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Article

Radiata Pine Wood Treated with Copper Nanoparticles: Leaching Analysis and Fungal Degradation

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Abstract: Radiata pine is the main wood species used in the Chilean construction industry, but it must be protected due to its low natural durability. Chemical protection of wood by impregnation allows for a more efficient utilization of the forest resources by extending its useful life. The use of nanoparticles in wood protection has garnered great interest during the last decade, due to their unique physicochemical properties, different from those of larger sized materials. In this research, the impregnation of radiata pine wood with copper nanoparticles (CuNP) was studied in terms of retention, penetration, leaching, and its protective effect against wood rot fungi growth according to EN 113, AWPA A3-91, A9-18, and E11-16. Penetration analysis confirmed a uniform distribution across the wood, with total penetration in the impregnated samples with the highest concentration solution of CuNP. Retention values of the impregnated wood increased proportionally with the concentration of nanoparticles evaluated by EDXRF. Leaching analysis showed copper removal during the first hours of the test, with a constant leaching rate up to 144 h. Impregnated wood mass loss (ML) due to exposure to *Gloeophyllum trabeum* and *Rhodonia placenta* fungi were significantly reduced regardless of the CuNP concentration or fungi tested, with an ML smaller than 5% and smaller than 14% for leached samples.

Keywords: copper nanoparticles; fungal degradation; *Pinus radiata*; wood preservation

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1. Introduction

Wood protection by impregnation can significantly increase wood durability, as has been reported since the 1950s [1], and even today it is an active field of research [2–4]. Most wood species commonly used in construction deteriorate if exposed to conditions that support the growth of wood degrading organisms. Thus, impregnation enables the more efficient use of forest resources by extending wood's service life. The effectiveness of the impregnation treatment depends on the tree species, the chemical products, and the impregnation method [5]. The degree of protection achieved depends basically on the preservative used and the appropriate penetration and retention of the chemicals involved.

Chromated copper arsenate (CCA) has been used as a major wood preservative for many applications; however, it is known for its high toxicity, since chromium and arsenic are toxic and carcinogenic [6,7], and the biggest drawback is the disposal of treated wood and the wood waste at the construction site (clippings, sawdust, dust, etc.). Being a partially biodegradable material with chemical treatment, there is a need to consider it as a dangerous waste, since it gradually releases the copper, chromium, and arsenic into the environment at levels that pose a risk of contamination of watercourses and soil [8].

In recent years, several highly effective wood preservatives have been banned in different countries as they harm human health and the environment [9]. New chromium- and arsenic-free formulations of wood preservatives have been introduced as replacements for CCA in many applications. These new wood preservatives contain copper and organic co-biocides such as quaternary ammonium compounds (quats), azoles, borates, and/or HDO [10–12]. However, a wood protection treatment which has attracted considerable interest from the scientific community during the last decade is wood impregnation with nanocompounds [13–18]. Nanometer-sized metal particles have different physical and chemical properties from their macroscale counterparts that alter their interaction with biological structures and physiological processes [19]. Nanocompounds penetrate into wood cell walls more easily, while they are more difficult to leach out and have a higher bio-durability. Metallic-organic nano formulations have also been reported, such as soy oil derived silver nanoparticles, which have effectively protected Scots pine from white rot decay [20]. The antimicrobial activity of copper nanoparticles (CuNPs) and their oxides, having a broad-spectrum biocide effect, has been reported in studies of growth inhibition of bacteria, fungi, and algae [21–23]. Akhtari and Ganjipour [24] investigated and compared the effects of three nanometals (copper, silver, and zinc oxide) on the resistance of *Paulownia fortunei* against white rot fungus. Their results showed, with a nanoparticle solution of 400 ppm with sizes between 10 to 80 nm and a chemical retention of 0.14 kg/m^3, that nano-copper, nano-silver, and nano-zinc oxide significantly increased Paulownia's resistance to decay against *Coriolus versicolor* by reducing wood weight loss from 28% to 2%. Bak and Németh [25] evaluated the efficacy of five different nanoparticles, including copper and copper borate, in two different wood species (beech and pine sapwood) exposed to the attack of *Coniophora puteana* and *C. versicolor* fungi. The concentration of the basic copper nano-suspension was 2% (m/m). The authors indicated that copper nanoparticles showed great potential as an effective treatment, but at higher concentrations (over 2% m/m).

Radiata pine (*Pinus radiata* D. Don) is the main wood species used in the construction industry in Chile. Currently, the area planted with this species is 2 million hectares and has a total availability of sawnwood of 8 million m^3, of which 78% is consumed in the country [26]. Radiata pine has been classified as a non-durable wood species [27]. Thus, according to the requirements the Chilean General Law for Urban Planning and Construction, radiata pine wood used in construction must be preserved. Therefore, the aim of this study was to investigate the impregnation of copper nanoparticles in terms of efficiency and penetration and its protective effect against wood rot fungi. The copper nanoparticle treatments were applied by impregnation (Bethell process) in radiata pine sapwood. A leaching study of the CuNPs-impregnated wood and a decay resistant test following the wood protection from the preservative against two brown rot fungi were conducted.

2. Materials and Methods

2.1. Materials

Radiata pine sapwood samples, free from cracks, knots, stain, or other defects and with at least three growth rings in the cross-section of each specimen, were prepared. The copper nanoparticle (CuNP) solution was provided by Nano Process Company (Antofagasta, Chile). CuNPs were characterized by a transmission electron microscope (TEM) and their size, polydispersity index, and z-potential were measured using a Zeta sizer (model ZS 90, Malvern Panalytical Ltd., Malvern, UK).

2.2. Wood Impregnation

Wood impregnation was performed in a pressure vessel at laboratory scale using the Bethell process (empty cell), simulating an industrial process. Wood samples were impregnated with CuNP solutions containing 1 g/L and 3 g/L [23], referred to in this study as group A and group B, respectively. Each sample set of 30 probes, placed within the securely closed impregnating vessel to prevent any leaking, was initially vacuumed to

−20 mmHg for 30 min and flooded with the assigned copper nanoparticle solution. Next, for 90 min, an impregnation pressure of 6 bar on the wood samples was applied. Finally, after the impregnation was ended and emptied from the remaining solution, a vacuum for 15 min at −20 mmHg was applied to remove excess solution. The retention for each treatment solution was calculated using Equation (1):

$$R = \frac{G \times C}{V} \times 10 \ (\mathrm{kg/m^3}) \tag{1}$$

where R is the retention ($\mathrm{kg/m^3}$), G is the treating solution absorbed by the samples (g), C is the concentration of the treating solution (%), and V is the volume of the samples.

It is known that CCA fixation is achieved after 48 h of storage in air following the ending of the impregnation process [28]. In our case, there is no information available about the CuNP fixation after the impregnation process in wood; therefore, a longer storage period of 72 h at a temperature between 20 and 25 °C was considered prior to the subsequent tests. To analyze the behavior of the impregnated nanoparticles in the wood, a penetration test was performed [29], which consists of a colorimetric test staining with chromium azurol solution, which is applied in the internal cross-section of the impregnated specimen.

2.3. Leaching Test and Copper Analysis during Leaching

Leaching tests are commonly used as a standalone indicator test for permanence of preservative components in treated wood. The leaching test was performed according to AWPA E11–16 [30]. Six cubes of treated wood samples were impregnated with 300 mL of distilled water, after 6, 24, and 48 h, and thereafter at 48-h intervals, the leachate was removed and replaced with fresh distilled water. For each treatment, the leachate was collected after the vacuum impregnation, after the 1st day of leaching and subsequently up to the 14th day for copper analysis.

The amount of copper in the leached solution was measured in an atomic absorption spectrometer (AAS) (Model ICE 3300, Thermo Scientific, Waltham, MA, USA) using the primary Cu spectral line of 324.754 nm. Each leached solution was previously attacked in acid digestion with approximately 10 mL of 70% HNO_3 and then treated with HCl (5 wt.%) for subsequent measurement in AAS. Analyses were performed in triplicate for each sample.

2.4. Copper Retention in Wood by Energy Dispersive X-ray Fluorescence Analysis (EDXRF)

The copper retention of each group, in impregnated and leached samples, was determined according to the procedure of AWPA A9–18 [31]. For the EDXRF calibration, radiata pine wood in powdered form was utilized as the solid matrix. Five calibration points were prepared using a copper standard solution (Centipur®, Merck, Darmstadt, Germany), which was diluted in water and mixed manually with the solid matrix. The mixture was dried to a constant weight to remove water content. Then, each point of the calibration curve was homogeneously pulverized, and 3 g of each sample was placed in a 32 mm XRF sample holder of open plastic double rings with Mylar® film on the base in plastic cylindrical XRF sample cups. Then, a low pressure of 250 in-lbs was applied on the sample for 30 s. The Cu contained in the samples was analyzed using an EDXRF spectrometer (Rigaku Nex QC, Austin, TX, USA).

Each wood sample, impregnated and leaching, was chipped by hand, milled in a knife mill and sieved to 30 mesh. The sample in powder form was dried in an oven at 105 °C to constant weight, then cooled in a desiccator. If the treated sample is not immediately irradiated, it should be kept in a desiccator to avoid variations in moisture content. Each sample was placed in a 32 mm XRF sample holder with Mylar® film, pressed, and measured in EDXRF.

2.5. Decay Test

The impregnated, leached, and untreated (control) radiata pine sapwood probes were exposed to fungal attack; hence, a wood decay test was performed following the general guidelines of EN 113 [32], with slight modifications. Two brown rot fungi (BRF) *Gloeophyllum trabeum* (Mad-617-R) and *Rhodonia placenta*, previously *Poria placenta* (Mad-698-R), were used. Both fungal stock cultures (deposited in the Bioprocess and Biotreatment Laboratory Culture Collection at Universidad del Bío-Bío) were maintained at 4 °C in PDA plates until use.

2.5.1. Fungal Activation

Fungal activation was performed in a three-step sequence as follows: first, a fungal 3-week pre-growth period for each species was performed, using sterile *Pinus radiata* sapwood wafers (35 mm $\times$ 25 mm $\times$ 3 mm), as a sole carbon source, placed in 20 cm petri dishes with 20 mL of agar (18 g/L) at room temperature (22 $\pm$ 1 °C). Then, a 5 mm diameter disk from that actively growing mycelium was transferred aseptically to 10 cm PDA petri dishes and incubated for 1 week a 22 $\pm$ 1 °C. Finally, a 5 mm diameter disk, of the latter actively growing mycelium, was transferred aseptically as the inoculum for MDA flasks (500 mL glass flasks with 10 mL MDA: 15 g/L malt extract + 10 g/L agar), and incubated for 2 weeks at 22 $\pm$ 1 °C.

2.5.2. Wood Decay Test

Treated and untreated probes were vacuum re-hydrated by immersion in distilled water for 2 min, autoclaved (121 °C, 1.5 atm), and placed under sterile conditions (laminar flow), inside of the formerly described MDA flasks, containing the 2-week pre-grown fungi in MDA (see Section 2.5.1). All these flasks were incubated for 16 weeks at 22 $\pm$ 1 °C. After incubation, all superficial mycelial growth was thoroughly removed from the probes. Probes were then oven-dried at 103 $\pm$ 2 °C to constant weight. Wood mean percent mass losses, ML%, of treated probes were calculated as shown in Equation (2).

$$\mathrm{ML}\ \% = \frac{\mathrm{m_0} - \mathrm{m_i}}{\mathrm{m_0}} \times 100 \tag{2}$$

where $\mathrm{m_0}$ is the probe dry weight before fungal exposure, and $\mathrm{m_i}$ is the probe dry weight after fungal attack.

2.6. Data Analysis

A descriptive analysis of mean and standard deviation was performed for the retention data and the fungal decomposition test. ANOVA was applied to verify the effect of treatment with CuNPs. Tukey's test was established with a 95% confidence level to determine the statistical difference between means.

3. Results and Discussion

3.1. CuNPs Characterization and Their Performance in Wood Impregnation

The CuNPs presented a homogeneous size and morphology as observed in the TEM image (Figure 1). The average diameter was 26.41 $\pm$ 1.32 nm; therefore, the copper nanoparticles were only in the nano-range. The diluted CuNPs had a mean z-potential of -15.0 ± 0.4 mV, indicating that the suspensions tend to aggregate.

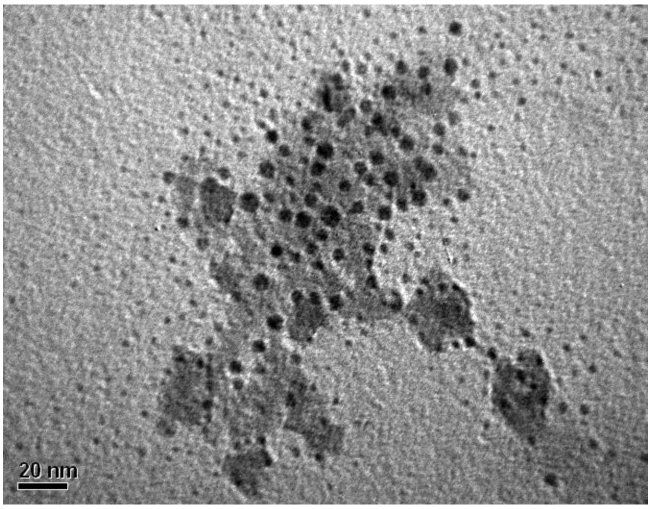


Figure 1. Transmission electron microscope image of copper nanoparticles used in this study.

An important feature of NPs is that they can be produced with a controlled particle size and have a demonstrated high dispersion stability; on the other hand, particle size in the range of 1–100 nm can improve the penetrability of chemical compounds (biocides) in wood. [33–35]. The degree of penetration and uniformity of distribution of particles into wood cellular structures is inversely related to the prevalence of large size particles [33]. Matsunaga et al. [36] found that copper nanoparticles can pass through southern pine pores in bordered pit membranes (an average diameter of 300–4000 nm) and even penetrate the cell walls.

The penetration depth of the preservative should also be taken to determine whether the wood has been well treated [3,37–39]. In this study, the performance of the impregnation of radiata pine wood with CuNPs was evaluated by means of penetration tests. The coloration test is a quick technique to visualize the behavior and dispersion of the CuNPs inside the wood cross-section, given the color change from transparent to red (low concentration impregnated) and/or blue (high concentration impregnated). In addition, the wood treated can be classified according to the penetration obtained, uses scales ranging from regular total penetration to zero penetration are used [40]. The results of penetration for both concentrations of copper nanoparticles are observed in Figure 2. In the group A samples, an Irregular Total Penetration (some red zones) is visualized, since there are very small sections in the penetrated area with areas of higher concentration. And other hand, in the group B samples (3 g/L) a Total Penetration is observed, where the sample is penetrated with a uniform concentration (blue color in all transversal section). In this study, it was expected that CuNPs would present a uniform distribution in the impregnated wood, given the average size of the synthesized nanoparticles (26.41 nm); they presented a polydispersity index (PDI) of 0.375, confirming their uniformity.

(**a**) (**b**)

Figure 2. Photographs of interior cross-section of impregnated wood samples with CuNPs with chromium azurol staining. (**a**) Group A sample impregnated with 1 g/L CuNPs and (**b**) group B sample impregnated with 3 g/L CuNPs.

3.2. Retention and Leachability of CuNPs in Radiata Pine Wood

Retention expressed as kilograms of preservative per cubic meter of wood, is one measurement used to estimate the degree of protection provided by a treatment, [38]. Cu retention in impregnated wood increased proportionally with nanoparticle concentration (Figure 3). It was observed that in impregnated wood samples, the CuNP retention from concentration in water solution was 0.64 kg/m^3 and 2.08 kg/m^3 for groups A and B, respectively. Figure 4b,d shows photographs of the tangential section of impregnated woods of groups A and B, respectively, where a color change to green can be observed with respect to the control sample. As expected, the green color was more prominent in samples treated with the solution with a higher concentration of CuNPs (group B).

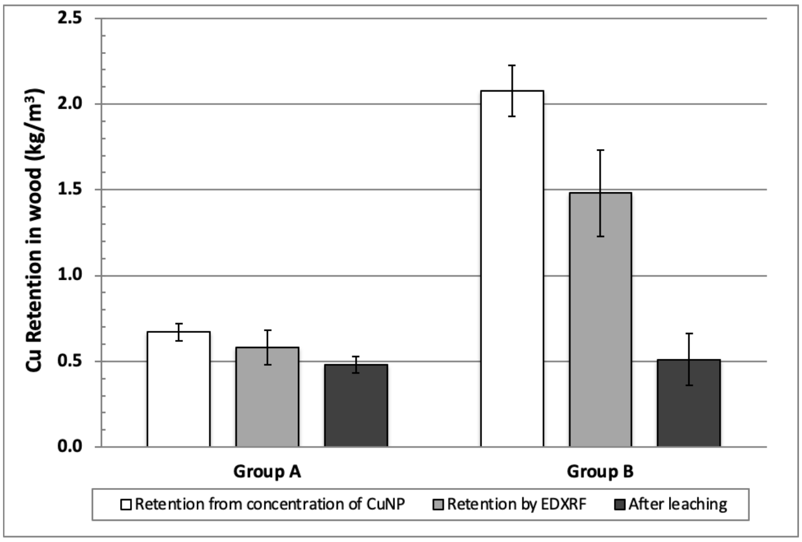


Figure 3. Copper retention in radiata pine wood impregnated with CuNPs. Group A woods were treated with a 1 g/L solution of CuNP and group B woods were treated with a 3 g/L solution of CuNP. White bars correspond to Cu retention in wood from the concentration of CuNPs in the solution, gray bars correspond to Cu retention in wood impregnated by EDXRF, and black bars correspond to Cu retention in wood leached by EDXRF.

Similar values have been reported in the literature for wood impregnation with CuNPs. Pařil et al. [23] impregnated Scots pine sapwood with copper and silver nanoparticles. They reported retention values of 0.71 kg/m^3 for samples impregnated with 1 g/L CuNP solution, and 2.08 kg/m^3 for samples impregnated with 3 g/L CuNP solution (values determined from the respective aqueous solutions). They also reported that the average particle size of copper used in the treatments was 16.5 nm. It has been reported in the literature that there is an advantage in the use of nanoproducts in wood impregnation, given their ability to enter deeply into the wood structure, hence improving the penetration and retention of the nanoparticle solution used as a preservative [41,42].

Copper content in the impregnated wood samples was determined by EDXRF: the values obtained were of 0.57 kg/m^3 and 1.68 kg/m^3 for groups A and B, respectively (grey bars, Figure 3). These values were lower than the retention values calculated from the CuNP concentration solution (white bars, Figure 3), since they are considered as the absorption values of the preservative solution in the wood block after impregnation [43]. The use of the EDXRF-based technique allows several trace elements to be measured simultaneously, and the retention values are calculated based on instrumental calibration, calculation of the incident intensity spectrum, sample density calculations, and element concentration calculations [44]; for this reason, EDXRF is a technique strongly recommended for the wooden sector [45].

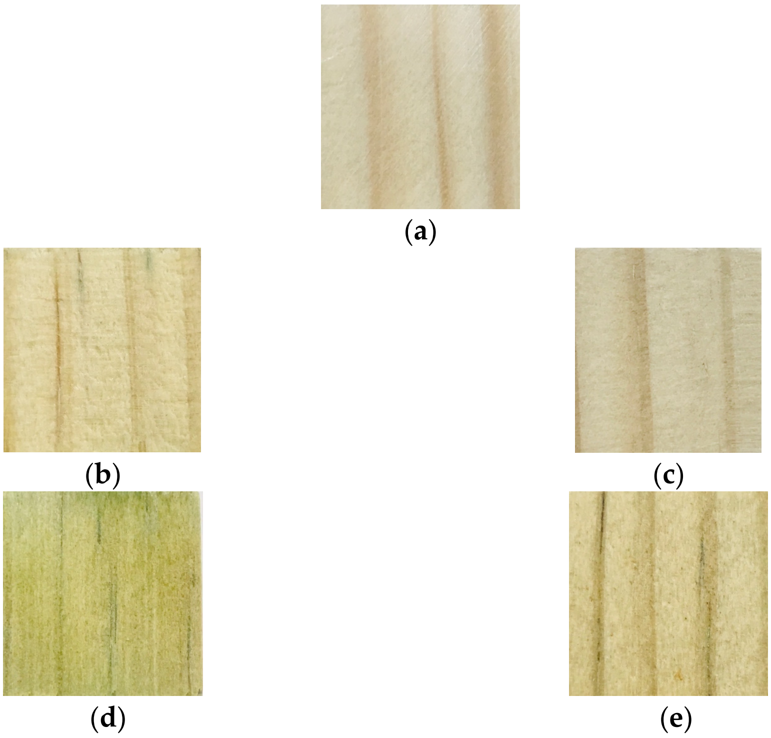


Figure 4. Photographs of control, impregnated, and leached radiata pine wood samples. (**a**) Control sample; (**b**) group A, wood impregnated with CuNP 1 g/L and (**c**) leached; (**d**) group B, wood impregnated with CuNP 3 g/L and (**e**) leached.

Studying the leaching of the nanoparticles from the wood material is an important aspect that must be considered, since it determines the subsequent efficiency of the wood put into service. After the leaching test on the impregnated woods, the amount of copper retained in the wood was determined by EDXRF; values of 0.48 kg/m^3 and 0.51 kg/m^3 were observed for samples of groups A and B, respectively (Figure 3), and no significant differences ($p < 0.05$) were observed between the results. As in the impregnated samples, the leached wood samples showed a color change with respect to the control wood, but with less intensity, evidencing the leaching of copper from the wood block (Figure 4c,e). Figure 5 shows the amount of copper leached from the impregnated wood, present in the test water, in both groups over time. It is evident for both groups that the amount of copper leached was highest in the first few hours of the test with a constant leaching rate up to 144 h and subsequently decreased with time. Similar trends throughout the leaching process have been reported by Thaler and Humar [46] for copper-based preservatives in *Picea abies* (L.) Karst and reported by Pařil et al. [23] for copper nanoparticles in *Pinus sylvestris* L. These authors agree that the accessibility of the deposited copper to a higher concentration in the surface layer of the wood is considered the main reason for the high initial values in the leaching process. Temiz et al. [12] comment that conventional leaching tests (AWPA E11 and EN 84) are very aggressive compared to natural rainfall exposure and/or normal wood sample size in service, so they should not be considered representative of reality, but only as reference values on the permanence of preservatives in treated wood. In general, the leaching and mobility of metallic components depend on different factors, such as exposure time, wood volume and water flow, and the exposed surface [47,48].

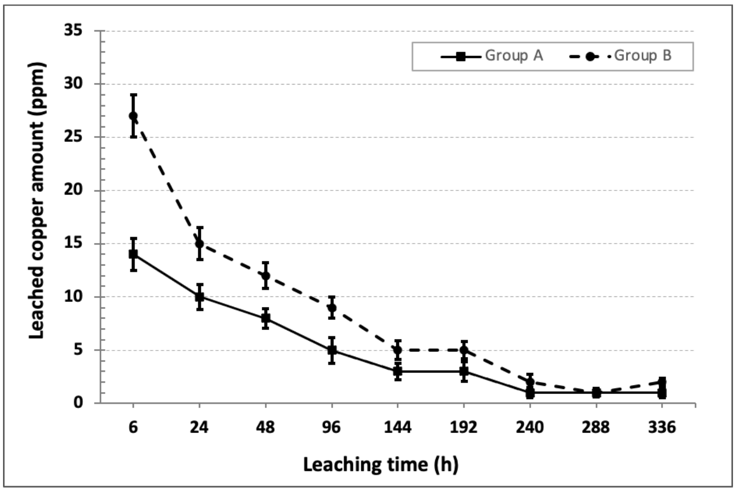


Figure 5. Element amount leached out from radiata pine wood samples.

3.3. Biodeterioration Assay Analysis

Mass loss of impregnated and leached wood samples due to brown-rot fungus (exposed to either *G. trabeum* or *R. placenta*) are presented in Figure 6. The mass loss (ML) due to fungal decay was significantly reduced for the impregnated woods, independently of the solution concentration or fungus specie. For samples impregnated with CuNPs, ML values of 2.70% and 1.19% against *G. trabeum* were obtained in both group A and B, respectively (1 and 3 g/L), and ML values of 4.02% and 0.70% against *R. placenta* were obtained in both group A and B, respectively. According to EN 113, the appropriate value of mass loss in wood after the biodeterioration test must be less than 3%; therefore, group A samples against *R. placenta* are out of range (ML 4.02%).

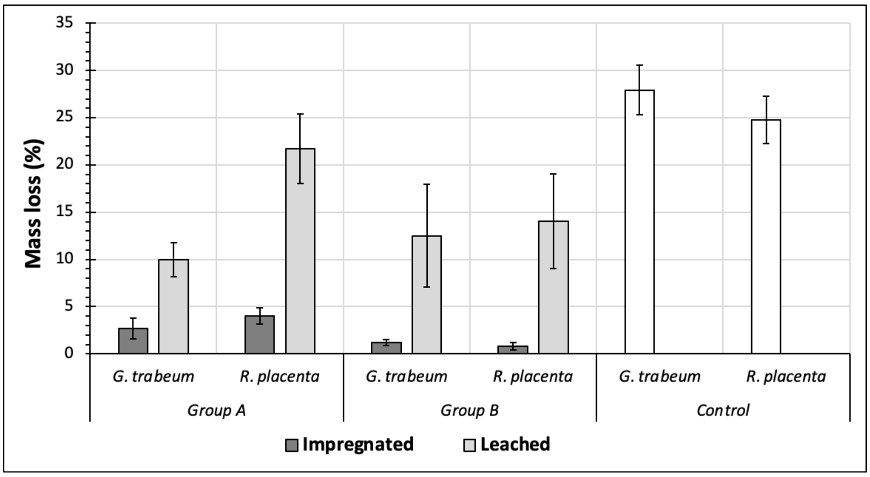
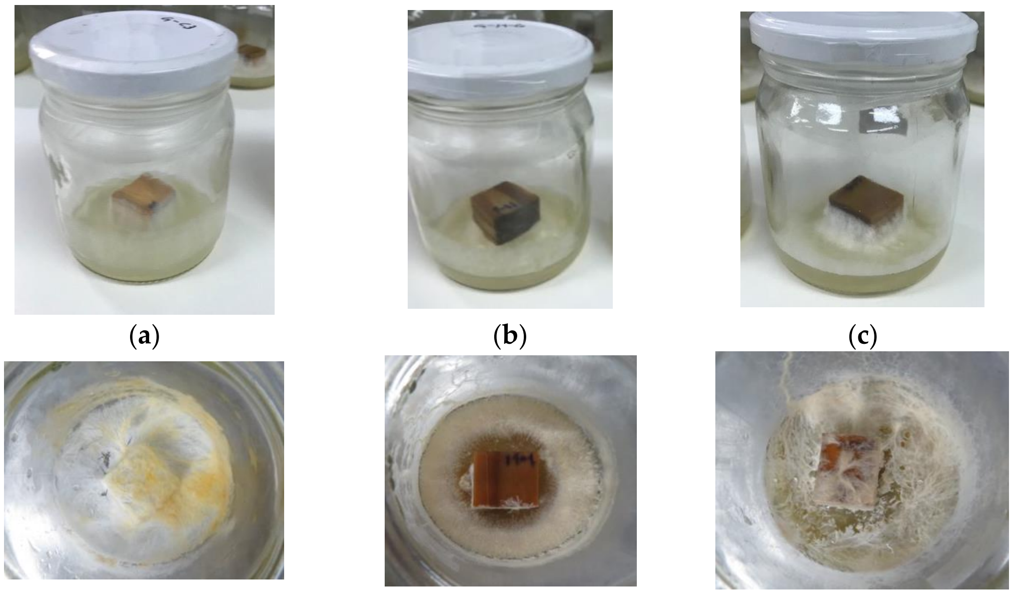


Figure 6. Mass loss, expressed in percent, in impregnated and leached specimens of radiata pine wood treated with CuNP after exposure to *Gloeophyllum trabeum* and *Rhodonia placenta*. The maximum standard error for mass loss is 3.6%.

In general, brown rot fungi represent approximately 10% of all wood rot fungi and primarily attack softwoods [49]. Hence, mainly brown-rot is found in planted coniferous forests and in naturally occurring conifer ecosystems as well. Several studies have indicated that copper tolerance is associated with brown-rot fungi [23,34,50–52] and biodeterioration on CuNP-impregnated wood, which presented mass loss values similar to those reported in this study. Kartal et al. [34] reported that nano-copper impregnation of southern yellow pine wood significantly improved resistance to decay against *G. trabeum*, with a mass loss smaller than 10%. In another study, reported in Scots pine wood treated with copper nanoparticles against *R. placenta* (reported at that time as *P. placenta*), and at the same concentrations to those of this study (1 and 3 g/L), a mass loss smaller than 5% was found [23].

Due to their biocidal activity, some metals can be toxic to most microorganisms at low concentrations. Despite this long-standing evidence, the mechanisms that explain such toxic effects are not yet fully elucidated. However, depending on the metal property, biocidal behavior can be triggered by the reduction potential of the metal and by the selectivity of the metal donor atom and/or speciation [53]. In some cases, fungi may not be able to recognize copper nanoparticles. Once the nanoparticles enter the fungal cell wall, they form reactive oxygen species within the fungal cell. In addition, nanoparticles can also undergo dissolution and thus interfere with homeostatic processes within the fungal cell [53,54]. In this way the nanoparticles in a biocidal solution may present a higher effectiveness compared to other particles. Indeed, at the cellular level Karlsson et al. [55] have indicated that micrometer-sized metallic copper does not cause cell damage, as compared to copper nanoparticles in lung cells.

Photographs of the radiata pine samples, shown in Figure 7, display the CuNPs' effect on the colonization rate at 4 weeks (Figure 7b,e) and 16 weeks (Figure 7c,f) of exposure to the *G. trabeum* fungus in both the impregnated and leached samples. Moreover, the leached samples (Figure 7a,c) presented an almost comparable mycelial growth to that observed in the control samples (Figure 7d,f).



(**a**) (**b**) (**c**)

(**d**) (**e**) (**f**)

Figure 7. Photographs of radiata pine samples exposed to biodeterioration. (**a**,**d**) control sample; (**b**,**e**) sample impregnated with CuNPs; and (**c**,**f**) leached sample. Images (**a**–**c**) correspond to samples at week 4 of exposure to *G. trabeum*. Images (**d**–**f**) correspond to samples at week 16 of exposure to *G. trabeum*.

After leaching, samples exposed to biodeterioration compared to non-leached samples presented a higher ML. The impregnation effectiveness of the treatment was significantly reduced for both groups and the tested fungi (Figure 6, gray bars). ML in samples of groups A and B inoculated with *G. trabeum* were 10.5% and 12.5%, respectively, and ML in samples of groups A and B inoculated with *R. placenta* were 22.4% and 14.0%, respectively. The low CuNP fixation on the leached samples, due to the removal of a large part from the treated wood, led to an increase in ML after biodeterioration tests. Freeman and McIntyre [33] indicated that copper-based biocide fixation during the wood impregnation treatment occurred by simple deposition on the wood fiber and not by the formation of a covalent bond. Therefore, there is a portion of free Cu highly available for leaching once the wood is put into service. ML in samples from group A as compared to its control, both inoculated with *R. placenta*, with 22.4% and 24.7%, respectively, was non-statistically significant. These results show that the preservation treatment is effective only in non-leached CuNP treated wood, where mass loss in samples of groups A and B, both inoculated with *G. trabeum* and *R. placenta*, was lower than 5%.

4. Conclusions

In this work, the behavior of radiata pine wood impregnated with a copper nanoparticle solution was investigated by leaching analysis and the antifungal effect against two brown rot fungi was determined. Samples impregnated with higher copper concentration exhibited, as observed by EDXRF, higher copper retention. The penetration analysis confirmed a uniform copper distribution inside the wood. The leached copper amount from wood as a function of time showed the same trend in both copper concentrations used, where the highest content of elements removed was observed up to 144 h of the leaching test. Furthermore, it was demonstrated that copper nanoparticles in both concentrations are effective against the tested fungi, obtaining ML values lower than 5%. Additionally, in their respective leached samples, ML was close to 10%. The challenge for future research is to decrease the impregnating solution leachability through the fixation of the copper particles inside the wood, and thus improve the performance of the impregnated wood as a final product.

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Conflicts of Interest: The authors declare no conflict of interest.

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