Research Article

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Termiticidal activity of chitosan against the subterranean termites *Reticulitermes flavipes* and *Reticulitermes virginicus*†

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Abstract

BACKGROUND: Chitosan is a derivative form of chitin, which is the major component of exoskeletons of arthropods and the cell walls of fungi. The antimicrobial activity of chitosan against lepidopterans, aphids, fungi and bacteria has been extensively investigated, but only one report on the termiticidal effect of chitosan on termites has been published. In this study, we examined the termiticidal activity of chitosan by exposing single colonies of *Reticulitermes flavipes* (Kollar) and *Reticulitermes virginicus* Banks to wood treated with six different concentrations of chitosan solutions. Termite mortality and percent mass loss of wood samples after exposure to termites for 4 weeks were calculated.

RESULTS: High termite mortality (≥ 94%) occurred during exposure of *R. flavipes* termites to chitosan-treated wood with ≥38 mg g^{-1} treatment concentrations (≥ 2% chitosan), while <50% termite mortality was observed at lower treatment concentrations (11–15 mg g^{-1}; 0.5% and 1% chitosan). For *R. virginicus*, 100% mortality was observed at all levels of treatment concentrations. A decrease in the percent mass loss of the wood sample was apparent in samples treated with solutions with an increasing chitosan concentration, with a significant difference ($P < 0.05$) between lower and higher treatment concentrations. Treatment retention in wood samples upon leaching was also determined and showed retention levels of between 0 and 30 mg g^{-1} chitosan retention.

CONCLUSION: This study investigated the exposure of subterranean termites to chitosan as a wood preservative. The results show that chitosan treatments at sufficiently high loadings could protect wood against termites, preferably under non-leaching conditions.

Keywords: natural products; chitosan; subterranean termites; resistance; mortality; toxicity

1 INTRODUCTION

Wood is a major biodegradable and biorenewable construction material, and as such requires protection against wood-degrading organisms. Currently, most wood protection preservatives contain copper, which can have a negative effect on the ecosystem.[1–3] As public preference for sustainable, ecofriendly products has increased, wood protection scientists are looking more closely into alternatives to traditionally used preservative systems. Among the examined alternative substances, chitosan showed effective fungicidal properties[4,5] and has been described as a potentially effective wood preservative,[6] but its effects on subterranean termites, *Reticulitermes flavipes* (Kollar) and *Reticulitermes virginicus* Banks, both major wood-destroying insects in the USA, have not been investigated.

Chitosan is made up of β-1,4-linked D-glucosamine and N-acetyl-D-glucosamine units. It is derived from an aminopolysaccharide, chitin, which is a building material of arthropod exoskeletons and the cell walls of fungi. Chitin is biosynthesized in annual yields similar to the amounts of cellulose,[7,8] with 1.5 million tons available for commercial use.[9] It is isolated from the exoskeleton through a demineralization process with diluted acid or deproteinization in hot base solutions, and further deacetylated in concentrated sodium hydroxide solution to yield chitosan of varying degrees of deacetylation and molecular weights.[10]

Chitosan is of low toxicity to non-target organisms. A Norwegian purified chitosan product, ChitoClear (Primex Ingredients ASA, Norway), has reportedly attained a self-affirmed safe substance status in US markets.[11] Although it has not been issued the generally recognized as safe (GRAS) designation by the United States Food and Drug Administration (FDA), chitosan is used in bandages,[12] drug delivery systems,[13] and the winemaking process.[14]

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As a biopesticide, chitosan is used in agriculture and horticulture and has a greater antimicrobial effect than chitin.[15] It inhibits the growth of several plant and human pathogenic fungi.[16–18] Chitosan at 0.3–0.4% (w/v) in nutrient media has also been shown to have fungistatic activities against tree pathogens, *Leptographium procerum* (Kendrick), and *Sphaeropsis sapinea* (Fries).[19] In the case of Basidiomycota, which includes the wood decay fungi, chitosan has showed promise as a wood preservative against both brown and white rot fungi.[4,20–22] For example, growth of *Poria placenta* (Fries), *Coniophora puteana* (Schumacher ex Fries) and *Trametes versicolor* (Linnaeus) was completely inhibited on nutrient media with 1% chitosan.[4]

Effects of chitosan on insects were reported in several publications which included studies on Lepidoptera (cotton leafworm, corn earworm, and diamondback moth) and aphids (oleander aphid, rose-grain aphid, green peach aphid, melon aphid, mealy plum aphid, bird cherry-oat aphid, and grain aphid).[23–25] All of the studies reported chitosan as an effective insecticide within a concentration range of 5–11 mg g^1 chitosan in artificial diet, resulting in 15–100% insect mortality depending on aphid species.[23,25] Chitosan as a termiticide has not been investigated, but the effect of chitosan as an agent for fixation of copper and zinc in wood to protect it against Coptotermes formosanus Shiraki has been reported.[26] The results indicated that chitosan improved fixation of metals in wood and overall increased treatment effectiveness.

Therefore, the aim of this study was to investigate the activity of chitosan-treated wood against subterranean termites using a laboratory bioassay method. An effective retention for wood treatment was determined by treating wood with six different concentrations of chitosan solutions and measuring their effects on termite mortality and wood percent mass loss after 4 weeks of termite exposure. In addition, the change in mass of chitosan-treated wood after a 19-day water extraction was measured to evaluate chitosan leachability from wood.

2 MATERIALS AND METHODS

2.1 Termites

Subterranean termites used in this study were obtained from single colonies collected at the United States Department of Agriculture, Forest Service Harrison Experimental Forest, Saucier, MS (colony 1), and from Mississippi State University Dorman Lake Test Site, Starkville, MS (colony 2). Termite-infested logs were cut into smaller sections and kept in a covered metal bin at room temperature with sufficient moisture until they were utilized, which was within 3 months of collection.

Species identification of the termites used was based on the DNA sequence of the mitochondrial AT-rich region described by Foster *et al.* in 2004.[27] Genomic DNA from five termite soldier heads per colony was extracted using the MasterPure™ DNA/RNA Extraction kit (Epicentre, Madison, WI, USA). The species-specific region was amplified from the genomic DNA template in a polymerase chain reaction (PCR) using the forward and reverse primer sequences previously reported.[27] The amplified PCR product (~400 bp) was cloned into the pGEM T-Easy Vector System (Promega, Madison, WI, USA) and four clones per colony were sent to Eurofins Genomics (Louisville, KY, USA) for sequence determination. The returned DNA sequences were aligned using DNASTAR Lasergene v8 software (DNASTAR, Madison, WI, USA) and then compared against the NCBI-nr nucleotide database using BLAST®, [28,29] for species determination.

2.2 Chitosan solution preparation and wood treatment

Low-molecular-weight chitosan powder (50–190 kDa) was purchased from Sigma-Aldrich Co. (St Louis, MO, USA). Chitosan solutions of 0.5, 1, 2, 3, 4 and 5% (w/v) were prepared in 25% aqueous acetic acid (w/v), pH 1.85. The solutions were agitated overnight using a laboratory magnetic stirrer in a laminar flow cabinet until the chitosan was completely dissolved. Using an AR 1500 Rheometer (TA Instruments, New Castle, DE, USA), the viscosity of the chitosan solutions was measured at a shear rate of 10 s^{-1} and at 25 °C (room temperature). Chitosan concentrations >5% were not tested for wood treatment because of the very high solution viscosity (Table 1).

Southern yellow pine, *Pinus* spp. (Linnaeus), sapwood samples of dimensions 25 × 25 × 6 mm (tangential × radial × longitudinal) were dried in a laboratory oven (50 °C) to constant mass. Wood samples were sawn from the same board and used for both termite experiments. Treatments were randomly assigned to the wood samples. Oven-dried wood samples, with known mass, were submerged in each chitosan solution (one solution prepared per treatment) and kept under vacuum (29.8 mm Hg) for 3 h, and subsequently equilibrated in the solutions under atmospheric pressure for 24 h. Two sets of control samples were treated similarly but in 25% acetic acid solution (w/v) and distilled water. Samples were removed from the solutions, gently wiped, initially dried at room temperature for several hours, and finally dried in a laboratory oven (50 °C) to constant mass. Treated weights were recorded. Treated-wood samples were used for the laboratory bioassay to evaluate termite resistance and preservative leaching. Concentration (mg chitosan g^{-1} oven-dried wood) was calculated as follows:

$$\text{Treatment concentration } \left(mg\ g^{-1}\right) = \frac{\left(m_{0t} - m_0\right)}{m_0} \times 1000$$

where m_{0t} (g) is the oven-dry mass of samples after chitosan treatment and m_0 (g) is the oven-dry mass of samples before chitosan treatment.

2.3 Termite no-choice exposure laboratory bioassay

The no-choice test of American Wood Protection Association Standard E1[30] was chosen for this study in order to determine the toxicity of chitosan to termites. The choice test, also outlined in this standard, is applicable for determination of the acceptance of treated wood as a food source to termites, which was out of the scope of this study. The test standard was followed with minor modifications. The amount of termites used was changed from the recommended 400 to 100 individual termites, and the moisture content of soil and test temperature were chosen according to the appropriate conditions for the termite species.

Two experiments were set up, one for colony 1 and the other for colony 2. The termite treatments were: water-treated wood, acetic acid-treated wood, 0.5% chitosan-treated wood, 1% chitosan-treated wood, 2% chitosan-treated wood, 3% chitosan-treated wood, 4% chitosan-treated wood and 5% chitosan-treated wood. Each treatment group consisted of five sample replicates, where one replicate was defined as one treated-wood sample. Treated-wood samples were placed individually on top of play sand (150 g, dried at 121 °C and cooled) in autoclaved Qorpak® (Bridgeville, PA, USA) round-bottom glass jars, with the addition of autoclaved distilled water (24 ml) for a 16% moisture content. The sand (Sakrete, Charlotte, NC, USA) was purchased from a local home improvement store. One hundred

Table 1. Shear stress and viscosity of prepared chitosan solutions

Solution	Shear stress (Pa)	Shear rate (s^{-1})	Viscosity (Pa s)	Temperature (°C)
Water	0.07474	10	0.007	25
1% chitosan	0.8159	10	0.082	25
2% chitosan	2.975	10	0.298	25
3% chitosan	22.68	10	2.268	25
4% chitosan	115.2	10	11.52	25
5% chitosan	246	10	24.60	25

cleaned termites (99 workers and one termite soldier) were placed in each jar, away from the treated-wood sample. The jars were kept in a laboratory incubator (28 °C) for 4 weeks, then disassembled to assess termite mortality, which was estimated as follows:

$$\text{Mortality } (\%) = \frac{T_D}{T} \times 100$$

where T is the total number of termites (100) used in each jar and T_D is the calculated number of dead termites per jar after 2 weeks. The number of dead termites was calculated by counting the number of live termites at the conclusion of the test, and then subtracting from the total number of termites used at the beginning of the test.

Termite-exposed wood samples were dried in a laboratory oven (50 °C) to constant mass. Wood percent mass loss caused by termite activity was calculated as follows:

$$\text{Mass loss } (\%) = \frac{(m_{0t} - m_{1t})}{m_{0t}} \times 100$$

where m_{0t} is the oven-dry mass of samples after chitosan treatment and m_{1t} is the oven-dry mass of samples after termite exposure.

2.4 Leaching test

Chitosan leaching was performed following the AWPA Standard E11[31] with one modification: the volume of water used for leaching was decreased based on the dimension of the wood samples used in this study. For this study, a replicate is defined as one treated-wood sample leached in water. Wafers (five per treatment) were submerged in a beaker containing deionized water (300 ml), then placed on a laboratory orbital shaker (100 rpm) for 6 h. The water containing leachate was removed, discarded and replaced with new deionized water (150 ml) and shaken for 1 day, and the process repeated every 48 h thereafter. The entire leaching procedure lasted for a total of 19 days.

The leached wood samples were dried at room temperature for several hours, and then in a laboratory oven (50 °C) to constant mass. The amount of chitosan left in the samples was adjusted for the amount of lost extractives. Lost extractives were calculated from the difference between the oven-dried masses of water-treated samples before and after leaching.

$$\text{Post-leaching retention } \left(\text{mg g}^{-1}\right) = \frac{(m_{0c} - m_0)}{m_0} \times 1000$$

where m_{0c} (g) is the oven-dry mass of samples post-leaching, corrected for lost extractives, and m_0 (g) is the oven-dry mass of samples pre-treatment.

2.5 Statistical analysis

Statistical Analysis Software (SAS Institute Cary, NC, USA) was used to assess homogeneity of variance within sample groups using Levene's test. Results for treatment concentrations and post-leaching retentions were analyzed separately. Mortality and mass loss values were statistically analyzed by species. The assumption of homogeneity of variance was met for the response variables, concentration of treated samples, and *R. flavipes* mortality. One-way ANOVA and Tukey post hoc tests were performed in these cases to determine if the difference between treatments was significant. For the other three response variables (percent mass loss, *R. virginicus* mortality, and retention of leached samples), the assumption of homogeneity of variance was not met. Therefore, Welch's ANOVA was used in these cases. If the treatment effect was found to be significant, the Games–Howell post hoc test was performed. All results were interpreted at the 5% significance level and an alpha level of 0.05 was used to determine statistical significance.

3 RESULTS

3.1 Species identification of subterranean termites

Comparison of the consensus sequences from the alignment against the NCBI-nr nucleotide database revealed that colony 1 termites were *R. flavipes* (100% coverage and 99% identity) and colony 2 termites were *R. virginicus* (100% coverage and 99% identity).

3.2 Viscosity of chitosan solutions and retention of chitosan in treated-wood samples

The relationship between the concentration of chitosan solution and its viscosity was fairly linear ($R^2 = 0.8205$), as the data points in the residual plot (not shown) were randomly dispersed around the horizontal axis. The average retention of chitosan in treated wood increased with higher concentrations of chitosan solutions, as expected. It is important to note that the acetic acid treated controls retained approximately 1.2 mg of acetic acid per gram of oven-dried wood (Figure 1), and it is therefore expected that the chitosan treaments also contained trace residual amounts of acetic acid. The 0.5% chitosan-treated samples increased their dry mass by an average of 11 mg g^{-1}, which was not significantly different from retention amounts of 1% chitosan-treated samples (15 mg g^{-1}). Retention in both 0.5 and 1% chitosan-treated samples was significantly lower than retention in other chitosan-treated samples. The 2% chitosan-treated samples increased their dry mass by an average of 38 mg g^{-1}, which was significantly lower than the retention in 3% chitosan-treated samples (48 mg g^{-1}). Treatment concentrations for 4% chitosan-treated samples (67 mg g^{-1}) and for 5% chitosan-treated samples (75 mg g^{-1}) were not significantly different from each other.

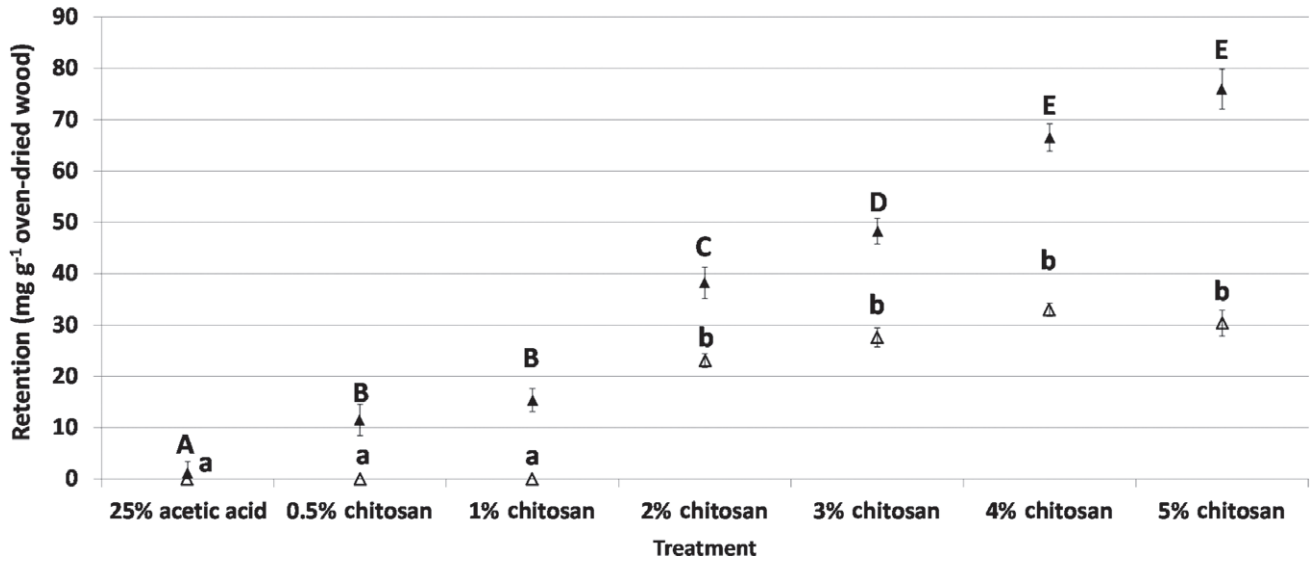

Figure 1. Chitosan retention (filled triangle, post treatment; clear triangle, post leaching) of wood samples treated with different concentrations of chitosan (bars denote standard error; $n = 5$). Markers with different letters are significantly different ($P < 0.05$); uppercase for retentions after treatment, lowercase for retentions after leaching.

3.3 Leachability of chitosan from different chitosan-treated wood samples

As shown in Figure 1, acetic acid treated samples leached all acetic acid during the leaching procedure. The 0.5 and 1% chitosan-treated samples also showed no residual levels of acetic acid or chitosan. Samples treated with the higher chitosan concentration solutions showed approximately 23–30 mg g^{-1} in 2–5% chitosan-treated samples, values not significantly different from each other.

3.4 Percent mass loss of wood samples as affected by different chitosan treatments

The average percent mass loss of chitosan-treated samples caused by termite feeding generally decreased with increased chitosan concentration (Figure 2). A higher percent mass loss was observed in samples exposed to *R. flavipes* (4.6–11.7%), compared with the percent mass loss of *R. virginicus*-exposed samples (3.7–8.9%). For *R. flavipes*, percent mass loss was significantly different between two treatment groups. Wood treated with a higher concentration of chitosan (2–5%) showed significantly lower percent mass loss compared with wood treated with a low concentration of chitosan (0.5 and 1%) or no chitosan (water and 25% acetic acid).

For *R. virginicus*, in contrast, statistical analysis revealed a different pattern. Treatments in ascending order of percent mass loss were: 3, 2, 4, 5, 1 and 0.5% chitosan, 25% acetic acid, and water. The lowest percent mass loss was observed for wood treated with 3% chitosan, which was significantly different from all other groups except for the 1, 2 and 4% treatments. The 2 and 4% treatments were only significantly different from the controls (water and 25% acetic acid) and the 0.5% chitosan treatment. Wood from the 5% chitosan treatment was significantly different from the 3 and 0.5% treatments and both controls (water and 25% acetic acid). The 1% chitosan treatment showed the most overlap and only showed a significant difference from the water treatment. The 0.5% chitosan treatment was significantly different from all other chitosan treatments except for the 1% chitosan and 25% acetic acid control treatments. The 25% acetic acid treatment was only significantly different from treatments with 2% or more chitosan. The water control, however, was significantly different from all treatments with chitosan. Overall, results for both termite species showed that chitosan-treated samples generally exhibited significantly lower percent mass loss compared with controls.

3.5 Termite mortality as affected by different chitosan treatments

Termite mortality increased with an increase in chitosan concentration for *R. flavipes*, which exhibited ≥94% mortality when termites were exposed to samples with ≥38 mg g^{-1} treatment concentrations (≥ 2% chitosan). No significant difference in mortality was found among the treatments with ≥2% chitosan, which was significantly higher than the mortality of termites subjected to lower retentions of chitosan (0.5 and 1% chitosan) and controls (Figure 3). Termites exposed to samples with ≤15 mg g^{-1} retentions (0.5 and 1% chitosan) exhibited mortality of 38–46%. Termite mortality for the 1% chitosan treatment was significantly different from all treatments except the 0.5% chitosan treatment, while the 0.5% chitosan treatment was only significantly different from the treatments with ≥2% chitosan. Mortality values observed in control treatments, water and 25% acetic acid were 24 and 28%, respectively, with no significant difference between them. For *R. virginicus*, every concentration level of chitosan treatment resulted in death of all termites after 4 weeks. The observed 100% mortality of *R. virginicus* exposed to the 4 and 5% chitosan-treated wood samples, however, probably happened during the first 2 weeks of the study, as no activity of termites could be observed at this time. Water and acetic acid control samples exposed to *R. virginicus* exhibited mortality values of 37 and 55%, respectively, which were not statistically different from each other.

4 DISCUSSION

Vacuum treatment of wood with chitosan solutions yielded average retention levels in the range of 11–75 mg g^{-1}. As we did not actually quantify the amounts of retained chitosan or acetic acid in the wood, retentions based on mass change contained both chitosan and acetic acid. The complete removal of acetic acid from

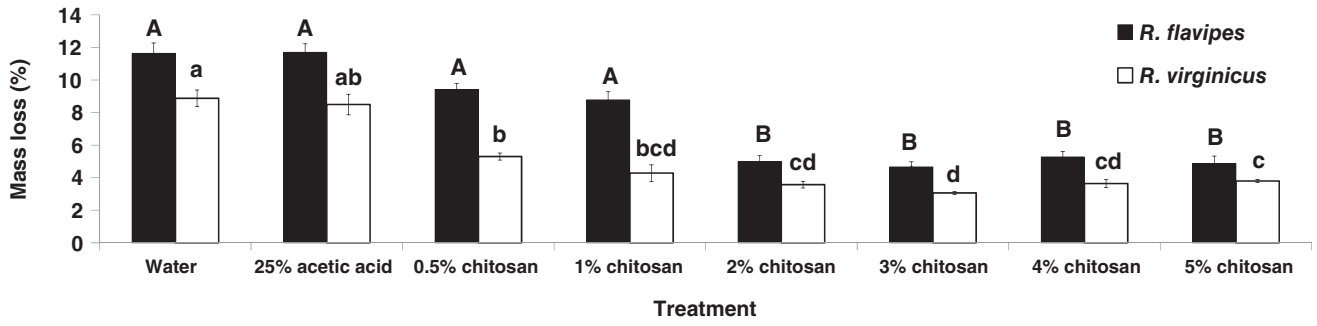

Figure 2. Percent mass loss of wood samples as affected by chitosan treatments and termite exposure (bars denote standard error; $n = 5$). Bars with different letters are significantly different ($P < 0.05$); uppercase letters for the *R. flavipes* experiment, lowercase letters for the *R. virginicus* experiment.

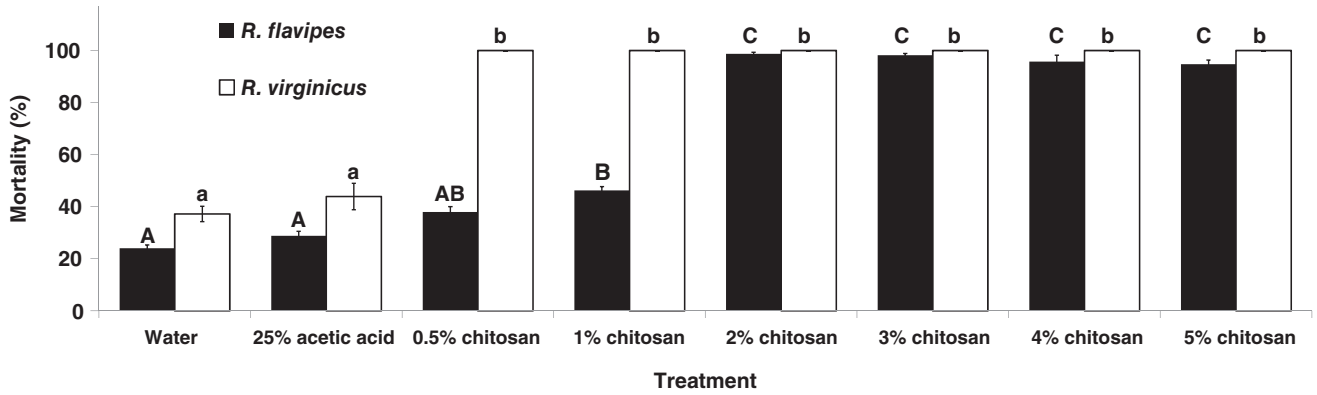

Figure 3. Termite mortality in chitosan and control treatments (bars denote standard error; $n = 5$). Bars with different letters are significantly different ($P < 0.05$); uppercase letters for the *R. flavipes* experiment, lowercase letters for the *R. virginicus* experiment.

wood upon acetylation has been shown to be incomplete through end-vacuum or drying.[32] Retentions ranging from approximately 20 to 45 mg g^{-1} have been reported in the literature depending on treatment method, solution strength, wood species and molecular weight.[4] Eikenes *et al.* obtained approximately 20 mg g^{-1} retention in Scots pine sapwood when vacuum/pressure-treating wood with 2.5% chitosan (35–215 kDa in acetic acid), with no difference in retention among different molecular weights.[4] However, they found that an increase in chitosan concentration did have an effect on retention, with 5% concentrations having higher retention than 2.5% chitosan concentration treatments.[4] Our results also indicate that retention increases in wood treated with solutions of 1–4% chitosan concentrations. However, as no significant difference in retention was found between treatments with 4 and 5% chitosan concentrations, the further uptake of low-molecular-weight chitosan above 5% is improbable in southern pine. The uptake, though, could be enhanced by applying pressure in the vacuum treatment, as was shown in a previous study.[33] Molecular weights did not have a significant effect on treatment uptake when using higher molecular weights chitosan.[4,33,34] However, it is expected that use of chitosan oligomers would give higher retentions and better penetration into microvoids of cell walls, as was found for *Pinus radiata* (David Don).[35]

Although a trend of a decrease in percent mass loss with increased treatment concentration was observed in our study, the differences in percent mass loss were not always statistically significant among wood samples treated with 2–5% chitosan solutions for both species. Also, percent mass losses of samples treated with 0.5 and 1% chitosan solutions were not statistically different from each other. It is improbable that a prolonged exposure time may increase the percent mass loss from *R. virginicus*-exposed samples, because the lowest level of chitosan retention (11 mg g^{-1}) resulted in 100% mortality. It is possible that *R. virginicus* cannot tolerate low levels of chitosan exposure, although it would be prudent to test multiple colonies of *R. virginicus* to confirm this hypothesis. As for *R. flavipes*, a longer feeding time at low concentrations could possibly increase the sample percent mass loss, because termites exposed to wood samples treated with 0.5 and 1% chitosan solutions exhibited 38–46% mortality after 4 weeks of exposure.

The insecticidal activity of chitosan against termites seems to be greater than that against moth larvae. Approximately 15% mortality was observed for cotton leafworm third-instar larvae after 5 days of exposure to 5 mg g^{-1} chitosan (50–190 kDa) in artificial diet, [24] compared with 100% and 38% mortality observed in our study, respectively, for *R. virginicus* and *R. flavipes*, when exposed to 11 mg g^{-1} chitosan (50–190 kDa) in wood. Different molecular weights of chitosan can also impact mortality. Cotton leafworm showed mortality rates of up to 50% when exposed to high-molecular-weight chitosan (227–947 kDa) at 4 mg g^{-1} artificial diet after 7 days.[23] Higher molecular weight chitosan (300 kDa) also showed higher insecticidal activity against corn earworm when compared with chitosan oligomers (~3.5 kDa) with an average degree of polymerization of 20.[25] Comparison of the effectiveness of chitosan against termites and aphids is difficult to make as the aphid studies used dip or sprayed-leaf methods instead of incorporating the chitosan into the diet. However, when comparing aphid species, it is obvious that species show a very wide range of susceptibility to the same levels of chitosan solution concentrations, as is the case in our study with subterranean termites.[25]

No results for the effect of chitosan-treated wood on *Reticulitermes* spp. have been published previously. However, a single study was performed on the effect of chitosan on *C. formosanus* termites exposed to 9.85 mg g^{-1} chitosan-treated wood samples .[26] After 3 weeks, *C. formosanus* exhibited 10.5% average mortality and 15.5% average wood mass loss. In our study, termites exposed to similar treatment concentrations in wood (11 mg g^{-1}; 0.5% chitosan solution treatments) showed higher mortality and lower percent mass loss: 100% mortality and 5% mass loss for *R. virginicus* and 38% mortality and 9% mass loss for *R. flavipes* after 4 weeks. It is possible that the extra week of termite exposure in our study caused the higher mortality, but the termite species and/or molecular weight of chitosan used could also have contributed to the observed differences. It was shown previously that *C. formosanus* has more aggressive feeding habits compared with *Reticulitermes* spp.[36] Also, Kobayashi *et al.* utilized lower molecular weight chitosan (10–30 kDa) in comparison to our chitosan (50–190 kDa).[26] A positive trend between chitosan effectiveness and molecular weights in the range 35–215 kDa was also reported in the case of wood-degrading fungi, although a significant difference was not found.[4,20] However, Hussain *et al.* found that antifungal effects of chitosan oligomers on wood-degrading fungi decreased with an increase in the degree of polymerization for chitosan oligomers.[37]

In a comparison of the termiticidal effectiveness of chitosan to that of disodium octaborate tetrahydrate (TIM-BOR®, Nisus, Rockford, TN, USA), currently one of the most effective termiticidal wood preservatives, considering strictly mortality rates and preservative retentions, chitosan seems to underperform at least in the case of *R. flavipes*. *Coptotermes formosanus* exposed to 2.9 mg g^{-1} TIM-BOR-treated wood for 3 weeks showed 100% mortality and 8.4% wood mass loss,[38] while for *R. flavipes*, the lowest retentions of 11 mg g^{-1} chitosan-treated wood showed 38% mortality and 9.4% mass loss. Also, exposure of *R. flavipes* to 7.2 mg g^{-1} TIM-BOR-treated wood for 4 weeks resulted in 100% termite mortality and 75.7 mg wood mass loss,[39] while exposure of the same termite species to 11 mg g^{-1} chitosan-treated wood resulted in 38% mortality and 180 mg mass loss. However, borate treatments, similar to chitosan, have a tendency to leach and can be completely removed after 8 days, regardless of treatment solution concentration.[40] Although wood treated with higher concentrations of chitosan solutions should retain sufficient amounts of chitosan to provide protection against *R. virginicus* termites based on the mortality results of the lower chitosan retentions tested, retained amounts of chitosan are inadequate amount to protect against *R. flavipes* termites. As shown, treatment concentrations of approximately 38 mg g^{-1} oven-dried wood (2% chitosan), which is comparable to retentions of leached samples, caused approximately 46% termite mortality in a 4-week period for *R. flavipes* and 100% mortality in *R. virginicus*.

Despite the confirmation that highly concentrated chitosan solutions generally yield samples with higher chitosan amounts before and after leaching,[4,34] the issue of overall leaching of chitosan remains a challenge which could possibly be addressed by treating wood with chitosan oligomers, which penetrate the cell walls and enhance adhesion, as suggested by the work of Singh *et al.* in 2010.[35]

5 CONCLUSIONS

The consensus sequence from the alignment confirmed that the species identities of the tested colonies were *R. flavipes* and *R. virginicus*. Results of this study show that chitosan has a termiticidal effect on both subterranean termites at varying retention levels. Approximately 40–100% of chitosan retained in treated wood was leached, depending on the initial retention. Termite mortality increased when termites were exposed to samples treated with higher chitosan concentration solutions. Post-leaching results indicate that chitosan is not suitable for protection against both subterranean termites in outdoor conditions. Further studies need to be conducted to understand chitosan toxicity mechanism in termites and to prevent its leaching from treated wood.

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AUTHOR CONTRIBUTIONS

Conception and design: DJ and JDT; investigations and acquisition of data: OR and TT; methodology: OR, TT, DJ and JDT; data analysis and interpretation: OR and DJ; writing – original draft preparation: OR, DJ and JDT; writing – review and editing: all authors.

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