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2 **initial onset of brown rot decay of Scots pine (*Pinus sylvestris L.*)**

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11

12 **Abstract**

13 Microbiological degradation of wood by decay fungi can cause a rapid change in the
14 structural properties of timber which can result in both strength and mass loss.
15 Traditional techniques for the evaluation of decay (e.g. mass loss) lack the sensitivity to
16 evaluate the effects of the very first stages of the decay process. This paper describes the
17 effects of initial brown rot decay, defined by the amount of *Poria placenta* genomic DNA
18 (gDNA) present in the samples, on the dynamic mechanical properties of the timber. It
19 was found that there is a correlation between the mean storage modulus of the timber
20 and the amount of *P. placenta* gDNA present, and therefore the level of decay. This
21 shows that using dynamic mechanical analysis is a viable technique that can be used to
22 study initial decay processes.

23

24 **Keywords:** decay, *Poria placenta,* viscoelastic behaviour, dynamic mechanical analysis
25 (DMA),

26

1. Introduction

28 Microbiological degradation of wood by fungi can cause a rapid change in the structural
29 properties of timber (Jellison et al., 2013), however traditional techniques for the
30 evaluation of the decay (e.g. mass loss) lack the sensitivity to evaluate the effects of
31 decay in the very first stages of the decay process.

32 The mechanism for the decay of timber via brown rot is through oxidative and
33 enzymatic reactions (Goodell et al., 1997; Arantes et al., 2012; Alfredsen et al., 2015,
34 Zelinka, 2016). The fungus initiates the production of hydroxyl radicals by secreting
35 hydrogen peroxide and reductants in to the cell wall. The reductants reduce the ferric
36 iron (Fe^{3+}) to ferrous iron (Fe^{2+}) (Goodell et al., 1997; 2006), which then reacts with the
37 hydrogen peroxide to form hydroxyl radicals in the Fenton reaction. The hydroxyl
38 radicals depolymerize hemicelluloses and cellulose, modify lignin and generate
39 sufficient rearrangements in the cell wall to allow the hydrolysing enzymes to diffuse in
40 to the wall and degrade the polysaccharides (Goodell et al., 1997; Baldrian and
41 Valaskova, 2008; Arantes et al., 2012). Ray et al. (2010) terms the above mechanism the
42 Early Stage Decay Mechanism (ESDM) and suggest that it can act both rapidly and
43 extensively. This mechanism has also been referred to as chelator-mediated Fenton

(CMF) reaction (Arantes and Goodell 2014). Green et al. (1997) and Cowling (1961) both noted that whilst the reduction in the degree of polymerization is rapid there is low mass loss at this stage of decay. The study of early onset decay has, in recent years, been furthered by the use of molecular techniques. gDNA quantification has shown to be more sensitive than ergosterol and kition assays for estimation of fungal biomass in early stages of decay both in sterile laboratory samples (Eikenes et al. 2005) and for field samples (Pilgård et al. 2011). Hietala et al. (2014) found that the suppressive effect of suboptimal temperature on wood decay caused by *P. placenta* appeared more pronounced in Scots pine heart wood than in sapwood. At 30°C heartwood showed no mass loss, poor substrate colonization (gDNA quantification) and marker gene transcript level profiles indicating a starvation situation.

Alfredsen et al. (2015) reviewed the role of quantitative gDNA and gene expression studies in the understanding of the mode of action of brown rot decay on modified wood, charting the method development and recent studies. Based on gene profiles the studies provide indications of a possible shift toward increased expression, or at least no down regulation, of genes related to oxidative metabolism and no reduction, or concomitant reduction, of genes related to the enzymatic breakdown of polysaccharides in modified wood compared to untreated control. Using gene expression Ringman et al. (2014) showed that *P. placenta* was present in acetylated, DMDHEU-treated and thermally modified wood after 2 days while at 6 days, the gene expression levels were significantly different from zero. Alfredsen et al. (2016) found *P. placenta* gDNA in acetylated samples with high treatment level (22% acetyl content) after four weeks while initial mass loss was not detected until 20 weeks. It is interesting to note that the time to mass loss of the acetylated timber was in agreement with other researchers (Hill et al., 2006). However, fungus was present in the timber much earlier in the test, which suggests a good efficiency of acetylation in the prevention of decay.

Compositional data and structural properties of wood have also been used in investigating the early stages of brown rot decay. Monrroy et al. (2011) assessed the change in the degree of polymerization of the cellulose (of *Pinus radiata*), the crystalline structure of the cellulose and the hemicellulose content of the wood over a decay period of 8 weeks (using 20mm x 25mm x 5mm chips). Different species of brown rot were assessed with *Gloeophyllum trabeum* showing the highest rate of cellulose degree of polymerisation reduction and *Laetiporus sulphureus* showing a comparable loss in cellulose crystallinity. Curling et al. (2002) examined the effect on the hemicellulose content and the mechanical properties of southern yellow pine exposed to brown rot fungi. Their data showed that during initial decay, where there was negligible weight loss (up to 20 days exposure), there were significant changes in hemicellulose content associated with up to a 20% loss in modulus of elasticity, 40% loss in modulus of rupture (4 point bending strength) and 80% loss in work to maximum load. This indicates that physical properties may be a more appropriate method of studying early stage decay than simple mass loss. Their method used static mechanical tests to derive the data, although there is another physical characterisation method, Dynamic Mechanical Analysis (DMA), which has become a well-established technique for dynamically measuring the viscoelastic behaviour of polymers and polymer composites. The earliest examples of DMA studies on wood were in the 1960s using torsional pendulum apparatus (Norimoto and Yamada, 1966; Becker and Noack, 1968). DMA is often used to measure the response of a material to changes at the molecular or microstructural level (often but not always brought on by a change in temperature). In

their paper, Birkinshaw et al. (1986) assess the response of 10 timber species to dynamic flexing whilst being subjected to a temperature gradient raising from 10 °C to 100 °C at 10 °C h^{-1}. The species were selected for their variety in densities and in microstructure. It was noted that whilst the absolute values had a large variation the general shape of the shear storage modulus curve and the loss modulus (tan δ) were similar. Much research has been undertaken to determine the response to changes in both temperature and humidity; the effect of the humidity and moisture content of the wood on the glass transition temperature (T$_g$) and other relaxations of the lignin and hemicelluloses of wood; and the effect on the mechanical properties of the timber (Hillis and Rossa, 1978, 1985; Kelley et al., 1987; Salmén and Olsson, 1998; Olsson and Salmén 2004).

An important factor in the use of DMA is the determination of the Linear Viscoelastic Response region (LVR), and experiments should be conducted within this region. Within this region the strain is directly proportional to the stress and therefore the polymer packing is not altered by the stress applied; however, once the response becomes non-linear the polymer packing is being significantly and irreversibly altered. Sun et al. (2007) used DMA to assess the dynamic response of timber at low moisture content (<1%); they noted that the LVR was very low, between 0.03% and 0.16% strain although values were greater when bending perpendicular to the grain.

McCarthy et al. (1991) studied the fungal degradation of wood via DMA. Small samples were exposed to the brown rot fungus *Coniophora puteana* over a six week period on a weekly basis in accordance with ASTM D2017 and DIN 50 008. The response to dynamic loading was measured between -100 °C and 250 °C at a heating rate of 5 °C min^{-1}. The researchers showed that the shear modulus fell with increased amounts of decay at all temperatures. It was noticed that whilst the absolute values for the modulus changed with the increase in decay, tan δ and the shape of the shear modulus curve did not change significantly. It should be noted that this is in agreement with the earlier work of Birkinshaw et al. (1986), in that there are changes in the absolute values but not the location of the tan δ peak. Whilst we understand that the oxidative reaction depolymerizes the wood holocelluloses prior to the components being utilized by the fungi (which does not lead to a mass loss), the implication of this research is that the ultimate degradation of the polymers responsible for the stiffness of the wood is relatively uniform in rate.

The investigations undertaken to date have focused on the assessment of wood's ability to respond to a dynamic temperature range and the effects of altering the molecular and microstructure of the wood. The objective of this study was however, to determine the potential of DMA, under isothermal conditions, as a method for the assessment and quantification of initial onset brown rot decay.

2. Materials and methods

Scots pine (*Pinus sylvestris L.*) from South Norway was supplied by Norwegian Institute of Bioeconomy Research Norway. The pine was straight grained and had an average growth ring distribution of 10 rings/cm. The Scots pine was then machined to 2 mm x 2 mm strips and samples were selected to achieve 1 single latewood band through the centre of the strip. All samples were conditioned to 65% RH at 20 °C before testing.

2.1. Fungal exposure

138 Fungal cultures of *Poria placenta* ((Fries) Cooke sensu J. Eriksson) (FPRL 280)
139 (originally from the European recognized holding lab as defined in EN 113) were grown
140 on 90 mm Petri dishes containing 4% malt agar (Malt extract powder 40 g; Agar 20 g;
141 Deionised water 1 litre). *P.placenta* (FPRL 280) is a fungal isolate stipulated by a number
142 of European testing standards for example EN113 (CEN 2004). Once the fungal culture
143 had grown to cover the entire surface of the plate the cultures were ready for samples.
144 Wood samples (2 mm x 2 mm x 80 mm) were cleaned by immersing them in ethanol for
145 1 minute. The samples were then dried and vented aseptically using a laminar flow
146 bench. Once dry, the samples were aseptically placed onto the pre-prepared fungal
147 cultures as follows: two samples were placed in direct contact with the agar/fungus to
148 act as supports, with three further samples placed perpendicular and on top of the
149 supports. This was intended to prevent water logging which may have prevented decay.
150 The cultures were then incubated at 22 °C ±2 at 70% RH ±3 with replicate samples
151 (n=6) removed (aseptically) from incubation after 4, 8, 11 and 17 days. Upon removal
152 from test the wood samples were dried for 12 hrs at 50 °C in order to stop fungal growth
153 without degrading any DNA. Dried samples were stored in sterile tubes before
154 subsequent DNA isolation and testing.

155 *2.2. Assessment of the decay*

156 As weight loss is not an accurate measure of the early onset of brown rot decay, gDNA is
157 used as a measure of fungal activity and the storage and loss modulus at different early
158 decay stages are compared against gDNA quantification.

159

160 *2.2.1. Genomic DNA extraction*

161 From each decay duration a 0.4 g sample of wood was subsampled from the total
162 population (n=3). This sample was then cut to small pieces and then manually ground
163 with a pestle and motar under liquid nitrogen. The samples were then dismembrated for
164 2 x 2 minutes at full speed (with an intermediate liquid nitrogen freezing step) to ensure
165 a consistent powder was achieved (Retsch 300 mill, Retsch GmbH, Haan, Germany).
166 Aliquots of 20 mg were prepared from the powdered material and total gDNA was
167 extracted using DNeasy Plant Kit (Qiagen, Hilden, Germany) which works well for
168 extracting fungal DNA. The manufacturer's protocols were followed. Extractability of
169 gDNA from environmental samples, such as wood, can vary from sample to sample.
170 Hence, to normalise for this potential variation, 0.5 ng of reference DNA pGEM plasmid
171 (pGEM-3Z Vector, Promega, Madison, Wisconsin, USA) was added to each sample at the
172 start of the gDNA isolation (Coyne et al. 2005). The extracted gDNA was eluted in 50 µl
173 of buffer AE and stored in a fridge overnight.

174 *2.2.2. qPCR anlaysis*

175 gDNA from *P. placenta* was quantified using quantitative polymerase chain reaction
176 (qPCR) with the ViiA 7 (Applied BioSystems, Forster City, California, USA). A 10 µl PCR
177 reaction using TaqMan®SYBR®green mix (Applied Biosystems, Foster City, CA, USA)
178 and 300 nM concentration for the forward primer ACGCCCTGCTCTTCCATTC and
179 reverse primer AAACAGCATCCCCCGTTAGA targeting the *P. placenta* internal
180 transcribed spacer (ITS) gene (GenBank accession EF524035). A 2 µl template of DNA
181 solution was used both for experimental samples and standard curve. Standard PCR
182 cycling parameters, according to ViiA 7 (Applied BioSystems, Forster City, California,
183 USA), were applied.

184 Duplicate runs were performed for undiluted, 10- and 100-fold dilutions of all samples.
185 Samples for standard curve was included in each plate.

186 The internal standard pGEM was quantified in a 10 µl PCR reaction with a 300 nM
187 concentration of the forward primer CCCAGTCACGACGTTGTAAAACG, reverse primer
188 TGTGTGGAATTGTGAGC GGA and the FAM-labelled TaqMan® probe (Applied
189 Biosystems, Foster City, CA, USA) CACTATAGAATACTCAAGCTTGCATGCCTGCA (Coyne et
190 al. 2005) and qPCR was performed on the samples (ViiA 7, Applied BioSystems, Forster
191 City, California, USA).

192 After amplification the data was analysed and plotted using the ViiA 7 software v1.2.4
193 (Applied BioSystems, Forster City, California, USA). The extent of amplification was
194 calculated as the mean of 2 replicates from each sample. The yield of *P. placenta* gDNA
195 was adjusted in relation to the recovery rate of the pGEM reference DNA (Hietala et al.
196 2014). The same trend was found for undiluted, 10-fold and 100-fold dilutions, but the
197 100-fold gave a slightly better yield and is presented in the results chapter.

198

199 **2.3.** *Dynamic Mechanical Analysis*

200 Dynamic Mechanical Analysis (DMA) was performed on a Triton Technology DMA, using
201 a three point bending mode. The samples were simply supported with a span length of
202 35mm. A preload force of 0.6 N was applied to ensure that the sample remained under
203 loading throughout the oscillation of the applied cyclic force. The amplitude of
204 displacement was 0.05 mm at a frequency of 1 Hz. This displacement equated to a strain
205 of 0.048 to 0.053%, which is well within the LVR for pine as reported by Sun et al.
206 (2007). The experiment was conducted under isothermal conditions at nominally 25 °C.
207 During the experiments, the temperature showed an increase of no more than 1.0 °C.
208 The isotherm was maintained for 90 minutes.

209 Samples of 43 mm (±2 mm) length were cut from the samples exposed to the test fungi
210 as described above. The approximate dimension of cross section was 2 mm x 2 mm,
211 however each was measured using a digital calliper (2 d.p.) after drying prior to loading
212 to the DMA, to ensure precise dimensions were used in the calculation of moduli. The
213 sample was aligned in the machine such that the tangential orientation was parallel to
214 the axis of the applied load. The samples were prepared by drying overnight at 50 °C,
215 and their weight recorded prior to test and at the end of the DMA run. The moisture
216 uptake by the samples over the test duration was noted.

217 The TTDMA recorded Storage Modulus (E') and Loss Modulus (E") at intervals of 11 s.
218 The tan δ value was also calculated as the ratio of Loss Modulus to Storage Modulus. To
219 quantify the mechanical changes, complex modulus (E*) was chosen as the analysis
220 criterion, where E* = E' + n E" and n was assumed to be unity.

221

222 **3. Results and Discussion**

223 *3.1. Assessment of early onset decay*

224 qPCR analysis was used to quantify the presence of *P. placenta* gDNA within the wood
225 samples. Figure 1 shows the presence of the *P. placenta* gDNA in the decayed samples,
226 and an increasing trend in the amount of gDNA as the exposure time increases, this is in
227 agreement with the recent work of Ringman et al. (2014) and Alfredsen et al. (2015,
228 2016).

229

230

231

Figure 1: Quantity of fungal gDNA isolated (calculated from 100 factor dilution) from wood sample exposed to *P. placenta* for varying time periods

234

235

3.2. Dynamic Mechanical Analysis

237

The Dynamic Mechanical Analysis of decay timber has been the subject a very limited studies with only the studies of Birkenshaw et al. (1986) and Monrroy et al. (2011) being prominent within the literature. Further, neither of the two studies deal with initial on-set decay and therefore are not comparable to this study.

242

A typical DMA output for this isothermal experiment is shown in Figure 2. The control samples, and the majority of decayed samples showed similar behaviour over the 90 minute experimental period. The initial value of E' was low, and E' increased relatively rapidly over the first few minutes of the experiment. Thereafter the rate of increase slowed and the value tended towards a plateau value, however continuous small upward change was seen throughout the 90 minute period. The increase in E' was accompanied by a decrease in loss modulus (E"), and a corresponding decrease in the tan δ value. This behaviour has not been previously reported, but isothermal DMA studies of wood are relatively unexplored. It is suggested that the increase in observed stiffness may be related to changes in dimensions which accompany alteration of moisture content, or to testing outside the linear viscoelastic response region (LVR).

254

In this study, the moisture content was observed to increase by a small amount during the test period. Values of 1.3 to 3.2% M.C. were determined at the end of the DMA run. This corresponds with gain of moisture from the atmosphere, and would conventionally be related to a decrease in stiffness (Wilson 1932), rather than the increase observed here. However, moisture content change is likely also to cause small dimensional changes in the cross section of the sample. The change in observed stiffness over time, and any correlation with dimensional change, or effects of moisture sorption within the sample during this period is likely to be complex, and worthy of additional study beyond this present work.

264

265

Figure 2. Storage Modulus, Loss Modulus and tan δ throughout the isothermal DMA experiment, control sample 3.

268

Due to the evolution of E' and E" values throughout the test period, data from different time intervals was compared. The average of the initial five data points was calculated as the start value; in addition the value at 60 s was used, and values at 1000 s, and at 3600

272 s within the plateau region were used. It was found that similar trends occurred in the
273 storage modulus and complex modulus data from the start, middle and plateau stages of
274 the experiment, whereas greater variation was seen in the viscoelastic components (loss
275 modulus and tan δ). This may indicate that further experimentation using temperature
276 scan experiments would provide additional information about changes within the
277 amorphous cellulose and hemicellulose regions of the wood cell wall, relating to
278 viscoelastic response of the material. This will be the subject of further study, however
279 within this paper the elastic component (E') will be considered.

280 The storage modulus and complex modulus showed similar trends for the samples
281 tested (Figure 3). The values for unexposed control samples had an average value of
282 1.75 GPa and 1.85 GPa for E' and E* respectively. No statistically significant difference
283 (p=0.05, by t-test) could be found between this and the 4, 8 or 11 day exposure period,
284 however the 4 day exposed samples appeared to gain rather than lose strength. The
285 longest exposure period, 17 days, appeared to lose strength relative to the control and
286 the shorter decay intervals.

287

288 [Figure 3a]

289

290 [Figure 3b]

291 **Figure 3 (a) Loss Modulus and (b) Complex Modulus for samples exposed to *Poria***
292 ***placenta* for 0, 4, 8, 11 and 17 days. Average of three samples per exposure period,**
293 **and error bars represent standard deviation.**

294

295

296

297 Comparison of the change in mean storage modulus at each exposure duration with the
298 mean quantity of fungal gDNA isolated shows a negative correlation between the two
299 variables (Figure 4, R^2 = 0.9776). While the standard deviation for both *P. placenta* gDNA
300 and modulus data remains high, this indicates that further study with increased
301 replication may yield further insight into the initial brown rot decay and change in
302 mechanical properties.

303

304

305 **Figure 4: Comparison of storage modulus with amount of fungal DNA isolated for**
306 **the four exposure periods. Error bars represent one standard deviation of the**
307 **mean.**

308

309 **4. Conclusion**

310 The paper reports the effects of initial on-set decay and the presence of fungal DNA
311 within the timber on the storage modulus of the timber. A correlation between observed
312 stiffness and the onset of decay as indicated by the fungal gDNA quantification technique

313 has been found. This paper establishes dynamic mechanical analysis as a technique for
314 the assessment of the effects of early on-set decay on the storage modulus of timber.

315

316

327

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