The number of expressed gene transcripts was limited, but the findings supported the results of previous studies: W_{Ac} seems to have some resistance against oxidative mechanisms, which are part of the metabolism of *P. placenta*. This leads to a delay in decay initiation, a delay in expression of genes involved in enzymatic depolymerisation, and a slower decay rate. The magnitudes of these effects are presented for each acetylation level. The data also imply that there is no absolute decay threshold at high acetylation levels, but instead a significant delay of decay initiation and a slower decay rate.

Keywords: acetylation, carbohydrate metabolism, equilibrium moisture content, genomic DNA (gDNA), gene expression, mass loss, oxidative metabolism, quantitative real-time polymerase chain reaction (PCR), southern yellow pine, weight percent gain

Introduction

Wood is a renewable material with good thermal, acoustic, and mechanical properties. However, wood is also susceptible to decay in outdoor utilisation, when wood may have high moisture content (MC). Hence, to deal with an increasing demand for long-lived products, non-durable wood species have to be protected (Hill 2011), for example by acetylation (Stamm and Tarkow 1947). Acetylation via acetic anhydride (Ac_2O) is an established technique (Rowell 1983; Hill 2006). In the course of the reaction, the accessible OH groups are esterified with one acetyl group while another acetic acid becomes free. Hill (2006) gives a summary of decay studies on acetylated wood (W_{Ac}): a weight percent gain (WPG) in the order of 20% is required to protect wood against brown-rot fungi, while for the protection of softwoods against white rot and against hardwoods, 10% WPG and 20% WPG are needed, respectively. Data from long-term laboratory and field trials are sparse, but Larsson-Brelid and Westin (2010) found that the resistance of W_{Ac} (WPG 20%) to fungal decay, after 18 years in soil contact, was of the same order of magnitude as it was for chromate copper arsenate (CCA)-treated wood at a high retention level (10.3 kg m^3). W_{Ac} shows an anti-shrinking/swelling efficiency of up to 80% (Goldstein et al. 1961; Rowell and Plackett 1988), with minor change of the mechanical strength and modulus of elasticity (MOE) (Goldstein et al. 1961; Militz 1991; Akitsu et al. 1993;

***Corresponding author: Gry Alfredsen,** Division of Forest and Forest Resources, Department of Wood Technology, Norwegian Institute of Bioeconomy Research, Box 115, NO-1431 Ås, Norway, e-mail: gry.alfredsen@nibio.no
Annica Pilgård: SP Technical Research Institute of Sweden, Brinellgatan 4, SE-501 15 Borås, Sweden; and Technical University Munich, Holzforschung München, Winzererstraße 45, DE-80797 München, Germany
Carl Gunnar Fossdal: Division of Biotechnology and Plant Health, Department of Forest Health, Norwegian Institute of Bioeconomy Research, Box 115, NO-1431 Ås, Norway

Larsson Brelid 1998). The aquatic toxicological effects of W_{Ac} are low (Van Eetvelde et al. 1998). Among the theories explaining the positive effects of acetylation (Hill 2009; Ringman et al. 2014a), two are prominent: (1) the biochemical effect meaning that fungi do not recognise the substrate and (2) the physical explanation meaning that the micropores in the cell wall are blocked and the MC is reduced.

The first hypothesis says essentially that various specific enzymes are no longer capable of recognising the substrate when OH groups are converted to OAc groups (Stamm and Baechler 1960; Takahashi et al. 1989; Takahashi 1996). However, such enzymes are too large for penetrating the cell wall of sound wood (Hill 2009). Therefore, such mechanism would be mainly important for erosion attack from the lumen inwards (Hill 2006). But it is also possible that the action of low molecular wood-degrading substances is chemically or sterically prevented or delayed from reaching the reactive OH groups (Hill 2006).

Concerning the physical effects, Hill et al. (2005) investigated cell wall micropore blocking on Corsican pine (*Pinus nigra*) sapwood modified with Ac_2O or hexanoic anhydride at different WPG levels. There was a decrease in the cell wall microporosity as the level of hydroxyl substitution increased, but a high level of accessibility remained even at a WPG of 20%. Hence, the hypothesis of a micropore blocking mechanism alone cannot explain the positive effects of acetylation.

The lowered equilibrium MC (EMC) in W_{Ac} seems to be very important. Hill et al. (2005) found that the reduction in fibre saturation point (FSP) may be attributable to the bulking of the cell wall by the OAc groups, but the level of substitution is not the primary mechanism of decay protection. Hill et al. (2009) and Hill and Kwon (2009) expanded their work to include sapwoods and heartwoods of Japanese larch (*Larix kaempferi*), Korean pine (*Pinus koraiensis*), European beech (*Fagus sylvatica*) and oriental white oak (*Quercus aliena*). They found that decay resistance is not a direct function of the modification degree with Ac_2O and hexanoic anhydride. The plots, mass loss (ML) vs. WPG, were species-dependent, and the authors concluded that there are differences in distribution of the OAc groups at the cell wall level and that there are several mechanisms operative.

Hill (2006) pointed out that modified wood might cause a time lag effect but an absolute protection is not given, i.e. if the exposure time is long enough, the decay mechanism might be effective at some point. Importantly, the decay onset is dependent on more factors than simply the WPG. Alfredsen et al. (2013) found that the WPG could explain approximately 50% of the performance for Ac_2O-treated wood. Wood species or the type of fungus could reduce the variance in performance by additional 15%.

Baldrian and Valaskova (2008) provided a review of the degradation of cellulose by basidiomycetous fungi, and the brown-rot decay mechanisms were reviewed by Arantes et al. (2012) and Arantes and Goodell (2014). The recognition of the non-enzymatic chelator-mediated Fenton system – that rapidly depolymerises polysaccharides and lignin in initial decay stages (Goodell et al. 1997; Arantes et al. 2011; Eastwood et al. 2011) – was an important step toward understanding the decay mechanism. Martinez et al. (2009) studied the *Postia placenta* genome, transcriptome, and secretome and detected its extracellular enzyme systems including an unusual repertoire of extracellular glycosidase hydrolases. Molecular studies can provide some answers concerning the interaction between fungi and modified wood. Kang et al. (2009) studied gene expression of selected decay enzymes of *Phlebia radiata* produced during biodeterioration of pine, cedar, and pine protected by alkaline copper quaternary (ACQ) salt. The authors concluded that the ACQ treatment, one way or another, is able to inhibit the effectiveness of the enzymes and found that woods with different decay resistances show different effects concerning microbial colonisation and enzyme activity. Tang et al. (2013) tried to understand the effects of copper tolerance and wood decay by analysing the gene expression of *Fibroporia radiculosa* in southern yellow pine (SYP) treated with micronised copper quaternary (MCQ) and "identified several genes that appear to be coregulated with putative functions related to copper tolerance and/or wood decay". Ringman et al. (2014b) studied *P. placenta* decay of Scots pine sapwood (*Pinus sylvestris*), which was acetylated, protected by 1,3-dimethylol-4,5-dihydroxy ethylene urea (DMDHDEU), and thermally modified (one treatment level for each modification) after 2, 6, 10, 14, and 56 incubation days. No ML was found in the modified samples. The authors concluded that the response of *P. placenta* during initiation of decay of modified wood seems to up-regulate the expression of the oxidative degradation machinery. So far no studies have been performed on gene expression during long-term decay tests of modified wood.

The aim of the present study is to fill the gap via investigation of the question, how different treatment levels of acetylation of SYP influence *P. placenta* colonisation and decay at five exposure times up to 36 weeks. The analytical methods will include (1) ML determination, MC and initial EMC (EMC_{in}) determination, (2) *P. placenta* genomic DNA (gDNA) quantification, and (3) gene expression targeting of six different *P. placenta* genes.

Materials and methods

Wood material: Three SYP sapwood boards were analysed, and the boards were cut into four sections. One section of each board remained untreated while the other sections were treated with three intensities (described by percentages of OAc groups): low level (L) 11.7%±0.3% OAc content, 1.0%±0.3% free acid; medium level (M) 17.0%±0.1% OAc content, 0.97%±0.4% free acid; high level (H) 22.0%±0.3% acetyl content, 0.96%±0.3% free acid. The wood materials were provided by Eastman Chemical Company, Kingsport, TN, USA. Mini-block samples, 5×10×30 mm^3 (Bravery 1979), were prepared from the boards, avoiding the outer 2 mm. All samples were exposed to a leaching procedure according to EN 84 (CEN 1997a). Thereafter the samples were conditioned at 65% relative humidity (RH) and 20°C for until stable weight was achieved, and the weight was measured in order to calculate EMC_{in}. Thereafter, the samples were wrapped in sealed plastic bags before sterilisation by gamma irradiation of 25 kGY at the Norwegian Institute for Energy Technology.

Decay test was performed with *P. placenta* (Fr.) M.J. Larsen and Lombard strain FPRL 280, which is recommended in the European test method EN 113 for determining the protective effectiveness against wood destroying basidiomycetes (CEN 1997b). The fungus was first grown on 4% (w/v) malt agar, and plugs from actively growing mycelia were transferred to a liquid culture containing 4% (w/v) malt. After 2 weeks, it was homogenised with a tissue homogeniser (Ultra-turrax T25, IKA Werke GmbH & Co. KG, Staufen, Germany) and 2.5 ml inoculum of liquid culture was added to each sample.

A modified E10-12 soil-block test (AWPA 2012) was used. The soil (2/3 ecological compost soil and 1/3 sandy soil) was adjusted to 95% of its water holding capacity according to ENV 807 (CEN 2001). French square bottles served as test jars and the soil was sterilised at 121°C for 2×60 min. A plastic mesh was separating the test samples from direct contact with the soil. To ensure similar conditions for fungal colonisation, the samples were placed in pairs in each jar, i.e. two samples of the same treatment (Junga and Militz 2005). The weight of the jars (including soil and wood specimens) was measured when the test started and thereafter every second week. Sterile water was added when needed in order to ensure stable MC of the soil. The samples were harvested after 4, 12, 20, 28 and 36 weeks of incubation at 22°C and 70% RH. Fifteen samples of each treatment were incubated without fungi to get reference material indicating the changes without fungus.

A total of 36 samples (12 samples from each board, three boards) were provided for each treatment and harvesting point. At each harvesting point, the mycelia covering the samples were manually removed. Five samples from each board (from separate jars), 15 samples in total for each treatment and harvesting point, were dried to obtain ML and MC. One sample from each board, three for each harvesting point and treatment, was kept frozen for further studies. The remaining 18 samples were frozen at -80°C. Wood powder from the frozen samples was obtained by drilling (3 mm bit diameter, Einhell SB 401/1, Landau/Isar, Germany) followed by grinding in a Retsch MM300 agitation mill (Retsch GmbH, Haan, Germany). During both processes, liquid nitrogen served to keep the samples frozen. Only the inner parts of the sample were analysed, avoiding the surface.

Genomic DNA levels of *P. placenta* indicative of fungal biomass were quantified by quantitative polymerase chain reaction (qPCR) (Hietala et al. 2014). Three biological replicates from the same board were pooled, resulting in six replicates (two replicates from each board) from each treatment and harvesting point. gDNA isolation was done from 20 mg of wood powder by means of a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany), which also works well for extracting fungal DNA. The manufacturer's instruction was followed. Extractability of gDNA from environmental samples, such as wood, can vary from sample to sample, and to normalise for this variation, 0.5 ng of an external reference DNA composed of pGEM plasmid (pGEM-3Z Vector, Promega, Madison, WI, USA) was added to each sample upon DNA isolation (Coyne et al. 2005). The gDNA was eluted in 50 µl of buffer. Duplicate runs were done for 10- and 100-fold dilutions of all the samples. Samples for a standard curve were included in each plate. *P. placenta gDNA* was quantified using qPCR with the 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with a 25 µl PCR reaction for the experimental samples and standard curve using TaqMan®SYBR®green mix (Applied Biosystems, Foster City, CA, USA) and 300 mM concentration for the forward primer ACGCCCTGCTCTTCCATTC and reverse primer AAACAGCATCCCCCGTTAGA targeting the *P. placenta* internal transcribed spacer (ITS) gene (GenBank accession EF524035). As a template, 3 µl of the DNA solution was used for each reaction, including standard curve and experimental samples. Standard PCR cycling parameters, according to 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA), were applied. The yield of fungal gDNA was adjusted in relation to the recovery rate of the pGEM reference DNA included to adjust for extractability of gDNA from the samples (Hietala et al. 2014). The internal standard pGEM was quantified in a 25 µl PCR reaction with a 300 nM concentration of the forward primer CCCAGTCACGACGTTGTAAAACG, reverse primer TGTGTGGAATTGTGAGC GGA and the FAM-labelled TaqMan® probe: (Applied Biosystems, Foster City, CA, USA) CACTATAGAATACTCAAGCTTGCATGCCTGCA described by Coyne et al. (2005). Real-time PCR (7500 Real-Time PCR System, Applied Biosystems, Foster City, CA, USA) was performed on DNA samples.

Gene expression: The qRT-PCR primers for the determination of the transcript levels were designed with Primer3 software (Rozen and Skaletsky 2000) based on the following criteria: melting temperature at 60°C and product size inferior to 120 bp. The target specificity of each primer set was examined by melting point analysis. Only primer pairs that gave a single melting peak and PCR product is presented in this study.

The total RNA was isolated from pooled biological samples from each of the tree boards (n=3). RNA was extracted from 100 mg of wood powder with Spectrum™ Plant Total RNA Kit 50 Prep (Sigma-Aldrich, St. Louis, MO, USA). DNA was removed from the samples by DNA-Free kit (Ambion®, Life Technologies, Foster City, CA, USA). RNA concentration was measured with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). RNA was converted into cDNA by TaqMan® reverse transcription system with Oligo d(T)16 (Applied Biosystems®, Life Technologies, Foster City, CA, USA) and amplified with PCR (GeneAmp PCR System 9700, Applied Biosystems, Foster City, Foster City, CA, USA). The list of genes analysed and their primer sequences are shown in Table 1. Real-time PCR (7500 Real Time PCR System, Applied Biosystems, Foster City, CA, USA) was performed on cDNA samples with 12.5 µl of 1× TaqMan®SYBR®green mix (Applied Biosystems, Foster City, CA, USA) and 250 nM of each primer. Standard curve was included in each plate. The following quantitative reverse transcription (qRT)-PCR cycling parameters were applied in the absolute quantification mode: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of melting at 95°C for 15 s and annealing and replication at 60°C for 1 min, ending with a terminal melting-curve determining step, where the

Table 1: Target genes and endogenous controls, protein ID, primer abbreviation and forward/reverse primers are listed.

Gene	Protein ID	Primer abbreviation	Forward/reverse primer (5′-3′)
Endogenous controls			
β-Tubulin	113871	βT-F/βT-R	CAGGATCTTGTCGCCGAGTAC/CCTCATACTCGCCCTCCTCTT
Target genes			
Oxidative depolymerisation			
Alcohol oxidase	118723	AlOx1-F/AlOx1-R	CATCAAGAGCGCCAATCCAT/GGCGCAAAGTCAGCCTTGT
Laccase 2 Pplcc2	89382	Lac2-F/Lac2-R	CGGTGCTCTTGGCCACTTAG/CCATTGGTTATGGGCAGCTC
NADH-quinone oxidoreductase	124517	QRD-F/QRD-R	CGACGACAAGCCCAACAAG/GATGACGATGGCGATTTTAGG
Enzymatic depolymerisation of holocellulose			
Endo-β-1,4-glucanase (GH5)	117690	GH5-F/GH5-R	GTTCAGGCCGCATTGTCCT/TTCCACCTGGCGTAATTGTG
Putative β-glucosidase (GH3)	112501	βGlu2-F/βGlu2-R	TGCGCACGAATGAGTTGATAG/CGCCTGCACACACACAACA
Cytochrome P450 monooxygenase			
Cytochrome P450	130132	P450 1-F/P450 1-R	GCCGTCCTTGACCACCTTT/TCATCATCCTCGGCCAATG

All *Postia placenta* sequences are from the Joint Genome Institute (JGI) database (http://genome.jgi-psf.org).

temperature was increased linearly from 55°C to 95°C for each PCR amplicon. A no-template control was run for each primer pair in each run to ensure that the primers did not form primer dimers or other PCR artefacts. Duplicate qRT-PCR runs were performed on all samples. All procedures above were performed according to the protocol provided by the manufacturers. All gene expressions (transcript levels) were calculated by means of the formula of Pfaffl (2001) with β-tubulin as reference transcript.

Statistics: All statistical analyses were performed in JMP (Version 10, SAS Institute Inc., Cary, NC, USA). The mean values were compared by a single-step multiple comparison procedure, Tukey's test: (1) between harvesting points of the same treatment for *P. placenta* gDNA quantification, (2) between harvesting points of the same treatment for gene expression, and (3) between treatments with statistically similar ML (control week 4 – C_{w4}, low level week 12 – L_{w12}, medium level week 20 – M_{w20}, and high level week 36 – H_{w36}) for gene expression. In the figures, data points not connected with the same letter are significantly different in the Tukey's test and comparisons between treatments are given in italics. A probability of ≤ 0.05 was the statistical type-I error level. Test of homogeneity of variance was done by the O'Brien test. When the homogeneity of variance assumption was not fulfilled, this is indicated in the figure and the P-value is given. Regression analysis served for studying the effect of EMC_{in} vs. ML.

Results and discussion

Mass loss and wood moisture content

The ML reveals a clear effect of treatment level and time of exposure (Figure 1a). Initial ML was detected after 4 weeks of incubation for the control C (mean ML 2.6%±1.2%), L (1.8%±2.6%), and M (0.01%±0.04%). In H, initial decay was found after 20 weeks (0.61%±1.56%). The rate of the decay was clearly affected by the treatment level. For L, there is a rather fast increase in ML over time, for M the increase is slower, and for H there are no significant differences in the mean ML of the weeks 20, 28, and 36. The latter seems to support the hypothesis of Hill (2006) concerning the time lag effect instead of an absolute decay threshold. ML was not recorded in all of the 15 H specimens at the three last harvesting points, but three show ML in week 20, five in week 28, and five in week 36. The H samples with the highest ML also have slightly higher EMC_{in} than the mean EMC_{in} (Table 2). However, the span in both ML and EMC_{in} data is low, and a regression analysis of EMC_{in} vs. ML for H shows only poor correlation (R^2=0.012, P=0.349, F ratio 0.888).

Individual measurements of the exact WPG of each sample were not done, but as shown in Table 2, EMC_{in} gave a good prediction for ML. The results of the regression analysis of EMC_{in} vs. ML at each harvesting point show that EMC_{in} explained 74% of the variation in the ML data after 12 weeks and reached 95% after 28 weeks. For 20 and 36 weeks, 84% and 85% of the ML were explained by EMC_{in}, respectively. Drop week 36 can be explained, at least partly, by low ML in three of the control (C) samples (i.e. the C samples with high EMC_{in} and low ML 26.3%, 37.8%, and 44.7% makes the trend less obvious). Generally, the MC after harvesting roughly reflects the treatment levels (Figure 1b), i.e. the higher the treatment level, the lower the MC. No clear MC trend is seen as a function of exposure time.

The presented data show that the different treatment levels clearly affect decay resistance and that decay protection can be indirectly predicted well by EMC_{in}. It is important to keep in mind that: (1) a lowered FSP will not affect the amount of free water in the lumens at a given EMC (Verma and Mai 2010), and (2) it is difficult to separate the mechanisms of EMC decrement and physical blocking (Hill 2006). The present data do not provide

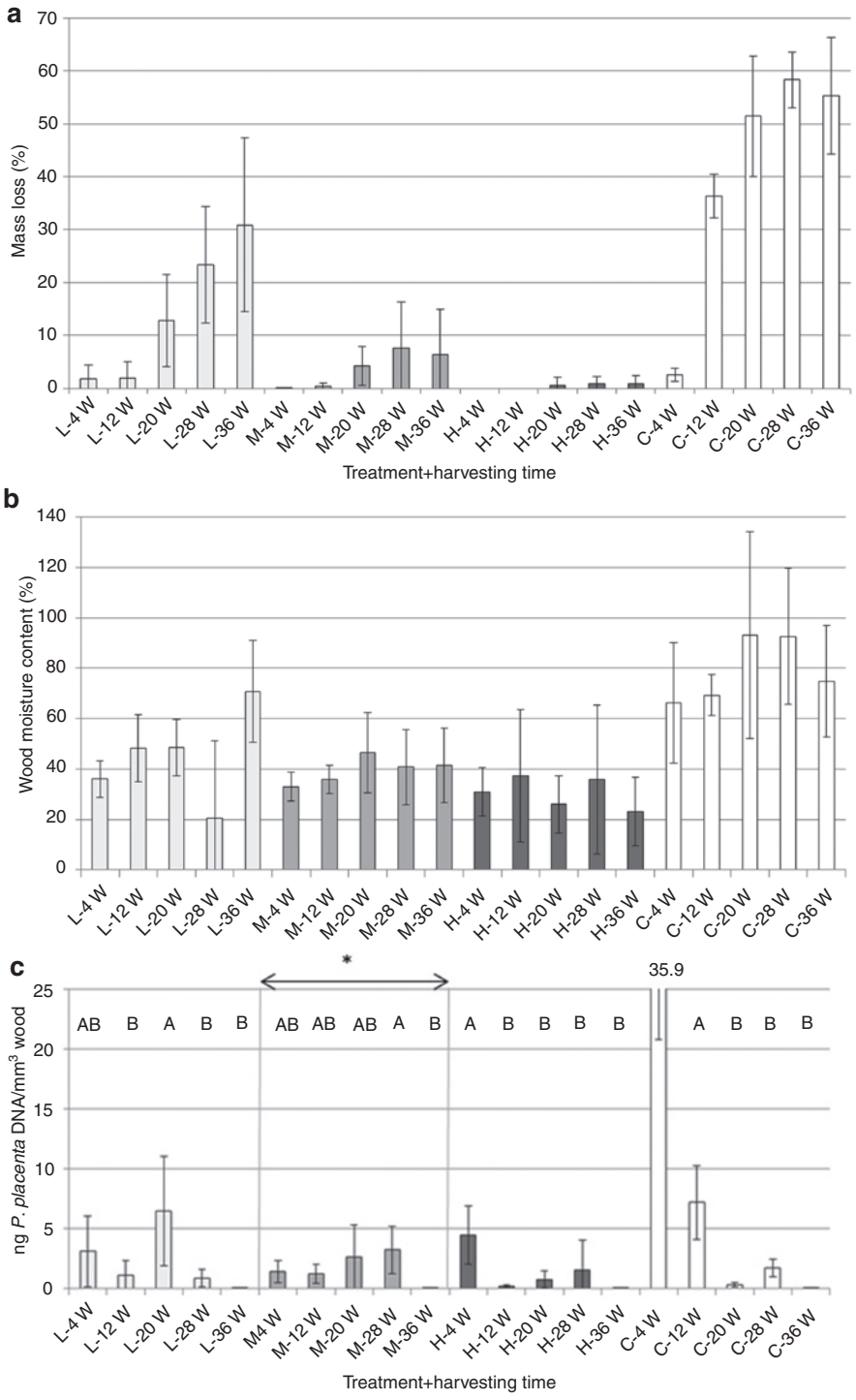

Figure 1: (a) Mean ML and SD, n=15, (b) mean MC content and SD, n=15, (c) amount of *Postia placenta* DNA (ng mm^{-3}), n=3, for the four different treatments at five different harvesting points.
Treatment levels: L=low (11.7%), M=medium (17.0%), H=high (22.0%), C=untreated SYP. Incubation with *P. placenta* after 4, 12, 20, 28, and 36 weeks. *Significant differences between the variances; O'Brian test, P=0.0445.

any information about the MC distribution within the wood (i.e. the utilisation degree of the fungi), or the degree of physical blocking (i.e. the effects of lowering the EMC_{in}).

Quantification of *P. placenta* gDNA

Fungal gDNA quantification (Figure 1c) identified colonisation in all samples, also at harvesting points, where no

Table 2: Regression analysis of EMC_{in} at 65% RH and 21°C vs. ML, n=60.

	R^2	R^2 Adj	P	F ratio
4 Weeks	0.444	0.434	<0.0001	46.28
12 Weeks	0.741	0.737	<0.0001	166.33
20 Weeks	0.842	0.839	<0.0001	309.19
28 Weeks	0.948	0.947	<0.0001	1063.50
36 Weeks	0.849	0.847	<0.0001	326.43

Mean EMC_{in}: C=11.1±0.7, L=6.7±1.4, M=4.2±0.5, H=3.2±0.7

Also given is the mean EMC_{in} for each batch of treatments and their SD, n=75.

ML was detectable (H_{w4} and H_{w12}). The decrease in quantified *P. placenta* gDNA in the untreated samples after 4 weeks reflects advanced stages of decay (>35% ML), i.e. depletion of nutrition source leading to starvation and reduced fungal biomass resulting in the lower level of fungal gDNA.

All harvesting points within each treatment were compared. For L, there was a significantly higher gDNA content at week 20 compared to weeks 12, 28, and 36. However, there was no significant difference between week 4 and week 20. For M, the only significant difference was found between weeks 28 and 36. However, a significant difference in variance was found and the comparison is therefore only a hint. For H, there was a significantly higher level at week 4 compared to the following harvesting points. For the controls, week 4 had a significantly higher level than the four later harvesting points. When leaving the high value of week 4 out of the comparison, week 12 had a significantly higher DNA content than the three following weeks (Figure 1c).

Jasalavich et al. (2000) detected decay fungi in wood by PCR before measurable WL occurred. Pilgård et al. (2010) studied early *Trametes versicolor* colonisation of modified wood and found an initial peak after 2 weeks in the modified wood; thereafter, the gDNA level of the white-rot fungus tended to level out during the next weeks of incubation (4, 6, and 8 weeks). In the current study, there is an initial peak in the most and the least durable samples. There was no such peak for the L and M treatment levels. Mohebby (2003) found that fungi could easily colonise some regions of W_{Ac}, preferably vessel lumina and rays at early stages. Peterson and Thomas (1978) observed by microscopy after 6 weeks of exposure that hyphal counts were substantially lower in W_{Ac} samples compared to control samples (sample size 10×10×5 mm^3). The fungal viability test showed that W_{Ac} was not fungitoxic as, in most instances, each fungus grew out of the wood to an agar surface in culture plates.

The decay test included an inoculum and soil, which might contribute to the fungal nutrition. This means that the fungi can potentially grow in the modified wood without actually feeding the woody mass. However, the soil test has several advantages compared to malt agar tests. Malt agar will be high in accessible sugars and it will only last up to 10–12 weeks before the medium starts drying out. The soil test avoids desiccation and it better reflects outdoor conditions than malt agar. Inoculation of the soil has previously proven to give a larger variance in ML than direct inoculation of the sample with liquid culture (data not shown); hence, the latter was preferred.

Quantification of *P. placenta* gene expression

The results from the expression of the selected *P. placenta* genes are given in Figures 2, 3 and 4. Statistical comparison is performed only between harvesting points within each treatment, not between treatments. In addition, for the tentative comparison of gene expression between different treatments with similar ML levels, the comparison included C_{w4}, L_{w12}, M_{w20} and H_{w36}. This was not the main focus of the paper; otherwise a different test design would have been required.

Oxidative depolymerisation: The genes related to oxidative depolymerisation tended to be up-regulated in W_{Ac} compared to untreated wood (Figure 2). The gene expression of the alcohol oxidase AlOx1 (Pp118723) and the NADH-quinone oxidoreductase QRD (Pp124517) tended to increase with increasing incubation time of the treated samples. The AlOx1 (Pp118723) is similar to *Gloeophyllum trabeum* methanol oxidase (GenBank DQ835989), with >85% of amino acid identity over the full sequence length (Martinez et al. 2009). Immunolocalisation studies by Daniel et al. (2007) identify the *G. trabeum* alcohol oxidase as a potential source of H_2O_2 to support Fenton chemistry and Martinez et al. (2009) suggested a similar role in *P. placenta*. In the brown-rot fungus *G. trabeum*, a QRD may drive extracellular Fenton systems via redox cycling of secreted fungal quinones (Baldrian and Valaskova 2008). For Lac2 (Pp89382), the gene expression was rather stable in the treated samples during 36 weeks of incubation. Martinez et al. (2009) demonstrated laccase-catalysed oxidation of lignin model substrate.

Also without detectable ML, the fungus actively expresses genes related to oxidative depolymerisation, a process that is believed to be the initial stage of brown-rot decay (Arantes et al. 2012; Arantes and Goodell 2014). Ringman et al. (2014b) found by means of the same primers a similar trend for acetylated, DMDHEU-treated,

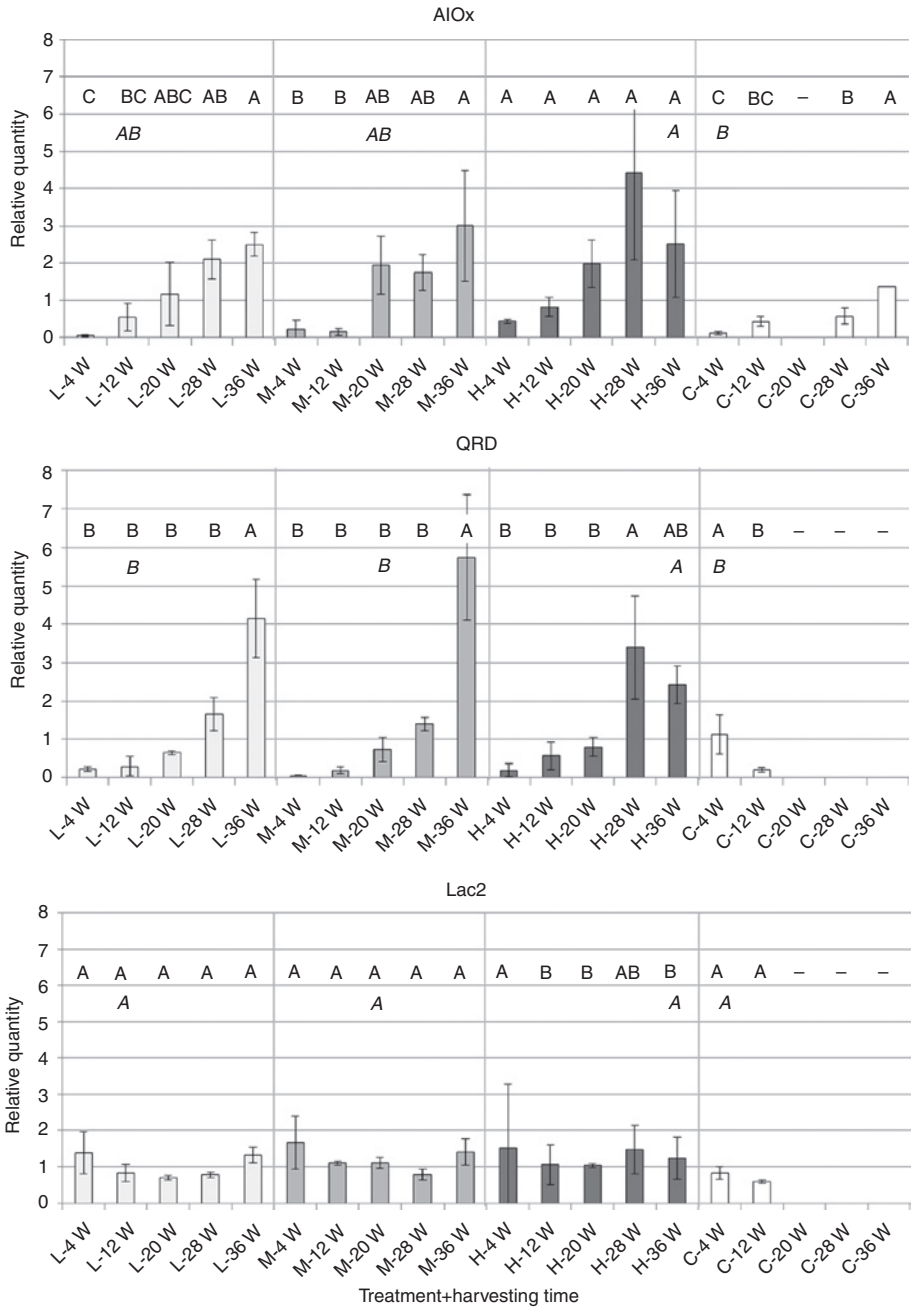

Figure 2: Mean and SD for genes involved in oxidative depolymerisation of polysaccharides.
Treatment levels: L=low (11.7%), M=medium (17.0%), H=high (22.0%), C=untreated SYP. Incubation with *Postia placenta* after 4, 12, 20, 28, and 36 weeks; n=3.

and thermally modified wood during 8 weeks of exposure to *P. placenta*. When similar ML levels were compared (C_{w4}, L_{w12}, M_{w20} and H_{w36}), a significant up-regulation was found in H_{w36} compared to C_{w4} for AlOx. QRD H_{w36} had a significantly higher expression than the other treatments. The elevated levels in H_{w36} could be explained by the onset of decay. No significant difference was seen between treatments with similar ML for Lac2. The data from these genes involved in oxidative depolymerisation indicate a delay in decay initiation rather than a difference in the initial decay mechanism.

Enzymatic depolymerisation: The two genes related to carbohydrate metabolism, the putative β-glucosidase βGlu2 (Pp112501) and the endo-β-1,4-glucanase GH5 (Pp117690), tended to be up-regulated in untreated wood compared to the treated samples (Figure 3). There was a tendency for slight up-regulation in the L and M treatment levels with increasing incubation time. When comparing

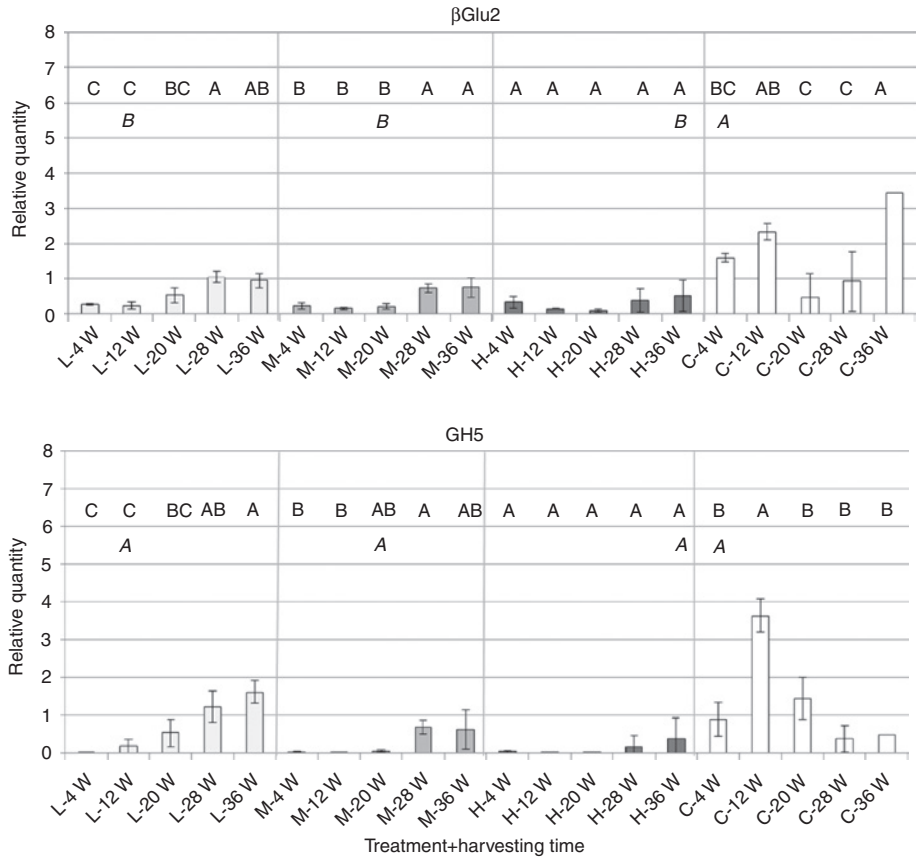

Figure 3: Mean and SD for two genes involved in enzymatic depolymerisation of polysaccharides.
Treatment levels: L=low (11.7%), M=medium (17.0%), H=high (22.0%), C=untreated SYP. Incubation with *Postia placenta* after 4, 12, 20, 28, and 36 weeks; *n*=3.

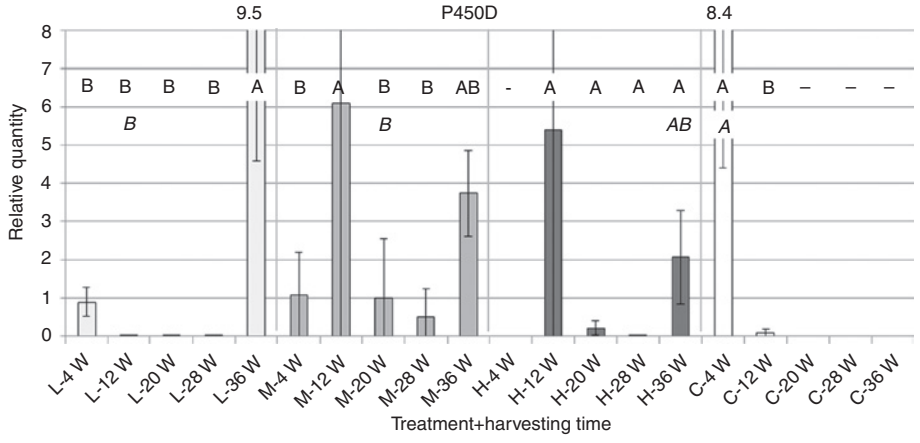

Figure 4: Mean and SD for a cytochrome P450 monooxygenase.
Treatment levels: L=low (11.7%), M=medium (17.0%), H=high (22.0%), C=untreated SYP. Incubation with *Postia placenta* after 4, 12, 20, 28, and 36 weeks; *n*=3. – = No data available.

harvesting points, where the ML was at similar levels for the different treatments, the gene expression in the treated samples was down-regulated compared to control for βGlu2. For GH5, no significant differences were found between treatments.

The *P. placenta* genome contains surprisingly few conventional cellulases (no glycoside hydrolase of families GH6 and GH7 nor any cellobiose dehydrolase); the recognisable cellulolytic enzymes in *P. placenta* seems to be limited to two endoglucanases (1,4-β-glucanases)

and several β-glucosidases (Martinez et al. 2009). Tang et al. (2013) found that when MCQ lost its effectiveness, a number of genes were up-regulated, among them genes related to degradation of polysaccharides and pectin. This finding can be related to the tendency found in the current study: as long as the wood is protected, one way or another, the genes related to the enzymatic depolymerisation of polysaccharides are down-regulated. It is hypothesised that the delay of the enzymatic depolymerisation is linked to the delay of the oxidative mechanisms.

The same primers were used for AlOx (Pp118723), QRD (Pp124517) and βGlu2 (Pp112501) in the present study and in that of Ringman et al. (2014b). The gene expression levels of *P. placenta* in W_{Ac} are higher for AlOx, QRD, and βGlu2 in the current study than in Ringman et al. (2014a). The differences are believed to mainly reflect the differences in exposure time to *P. placenta*. This is supported by the findings in this study that the expression of the respective genes increases with increasing incubation time. Tang et al. (2013) compared MCQ preservative treated wood with untreated wood and found much greater change in GH5 expression levels than observed in the current study. Gene expression was calculated differently. Tang et al. (2013) calculated expression of the three last harvesting points relative to the first harvesting point, and expression values were normalised against 18S rRNA. However, it is more likely that differences in test design can explain the differences, including different wood protection systems, different wood species, different test fungi and different incubation times. The magnitude of difference in expression levels indicates that copper protection may have a more direct effect on the fungus and thereby induce greater transcriptional effects than W_{Ac}.

From the cytochrome P450 monooxygenase P450D (Ppl128850), no clear tendencies could be seen as variations are very high in the gene expression in the treated samples (Figure 4). Compared to similar ML levels, the C has a significantly higher expression level than L and M. In Martinez et al. (2009), the gene encoding for Ppl128850 was significantly up-regulated in a cellulose-containing medium. The *P. placenta*, genome features an impressive set of P450 genes, and the P450s have various roles in secondary metabolism and are thought to be involved in biodegradation of breakdown products from lignin and various xenobiotic compounds (Martinez et al. 2009).

Future research demand

The limited number of genes observed here already gives some indications concerning the protective mechanisms of W_{Ac} against brown-rot decay fungi. Special attention should be paid in the future to factors influencing the chelator-mediated Fenton system. Lately, a new group of enzymes has been detected, the lytic polysaccharide monooxygenases (LPMOs), causing oxidative depolymerisation of crystalline polysaccharides (Vaaje-Kolstad et al. 2010). These enzymes should be taken into consideration in future studies focusing on decay mechanisms of brown-rot fungi. Obviously, the entire gene expression needs to be analysed, rather than only selected genes as in this study, and one should also study the proteins produced by the different genes. A critical discussion of the physics of wood-water interactions was given by Engelund et al. (2013). However, only a few studies focused on the moisture distribution within the cell wall of modified wood (e.g. Hill et al. 2005; Hill 2008; Engelund et al. 2010; Thygesen et al. 2010). Moisture dynamics in the cell wall during decay should be considered in the context of anatomical and chemical changes. The highest degree of treatment, which provides the longest protection should be ascertained. The decay difference between fungi species should also be explored in more detail.

Conclusions

Expectedly, the treatment level is of crucial importance for durability and that the lowered MC plays an important role in the protection mechanism. EMC_{in} could explain up to 95% of the ML. gDNA, ML and RNA quantification revealed fungal colonisation and gene expression before ML was detectable for the highest treatment level (22.0% of OAc content) at weeks 4 and 12. The initial decay detected from week 20 for the highest treatment level indicates that there is a time lag effect rather than an absolute protection threshold for W_{Ac}. Genes involved in oxidative metabolism tend to be up-regulated in treated samples compared to untreated samples, while the opposite seems to be the trend for genes involved in enzymatic metabolism. The initial oxidative metabolism is delayed in W_{Ac}. The magnitude of the delayed initiation of decay, the delayed expression of enzymes related to carbohydrate metabolism, and the reduced decay rate depend on the treatment level.

Acknowledgments: We would like to thank Sigrun Kolstad (NIBIO) for her help with the decay test and with the molecular analysis. We are very grateful for the valuable and open discussions with Jimmy Dickerson and his group at Eastman Chemical Company. The authors gratefully acknowledge financial support from the EcoBuild

Institute Centre of Excellence (337008-2), Norwegian Forest and Landscape Institute (335008) and The Research Council of Norway (243663/E50).

References

Alfredsen, G., Flæte, P.O., Militz, H. (2013) Decay resistance of acetic anhydride modified wood – a review. Int. Wood Prod. J. 4:137–143.

Akitsu, H., Norimoto, M., Morooka, T., Rowell, R.M. (1993) Effect of humidity on vibrational properties of chemically modified wood. Wood Fiber Sci. 25:250–260.

Arantes, V., Milagres, A.M., Filly, T.R., Goodell, B. (2011) Lignocellulosic polysaccharides and lignin degradation by wood decay fungi: the relevance of nonenzymatic Fenton-based reactions. J. Ind. Microbiol. Biot. 38:541–555.

Arantes, V., Jellison, J., Goodell, B. (2012) Peculiarities of brown-rot fungi and biochemical Fenton reaction with regard to their potential as a model for bioprocessing biomass. Appl. Microbiol. Biotechnol. 94:323–338.

Arantes, V., Goodell, B. (2014) Current understanding of brown-rot fungal biodegradation mechanisms: a review. In: Deterioration and Protection of Sustainable Biomaterials. Eds. Nicholas, D.D., Goodell, B., Schultz, T. ACS Series. Oxford University Press. pp. 3–21.

AWPA. E10-12. Standard Method of Testing Wood Preservatives by Laboratory Soil-Block Cultures. American Wood Protection Association, Birmingham, AL, 2012, p. 12.

Baldrian, P., Valaskova, V. (2008) Degradation of cellulose by basidiomycetous fungi. FEMS Microbiol. Rev. 32:501–521.

Bravery, A.F. (1979) A miniaturised wood-block test for the rapid evaluation of preservative fungicides. Proceedings of a special seminar held in association with the 10th annual meeting of the IRG, Peebles, Rep. No. 136. Swedish Wood Preservation Institute, Stockholm.

CEN. (1997a) EN 84. Wood preservatives. Accelerated Ageing of Treated Wood Prior to Biological Testing. Leaching Procedure. European Committee for Standardization (CEN), Brussels, Belgium.

CEN. (1997b) EN 113. Wood preservatives. Test method for determining the protective effectiveness against wood destroying basidiomycetes. Determination of the toxic values.

Coyne, K.J., Handy, S.M., Demir, E., Whereat, E.B., Hutchins, D.A., Portune, K.J., Doblin, M.A., Cary, S.C. (2005) Improved quantitative real-time PCR assays for enumeration of harmful algal species in field samples using an exogenous DNA reference standard. Limnol. Oceanogr-meth. 3:381–391.

Daniel, G., Volc, J., Filonova, L., Plíhal, O., Kubátová, E., Halada, P. (2007) Characteristics of *Gloeophyllum trabeum* alcohol oxidase, and extracellular source of H_2O_2 in brown rot decay of wood. Appl. Environ. Microb. 73:6241–6253.

Eastwood, D.C., Floudas, D., Binder, M., Majcherczyk, A., Schneider, P., Aerts, A., Asiegbu, F.O., Baker, S.E., Barry, K., Bendiksby, M., Blumentritt, M., Coutinho, P.M., Cullen, D., de Vries, R.P., Gathman, L., Goodell, B., Henrissat, B., Ihrmark, K., Kauserud, H., Kohler, A., LaButti, K., Lapidus, A., Lavin, J.L., Lee, Y.-H., Lindquist, E., Lilly, W., Lucas, S., Morin, E., Murat, C., Oguiza, J.A., Park, J., Pisabarro, A.G., Riley, R., Rosling, A., Salamov, A., Schmidt, O., Schmutz, J., Skrede, I., Stenlid, J., Wiebenga, A., Xie, X., Kües, U., Hibbett, D.S., Hoffmeister, D., Högberg, N., Martin, F., Grigoriev, I.V., Watkinson, S.C. (2011) The plant cell wall–decomposing machinery underlies the functional diversity of forest fungi. Science 333:762–765.

Engelund, E.T., Thygesen, L.G., Hoffmeyer, P. (2010) Water sorption in wood and modified wood at high values of relative humidity – part 2. Theoretical assessment of the amount of capillary water in wood microvoids. Holzforschung 64:325–330.

Engelund, E.T., Thygesen, L.G., Svensson, S., Hill, C.A.S. (2013) A critical discussion of the physics of wood–water interactions. Wood Sci. Technol. 47:141–161.

ENV 807. (2001) Wood preservatives. Determination of the Effectiveness against Soft Rotting Micro-fungi and other Soil Inhabiting Micro-organisms. European Committee for Standardization (CEN), Brussels, Belgium.

Goldstein, I.S., Jeroski, E.B., Lund, A.E., Nielson, J.F., Weaver, J.W. (1961) Acetylation of wood in lumber thickness. Forest Prod. J. 11:363–370.

Goodell, B., Jellison, J., Liu, J., Daniel, G., Paszczynski, A., Fekete, F., Krishnamurthy, S., Jun, L., Xu, G. (1997) Low molecular weight chelators and phenolic compounds isolated from wood decay fungi and their role in the fungal biodegradation of wood. J. Biotechnol. 53:133–162.

Hietala, A, Stefanczyk, E, Nagy N.E., Fossdal, C.G., Alfredsen, G. (2014) Influence of wood durability on the suppressive effect of increased temperature on wood decay by the brown-rot fungus *Postia placenta.* Holzforschung 68:123–131.

Hill, C.A.S. Wood Modification: Chemical, Thermal and other Processes. John Wiley & Sons, Ltd., New York, 2006.

Hill, C.A.S. (2008) The reduction in the fibre saturation point of wood due to chemical modification using anhydride reagents: a reappraisal. Holzforchung 62:423–428.

Hill, C.A.S. (2009) Why does acetylation protect wood from microbiological attack? Wood Mater. Sci. Eng. 1–2:37–45.

Hill, C.A.S. An Introduction to Sustainable Resource Use. Earthscan Ltd, London, 2011.

Hill, C.A.S., Kwon, J.H. (2009) The influence of wood species upon the decay protection mechanisms exhibited by anhydride modified wood. In: Proceedings 4th European Conference on Wood Modification (ECWM4). Eds. Englund, F., Hill, C.A.S., Militz, H., Segerholm, B.K. Stockholm, Sweden. pp. 95–102.

Hill, C.A.S., Forster, S.C., Farahani, M.R.M., Hale, M.D.C., Ormondroyd, G.A., Williams, G.R. (2005) An investigation of cell wall micropore blocking as a possible mechanism for the decay resistance of anhydride modified wood. Int. Biodeterior. Biodegrad. 55:69–76.

Hill, C.A.S., Curling, S.F., Kwon, J.H., Virginie, M. (2009) Decay resistance of acetylated and hexanoylated hardwood and softwood species exposed to *Coniophora puteana*. Holzforschung 63:619–625.

Jasalavich, C., Otsrofsky, A., Jellison, J. (2000) Detection and identification of decay fungi in spruce wood by restriction fragment length polymorphism analysis of amplified genes encoding rRNA. Appl. Environ. Microbiol. 66:4725–4734.

Junga, U., Militz, H. (2005) Particularities in agar block tests of some modified woods caused by different protection and decay principles. In: Proceedings of the 2nd European Conference on Wood

Modification. Eds. Militz, H., Hill, C.A.S. Göttingen, Germany. pp 354–362.

Kang, Y.-M., Prewitt, M.L., Diehl, S.V. (2009) Proteomics for biodeterioration of wood (*Pinus taeda* L.): Challenging analysis by 2-D PAGE and MALDI-TOF/TOF/MS. Int. Biodeterior. Biodegrad. 63:1036–1044.

Larsson-Brelid, P., Westin, M. (2010). Biological degradation of acetylated wood after 18 years in ground contact and 10 years in marine water. In: Proceedings 41st Annual Meeting of the International Research Group on Wood Protection. Biarritz, France. IRG/WP 10-40522.

Martinez, D., Challacombe, J., Morgenstern, I., Hibbett, D., Schmoll, M., Kubicek, C.P., Ferreira, P., Ruiz-Duenas, F.J., Martinez, A.T., Kersten, P., Hammel, K.E., Wymelenberg, A.V., Gaskell, J., Lindquist, E., Sabat, G., BonDurant, S.S., Larrondo, L.F., Canessa, P., Vicuna, R., Yadav, J., Doddapaneni, H., Subramanian, V., Pisabarro, A.G., Lavín, J.L., Oguiza, J.A., Master, E., Henrissat, B., Coutinho, P.M., Harris, P., Magnuson, J.K., Baker, S.E., Bruno, K., Kenealy, W., Hoegger, P.J., Kues, U., Ramaiya, P., Lucas, S., Salamov, A., Shapiro, H., Tu, H., Chee, C.L., Misra, M., Xie, G., Teter, S., Yaver, D., James, T., Mokrejs, M., Pospisek, M., Grigoriev, I.V., Brettin, T., Rokhsar, D., Berka, R., Cullen, D. (2009) Genome, transcriptome, and secretome analysis of wood decay fungus *Postia placenta* supports unique mechanisms of lignocellulose conversion. Proc Natl Acad Sci 106:1954–1959.

Militz, H. (1991) Die Verbesserung das Schwind- und Quellverhaltens und der Daurhaftigkeit von Holz mittels Behnadlung mit unkatalysiertem Essigsäureanhydrid. Holz Roh. Werkst. 49:147–152.

Mohebby, B. Biological Attack of Acetylated Wood, PhD thesis, Institute of Wood Biology and Wood Technology, Faculty of Forest Sciences and Forest Ecology, Georg-August-Universität Göttingen, Göttingen, 2003.

Peterson, M.D., Thomas, R.J. (1978) Protection of wood from decay fungi by acetylation – an ultrastructural and chemical study. Wood Fiber 10:149–163.

Pfaffl, M.W. (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29:2002–2007.

Pilgård, A., Alfredsen, G., Hietala, A. (2010) Quantification of fungal colonization in modified wood – qPCR as a tool for studies on *Trametes versicolor*. Holzforschung 64:645–651.

Ringman, R., Pilgård, A., Brischke, C., Richter, K. (2014a) Mode of action of brown rot decay resistance in modified wood: a review. Holzforschung 68:239–246.

Ringman, R., Pilgård, A., Richter, K. (2014b) Effect of wood modification on gene expression during incipient *Postia placenta* decay. Int. Biodeterior. Biodegrad. 86:86–91.

Rowell, R.M. (1983) Chemical modification of wood. For. Prod. Abstr. 6:363–382.

Rowell, R.M., Plackett, C.V. (1988) Dimentional stability of flakeboards made from acetylated *Pinus radiata* heartwood and sapwood flakes. New Zeal. J. For. Sci. 18:124–131.

Rozen, S., Skaletsky, H. (2000) Primer3 on the WWW for general users and for biologist programmers. Methods Mol. Biol. 132:365–86.

Stamm, A.J., Tarkow, H. (1947) Dimensional stabilization of wood. J. Phys. Colloid Chem. 51:493–505.

Stamm, A.J., Baechler, R.H. (1960) Decay resistance and dimensional stability of five modified woods. For. Prod. J. 10: 22–26.

Takahashi, M., Imamura, Y., Tanahashi, M. (1989) Effect of acetylation on decay resistance of wood against brown-rot, white-rot and soft-rot fungi. In: Proceedings 20th Annual Meeting of the International Research Group on Wood Preservation. Lappeenranta, Finland. IRG/WP 3540.

Takahashi, M. (1996) Biological properties of chemically modified wood. In: Chemically Modified Wood and Lignocellulosic Materials. Ed. Hon D.N.S. Marcel Dekker, New York. pp. 331–361.

Tang, J.D., Parker, L.A., Perkins, A.D., Sonstegard, T.S., Schroeder, S.G., Nicholas, D.D., Diehl, S.V. (2013) Gene expression analysis of copper tolerance and wood decay in brown rot fungus *Fibroporia radiculosa*. App. Environ. Microbiol. 79:1523–1533.

Thygesen, L.G., Engelund, E.T., Hoffmeyer, P. (2010) Water sorption in wood and modified wood at high values of relative humidity: Part I: Results for untreated, acetylated, and furfurylated Norway spruce. Holzforschung 64:315–323.

Vaaje-Kolstad, G., Westereng, B., Horn, S.J., Liu, Z., Zhai, H., Sørlie, M., Eijsink, V.G.H. (2010) An oxidative enzyme boosting the enzymatic conversion of recalcitrant polysaccharides. Science 330:219–222.

Van Eetvelde, G., De Geyter, S., Marchal, P., Stevens, M. (1998) Aquatic toxicity research of structural materials. In: Proceedings 29th Annual Meeting of the International Research Group on Wood Preservation, Maastricht, The Netherlands. IRG/WP 98-50114.

Verma, P., Mai, C. (2010) Hydrolysis of cellulose and wood powder treated with DMDHEU by a hydrolase enzyme complex, Fenton's reagent, and in a liquid culture of *Trametes versicolor*. Holzforschung 64:69–75.