## ORIGINAL ARTICLE

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# **Comparison of chitosans with different molecular weights as possible wood preservatives**

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Abstract Fungi cause serious problems in wood utilization, and environmentally benign wood protection is required as an alternative to traditional chemicals. Chitosan has shown promising antimicrobial properties against several microorganisms. In this study, we present the characterization of and antifungal properties of a commercial chitosan formulation developed for impregnation of wood. A broad range of chemical and mycological methods were used to evaluate the uptake, fixation, and antifungal properties of chitosan for wood preservation. The results show that the higher the uptake of chitosan the lower the relative recovery of chitosan in wood after leaching, and the higher the molecular weight of chitosan the higher the recovery. Chitosan with high molecular weight proved to be more efficient against decay fungi than chitosan with low molecular weight. The fungi tested on chitosan-amended nutrient agar medium were totally inhibited at 1% (w/v) concentration. In decay studies using small wood blocks, 4.8% (w/v) chitosan concentration gave the best protection against brown rot fungi.

**Key words** Antifungal activity · Chitosan · Decay fungi · Impregnation · Molecular weight

## Introduction

There has been enhanced focus from environmental organisations, consumers, and governments on environ-

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mentally sustainable treatments of wood for preservation against wood-decaying organisms, due to the fact that use of traditional preservatives containing chromium and arsenic has been banned in several European countries and the USA. Application of fungicides is the most widely used method to control fungal decay in wood. However, chemical control may induce biocide resistance in fungi and there are also health risks to consider when using fungicides. Thus, there is a growing need to develop antifungal chemicals that are not toxic to humans and the surrounding environment.

Chitin, a 1–4 linked polymer of 2-acetamido-2-deoxy- $\beta$ -D-glucose, is the most abundant natural nitrogen-containing polysaccharide and its annual production is estimated to be almost as much as cellulose.<sup>1</sup> It has been estimated that chitin is synthesized in nature at a level of up to 1-10 billion tonnes per year.<sup>2</sup> The major source of chitin is crustacean shells, which are a by-product of the seafood refining industry, although chitin is also found in the skeletons of several insects and in the cell walls of several fungi.<sup>3</sup> Chitosan is partially deacetylated chitin and is mainly produced from chitin by hydrolysis of the amide C-N bond by strong alkali.<sup>4</sup> In contrast to chitin, which is completely insoluble in water, chitosans are generally water soluble under acidic conditions, and chitosans with  $F_A$  (fraction of N-acetylated residues) of around 0.5 are also soluble under alkaline conditions.<sup>5,6</sup> Because chitosans are derived from a natural product, they vary in both relative content of the monomers, i.e.,  $F_A$  and in molecular size of the polymer (average molecular weight).

The antifungal activity of chitosan has been documented by several authors, e.g., Allan and Hadwiger,<sup>3</sup> Reddy et al.,<sup>7</sup> Benhamou and Theriault,<sup>8</sup> Roller and Covill,<sup>9</sup> and Li and Yu.<sup>10</sup> Much interest in the antimicrobial properties of chitosans is focused on its possible role in plant protection. It has also been claimed that chitosans may act as elicitors of defensive metabolism in pines.<sup>11</sup> However, there are only a few reports describing its antifungal activity against forest pathogenic fungi and wood-decaying fungi.<sup>12–15</sup> In general, chitosans are believed to act both as fungistatic agents and as fungicides at higher concentrations.<sup>7</sup> Laflamme et al.<sup>12</sup>

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demonstrated that chitosan is not only effective in reducing the radial growth of fungi, but also induces severe morphological and ultrastructural changes in the fungi. The precise mechanisms by which chitosans exert their antifungal activities are not yet elucidated and a number of hypotheses have been suggested over the years. At least two concepts have been presented to explain the mode of action of chitosans:

- 1. Chitosan, which is a polycationic polymer, interferes with the charged phospholipids in the fungal cell wall membranes, causing alterations in the permeability of the membrane. Such an interaction would trigger internal osmotic imbalances,<sup>16,17</sup> and cause leaching of electrolytes and proteins from the fungus leading to pronounced cell disorganization and ultimately to plasmolysis.<sup>7,12,18</sup>
- 2. Chitosan may enter the fungal cell, interact with DNA and alter its conformation, thus inhibiting the synthesis of mRNAs and proteins.<sup>19</sup> Jung et al.<sup>20</sup> and Shin et al.<sup>21</sup> showed that the effect of chitosans on microbial activities does not always reveal a linear dose–response curve. For some species, microbial activities did not change much even at higher concentrations after reaching a stable state of inhibition.

Several authors claim that all fungi, except those containing chitosan as a major cell wall compound, i.e., Zygomycetes, are vulnerable to chitosan.<sup>3,17</sup> Other authors<sup>9,18</sup> have questioned this generalization given results of antifungal activity also among Zygomycetes.

The electrostatic interaction between chitosan and the negatively charged surface cellulose is important for the adsorption of chitosan to cellulose. Roberts<sup>22</sup> reported that there is a positive correlation of both pH and the degree of acetylation of chitosan with the adsorption of chitosan to wood. The pH at maximum adsorption of chitosan to wood<sup>22</sup> was used by Frederiksen<sup>14</sup> in an attempt to fixate chitosan in wood, which was, however, unsuccessful. Kobayashi and Furukawa<sup>23</sup> studied the formation of chitosan has been tested both as a pretreatment before impregnation with chrome–copper–arsenic (CCA) salts<sup>24,25</sup> and in combination with zinc or copper.<sup>26</sup>

The mass loss in *Cryptomeria japonica* treated with 0.6% CCA had a mass loss of 12.8% after a 3-month exposure to *Tyromyces palustris*. This was reduced to a mass loss of 1.7% by pretreatment with chitosan.<sup>25</sup> In a study using scanning electron microscopy with wave dispersive X-ray analysis (SEM-WDX), it was found than chitosan formed a membrane at the lumen surfaces in chitosan-treated *C. japonica*. Furthermore, the absorption of CCA was greater for samples pretreated with chitosan than for samples without chitosan pretreatement.<sup>24,25</sup> Weathering did not easily remove the CCA elements from the chitosan membrane and the cell walls.<sup>24</sup>

In this study we present a commercial product developed for wood preservation based on chitosan. The main objective of this work has been to evaluate the efficacy of this chitosan formulation for wood preservation with varying average molecular weights and concentrations. Focus was put on impregnation studies, uptake and leaching of chitosan, and fungal assays of wood treated with chitosan.

## **Materials and methods**

#### Chitosan solutions

Four aqueous 5% (w/v) solutions of commercial chitosan products (M302–M305), with additives for fixation as described by Mikalsen et al.,<sup>27</sup> were provided by Multimar AS (Haugesund, Norway). The chitosan in the solutions was of different molecular weight (MW), M302 having the highest MW, and M305 the lowest MW. The solutions were diluted with tap water to 2.5% (w/v) and 1% (w/v). The pH of each solution used for impregnation was determined with a Sentron pH Meter (Type Argus, Sentron Red-Line probe) using a two-point calibration.

The molecular weight distributions were analyzed by size-exclusion chromatography combined with on-line multiangle light scattering as described by Fredheim and Christensen.<sup>28</sup> The chemical compositions of the chitosans where determined by <sup>1</sup>H nuclear magnetic resonance (NMR) spectroscopy as described by Vårum et al.<sup>5</sup>

#### Preparation and impregnation of wood samples

Wood samples were prepared from Scots pine (*Pinus sylvestris* L.) sapwood. All samples were dried at 103°C for 18h before the initial dry weight was recorded. For each treatment, 50 miniblock samples of  $10 \times 5 \times 30 \text{ mm}^{29}$  were randomly selected and impregnated by placing the samples in the impregnation solution and applying 4kPa (30 Torr) vacuum for 30min followed by 0.8MPa (6000 Torr) pressure for 2h. The weight of each wood sample after impregnation was recorded. The samples were stored at 65% relative humidity and 22°C for 4 weeks until further use. Eight miniblock samples for each treatment were then analyzed for uptake of chitosan.

Leaching of impregnated wood samples

Twenty-four wood samples for each treatment, including control samples (water-impregnated), were placed in separate test tubes of  $16 \times 150$  mm with a 10-mm glass sphere on top of the wood sample. Then 7.5 ml of deionised water was added to each sample before vacuum at 4kPa (30 Torr) was applied for 20min. After the pressure was normalized, the sample tubes were covered with aluminum foil. The water change was performed nine times with 7.5 ml deionised water. The water was changed daily except at the weekend. For six wood samples from each treatment, the water samples were stored at 4°C until further use for analysis of chitosan content. These wood samples were also analyzed for chitosan content. All remaining wood samples were packed in air-tight plastic bags and sterilized by Gamma irradiation of 25 Gy at the Institute for Energy Technology at Kjeller, Norway.

Quantification of chitosan in wood and water samples

Both wood and water samples were analyzed for the content of chitosan by a method described by Eikenes et al. (Eikenes, 2004, personnal communication). The wood samples were ground in a Retch mill to fine wood powder. Each ground wood sample (100 mg) was hydrolyzed by 1.5 ml of 6 M hydrochloric acid in a 2-ml centrifuge tube for 64–70h, and then filtered and stored at 4°C until further use. For each water sample, 0.5 ml was transferred to a 2-ml centrifuge tube and 1.0ml of 9M hydrochloric acid was added. The sample was then hydrolyzed at 100°C for 24h, diluted with deionised water, and stored at 4°C until further use. An appropriate internal standard was used for all samples. The hydrolyzed samples containing glucosamine were then analysed by high performance liquid chromatography (HPLC) with fluorimetric derivatization and detection. The results of the chemical analysis expressed in micromoles per liter were converted to mass of chitosan by the use of an average molar mass of 170.4 g mol<sup>-1</sup>, based on an assumption that the fraction of N-acetylated residues  $(F_{\rm A})$  was 0.22.

### Growth rate on chitosan-supplemented media

Fungi used in the experiments were (1) Poria placenta (Fries) Cook sensu J. Eriksson. (FPRL 280) - Basidiomycete - brown rot, (2) Coniophora puteana (Schumacher ex Fries) Karsten (BAM Ebw. 15) - Basidiomycete - brown rot, and (3) Coriolus versicolor (Linnaeus) Quélet (CTB 863 A) – Basidiomycete – white rot. The stock solutions of chitosan (M302-M305) were added to sterile molten 4% (w/ v) malt extract-agar medium (MA) to obtain the final concentrations of each solution at 0.01%, 0.1%, and 1% (w/v). For each fungus, chitosan solution, and chitosan concentration, five standard 9-cm Petri dishes containing 20ml chitosan-supplemented medium were inoculated with a 3mm fungal plug. The dishes were sealed with parafilm and incubated in the dark at 21°C. For each fungus, five dishes of plain malt agar were used for control. The radial growth rates in four directions were recorded daily until the controls reached the edge of the dish. Growth inhibition was recorded as the reduction in fungal growth in chitosanamended plates compared with the control. The antifungal activity (AFA) was expressed as the growth rate of the respective fungus on chitosan-supplemented medium relative to the fungal growth in the control dish:

$$AFA(\%) = [(GC - GT)/GC] \times 100$$

where GC is the hyphal growth (mm) on the control medium without test solution and GT is the hyphal growth (mm) on the test medium.

Miniblock assay

Petri dishes (diameter 9cm, height 2cm) containing 20ml 4% (w/v) malt agar were inoculated with *P. placenta* or *C.* puteana, two brown rot fungi recommended by van Acker et al.<sup>30</sup> A plastic mesh was used to separate the samples from direct contact with the media, and each dish contained one treated and one untreated control sample. Six replicates for each impregnation and fungus were used. For each chitosan treatment, six Petri dishes containing one treated and one untreated wood sample on media without fungi were included to obtain correction values for calculation of mass loss as described in EN113.<sup>31</sup> For both fungi, four Petri dishes containing two untreated wood samples were included to measure the viability of the fungal strains. The incubation time was 8 weeks at 22°C and 65% relative humidity. Calculations of decay (mass loss) and water content after decay were performed as described in EN113.<sup>31</sup>

## Results

#### Chitosan solutions

Initial experiments indicated that the samples were highly aggregated. They were therefore diluted and subjected to ultracentifugation prior to analysis by size-exclusion chromatography combined with multiangle light scattering (SEC-MALLS). The SEC concentration profiles [refractive index (RI) detection] gradually shifted to higher elution volumes in the order M302 < M303  $\approx$  M304 < M305, reflecting the decrease in molecular weight which follows from increasing extents of depolymerization (Fig. 1). Furthermore, the profiles are quite broad, reflecting the polydispersity of the samples (Table 1).

The molecular weight at each point  $(M_i)$  of the elution curve was calculated on the basis of the combined detector outputs using the standard Zimm equation:

$$\frac{Kc_i}{R_i} = \frac{1}{M_i} + 2A_2c_i$$



**Fig. 1.** Elution profiles and molar mass versus volume obtained from size-exclusion chromatography with on-line multiangle light scattering (*SEC-MALLS*) of the four chitosans M302–M305 and two chitosan standards

**Table 1.** Results from the <sup>1</sup>H nuclear magnetic resonance spectroscopy and size-exclusion chromatography with multiangle light scattering for the chitosans M302–M305 and pH measurements of the impregnation solutions of M302–M305 at 5%, 2.5%, and 1% (w/v) concentration

Chitosan solution	Fraction of <i>N</i> -acetylated residues $(F_A)$	$M_{\rm W}$ (daltons)	M <sub>n</sub> (daltons)	Polydispersity $M_{\rm w}/M_{\rm n}$	$pH^{a}$		
					5%	2.5%	1%
M302	0.22	215 k	93 k	2.3	_	3.3	_
M303	0.22	70 k	41 k	1.7	3.0	3.4	3.5
M304	0.22	67 k	37 k	1.8	3.0	3.4	3.6
M305	0.22	35 k	24 k	1.5	-	3.4	-

<sup>a</sup>M302 and M305 were tested only at 2.5% concentration

where  $c_i$  is the concentration (given by the RI detector),  $R_i$  is the Rayleigh factor extrapolated to zero angle, and K is the optical constant.

The calculated  $M_i$  values are included in Fig. 1. For perfectly dispersed chains of the same type, only differing in molecular weight, all data should ideally fall on a single line, corresponding to the calibration curve obtained with monodisperse standards. It was indeed observed that the two chitosan standards, that were included in the analysis for comparison, behaved normally by having totally overlapping curves. However, the samples M302-M305 generally showed  $M_i$  curves that were nonlinear, and to various extents shifted to higher molecular weights. Such behavior may be attributed to the presence of contaminants or aggregates which contribute with additional scattering. To compensate for this behavior, further calculations for samples M302–M305 were performed on the basis of the calibration curve obtained with the chitosan standards. Samples M303-M305 showed small but distinct peaks eluting near the exclusion limit. It is assumed these peaks are due to contaminating material and were not included in the calculations.

The weight average MW of the chitosans varied from 215 k dalton for M302 to 35 k dalton for M305 (Table 1). The pH was 3.0 for the 5% solutions, 3.3–3.4 for the 2.5% solutions, and 3.5–3.6 for the 1% solutions (Table 1). The fraction of *N*-acetylated residues ( $F_A$ ) was 0.22 for all samples, corresponding to a degree of de-*N*-acetylation of 78%. The chitosan concentrations in the impregnation solutions were determined by chemical analysis and ranged from 4.8% (w/v) for 5% M303 to 0.7% (w/v) for 1% M304 (Fig. 2). The 2.5% dilutions of M302–M305 all contained chitosans in the range of lay 2.1%–2.3% (w/v). The 5% M304 dilution was determined to contain 3.8% (w/v) chitosans.

#### Uptake and leaching

The higher the concentration of chitosan in the impregnation solutions (Fig. 2) the higher the concentration of chitosan in impregnated wood (Fig. 3). The chitosan content in the wood samples after leaching show that higher content of chitosan in wood resulted in more chitosan in wood after leaching than did low content (Fig. 3). However, the relative recovery of chitosan after leaching (content of



**Fig. 2.** Chitosan concentration (% w/v) in the chitosan solutions used for impregnation of wood samples ( $n = 3, \pm 1$  SD)



**Fig. 3.** Chitosan content in wood samples before (*black bars*, n = 8) and after leaching (*gray bars*, n = 6). The results are presented as millgrams of chitosan per gram of wood ( $\pm 1$  SD). The calculation from the HPLC analysis (micrograms per gram of wood sample) includes an average molecular weight of the monomer mixture of chitosan ( $F_A$  0.22) of 170.4 g mol<sup>-1</sup>

chitosan in wood after leaching divided by content before leaching) decreased with increasing content of chitosan in the wood samples (data not shown). For the high concentrations (5% M303 and 5% M304) the recovery was approximately 60%, while for the low concentrations (1% M303 and 1% M304) the recovery rates were approximately 90%. For the 2.5% solutions, the recovery rates decreased with decreasing MW.

#### Fungus tests

All the fungi were totally inhibited in agar plates supplemented with 1% chitosan solutions (Fig. 4). *Poria placenta* and *Coniophora puteana* were totally inhibited using 0.1%



Fig. 4. Reduction in colony size for three concentrations of chitosan solutions. *Black bars* for *Poria placenta, gray bars* for *Coniophora puteana*, and *white bars* for *Coriolus versicolor* 

M302 and *P. placenta* was also inhibited with 0.1% M303. The chitosan solutions showed a tendency of decreasing antifungal activity (AFA) with decreasing MW. M304 showed the lowest AFA of all three fungi both for the 0.1% and 0.01% concentrations. The 0.01% concentration generally made a minor impact on the growth rate. The 0.1% solution of M304 and all chitosan solutions at 0.01% concentration showed negative growth inhibition effects on *C. puteana*.

*Coniophora puteana* caused more decay in the samples than *P. placenta*; the average mass loss of the virulence samples of *C. puteana* was 44.7% while for *P. placenta* the average mass loss was 28.9%. The untreated control samples showed a slightly higher decay rate than the virulence samples. Mean mass loss with *C. puteana* was 53.1% for the leached control samples and 57.0% for the unleached control samples (Fig. 5). For *P. placenta* the mean mass loss for leached control samples was 40.1% and 36.4% for unleached control samples (Fig. 5). Generally, there was an inverse correlation between the decay rate of the treated samples and the untreated control samples.

There was a common effect of all chitosan formulations at all concentrations tested on wood-decaying fungi. For the unleached chitosan-treated samples at 5% and 2.5%, the decay caused by both fungi was below 5% mass loss, except for the 2.5% concentration of M304 where the mass loss was slightly higher. The M302, M303, and M305 solutions at 2.5% and M303 and M304 solutions at 5% concentration inhibited the decay to below 3% mass loss for both fungi, which according to EN113<sup>31</sup> is acceptable for wood preservatives. For the leached samples, the decay was more





Fig. 5. Decay results as average mass loss (n = 6) after 8 weeks incubation time at 22°C and 70% relative humidity for **a** *Coniophora puteana* on unleached wood samples, **b** *C. puteana* on leached wood samples, **c** *Poria placenta* on unleached wood samples, and **d** *P. placenta* on

leached wood samples. The *black bars* are for treated samples and the *gray bars* for untreated control samples. *Error bars* represent one standard deviation



Fig. 6. Relative moisture content in chitosan-treated wood samples (n = 6) after 8 weeks incubation at 22°C and 70% relative humidity with a *Coniophora puteana* and b *Poria placenta* on unleached (*black bars*) and leached (*gray bars*) samples. Error bars represent 1 standard deviation

severe. M302 performed best among the solutions at 2.5% concentration. Both 5% solutions showed low decay after leaching, but again the best results were obtained by 5% M303. Mass loss was 1.6% using C. puteana and 0.1% using P. placenta. M304 at 5% concentration resulted in 3.5% mass loss with C. puteana and 6.8% mass loss using P. placenta. The 1% solutions showed only limited effects on the decay rate caused by the fungi. The average moisture content relative to dry weight for all samples decayed by fungi (both leached and unleached) was above 30% (Fig. 6). For P. placenta (Fig. 6b), all leached and unleached samples were below 80% moisture content, except for both the unleached samples treated with 1% concentrations of M303 and M304. For C. puteana (Fig. 6a), all leached chitosantreated samples were below 80% water content after decay. The virulence samples had 92.8% water content, and waterimpregnated control samples with and without leaching had 83.4% and 85.1% water content, respectively. The unleached samples treated with 2.5% and 5% chitosan had water content after fungal decay with C. puteana between 90.8% and 128.3% (Fig. 6). The 1% unleached samples of M303 had 74.4% moisture and M304 had 88.3%.

The impregnated samples, which were placed on agar without fungi during the decay period, had an average water content for all leached and unleached samples slightly above 30%, except for the unleached M303 and M304

samples that, for the 5% concentration, had just above 60% water content.

## Discussion

The higher the concentration of chitosans in a solution, the higher the viscosity of the solution becomes, and it becomes more difficult to dissolve the chitosans. Therefore we limited the highest concentrations of chitosan in this study to 5% (w/v).

The results of uptake of chitosan in wood are well correlated with the concentration of chitosan in the impregnation solutions. There is no indication that there are any differences in penetration for these small samples with regard to MW. However, preliminary results with larger wood samples show that there is a gradient in the penetration of chitosan with high MW during impregnation (Eikenes, 2004, personal communication).

The chitosan solutions had a very broad range of average MW. The results clearly show that the chitosan with high MW are superior to lower-MW chitosan for impregnation solutions due to the fact that they tend to fixate better in wood, and hence prevent leaching during service. There is a positive correlation between the total amounts of chitosan in the leaching water samples and the difference in uptake versus recovery of chitosan. This verifies that the choice of method was suitable for quantification of chitosan.

The differences in relative recovery of chitosan after leaching with respect to uptake indicate that there is an interaction between the chitosan and the wood cell wall. The cell wall's ability to interact with chitosan seems to decrease with increasing concentration. This indicates a certain saturation of chitosan adsorbed to the cell wall of impregnated wood at higher concentrations of chitosan. Alternatively the fixation of chitosan may be less effective at higher concentrations due to less effective oxidation of the sulfuric compounds to sulfates,<sup>27</sup> which will precipitate and fixate chitosan, by oxygen in air. One possible explanation for this is that when the wood sample is drying the chitosan precipitates, and complete fixation of the chitosan is prevented by an air-tight coating shield of chitosan.

Decreasing fungal growth rate with increased chitosan concentration in agar plates has been observed in several previous studies (Laflamme et al.,<sup>12</sup> Chittenden et al.,<sup>15</sup> El Ghaout et al.,<sup>18</sup> Bell et al.<sup>32</sup>). The results showed no apparent difference between white rot, brown rot, and mould fungi. The effect of the 0.1% chitosan-amended media on decay fungi seems to be in accordance with the work of Frederiksen.<sup>14</sup> He found that this concentration totally inhibited Serpula lacrymans, and delayed growth of Coniophora puteana and Coriolus versicolor. Morphological changes of fungal strains on chitosan were reported earlier by Roller and Covill,9 Laflamme et al.12 and Chittenden et al.<sup>15</sup> In this study, Poria placenta tended to make fewer and thicker hyphae on chitosan supplemented media and C. versicolor made denser hyphal mats than on control media with more aerial mycel.

The results from the 0.1% chitosan solutions demonstrate that the growth reduction may be a function of MW; the higher the MW the larger the reduction in growth rate. These results are the opposite of the results reported by Chittenden et al.,<sup>15</sup> who studied three different molecular weights of chitosan against mould and sapstain fungi. In contrast to this, Frederiksen<sup>14</sup> reported no differences in the results with varying MW in his studies of Aspergillus niger growth on filter papers. Interestingly, Shin et al.<sup>21</sup> found that chitosan with high MW was more effective in inhibiting bacterial growth on cotton fabrics than chitosan with low MW. By studying chitosan oligomers, Kendra and Hadwiger<sup>33</sup> reported that antifungal ability against Fusarium solani increased as the oligomer MW increased. The heptamer was on a level with high MW chitosan with regard to antifungal ability. Matsubara and Kuroda<sup>34</sup> reported that the elicitor activity of the chitosan hexamer and octamer had almost the same activity as commercially available chitosan.

The slight enhancement of *C. puteana* growth (faster growth rate than the control) was present for all chitosans at 0.01% concentrations as well as for 0.1% M304. Therefore, this is not regarded as a methodical artifact. One explanation is that low concentrations of the chitosan might act as a growth promoter for *C. puteana* rather than as a growth inhibitor, with the chitosan and/or additives having a fertilizing effect.

Comparison of the 2.5% solutions of the leached wood samples showed an increasing decay rate with decreasing MW. These results are supported by the agar plate growth rate study. The results from the leaching experiments also support this, because there was a positive correlation between the MW and the relative recovery of chitosan after leaching. Mehrtens<sup>13</sup> and Frederiksen<sup>14</sup> both tested the performance of chitosan by a fungal decay test. Both tested different chain lengths and fixation methods, but found no effect for either of the parameters. The lack of effect in both cases might be caused by problems with the test system or the chitosan concentrations.

Wood samples impregnated with chitosan solutions at 1% (w/v) concentration showed only a small effect in reducing wood fungal decay. The results are nearly the same as those for wood samples treated with tap water. However, at higher chitosan concentrations wood treated with these chitosan solutions seems to have been very effectively protected, with only minor mass loss after decay. A concentration of 5% seems adequate for total protection of wood against these brown rot fungi. However, further studies with larger wood samples in the laboratory and the field will have to be performed to provide results to support these effects gained from testing small samples.

Frederiksen<sup>14</sup> found no effect of chitosan compared with the control before or after leaching, using *C. puteana* and *C. versicolor* in a modified EN113<sup>31</sup> test for the concentration used. The maximum concentration was 0.4% (w/v), which is approximately tenfold lower than what seems to be necessary according to our results. Kobayashi and Furukawa<sup>26</sup> found in a study using chitosan-metal salts as wood preservative that retention of 11.6kgm<sup>-3</sup> chitosan was ineffective in controlling the growth of the brown rot fungi *Tyromyces palustris*. This should resemble a 1.5%–2.0% (w/v) chitosan solution. Concentrations of this level were also found to be insufficient to make a substantial impact on fungal growth rate in the present study.

These tests confirm that chitosan exerts some effect when impregnated in wood, even though the leaching procedure was performed according to a down-scaled EN84,<sup>35</sup> which is a relatively rough procedure on these small samples. The decay in the virulence and untreated control samples was also significantly higher than required in EN113,<sup>31</sup> proving the activity of the fungi used.

The moisture content values in all the leached chitosantreated wood samples after the fungal decay tests are within the limits of the moisture content required for fungal decay. However, some of the treatments showed significantly higher moisture content in wood for the unleached samples. This is probably due to the hygroscopic ingredients in the formulation that assists in fixating chitosan in wood. Some samples, like the 1% treatment, water-treated, and virulence samples, showed relatively high moisture contents and also high degrees of decay. The high moisture content itself can also be a result of the decay process. Shin et al.<sup>21</sup> also found that the moisture gain increased as the treatment concentration and the MW of the chitosan were increased. Their explanation was that this was due to the amino and hydroxyl groups, which provide reactive sites for moisture. Furthermore, a coating of chitosan of high MW on the surface of the cotton fibers made it easier for water molecules to approach reactive sites.

In conclusion, results of the decay tests and leaching experiments showed that a 5% solution of high-MW chitosan was adequate for preserving the small miniblock samples. However, further work needs to be done to test whether this is also valid for larger samples and longer exposure time in the field. An improvement of the fixation process will further enhance the properties of chitosanimpregnated wood exposed to leaching in water.

The differences in an effective concentration between the growth rate in agar plates and decay studies using solid wood were as expected. An inhibition of radial growth of fungi or a reduction of sporulation point to a biocidal action of chitosan; however, they do not provide an answer to the fungal ability to degrade impregnated wood.

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