Assessing the effectiveness and environmental risk of nanocopper-based wood preservatives

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ASSESSING THE EFFECTIVENESS AND ENVIRONMENTAL RISK OF NANOCOPPER-BASED WOOD PRESERVATIVES

A thesis submitted to attain the degree of

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2016
To Aunt Gina and Tenia
Because I miss you so much
Abstract

Nanotechnology is revolutionizing our lives, from consumer products to medicine, electronics, and building materials. In fact, nanoparticles are recognized to have properties that differ from their bulk counterparts. Despite the potentials of nanoparticles, their innovative properties may also cause specific nano risks. These occur under two circumstances: if nanoparticles are hazardous, and if humans and the environment are exposed to them.

In wood protection, nanomaterials have recently been developed to outperform conventional wood preservatives and lengthen the service life of timber structures. These nanoparticles-based wood preservatives are best known as “micronized copper”, which are composed of basic copper carbonate nanoparticles and a second organic biocide.

Copper-based nanoparticles are frequently associated with adverse health effects, however the environmental fate of micronized copper -which is already present in the market- is not clear yet. In this thesis, we investigate whether the use of nanoparticles in micronized copper used for wood protection has an added value, and we monitor, quantify, and visualize the fate of copper from its wood preservative formulation stage, to the impregnation of wood, and ageing of the structures. The majority of the studies on wood preservatives remobilization in the environment deal with the release of copper in water and soil. Here, we mostly focus on the air compartment. In fact, lungs are considered as the main entry portal for nanoparticles and inhalation of copper-based nanoparticles from wood preservatives may cause adverse health effects. In particular, two possible release pathways were assessed: wood dust produced by mechanical abrasion of treated wood surfaces, and release via spores of copper-tolerant wood-destroying basidiomycete fungi that colonize treated wood. In addition, preliminary in vitro cytotoxicity tests were conducted on lung epithelial cells and macrophages, representing a simplified human lung, to assess the potential acute hazard of wood dust from micronized copper-treated wood, and compare it with wood dust generated from wood treated with a conventional wood preservative.

On the basis of the experimental results, micronized copper effectively protected the wood from soilborne fungi and wood-destroying basidiomycetes, and homogeneously penetrated into easily treatable wood species. However, the treatment is not effective against copper-tolerant wood-destroying fungi, and refractory wood species are only poorly treated. Copper can be released in the air both via wood dust and spores, but not in a nanoparticulate form. Therefore, micronized copper does not pose a specific nano risk. Furthermore, the preliminary cytotoxicity assessment did not reveal a specific nano hazard. Further, the possibility of adverse health effects related to copper is likely only a risk for woodworkers, which are continuously exposed to wood dust.
Sommario

Le nanotecnologie stanno rivoluzionando le nostre vite, dai prodotti di consumo a medicina, elettronica e materiali da costruzione. Infatti, le nanoparticelle hanno proprietà che differiscono rispetto ai loro corrispettivi macroscopici. Nonostante le potenzialità delle nanoparticelle, le loro proprietà innovative possono anche causare specifici nano rischi. Questi possono verificarsi qualora le nanoparticelle fossero tossiche e l’uomo o l’ambiente fossero esposti a queste.

Nel campo della protezione del legno, nanomateriali sono stati recentemente sviluppati al fine di migliorare i preservanti convenzionali presenti e garantire una maggior durata delle strutture lignee. Questi preservanti a base di nanoparticelle sono meglio conosciuti come “rame micronizzato” e sono composti da particelle di carbonato di rame associate ad un secondo biocida organico.

Le nanoparticelle a base di rame sono spesso associate a esiti clinici avversi, tuttavia il destino ambientale del rame micronizzato -già presente nel mercato- non è ancora chiaro. In questa tesi viene valutato se l’uso di nanoparticelle contenute nel rame micronizzato ha un valore aggiunto al fine di proteggere il legno e viene monitorata, quantificata e visualizzata la sorte del rame a partire dallo stadio di formulazione come preservante, all’ impregnazione del materiale ligneo, fino all’ invecchiamento del materiale. La maggior parte degli studi sulla remobilizzazione dei preservanti nell’ambiente considera solamente l’ emissione di rame nell’ acqua e nel suolo. Qui viene considerato principalmente il rilascio nell’aria. Infatti i polmoni sono considerati come il principale portale per l’ ingresso di nanoparticelle e l’ inalazione di nanoparticelle a base di rame a causa dell’ uso di rame micronizzato può avere conseguenze negative sulla salute. In particolare, due possibili scenari per l’ emissione di nanoparticelle sono stati considerati: tramite polvere di legno generata dall’ abrasione meccanica di superfici lignee trattate e tramite spore di basidiomiceti rame-tolleranti in grado di colonizzare il legno trattato. In aggiunta, studi citotossicologici preliminari sono stati condotti in vitro su cellule epiteliali e macrofagi, al fine di riprodurre in maniera simplificata il polmone umano e valutare la potenziale tossicità acuta della polvere di legno generata da legno trattato con rame micronizzato e compararla con quella proveniente da legno trattato con preservanti convenzionali.

Sulla base dei dati sperimentali, il rame micronizzato protegge in maniera efficace da carie soffici e basidiomiceti, inoltre penetra in modo omogeneo in legni facilmente trattabili. Tuttavia, il trattamento non risulta efficace contro le carie del legno rame-tolleranti. Pergiunta, specie legnose refrattarie non risultano facilmente trattabili. Il rame può essere remobilizzato nell’ aria tramite polvere del legno e spore, ma non in forma nanoparticellare. Di conseguenza, il rame micronizzato non pone un nano rischio specifico. Inoltre, è probabile che gli effetti negativi sulla salute si possano verificare solamente in soggetti esposti in maniera prolungata alla polvere di legno, come falegnami.
Acknowledgments

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>% w/w</td>
<td>Mass Percent</td>
</tr>
<tr>
<td>ζ</td>
<td>Zeta</td>
</tr>
<tr>
<td>ABTS</td>
<td>2,2-Azino-Bis(3-ethylbenzoThiazoline-6-Sulfonic acid)</td>
</tr>
<tr>
<td>ACQ</td>
<td>Alkaline Copper Quaternary</td>
</tr>
<tr>
<td>ANOVA</td>
<td>ANalysis Of Variance</td>
</tr>
<tr>
<td>APS</td>
<td>Aerodynamic Particle Sizer</td>
</tr>
<tr>
<td>CC</td>
<td>Chromated Copper</td>
</tr>
<tr>
<td>CCA</td>
<td>Chromated Copper Arsenate</td>
</tr>
<tr>
<td>CdSO₄</td>
<td>Cadmium Sulfate</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon Dioxide</td>
</tr>
<tr>
<td>CPC</td>
<td>Condensation Particle Counter</td>
</tr>
<tr>
<td>CrO₃</td>
<td>Chromium Trioxide</td>
</tr>
<tr>
<td>CT</td>
<td>Computed Tomography</td>
</tr>
<tr>
<td>Cu</td>
<td>Copper</td>
</tr>
<tr>
<td>Cu²⁺</td>
<td>Divalent Copper</td>
</tr>
<tr>
<td>CuCl₂</td>
<td>Copper Chloride</td>
</tr>
<tr>
<td>CuCO₃·Cu(OH)₂</td>
<td>Basic Copper Carbonate</td>
</tr>
<tr>
<td>CuSO₄</td>
<td>Copper Sulfate</td>
</tr>
<tr>
<td>CuSO₄·5H₂O</td>
<td>Copper Sulfate Pentahydrate</td>
</tr>
<tr>
<td>Cys</td>
<td>Cysteine</td>
</tr>
<tr>
<td>DCF</td>
<td>2',7'-DiChloroFluorescein</td>
</tr>
<tr>
<td>DMA</td>
<td>Differential Mobility Analyzer</td>
</tr>
<tr>
<td>Empa</td>
<td>Eidgenössische MaterialPrüfungsAnstalt (Swiss Federal Laboratories for Materials and Technology)</td>
</tr>
<tr>
<td>EN ***, ENV ***</td>
<td>European Standard, followed by number</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked ImmunoSorbent Assay</td>
</tr>
<tr>
<td>ETH</td>
<td>Eidgenössische Technische Hochschule (Swiss Federal Institute of Technology)</td>
</tr>
<tr>
<td>EXAFS</td>
<td>Extended X-ray Absorption Fine Structure</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal Calf Serum</td>
</tr>
<tr>
<td>Fig.</td>
<td>Figure</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s Balanced Salt Solution</td>
</tr>
<tr>
<td>H₂DCF-DA</td>
<td>2',7'-DiChlorodiHydroFluorescein-DiAcetate</td>
</tr>
<tr>
<td>HNO₃</td>
<td>Nitric Acid</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen Peroxyd</td>
</tr>
<tr>
<td>HSD</td>
<td>Honestly Significant Difference</td>
</tr>
<tr>
<td>ICP</td>
<td>Ion-Coupled Plasma</td>
</tr>
<tr>
<td>K₂Cr₂O₇</td>
<td>Potassium Dichromate</td>
</tr>
<tr>
<td>LCA</td>
<td>Life Cycle Assessment</td>
</tr>
<tr>
<td>LCIA</td>
<td>Life Cycle Impact Assessment</td>
</tr>
<tr>
<td>LPS</td>
<td>LipoPolySaccharides</td>
</tr>
<tr>
<td>MC</td>
<td>Micronized Copper</td>
</tr>
<tr>
<td>MCA</td>
<td>Micronized Copper Azole</td>
</tr>
<tr>
<td>MCA_HTBA</td>
<td>Micronized Copper Azole with high amount of Tebuconzole</td>
</tr>
<tr>
<td>MCA_LTBA</td>
<td>Micronized Copper Azole with low amount of Tebuconzole</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>MTS</td>
<td>3-(4,5-diMethylThiazol-2-yl)-5-(3-carboxymethoxy phenyl)-2-(4-Sulfophenyl)-2H</td>
</tr>
<tr>
<td>MWCNT</td>
<td>Multi-Walled Carbon NanoTube</td>
</tr>
<tr>
<td>NTA</td>
<td>Nanoparticle Tracking Analysis</td>
</tr>
<tr>
<td>NP</td>
<td>Nanoparticle</td>
</tr>
<tr>
<td>OES</td>
<td>Optical Emission Spectrometry</td>
</tr>
<tr>
<td>PM</td>
<td>Particulate Matter</td>
</tr>
<tr>
<td>pH</td>
<td>Potential Hydrogenium</td>
</tr>
<tr>
<td>RH</td>
<td>Relative Humidity</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>S1</td>
<td>Outer Secondary Wall</td>
</tr>
<tr>
<td>S2</td>
<td>Central Secondary Wall</td>
</tr>
<tr>
<td>S3</td>
<td>Inner Secondary Wall</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning Electron Microscopy</td>
</tr>
<tr>
<td>SMPS</td>
<td>Scanning Mobility Particle Sizer</td>
</tr>
<tr>
<td>SYP</td>
<td>Southern Yellow Pine</td>
</tr>
<tr>
<td>TBA</td>
<td>Tebuconazole</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission Electron Microscopy</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor Necrosis Factor Alpha</td>
</tr>
<tr>
<td>TTM</td>
<td>Ammonium TetraThioMolybdate</td>
</tr>
<tr>
<td>UV</td>
<td>UltraViolet</td>
</tr>
<tr>
<td>XAS</td>
<td>X-ray Absorption Spectroscopy</td>
</tr>
<tr>
<td>XANES</td>
<td>X-ray Absorption Near Edge Structure</td>
</tr>
</tbody>
</table>
1. Introduction
1.1 Research question

The following dissertation focuses on the wood preservative “micronized copper” (MC): its effectiveness and possible remobilization in the air compartment.

Copper is an essential biocide for wood in contact with the soil (EN 335: use class 4) [1], due to its unique ability to inhibit soft rot and other soilborne fungi, thus it is considered to be a mainstay in wood protection [2]. However, the field of wood preservation is constantly developing new formulations, in order to overcome different issues: homogeneous penetration of wood preservatives into the wood structure, especially in poorly permeable (refractory) wood species; protection against wood-destroying fungi, especially if tolerant to Cu or azoles; reduced toxicity and risk for human health and the environment. The latest innovation in wood preservative formulation is best known as MC.

MC wood preservatives are composed of basic Cu carbonate (CuCO$_3$·Cu(OH)$_2$) particles with sizes that range from 1 nm to 250 µm and an organic co-biocide (either azoles or quaternary ammonium compounds) [3, 4]. MC is believed to have an added value compared to conventional wood preservatives, due to its novel impregnation chemistry, which should facilitate the impregnation of refractory wood species, e.g. Norway spruce, White fir, and Douglas fir, and provide a long-term protection due to the presence of a Cu reservoir, that constantly releases bioavailable Cu$^{2+}$ ions in wood against wood-destroying fungi [5].

Despite the misleading name and the broad particle size range, as measured in different studies [6, 7], MC is actually a nanomaterial according to the definition from the European Commission [8] recommendation that describes it as “a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm - 100 nm. In specific cases and where warranted by concerns for the environment, health, safety or competitiveness the number size distribution threshold of 50 % may be replaced by a threshold between 1 and 50 %.”. In addition, within the biological sciences larger particles can exert similar effects and are therefore also classified as nanomaterials [9].

Since its introduction in 2006, MC has developed into the largest end uses for wood products all over the world [10]: it is currently used in more than 75% of residential lumbers in the US [11] and a total of approximately 20 million m$^3$ of wood is treated worldwide [12]. This makes MC one of the largest scale commercial applications for nanotechnologies [10]. At present, MC systems are not used for wood protection in Europe, however, wood preservative companies are currently testing MC formulations to
seek approval in different European countries, e.g. France, Germany and Switzerland. The North American and European wood decking market greatly differ in term of wood species used. While the first mostly utilizes Southern yellow pine (SYP, like Pinus caribaea, Pinus echinata, Pinus palustris, and Pinus taeda), Central Europe mainly uses Norway spruce (Picea abies) and White fir (Abies alba), which are refractory to treatment. Thus, as the penetration and efficacy of MC in SYP or Norway spruce are not likely to be comparable, the investigation of these two aspects is fundamental.

Nanoparticles (NPs) and MC among them, are not dangerous per se, but despite their potential, the current knowledge on the human and environmental risks is limited. This can emerge if both exposure and hazard are observed. As the development and use of MC for wood protection advances, an increase in MC production and worldwide diffusion is likely to lead to an increase in the intentional and/or unintentional release of Cu-based NPs in the environment, and a subsequent increase in human and environmental exposure is a realistic scenario. NP risk is a function of NP hazard and NP exposure [13], and there is evidence for Cu-based NPs toxicity towards aquatic organisms [14–19], terrestrial plants [20], mammals [21-26], and humans [27–32]. Therefore, it is extremely important to determine if and how much humans and the environment could be exposed to Cu-based NPs if they are released from MC-treated wood. In particular, the respiratory tract is considered as the most sensitive entry portal for nanoparticles into the human organism [13, 33]. To date, the environmental fate of conventional wood preservatives and MC has mainly focused on leaching into the soil and water compartments, neglecting possible releases in the air. Due to these two facts, the current research monitored the fate of Cu-based NPs from MC formulation, to their penetration and interaction with wood, and finally their possible release into the air compartment. Two possible release pathways were investigated: the release during mechanical abrasion of treated wood, and copper uptake and dissemination by fungal spores. Human exposure pathway to copper and other toxic metals through abrasion of wood treated with conventional wood preservatives was already recognized by Townsend et al. [34]. Therefore, the abrasion of wood treated with MC or improper disposal of its debris may pose a further environmental and human health risk via the release of dust or wear debris containing Cu-based NPs. In addition, interactions between Cu-based NPs and wood-destroying Cu-tolerant fungi are poorly understood. Studies [35-37] revealed a mechanism of Cu deposition into mycorrhizal and ascomycete fungal spores as a consequence of exposure to Cu, which corresponds to an uptake of up to 40% w/w [38]. Fungal spores can account for up to 4-11% of the fine particle (<2.5 μm diameter) mass in rural and urban air [39, 40]; with 64% of these spores coming from basidiomycetes [41]. Therefore, the uptake of Cu due to inhalation of Cu-loaded spores may take place in large amounts.

The aim of this thesis was to determine whether MC is superior to conventional wood preservatives, especially for wood species that are commonly used for the European wood decking
market. This implies that the penetration into refractory wood species is facilitated, the efficacy of the treatment against wood-destroying fungi is enhanced, and that the formulation does not constitute a health and environmental risk. The focus was set on specific stages of MC’s life cycle. In accordance with what discussed during the international workshop “Lifecycle impacts of copper nanomaterials released from timber preserving impregnations”, held on the 22nd January 2016 at Ca’ Foscari University of Venice and organized by the EU FP7 SUN and ECONANOSORB projects, we suppose that the compounding, formulation and disposal phases are the least dispersive ones, due to the industrial control, and they are not discussed here. Therefore, we mainly assess the impregnation stage, which is fundamental for the future particle modification, and the use stage, which is believed to be widely dispersive. In order to fulfil the main aim and research question, the following objectives were set:

1. Particle characterization, and effectiveness of MC towards different wood-destroying fungi, namely soilborne fungi and basidiomycetes
2. Discrimination of ionic, nano and bulk Cu effects that could lead to a better MC performance against wood-destroying fungi
3. Visualization of MC penetration, interaction, and particle speciation into easily treatable and refractory wood species
4. Life cycle studies of Cu-tolerant wood-destroying fungi exposed to MC to determine if Cu can be released via spores and in which form
5. Characterization of the particles released from MC-treated wood during an abrasive process, in terms of size, morphology, and chemical composition
6. Preliminary lung toxicity assessment of MC and particles released from MC-treated wood

The work here proposed was carried out within the framework of the following working hypotheses:

1. Within MC particle size range, Cu-based NPs, rather than Cu-based microparticles, are the main responsible for wood protection against wood decomposing fungi
2. Cu-tolerant wood-decomposing basidiomycetes exhibit the same tolerance mechanisms towards Cu, independently from the form of Cu
3. Similarly to mycorrhizal fungi, Cu-tolerant wood-destroying basidiomycetes can accumulate Cu in their spores
4. Abrasion of MC-pressure-treated wood may lead to further dissemination of Cu-based NPs in the environment
5. Cu-based NPs from MC wood preservative formulations, if inhaled, can cause adverse effects due to specific nano effects.
1.2 Thesis overview

This thesis is structured in six chapters. Chapter 1 provides an introduction into motivation and objectives the research project. Chapter 2 provides an introduction and background information based on the existing literature available on the topic, and it contains a review article published in a peer-reviewed journal, and excerpts from the paper proposed for oral presentation at the 46th Annual Meeting of the International Research Group on Wood Protection. Chapter 3 highlights and explains the main materials and methods used to unravel the research question. Chapter 4 presents the main experimental findings and consists of 3 research articles, either published or under peer-review, and 2 additional studies. This chapter assesses the effectiveness of the MC treatment against soft rot and other soilborne fungi and wood-destroying basidiomycetes, its ability to penetrate refractory wood species, the particles released by mechanical abrasion of treated wood surfaces and their relative cytotoxicity towards lung cells, and the spore compartmentalization as a Cu release pathway. The discussion and interpretation of the results, followed by drawing conclusions and an outlook are presented in Chapter 5. The articles here proposed are reprinted with the permission of the respective publishers.

1.3 Publications and author contributions

1. Civardi C, Schlagenhauf L, Benz J, Hirsch C, Van den Bulcke J, Schubert M, Van Acker J, Wick P, Schwarze FWMR. Environmental fate of micronized copper. International Research Group on Wood Protection IRG/WP 15-50310, 9 pp. (Excerpts) Chiara Civardi summarized the results and the existing literature, and wrote the manuscript. Mark Schubert, Francis W.M.R. Schwarze, and Peter Wick provided expertise, guidance and critical reading. All authors contributed to finalize the manuscript.

2. Civardi C, Schwarze FWMR, Wick P. Micronized copper wood preservatives: An efficiency and potential health risk assessment for copper-based nanoparticles. Environmental Pollution 2015; 200: 126–132. Chiara Civardi summarized the existing literature and wrote the manuscript. Francis W.M.R. Schwarze, and Peter Wick provided expertise, guidance and critical reading. All authors contributed to finalize the manuscript.

Civardi, Mark Schubert, and Angelika Fey performed the experiments. Chiara Civardi and Mark Schubert analyzed the data. Chiara Civardi summarized the results and wrote the manuscript. Mark Schubert, Francis W.M.R. Schwarz, and Peter Wick provided expertise, guidance and critical reading. All authors contributed to finalize the manuscript.

Chiara Civardi and Jan Van den Bulcke conceived and designed the experimental plan. Chiara Civardi, Jan Van den Bulcke, Mark Schubert, Elisabeth Michel, and Maria Isabel Butron performed the experiments. Chiara Civardi, Jan Van den Bulcke, Mark Schubert, Matthieu N. Boone, and Manuel Dierick analyzed the data. Chiara Civardi and Jan Van den Bulcke summarized the results and wrote the manuscript. Mark Schubert, Matthieu N. Boone, Manuel Dierick, Joris Van Acker, Francis W.M.R. Schwarz, and Peter Wick provided expertise, guidance and critical reading. All authors contributed to finalize the manuscript.

Chiara Civardi, Lukas Schagenhauf, Jean-Pierre Kaiser, and Cordula Hirsch conceived and designed the experimental plan. Chiara Civardi, Lukas Schagenhauf, Jean-Pierre Kaiser, Cordula Hirsch, Claudio Mucchino, and Adrian Wichser performed the experiments. Chiara Civardi, Lukas Schagenhauf, Jean-Pierre Kaiser, and Cordula Hirsch analyzed the data. Chiara Civardi summarized the results and wrote the manuscript. Francis W.M.R. Schwarz, and Peter Wick provided expertise, guidance and critical reading. All authors contributed to finalize the manuscript.

1.4 References of Chapter 1


2. Background & literature review

In order to comment the potentials and pitfalls of MC wood preservatives, it is necessary to discuss first basic concepts of wood science. Wood is a natural product that has been used for centuries as a building material. In order to understand its potential applications and use it at its best, it is fundamental to maximize its service life, reduce any form of decay, and apply suitable protective systems. Therefore, it is essential to know about wood anatomy and composition, how decay can occur, and the wood preservative tools at our disposal. Further, it is necessary to know how wood preservatives can be remobilized and end up in the environment. All these aspects are discussed below.

2.1 Wood

Wood, or xylem, is defined as the secondary and permanent tissue of vascular plants and it mainly fulfills structural as well as water and nutrient transport functions. It is surrounded by the cambium and an inner and outer bark (phloem). Two different wood types can be identified: softwood and hardwood, depending on whether they belong to gymnosperms or angiosperms, respectively. The studies presented in this thesis only involve softwood species, therefore the discussion in this chapter will be limited to it.

Softwood is predominantly composed of longitudinal tracheids, and to a minor fraction of xylem ray tissue, longitudinal parenchyma, and, in some genera, resin canals lined with epithelial cells. Every wood cell is composed of a cavity, called lumen, surrounded by a cell wall structure that can be further subdivided into the outermost layer, the middle lamella, the primary wall, and the innermost secondary wall; in some cases, a warty layer next to the lumen can be identified. Three layers can be then distinguished within the secondary wall: the outer secondary wall (S1), the central secondary wall (S2), and the inner secondary wall (S3). The cellulose-free middle lamella is located between adjacent cells [1, 2]. For cell connection, three kinds of pits can be identified: simple pits, bordered pits, and cross-field pits. These connect two parenchyma cells, two tracheids, and parenchyma cells in rays with axial tracheids, respectively. The dominating type are the bordered pits, which are important for transport and exchange of substances between the lumina of contiguous dead cells [3]. These are cavities in the lignified cell walls, and their porous pit membrane lies in the center of each pit. Since pits are responsible for the passage of liquids (plant water transport) and the halt of gas voids (prevent from embolism) from one lumen to another, they provide resistance to the liquid flow [3], which then adds to the resistance encountered in the lumen. Therefore pits largely affect the permeability to liquids. In particular, when wood is dried, the bordered pit’s torus (central thickening) tends to move across the pit chamber and seal off one of the pit apertures. This phenomenon is called pit aspiration, and the degree of pit aspiration accounts for treatability of a certain wood species [4]. For instance, while the percentage of unaspirated pits in Norway spruce latewood is approximately 20-25%, it is up to 50% in Scots pine [5]. Further, the incrustation or
occlusion of pits due to extractives can heavily affect the liquid flow through pits. Furthermore, the number and morphology of cross-field pits heavily affect wood permeability [5]. While in easily treatable Scots pine, they are fenestrate and with a large thin membrane, in Norway spruce they are of the piceoid type, with smaller membrane and smaller dimensions [6, 7]. Resin canals, if present, are important pathways for liquids, too [8]. In particular, longitudinal resin ducts sometimes intersect with rays by means of smaller radial resin passages. In terms of size, for conifer wood the conduction of liquids occurs in the hollow tracheids (longitudinal) of 20-40 μm in diameter, parenchymal rays and their pits for what constitutes the radial direction [12-14]. From either of these, liquids can then penetrate into the cell through the pits, whose average diameter in conifers is 300 nm [12]. Wood cell wall capillary network allows the path flow of particles with diameters up to 10 nm [13-17]. In fact, whether fluids or particles can enter into the cell wall depends on their size. In addition, liquid polarity, viscosity, and surface tension affect the permeability, i.e. non-polar [18], low-viscosity [19], and high surface tension [20] liquids can penetrate more easily in wood.

Another factor influencing the penetration of liquids in wood is the amount of earlywood versus latewood. Softwood cells formed early in the growth periods have larger lumina and thinner walls, elements that favor the transport of liquids. These cells are known as earlywood tracheids. The rate of growth reduces later in the growth period, and the cells, densely packed, are characterized by a smaller diameter and thicker walls (latewood tracheids). The mechanical strength of wood is mainly provided by the thick-walled latewood cells. This last feature is the reason why latewood is generally more resistant to liquid flow than earlywood. Further, wood can be divided into sapwood (i.e. xylem tissue composed of dead cells that transport water) and heartwood, which is formed by the innermost layer of sapwood during the growth cycle. When correlated to their structures, it appears clear that sapwood is more permeable to liquids, while heartwood is more resistant to the passage of fluids. In conifers, this is mainly due to the changes that occur in the bordered pits. However, to which extent heartwood is less permeable than sapwood heavily depends on the wood species.

From a chemical perspective, wood is mainly composed of cellulose, hemicelluloses and lignin macromolecules, which altogether represent over 90% of the wood structure. These are not homogeneously distributed in the cell wall. Beside these major components, the remaining up to 10% of wood is composed of a percentage of non-structural constituents with low molecular weights, called extractives, like vegetable waxes and fats (1.4%), pectic substances (0.2-2%), proteins, terpenes, phenols, alcohols, organic acids, monosaccharides and disaccharides. Some of these, like certain terpenoids and phenolic compounds, are toxic against microorganisms or insects, thus play a role in the “the inherent resistance of wood to attack by wood-destroying organisms.” [21], or natural durability of wood [22, 23]. Whereas, pectic substances are responsible for the flexibility of the primary cell wall [24].
Cellulose is the major component of wood cell walls, and it provides strength to the cell wall. It forms crystalline fibrils that are aligned parallel and arranged to form separate layers in the secondary wall. This molecule is characterized by linear sequences of cellobiose, a disaccharide obtained by the reaction of two D-anhydroglucopyranose molecules. On the surface of the cellulose molecules, over the median plane of the pyranonic ring, there lie hydroxylic groups [C(1)-OH] that account for the linearity of the chains, and the formation of intra- and inter-chain hydrogen bonds with other cellulose molecules and with water. While a fraction of cellulose is present in amorphous state, another fraction results in highly ordered arrangements (monocline symmetry) of cellulose chains that then form crystal lattices. Therefore, cellulose can be defined as a paracrystalline polymer [25]. Cellulose chains are generally differentiated on the basis of their size: microfibrils of approx. 2.5 nm [26], and fibril aggregates (approx. 20-25 nm).

Hemicelluloses, which surround cellulose microfibrils and fibril aggregates, and being part of the latter, are relatively short (degree of polymerization <200), non-linear, heterogeneous polysaccharides. They act as a matrix, and are mainly formed by glucose, mannose, arabinose, and xylose. Because of the length of their chains, the presence of side branches, their non-crystalline structure, and the presence of many carboxylic groups, hemicelluloses are more soluble in water and more reactive than cellulose. There are several kinds of hemicellulose and the most common in softwoods is (galacto)glucomannan, a polymer that contains glucose and mannose units in the backbone structure, and galactose in side branches. The second most common hemicellulose in softwood is arabinoglucuronoxylan.

Lignin is an amorphous, hydrophobic and aromatic three-dimensional polymer that is mostly found in the middle lamella. This molecule is an encrusting substance responsible for the cells’ compressive strength, thus it is responsible for the transverse rigidity of the tracheids. Within the cell wall, lignin creates a three-dimensional network that is partly linked with hemicelluloses by covalent bonds. The chemical composition of lignin is variable and mainly depends on the plant species; however, the macromolecule can be described as a polymer of phenylpropene units and substantially formed by three cinnamic acids that react randomly: p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol. These three compounds are the precursors of p-hydroxyphenil, guaiacyl, and syringyl units, respectively.

In terms of chemical elements, the main constituents of wood are carbon (49-51%), oxygen (43-44%), hydrogen (6-7%) and nitrogen (0.1-0.3%). Other minor cations are magnesium, calcium, potassium, manganese, iron, sodium and aluminum; anions are present as sulfates, carbonates, phosphates and chlorides.

The main features that influence the physical properties and behavior of wood are its moisture content, capillary absorption ability and density. Moisture content refers to the quantity of water absorbed
by the inner wood substrate, present as liquid free water within the cell cavities, or as bound water within the cell walls, and is defined as follows:

$$\text{Moisture content (\%)} = \frac{\text{Mass of moist wood (g)} - \text{Mass of dry wood (g)}}{\text{Mass of dry wood (g)}} \times 100$$

The situation where the wood cell wall is fully saturated is called the fiber saturation point. Any change to less than this moisture content will affect the mechanical properties of the material, whereas variations above this point have little or no effect. The capillary absorption ability mainly depends on the permeability of the cell structure and is strictly linked to the moisture content.

Finally, density is defined as the ratio of the mass and volume of a substance. Because wood is variable in its composition and is a porous material, able to contain gaseous, liquid or solid matter, inter- and intraspecific differences in wood, and moisture content are the main factors influencing density. Density also plays a role in the permeability of wood to liquids. However, since moisture content can affect it, different definitions of wood density are available. In particular, two types of densities can be identified: oven-dry density, defined as the oven-dry mass (drying to constant weight at 103°C) per oven-dry unit volume; and air-dry (or raw) density, calculated as the mass of wood in equilibrium with atmospheric conditions per volume of wood in equilibrium with atmospheric conditions. In general, low density wood is more treatable, due to the higher porosity. In fact, the porosity of wood determines the maximum amount of liquid that can enter into the structure [27].

### 2.2 Wood-decay fungi

Wood is susceptible to deterioration and decay, in particular from biological origins. One of the major cause of decay is attack by fungi. Fungi, or Eumycota, are eukaryotic and heterotrophic organisms characterized by the presence of a rigid cell wall that is mainly composed of chitin. They are subdivided into five phyla: Chytridiomycota, Zygomycota, Glomeromycota, Ascomycota and Basidiomycota [28]. Fungi are the organisms most frequently involved in biodeterioration, because they are able to decompose a vast number of materials of different origins. They can directly interact with organic matter, which is used for trophic purposes, and can also exert their action indirectly on inorganic materials that can, however, contain organic fractions to support fungal growth. Moreover, many species are saprophytes and are generally characterized by adaptive capabilities that allow them to grow and live efficiently in various environments. Although the Kingdom of Eumycota is extremely heterogeneous, there are characteristics that are associated with the organisms belonging to this taxon: fungi are heterotrophic organisms, a feature that generally manifests as forms of parasitism, saprophytism or symbiosis; furthermore, fungal species are not photosynthetic and, like animals, the reserve/stored substance used to obtain energy is glycogen; finally, the cell wall is primarily composed of chitin.
Fungi are characterized by different developmental stages (spore, hypha, mycelium, fruiting body) that greatly differ in their morphology. Spores are small, reproductive cells of different shapes and colors, either unicellular or multicellular. Hyphae are rod-shaped eukaryotic cells with protoplasm and sometimes vacuoles; other features include mitochondria, ribosomes and endoplasmic reticulum. At another level, there is the mycelium, or thallus, which is formed by several hyphae [29] to enable maximization of the surface area in contact with the environment for uptake of raw materials. The mycelial structure forms a mat that can have a variety of morphological appearances (cottony, woolly, plumose, leathery etc.) and can grow indefinitely. Finally, the fruiting body originates from the mycelium and is responsible for the development and release of sexual spores. The characteristic feature of all basidiomycetes (or macrofungi) is the basidium, in which meiosis occurs. This process results in the formation of basidiospores (four for each basidium), which are sexual spores produced on sterigmata. The mycelium changes from monokaryotic into dikaryotic, as there are two different haploid nuclei within one hyphal compartment. In addition, the hyphae of basidiomycetes generally have dolipore septa to prevent the nuclei migrating between the different compartments.

The first stage in the life cycle of the basidiomycetes is dissemination of the basidiospores into the environment and their germination. Each of these spores contains a single haploid nucleus. Under suitable conditions, a spore will produce a filament, the germ tube, which repeatedly branches in order to create a monokaryotic primary mycelium. In this phase, the monokaryon can produce oidia that can either germinate to produce other monokaryotic colonies or work as fertilizers. Next, a combination of different monokaryotic mycelia of different mating types fuse together in a process called plasmogamy and dikaryotic (i.e. with two nuclei) hyphae develop. This phase is accompanied by an increase in the hyphal diameter and the formation of clamp connections. The function of the clamps is to ensure that both the original and daughter cells contain two genetically distinct nuclei. The projecting hooks snare one of the new nuclei produced and then two septa are formed: one between them and the original cells and the second between the old and new cells; subsequently, the clamps fuse with the new cell, releasing the nucleus. The dikaryon can then grow and produce a network of hyphae and eventually, under suitable environmental conditions, the fungus produces a fruiting body, in some cases referred to as a mushroom or bracket, in which the mycelium becomes more dense and firm. Afterwards, within the margins of the new structure, the spore-bearing layer (or hymenium) develops and is characterized by hyphae that form the basidia (Fig. 1). At the same time, the spore-producing layer becomes folded and forms either pores (Fig. 1) or lamellae. On the end of the basidia, four basidiospores develop after karyogamy and meiosis (Fig. 1). Finally, they are released to the environment and a new cycle begins.

Some fungal species are lignolytic, thus able to digest wood components, causing decay, referred to as rot. Fungi often require a minimum moisture content of 20% to start colonizing wood and then most
species require a level between 35% and 60% relative humidity (RH) to continue growing. However, this strictly depends on the species involved, e.g. *Serpula lacrymans* initially requires a wood moisture content of 30-40%, but then is able to grow at RH <20% and so its decay is commonly referred to as ‘dry rot’ [30, 31]. In any case, water is required as it is the indispensable medium for life systems, it is a reactant in the hydrolytic reactions involved in cell wall digestion, it is the solvent and diffusion medium for the decomposing enzymes released by the hyphae, and it acts as a swelling agent, facilitating the penetration of the hyphae and the digestive enzymes into the woody cell wall.

Temperature, as well as pH, directly affect fungal enzymatic and metabolic reactions, and subsequently the organism’s ability to directly colonize the woody substrate. Although interspecific variations occur, the optimum temperature for fungal growth is in the range of 20-35°C, and the best pH ranges from 3 up to 6. Light, on the other hand, does not particularly affect the vegetative development of the fungus: the mycelium usually grows in the dark, but basidiomycetes’ fruiting bodies only develop under direct daylight. The quantity of light required, however, is highly variable.

**Fig. 1. Section from a fruiting body of the basidiomycete fungus *R. placenta*.** Pores, basidia, and spores are highlighted.
Fungi are aerobic organisms and as such, require free oxygen to carry out their normal metabolic processes; even so, the quantity of oxygen required depends on the species involved. Organic and inorganic compounds are necessary nutrients, the intake of which is required to synthesize proteins and enzymes, chitin and other cell components. In order to fulfill this function, fungi require carbon (from the cellulose and hemicelluloses in wood), nitrogen, a series of essential cations (such as calcium, phosphor, potassium, sulfur, magnesium), vitamins (B1 and B6) and minor amounts of metals (iron, copper, zinc, boron, manganese) [32]. When fungi colonize the woody substrate, they modify the material in different ways: wood-destroying fungi frequently cause a high mass loss, with a subsequent reduction in strength, whereas stains and molds are only responsible for pitting, foxing and superficial damage. Wood decomposition occurs as fungi excrete enzymes that are able to decompose the structural elements of the woody cells in order to obtain nutrients. In 1874, Hartig [33] introduced the terms ‘brown’ and ‘white’ rot, in order to classify the different types of damage to wood caused by fungi, which can turn wood either darker or lighter in color. Later on, the term ‘soft rot’ was invented to define softening of the woody material. These different types of damage depend on the enzymes produced by the fungal species to convert cellulose, and hemicelluloses into simple sugars [34].

White rot is primarily caused by ascomycetes and basidiomycetes that start degrading lignin and then continue depolymerizing cellulose and hemicellulloses [35]; in this case, the decay is referred to as selective delignification. The decomposition of holocellulose and lignin can occur contemporaneously, which is referred to as simultaneous rot. In the first case, lignin is decomposed by hyphae growing on the inner secondary wall and then depolymerization proceeds through the secondary wall towards the middle lamella; in simultaneous rot, however, the erosion of the cell wall occurs in close proximity to the hyphae [35].

Brown rot fungi largely belong to the Basidiomycota and mainly break down the polysaccharidic component of wood (e.g. cellulose and hemicelluloses), leaving lignin almost unaltered, though sometimes modified by demethylation or oxidation processes [36]. This inability to attack lignin can be explained from an evolutionary point of view: brown rot fungi evolved from white rot fungi [37], and their main feature developed from simplification of the mechanisms to initially decompose cellulose, which allowed extinction of the energetically disadvantageous lignocellulose degrading apparatus [38]. It is noteworthy that brown rot fungi depolymerize holocellulose, generating degradation products faster than their rate of usage [39]. Decomposition is believed to occur via a Fenton system, although it is still unclear whether other mechanisms are involved [40]. In the first stage, degradation is conducted by hyphae growing on the cell wall and the enzymes then diffuse through the S3 layer until the target S2 region, which has a high content of cellulose, is reached. The result is a rapid degradation of the S2 layer, while the S3 and middle lamella remain almost intact [35]. Although the major target of brown rot decay is
conifer wood, this should not be seen as a more favorable substrate, because the correlation lies in the predominant distribution of brown rot species in northern regions where conifers are abundant [41]. Most of the Cu-tolerant fungi belong to this classification, e.g. *Poria* and *Antrodia* spp. [42], which is explained in evolutionary terms by their ability to produce more oxalic acid than white rot fungi: the latter secrete oxalic acid-decarboxylase enzymes that do not allow the accumulation of acids, resulting in less ability to develop resistance mechanisms and survive effectively at lower pH values [43].

Compared with the other two types of decay, the fungi causing soft rot have a relatively simple and slow mode of wood degradation. These fungi, which mainly belong to the Ascomycotina and Basidiomycotina Phyla and to mitosporic fungi [44, 45], are able to grow on damp or wet wood and their moisture tolerance frequently ranges from dry dormancy to active decomposition at levels of almost complete saturation [46]. The soft rot action is mainly exerted on the outer layers of wood and the attack begins from the wood surface, where the fungus enters via the xylem rays; the hyphae then penetrate depressions in the cell wall, from where they penetrate into the secondary wall to reach the cellulose-rich S2 layer, branching and secreting cellulase enzymes to create longitudinally aligned rhomboidal cavities (soft rot Type 1). Soft rot Type 2 fungi are also able to grow within the lumen, causing erosion through the cell [47]. Lignin is decomposed by soft rot only if of syringyl origin, whereas guaiacyl-rich layers are often resistant. In addition, soft rot fungi commonly require high amounts of nitrogen, either from wood or from the environment.

**2.3 Wood preservatives**

Wood preservatives aim to increase the durability of wood and lengthen its service life. Therefore, wood preservatives should satisfy a series of criteria, the main one being product performance. This means that the wood preservative can uniformly penetrate into the wood structure [48], which occurs according to the transport mechanisms described in Chapter 2.1, and that the product can effectively protect wood from decay. Besides, wood preservatives should guarantee low production cost (in terms of raw material extraction, material processing, and product manufacture), health and environmental safety. From a life cycle assessment (LCA) and life cycle impact assessment (LCIA) perspective, it means that the use of treated wood should be advantageous to the use of untreated wood.

Industrial pre-treatment of timber with wood preservatives can be conducted via different carriers, namely tar oils, solvents, or water (either with or without metals). Their different properties make them suitable for different wood applications, therefore wood preservatives can be distinguished on the basis of wood uses. According to the European regulations, 5 end use (or hazard) classes can be distinguished [49], and wood preservatives should prevent from the most common type of biological attack for each end use. These are listed below:
1. Interior, dry wood; mainly susceptible to biological attack by insects
2. Interior, damp; subject to decay from insects and decay fungi
3. Exterior, protected or unprotected; exposed to insects, decay fungi, and bluestain (disfiguring) fungi
4. In-ground or fresh water; where deterioration can be caused by insects, decay fungi, bluestain (disfiguring) fungi, and soft rot fungi
5. Marine; whose decay is caused by insects, decay fungi, bluestain (disfiguring) fungi, soft rot fungi, and marine borers.

For in-ground timber structures (hazard class 4), Cu is essential for its protection, as it has the unique ability to inhibit soft rot [50] and most white rot fungi [51]. The fungicidal activity of Cu and its potential in wood protection have long been known. The first account of chemical wood preservatives dates back to the 17th century, when Cu salts were used for the first time as phytomedicines against wood-decay fungi, together with mercury chloride and arsenates [52]. However, the so-called first generation of Cu-based wood preservatives arrived only two centuries ago, with the development of creosote, chromated Cu arsenate (CCA) in the 1930s, and then penta (pentachlorophenol) in the 1950s [53]. Of these, the features of CCA enabled it to become the predominant product in the market for the preservation of a wide variety of wooden objects for industrial and residential purposes. It is a low-cost, easy to use formulation and, being waterborne, CCA does not have a tang odor, and all of its components (Cu, chromium and arsenic) contribute actively to the effective and long-lasting preservation of wood. Following the same path of CCA, ammoniacal Cu arsenate and ammoniacal Cu zinc arsenate waterborne systems were developed to deal with refractory species in particular. Despite the advantages of these products and their wide usage, health and environmental concerns arose in the 1970s regarding air and water pollution caused by chromium and arsenic, and this resulted in a US review of these pesticides between 1978 and 1980, when they were classified as restricted use. In 2004 registration of CCA for residential applications was withdrawn [54], and from 1998 the use of CCA was forbidden in situations where human contact could occur in Europe [55]. Consequently, a new generation of preservatives was developed, as CCA disposal began. The second generation of products for wood protection was characterized by boron- or Cu-rich waterborne systems, combined with an organic co-biocide to act against Cu-tolerant fungi. Although boron is mainly in the form of boric acid or derivatives (borates), the Cu successors of CCA can be further distinguished into metal complexes or systems with uncomplexed Cu. This group of wood-preservative systems commonly has higher concentrations of Cu and does not contain either arsenic or chromium. However, in these preservatives the absence of chromium causes major leaching and corrosion issues [56, 57], resulting in ineffective preservation and chemical dispersal in the environment. These concerns lead to the development of third- and fourth-generation products, in
which totally organic systems or biocontrol strategies are used [57]. The chemicals used in these systems are azoles, mainly triazoles such as tebuconazole (TBA), chlorotalonil (2, 4, 5, 6-tetrachloroisophthalonitrile), and betaine. Even these products can lead to environmental problems, as not only do they leach but they can also evaporate or be depleted by chemical, biological and photo reactions [57, 58]. The non-biocidal preservation systems and biocontrol mechanisms, in contrast, have the potential to be environmentally safe, but are only at preliminary stages of development. MC consists of CuCO$_3$·Cu(OH)$_2$ particles with a size range of 1 nm-250 µm, according to manufacturers, combined with a second organic biocide that provides protection against basidiomycetes, either azole (MCA) or quaternary ammonium compounds (MCQ) [59, 60]. Although MC still contains Cu as main biocide, it has an impregnation chemistry that differs from conventional Cu-based wood preservatives: part of CuCO$_3$·Cu(OH)$_2$ immediately solubilizes during the impregnation process and is complexed by wood organic macromolecules, while another fraction does not react and acts as reservoir, i.e. Cu is solubilized afterwards [61]. Therefore, the issues related to leaching and corrosion are reduced [62], and a continuous protection over time is hypothesized [63].

### 2.4 Release of Cu from wood into the environment

The loss of wood preservative active ingredients from wood does not only result in the loss of protection against biodeterioration and biodegradation, but it also have a negative impact on human health and the environment. Due to the intrinsic nature of wood preservatives, pressure-treated-wood contains organic and inorganic components that can be toxic to non-target organisms, if released into the environment. More precisely, these components may be released in air, soil, or water as a consequence to the conditions where pressure-treated wood is located. The main substance of concern in (pressure-treated) in-ground timber structure is Cu.

One of the major cause of Cu migration is leaching, which can release Cu in soil, fresh and marine waters, with their relative proportions depending on the precise location. The speciation of Cu from leachates also depends on the characteristics of the environment where pressure-treated wood is located. Soil in the vicinity of treated wood, similarly to agricultural soils [64], is a repository of Cu and the metal is likely to deposit in the upper part, close to treated timber structures exposed to the soil. Here Cu$^{2+}$ ions and CuCO$_3$·Cu(OH)$_2$ particles leached from wood treated with MC or conventional wood preservatives can undergo speciation due to soil pH, organic and inorganic matter. These parameters play a key role in determining whether Cu would be mobile and/or bioavailable. In particular, soils high in pH or organic matter would complex free Cu$^{2+}$ ions [65] or precipitate the residual Cu carbonate nanoparticles.
[66, 67], which are likely to aggregate/agglomerate into bigger and stable CuCO$_3$·Cu(OH)$_2$ particles. In acid soils poor in organic matter CuCO$_3$·Cu(OH)$_2$ would dissolve and result in mobile, free, Cu$^{2+}$ ions.

If Cu is leached into water, the physicochemical properties of the water in which Cu is transported play a key role in determining the Cu speciation and transport. Still, it is possible to draw some general conclusions. In seawater, freshwater and brackish water, Cu mainly interacts with dissolved components, principally with organic ligands that reduce Cu bioavailability. Cu is seldom found free (Cu$^{2+}$ ions), inorganically complexed, or adsorbed to particulate matter. At the sediment-water interface the amount of bioavailable Cu can significantly increase, due to different oxygen levels and organic matter decomposition.

Cu could also be remobilized in the air compartment. The main cause of it is believed to be via production of wood dust that contains Cu [68]. Since wood dust can cause per se adverse health effects [69-73], in particular diseases to the respiratory tract, the presence of Cu may exacerbate the stress response.

2.5 References of Chapters 2.1-2.4


2.6 Spore compartmentalization of Cu

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Review

Micronized copper wood preservatives: An efficiency and potential health risk assessment for copper-based nanoparticles

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Abstract

Copper (Cu) is an essential biocide for wood protection, but fails to protect wood against Cu-tolerant wood-destroying fungi. Recently Cu particles (size range: 1 nm–25 \textmu m) were introduced to the wood preservation market. The new generation of preservatives with Cu-based nanoparticles (Cu-based NPs) is reputedly more efficient against wood-destroying fungi than conventional formulations. Therefore, it has the potential to become one of the largest end uses for wood products worldwide. However, during decomposition of treated wood Cu-based NPs and/or their derivate may accumulate in the mycelium of Cu-tolerant fungi and end up in their spores that are dispersed into the environment. Inhaled Cu-loaded spores can cause harm and could become a potential risk for human health. We collected evidence and discuss the implications of the release of Cu-based NPs by wood-destroying fungi and highlight the exposure pathways and subsequent magnitude of the health impact.

We assess the fungicidal activity of particulate copper wood preservatives and their possible release in the air by Cu-tolerant wood-destroying fungi.
Keywords: Micronized copper; Copper-tolerant fungi; Nanocopper exposure; Wood-destroying fungi; Wood preservatives

1 Introduction

Wood was one of the first materials used by humans for a wide variety of applications. Moreover, this natural product is still used because of its exceptional density–strength properties, as well as being economically advantageous, sustainable, and carbon dioxide neutral. Hence, it is believed to be one of the most preferred building materials in the future (Buchanan and Levine, 1999).

Despite its positive features, wood is susceptible to different factors that cause its decay, and various biodeterioration organisms such as fungi, cyanobacteria, bacteria, protists, and insects have the capacity to decompose wood. In particular, fungi, insects, and bacteria are considered to be the principal pests of timber. Therefore, methods to improve the durability of wood have been developed since early times (Unger et al., 2001) and the chemical preservation of wood products appears to be the most common and developed strategy, with three generations' history. Health and environmental concerns with the older 1st-generation preservative systems (arsenic, penta) led to rapid and profound changes on a worldwide basis as we moved to the 2nd and 3rd generation Cu rich preservatives.

Nanotechnology, which is currently one of the most investigated areas of research, has influenced the utilization of wood preservatives because it has enabled the development of innovative metal-based biocides (Clausen, 2007; Dorau et al., 2004; Green and Arango, 2007; Kartal et al., 2009; Kim and Kim, 2006; Akhtari et al., 2013a).

More precisely, the wood preservation industry has begun to profit from formulations using particulate Cu systems, also known as micronized Cu, that contain a considerable amount of nanosized Cu (Mcintyre, 2010), which can readily penetrate into the wood (Geers et al., 2014; Matsunaga et al., 2007). Since 2006 more than 11,800,000 m$^3$ of wood treated with micronized Cu has been sold (Griffin, 2011), which corresponds to an annual use of 79,000 tonnes of Cu systems (Evans et al., 2008), greatly exceeding the quantities estimated for titanium dioxide NPs (Gottschalk et al., 2009).

In this review, the term Cu-based NPs will be used to indicate formulations containing a Cu nanosized fraction, according to the current nanomaterials definition (Commission Recommendation, 2011) and to recent findings (Mcintyre, 2010; Geers et al., 2014; Matsunaga et al., 2007). Cu-based NPs can be manufactured from metallic Cu or Cu compounds (e.g. Cu carbonate). Although wood preservatives based on Cu-based NPs are currently available, surprisingly there is little information on their hazard potential and the interactions between this biocide and wood-destroying Cu-tolerant fungi are poorly understood.
The objectives of this review are the assessment of (i) the effectiveness and the risk of wood preservatives based on Cu-based NPs, (ii) to discuss the probability of Cu-tolerant fungi accumulating, and re-mobilizing Cu during their life cycle, and (iii) subsequently the magnitude of the health impact.

2 Wood preservative treatments

To date, the main fungicide used for treating wood in contact with the soil is Cu (Cu carbonate, Cu citrate), and currently there is no satisfactory alternative yet: Cu compounds are the only biocides that show a high efficiency against soft rot fungi and other soilborne fungi, which cause the greatest damage to wood products in contact with the soil (Hughes, 2004).

Cu is generally required at low concentrations by every organism in order to sustain metabolic processes for its survival, but at higher concentrations it causes serious, in some cases irreversible alterations in metabolic activities (Gadd, 1993). Furthermore, despite its excellent properties as fungicide, Cu is considered to be a toxicant with minimal effect on mammals (Lebow, 1996), although the effect on aquatic communities is relevant (Eisler, 1998; Roales and Perlmutter, 1980). The features of Cu allow it to act as a biocide, or as a growth or reproduction inhibitor for biodeteriogens. In addition, Cu is also able to cause genetic perturbation or mutation.

2.1 Potential of Cu-based NPs formulations as wood preservatives

We are currently experiencing a development of particulate-based wood preservatives, in terms of composition and size range of the particles, in order to maximize the biocidal effect and effectively protect wood in contact with the soil from biodeterioration. As discussed above, the Cu-based NPs formulations currently available have a mean particle size that is evidently nanoscale, as indicated in Fig. 1 that shows the particles from a Cu-based NPs preservative formulation.

Fig. 1 Waterborne micronized copper formulations for wood preservatives: impregnation of wood samples and TEM micrograph on particles from a micronized copper.
The use of Cu-based NPs instead of bulk Cu reputedly improves the durability of wood against fungal decomposition (Kartal et al., 2009; Cookson et al., 2010; McIntyre and Freeman, 2009; Akhtari et al., 2013b) because of the following properties of NPs: (i) ability to penetrate bordered pits because they are smaller than the mean opening (300 nm), (ii) increase in the effective surface area of Cu, and enhanced dispersion stability; (iii) less viscous formulations than bulk ones, and (iv) presence of a reservoir effect that allows a continuous protection over time (Xue et al., 2014; Freeman and McIntyre, 2013). These properties, which enable easier impregnation and deeper and more homogeneous uptake of the biocide into the wood, can be further improved by selecting specific supporting systems (e.g. surfactants) (Green and Arango, 2007). Moreover, the presence of chromium and arsenic is no longer necessary; however, leaching of the nanometal greatly depends on treatment procedure and product formulation (Kartal et al., 2009; Preston et al., 2008; Ding et al., 2013).

Finally, there is only little evidence to suggest that Cu-based NPs are more efficient against soilborne fungi or some Cu-tolerant wood-destroying fungi (Kartal et al., 2009; Cookson et al., 2010; Tang et al., 2013).

2.2 Fungicidal mechanisms of Cu-based NPs

Although the literature on the fungicidal properties of Cu-based NPs is sparse and fragmented, several studies investigated the toxic mechanisms of Cu ions.

In addition, the nanoparticles commercially available are generally poorly characterized (Altes, 2008). Furthermore, there is a substantial lack of knowledge in the underlying mechanisms on how Cu-based NPs wood preservatives function, namely whether the toxicity is exerted by the nanoparticles themselves or by the release of ions, or only in combination with secondary biocides. Thus at present the use of Cu-based NPs for wood protection is an insufficiently understood system, in regards to both the effectiveness of the treatment and its safety.

What is generally known is that the principal mode of action of Cu-based NPs can be identified as highly reactive due to their larger specific surface area (Chen et al., 2006; Oberdürster, 2000), which results in the production of reactive oxygen species (ROS), mainly peroxides, which induce a series of chain reactions and oxidative stress to the exposed organism (Heinlaan et al., 2008; Saliba et al., 2006) and cause DNA damages. As demonstrated for white rot fungi, these reactions may eventually result in the \textit{in vitro} inhibition of lignocellulose-degrading enzymes (Shah et al., 2010). In addition, particles may interact with proteins and mitochondria damaging or disrupting them (Chang et al., 2012). Moreover, a strong ability to interact with the cell wall has been suggested (Heinlaan et al., 2008; Shah et al., 2010) and homeostatic processes may be impaired by Cu dissolution (Chang et al., 2012). These lethal
interactions mentioned above are described in Fig. 2, where the toxic mechanisms for fungi colonising wood treated with Cu-based NPs are shown at the cellular level.

**Fig. 2** Cu-based NPs and their uptake and the toxic mechanisms for non Cu-tolerant fungi (left) and Cu detoxification mechanisms for Cu-tolerant fungi (right) colonizing wood treated with Cu-based NPs. In non Cu-tolerant fungi the Cu-based NPs can enter the cell (1) and form ROS (2) or have disruptive effects on mitochondria, proteins and DNA (2, 3); otherwise the Cu-based NPs may undergo dissolution (1) and interfere with cell homeostatic processes (2). In Cu-tolerant fungi, Cu-based NPs taken up by the cell can be complexed and precipitated (1) or sequestrated in vacuoles (1). To avoid further Cu penetration, the Cu uptake (both as NPs or as ions) from the surrounding environment is reduced (2) and the cell wall is able to bind Cu (2). By taking up glucose (1) to produce oxaloacetate (2), the production of oxalate (3) and its extracellular release (4) allow the complexation of Cu, forming non-bioavailable Cu oxalate (5).
Due to these features, Cu-based NPs may require lower concentrations of the metal with similar or even better efficacy than water based and dissolved conventional Cu formulations (Kartal et al., 2009; Mahapatra and Karak, 2009).

2.3 Fungal Cu-tolerance mechanisms

The continuous improvement of Cu-based formulations is mainly driven by the need to improve the efficiency of Cu biocides against Cu-tolerant fungi. Several brown rot fungi have the ability to grow and survive at Cu$^{2+}$ concentrations up to 1.6 mM (Hughes, 2004) or 100 mg/kg (Gadd et al., 2007), because of their resistance and tolerance mechanisms, which are depicted in Fig. 2, where the tolerance mechanisms for fungi colonising wood treated with Cu-based NPs are shown at the cellular level:

- Cu complexes and/or precipitates in the presence of extracellular products, which detoxify Cu by altering it, i.e. in presence of oxalate Cu$^{2+}$ ions are precipitated as non-bioavailable Cu oxalate (Clausen et al., 2000)
- metal binding to the cell wall, to avoid Cu penetration through the cell
- intracellular Cu complexes/precipitates, or modification of the Cu$^{2+}$ ions, to shift Cu in a non-bioavailable form
- vacuolar compartmentalization, which isolates Cu from the living organelles in the cell
- reduction in Cu uptake by fungi, which prevents Cu from entering the cell
- spore compartmentalization (Figs. 3 and 4) (Cornejo et al., 2013), to translocate Cu away from the cell

Fig. 3 Claroideoglomus claroideum spores from the rhizosphere of Imperata condensate growing for 6 months in a 450 mg Cu kg$^{-1}$ spiked-soil (A–C) or in a non-spiked soil (D) (Reproduced from Cornejo et al., 2013 with permission from Elsevier).
All these mechanisms, which allow fungi to grow and develop fruit bodies and spores, have different degrees of efficacy on the basis of the substrate composition (Gharieb et al., 2004), and the Cu compounds against which the fungi are tested (De Groot and Woodward, 1999; Köse and Kartal, 2010; Green and Clausen, 2005). In addition, inter (De Groot and Woodward, 1999; Köse and Kartal, 2010; Guillén et al., 2009; Sierra-Alvarez, 2007) and intraspecific (Clausen et al., 2000; De Groot and Woodward, 1999; Sierra-Alvarez, 2007; Collet, 1992; Duncan, 1958; Green and Clausen, 2003) differences have been observed, suggesting that individual survival strategies enable adaptation to Cu-polluted environments. As a result, Cu-tolerant fungi are one of the main causes for early failures of in-ground timber structures in the US and Europe (Lebow et al., 2003; Bollmus et al., 2012). Thus, in Germany 4.800.000 wood poles are in service and annually 1% (48.000) early failures are caused by Cu-tolerant fungi. The total annual expenses for replacing wood poles are estimated EUR 36.000.000 (BASF Wolman, personal communication).

3 Risk is defined by hazard and exposure

According to Oberdorster et al. (Oberdörster et al., 2005), there is a NPs risk if there is evidence for both exposure and hazard. Cu-based NPs-based wood preservatives are used in increasingly higher retentions, so potential exposure to humans and the environment is evident. However, precise estimations
on how much and in which form Cu is released are not possible because of the lack of quantitative data. In the case of NP hazards, additional aspects, compared with conventional chemicals, have to be taken into account: (i) uptake and bio-distribution are dissimilar to bulk or ionic forms, (ii) surface effects influence cellular metabolism and signaling cascades, and (iii) material properties differ, e.g. size, composition, degree of crystallinity, surface, modification of NPs after contact with the environment and the consequences of such alterations (Joner et al., 2008).

With regard to Cu-based NPs, studies of different formulations confirm their cytotoxic activity, but a thorough exposure assessment is still not possible, because of the lack of quantitative release data. Thus, at the moment the uncertainties do not enable precise predictions on the risk, and therefore a suitable risk management is not possible (Som et al., 2012).

3.1 Hazard identification and characterization

The hazard of Cu-based NPs for organisms belonging to different phyla has been highlighted in several studies, as the toxicity exerted is not species-specific and results in non-target effects.

Although part of the Cu-based NPs in the preservative formulation reacts with wood, forming complexes, a fraction of Cu-based NPs does not react and remains as insoluble basic copper carbonate particles (Xue et al., 2014).

What has generally been noticed is that non-dissolved NPs easily interact with the cell membrane (Oberdörster, 2000) and eventually penetrate it (Geiser et al., 2005), in contrast to bulk Cu, via a Trojan-horse-like mechanism (Limbach et al., 2009). Once inside the cell, depending on the formulation, the Cu-based NPs can either generate ROS via Fenton's reaction or go through intracellular dissolution (Studer et al., 2010) and cause cell damage via the creation of “hot spots” of Cu ions (Limbach et al., 2009; Karlsson et al., 2009; Midander et al., 2009).

Moreover, because of their dimensions, NPs can be inhaled (Geiser and Kreyling, 2010), may cross biological barriers and are transported via lymphatic and circulatory systems to different tissues and organs, where accumulation can result in severe injuries and damage to living cells (Buzea et al., 2007). First indications of the adverse effects of Cu-based NPs on the brain (Sharma and Sharma, 2007), the gastrointestinal (Altes, 2008; Meng et al., 2007) and respiratory tracts of mammals (Chen et al., 2006; Limbach et al., 2005; Stearns et al., 1994; Vallyathan and Gwinn, 2006), as well as ecotoxicity in aquatic ecosystems have been reported (Shah et al., 2010; Shaw et al., 2012; Shi et al., 2011).

Karlsson et al. (Karlsson et al., 2008a, 2008b) and Zhang et al. (Zhang et al., 2012) found Cu oxide NPs to be highly cytotoxic (MTS and ATP logEC₅₀ = 1.38 ± 0.03 μg/mL for human bronchial epithelial cells, MTS logEC₅₀ = 1.12 ± 0.04 μg/mL and ATP 1.16 ± 0.03 μg/mL for rat alveolarmacrophage cells) (Zhang et al., 2012), causing severe damage to DNA (concentration of 20
μg/cm²). There are only a few studies on the effect of Cu-based NPs on the cardiac system, although the effects caused by ultrafine particles have been widely investigated (De Hartog et al., 2003; Pope et al., 2004) and similar patterns of disease can be assumed. In addition, there is evidence that NPs (including Cu) cannot be removed easily by phagocytosis (Karlsson et al., 2008b).

However the main studies conducted were focused on the determination of acute toxicity at single dose, literature concerning low dose but chronic exposure and long-term disease is lacking to date (Limbach et al., 2009).

In addition to these negative effects, since fungal spores can cause adverse health effects when inhaled (Denning et al., 2006), their presence in concomitance with Cu-based NPs may cause additional stress responses.

3.2 Potential exposure scenario

At present, no research has been conducted to verify human or environmental exposure to Cu-based NPs from treated wood as a potential source, therefore it is impossible to predict and estimate the risk properly. From the risk assessment perspective, as long as there is a lack of accurate and quantitative data on the release and effects of Cu-based NPs in the environment, the precautionary principle should be applied. However, the presence in the market of wood preservatives based on Cu-based NPs acts against this principle; even more concerning is the fact that over 75% of the residential lumber produced in the USA is now treated with such formulations (Freeman and McIntyre, 2013), implying that domestic exposure could be already occurring, potentially.

Although it has been argued that the quantity of Cu disseminated in the environment should not exceed threshold concentrations (Velleux et al., 2012), the possibility of Cu-based NPs being released either alone or absorbed by fungal spores has not been considered so far. Spore uptake has already been reported for bulk Cu by Cornejo (Cornejo et al., 2013) et al. and Somers (Somers, 1963), and subsequent spread by this vehicle is evident. Cu-based NPs are hazardous when inhaled, and will mainly exert their toxicity on the respiratory tract of humans, the most sensitive entry portal (Krug and Wick, 2011).

The aspect of spores as a vector of Cu-based NPs should not be underestimated, because fungal spores can account for up to 4–11% of the fine particle (<2.5 μm diameter) mass in rural and urban air (Womiloju et al., 2003); 64% of these spores belong to basidiomycetes in which a range of Cu-tolerant wood-decay fungi are classified (Fröhlich-Nowoisky et al., 2009). The concentration of fungal spores is estimated to be approximately 10³–10⁴ m⁻³ on continental land surfaces, for a global emission of 5 × 10¹⁰ kg/year (Elbert et al., 2007), which implies that fungal spore production is one of the largest sources of organic aerosols.
As these formulations are already being used (e.g. the US market) a careful monitoring and fundamental understanding of the underlying mechanism how these Cu-based NPs behave is necessary. This knowledge could also be used to design or control their function and fate in order to create a safer product with similar efficiency.

4 Conclusions and recommendations

The current knowledge provides circumstantial evidence for the release of Cu-based NPs from treated wood into the environment. In addition to leaching (Ding et al., 2013), one likely release route may be during the colonization of treated wood by copper-tolerant wood-destroying fungi. The accumulation of Cu in spores of mitosporic fungi has already been identified for standard Cu salts and compounds.

The environmental fate of Cu-based NPs from wood preservatives is only poorly understood. Despite broad knowledge of Cu-based NPs toxicity, to date the assessment of the potential risk related with the introduction of Cu-based NPs for wood protection (thousands of tons annually) is impossible to make. Therefore, the quantification of accumulation rate and release of Cu is mandatory. This issue can be solved only if the nanoscience and the wood protection communities interact and collaborate, as already stated by Evans et al. (Evans et al., 2008) years ago. More specific, nanosafety experts and wood specialists have the skills, analytics and understanding in order to explore and assess the environmental fate and health impact of Cu-based NPs in wood preservatives.

Author contributions
The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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3. Materials & methods

In this chapter only the main techniques are described, in order to explain why a certain method was used. Specific details about material and methods can be found within the compiled papers.

3.1 Wood and wood preservatives

3.1.1 Wood species

The studies were conducted on defect-free sapwood and heartwood specimens from two different wood species, Scots pine (*Pinus sylvestris* L.) and Norway spruce (*Picea abies* (L.) Karst.). The first one provides a good example of an easily treatable wood species, widely used in North America, and standard in wood preservative effectiveness tests; whereas Norway spruce is a refractory wood species commonly used in Central Europe, due to its abundance [1]. The assessment of MC penetration in both species gives a clear overview on the suitability of the treatment for the two different wood markets, as well as highlight potential major differences in the impregnation chemistry.

3.1.2 Micronized copper

Two commercially available MC formulations were tested. Both contained tebuconazole (TBA) as co-biocide, defining them as MC azole (MCA) formulations.

The MC particles present in both formulations were comparable in terms of size, morphology, and zeta (ζ)-potential, according to both the manufacturer’s descriptions and the characterization conducted. The techniques used to characterize the nanoparticles mostly consisted in NP tracking analysis and transmission electron microscopy (TEM). The difference between the two formulations was the concentration of TBA, which accounted to 0.4% w/w or 5% w/w. The size range of the MC particles according to the manufacturer was between 1 nm and 250 µm, however the analyses conducted revealed narrower size distributions for both formulations: the mean diameters were 104 ±1.7 nm (mode: 87±2.2 nm) for one formulation and 174±5.9 nm (mode: 150±8.2 nm) for the other one.

The chromated Cu (CC) formulation used as reference adheres to the ENV 807 [2] guidelines and it is composed of 50% w/w copper sulfate pentahydrate (CuSO₄·5H₂O), 48% w/w potassium dichromate (K₂Cr₂O₇), 2% w/w chromium trioxide (CrO₃).

3.2 Wood preservative effectiveness

The effectiveness of MC-treated wood against fungal decay was assessed according to the guidelines provided by the European Standards EN 113 [3] and ENV 807 [2], tests that focus on the wood preservative effectiveness against wood-destroying basidiomycetes and soft rot fungi, respectively. The main outcome of these tests is the wood mass loss, which is the percentage change between the wood
mass before and after fungal exposure. These tests allow to correlate wood mass losses with preservative effectiveness. The wood preservative treatment (or its dilution) is considered effective against wood-destroying fungi when the wood mass losses are below 3%, otherwise the treatment is considered ineffective.

3.2.1 Wood impregnation

Wood block dimensions, initial mass, and dry mass (oven dried at 103 °C for minimum 18 h) were recorded, oven dry and raw densities were calculated based on these measurements.

The wood samples were subsequently immersed in different dilutions of the wood preservative and stored under vacuum for 20 minutes, shaking the container from time to time to avoid particle deposition, and at normal conditions for 2 hours. Afterwards, the samples were removed from the solutions, the excessive liquid on the wood block surface was removed with filter paper. Wood samples mass after impregnation was measured, solution uptake was calculated as the difference between the wood block mass after impregnation and its initial oven dried mass, preservative retention was calculated as follows:

\[
Preservative\ retention\ \frac{(kg)}{(m^3)} = \frac{Solution\ uptake\ (kg) \times Solution\ concentration}{Volume\ (m^3)}
\]

The samples were then stored in a conditioning chamber avoiding contact between specimens for 8 weeks, according to the indications provided by the MC manufacturers. The container was kept covered for 1 week, and then slowly opened within the 8 week drying period.

3.2.2 Effectiveness against soft rot fungi-ENV 807

Natural top soil without any agro-chemicals, with pH between 6 and 8, and a water holding capacity between 25% w/w and 60% w/w was used. The soil was distributed in test vessels, wetted with water to reach the 95% of its water holding capacity, thoroughly mixed, and the masses was recorded. Small wood stakes (100 x 10 x 5 mm) untreated, MCA- and CC-treated were exposed to leaching accelerated ageing procedure (EN 84) [4] and were then planted into the soil with 20 mm in length protruding and with 20 mm space in between the samples. The vessels were stored at 28°C and 85% RH for 32 weeks. After 16 and 32 weeks incubation, replicate sets of test specimens were removed, brushed free of soil particles, and weighted. The samples were subsequently oven dried (103 °C). The percentage of wood mass loss was calculated from the dry mass before and after soil plantation.

3.2.3 Effectiveness against wood-destroying basidiomycetes-EN 113

Fungal mycelia were grown in 9 cm diameter Petri dishes with 25 mL solid medium (autoclave sterilized) containing 4% (w/v) malt extract and 2.5% (w/v) agar at 22°C and 70% RH. After min. 2 weeks (time required for the fungus to colonize the whole Petri dish) mycelia plugs of 9 mm in diameter were
placed in Kolle flasks with 75 mL solid medium (autoclave sterilized) containing 4% (w/v) malt extract and 2.5% (w/v) agar. The fungi were allowed to grow in the Kolle flasks for 2 weeks at 22°C and 70% RH (time required for the fungus to colonize the whole Kolle flask). Afterwards a glass structure for support and the wood samples (50 x 25 x 15 mm) were placed into the Kolle flasks. The Kolle flasks were then stored at 22°C and 70% RH for 16 weeks. After incubation, wood blocks were removed from the Kolle flasks, brushed free of mycelium, weighted, and oven dried at 103 °C. The percentage of mass loss was calculated from the dry mass before and after fungal exposure. The wood mass loss values were then corrected by subtracting the wood mass loss values obtained from sterile control wood samples.

### 3.3 Mechanical abrasion

Different woodworking activities, such as sawing, wiping, or milling, are responsible for the generation and release of airborne wood dust. To measure the release of particles during the mechanical abrasion of wood, we used a Taber Abraser. This is a widely used device to simulate sanding processes and to study abrasion resistances of materials and coatings, and is recognized by various international standards; e.g. EN 13696 [5] and EN 14354 [6]. It provides a reproducible continuous abrasion process under defined conditions. Several studies have used the Taber Abraser to generate particles from nanocomposites, to measure the particle size distribution, and to check if nanoparticles were released [7-11]. An abrasion process generally releases particles in the size range from a few nm to several µm. The size, morphology, and Cu content of the wooden abraded particles were assessed. Based on lung penetration [12], asbestos-like pathogenicity [13, 14], and heavy metal accumulation [15], the parameters above mentioned would provide an indication on the likelihood of adverse health effect in case of inhalation of the airborne particles released. The full size range of the wood dust particles released was characterized by aerosol measurement devices directly after the abrasion process using a scanning mobility particle sizer (SMPS), to detect particles below 1 µm, and an aerodynamic particle sizer (APS), for the measurement of bigger particles (up to 10 µm). After collection on grids, the morphology of the abraded particles was analyzed by scanning electron microscopy (SEM). Finally, elemental analysis to quantify the Cu content in the wood dust was performed by means of ion-coupled plasma (ICP)-mass spectrometry (MS) and ICP-optical emission spectrometry (OES).

### 3.4 X-ray techniques

X-rays are electromagnetic radiations with energies in the range between 100 eV and 100 KeV. These allow the X-rays to interact with atoms’ core electrons and provide indications on the elements being hit by them; therefore they can be applied for qualitative and semi-quantitative analytical purposes. The interactions between the X-rays and matter can be ascribed to three basic phenomena: absorption, scatter, and fluorescence. These constitute the underlying principles behind X-ray methods.
The X-ray analysis performed originated from both laboratory-scale and synchrotron X-ray sources. X-ray computed tomography (CT) allowed to non-invasively investigate the distribution of copper in the wood, as well as the fungal decomposition pathways. Whereas X-ray absorption spectroscopy, (XAS) enabled the assessment of copper speciation and distribution in fungal structures (mycelia and spores).

3.4.1 X-ray computed tomography

X-ray CT allows the reconstruction of the internal structure of a material (or a portion of it) without physical cutting of the sections for the examination. This is achieved in a relatively short time, under ambient conditions, and almost no sample preparation is required. In X-ray CT imaging, X-rays are directed at an object from multiple orientations and the beam intensity is monitored. Changes in the intensity are caused by X-ray energy, X-ray beam path length, and the materials composing the sample (material linear attenuation coefficient). Afterwards, algorithms are used to reconstruct the distribution of X-ray intensities in the sample volume.

Large wood samples were analyzed by means of the multi-resolution micro-CT scanner Nanowood [16, 17] built at the Ghent University Centre for X-ray Tomography (Ghent University, Belgium). These samples were selected to assess the overall distribution of Cu in wood or to illustrate fungal decay patterns. The samples were scanned using a closed type microfocus X-ray tube at 100 kV and 80 µA, 1000 projections and 1000 ms exposure time per projection. Reconstructions were performed with the Octopus Reconstruction software package [18], a tomography reconstruction package for parallel, cone-beam and helical geometry licensed by InsideMatters (www.insidematters.be), resulting in reconstructed data with an approximated voxel pitch of 31 µm. The reconstructed volumes were analyzed using Octopus Analysis, previously known as Morpho+ [19] and also licensed by InsideMatters. For the assessment of Cu penetration, the entire wood block was segmented and Cu was separated and quantified within the block. Volumes were also rendered in 3D using VGStudio Max software.

Small wood samples were used to investigate the Cu distribution or fungal decay in wood at the cellular level. The samples were scanned using an open type nanofocus X-ray tube at 80 kV and 45 µA, 1000 projections and 1500 ms exposure time per projection. Reconstructions were also performed with the Octopus Reconstruction software package, resulting in volumes with an approximate voxel pitch of 0.8 µm.

3.4.2 X-ray absorption spectroscopy

XAS is an element-specific method that provides information on the near atomic environment (2-3 closest neighboring atoms’ shells) of the absorbing atoms from the selected element. This is achieved by characterizing the element’s electronic transitions and the photoelectron emissions. It is possible to
distinguish three sections in X-ray absorption spectra: the pre-edge region, the X-ray absorption near edge structure (XANES), and the extended X-ray absorption fine structure (EXAFS).

The speciation of Cu was examined using the microXAS beamline at the Swiss Light Source. The samples were placed on a sample holder with Kapton foil window. The materials were analyzed at room temperature. Energy calibration of the Si111 double crystal monochromator was obtained by measurements of a metallic Cu reference foil (in transmission mode) prior and after taking experimental. First inflection point of the metallic copper K-edge spectrum was set to 8979 eV. Due to low Cu concentrations, XANES spectra of the samples were collected in fluorescence mode using a single element Silicon Drift Diode detector (SDD, KETEK GmbH). The beamsize was defocused resulting in a 700 µm diameter beam. In addition to the wood samples, several reference materials were analyzed at room temperature. To examine the possibility of beam-induced damage, spectra were collected at least twice from the same location and the spectra were compared to determine if the beam had caused a change in speciation. The XAS data processing software Athena was used to analyze the XAS spectra [20]. For each individual sample, multiple spectra were collected and merged. The XANES spectra obtained were used to compare the Cu speciation. The linear combination fitting tool provided by the software Athena was used to determine the speciation of Cu.

3.5 Ion-coupled plasma-mass spectrometry and optical emission spectrometry

ICP-MS and ICP- OES are commonly used techniques to determine the total content of selected elements in a sample. The samples are introduced as liquids -if solid, acid digestion prior to the elemental analysis is performed- and turned by means of a nebulizer into an aerosol, which is sprayed into the center of the plasma. The latter vaporizes, atomizes, ionizes, excites and subsequently relases the aerosol’s particles, which are then detected and quantified by optical emission spectrometers or mass spectrometers. OES uses the emission spectrum of the material analyzed, whose wavelength is element-specific, while MS separates the ions based on their mass-to-charge ratio. Both MS and OES provide information on the concentration of the element of interest based on the signal intensity detected.

These two techniques were employed to quantify the amount of copper contained in wood, wood dust, cell eluates, and fungal structures. The solid samples underwent acid digestions before being analyzed. The detailed procedures differed for each samples and are summarized in the research articles within Chapter 4.
3.6 Acute toxicity for human lungs

Basic acute toxicity tests on a biological model representing the lungs were performed to obtain preliminary indications on the potential hazard of wood dust from MC-pressure-treated wood. The biological model consisted of A549 lung epithelial and human acute monocytic leukaemia THP-1 cell lines. The oxidative stress paradigm [21] was employed and the following parameters were assessed:

1. Reactive oxygen species (ROS) formation: overproduction of ROS can cause cell oxidative stress and damages to the cell structures. Therefore, the concentration of ROS within a cell is a good indicator of cellular fitness.
2. Cell viability: to assess if the cells are undergoing apoptosis.
3. Inflammatory response: release of the pro-inflammatory tumor necrosis factor TNF-α.

3.7 References of Chapter 3


4. Results & discussion
4.1 Wood preservative effectiveness

4.1.1 Micronized copper wood preservatives: efficacy of ion, nano, and bulk copper against the brown rot fungus Rhodonia placenta

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Micronized copper wood preservatives: efficacy of ion, nano, and bulk copper against the brown rot fungus Rhodonia placenta

Short title: Ionic, nano and bulk copper against Rhodonia placenta

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Abstract

Recently introduced micronized copper (MC) formulations, consisting of a nanosized fraction of basic copper (Cu) carbonate (CuCO$_3$·Cu(OH)$_2$) nanoparticles (NPs), were introduced to the market for wood protection. Cu NPs may presumably be more effective against wood-destroying fungi than bulk or ionic Cu compounds. In particular, Cu-tolerant wood-destroying fungi may not recognize NPs, which may penetrate into fungal cell walls and membranes and exert their impact. The objective of this study was to assess if MC wood preservative formulations have a superior efficacy against Cu-tolerant wood-destroying fungi due to nano effects than conventional Cu biocides. After screening a range of wood-destroying fungi for their resistance to Cu, we investigated fungal growth of the Cu-tolerant fungus Rhodonia placenta in solid and liquid media and on wood treated with MC azole (MCA). In liquid cultures we evaluated the fungal response to ion, nano and bulk Cu distinguishing the ionic and particle effects by means of the Cu$^{2+}$ chelator ammonium tetrathiomolybdate (TTM) and measuring fungal biomass, oxalic acid production and laccase activity of R. placenta.
Our results do not support the presence of particular nano effects of MCA against *R. placenta* that would account for an increased antifungal efficacy, but provide evidence that attribute the main effectiveness of MCA to azoles.

**Introduction**

Copper (Cu) has long been known for its fungicidal properties and it is an essential biocide for wood in contact with the soil, as it is the only active substance that hitherto successfully inhibits wood decomposition by soft rot fungi [1]. The efficacy of Cu-based wood preservatives against wood-destroying fungi is mainly exerted by Cu in its soluble form, as Cu$^{2+}$ ions [2–4]. However, an increased copper efficacy may be achieved at the nanoscale: several nanoparticles (NPs) have been shown to be more toxic to prokaryotes and eukaryotes than larger particles of the same chemical composition [5, 6] or dissolved ions [7–9]. In fungi, it is believed that NPs may enter the fungal cell through endocytosis [10] or, if smaller than the pores, across the cell walls eluding barrier and entering the plasma membrane [11, 12]. Afterwards, the NPs may be able to cross the cell membrane, enter into the fungal cell and get in direct contact with cell components. However, to date there is a lack of understanding of their specific mode of action in fungi, despite their key role in wood decomposition of treated wood or bioremediation of contaminated soils. Recently, basic CuCO$_3$·Cu(OH)$_2$ particulate systems for wood protection were introduced to the US market [13]. These wood preservatives, commonly known as micronized copper (MC) formulations, contain a considerable amount of nanosized CuCO$_3$·Cu(OH)$_2$ with low water solubility, and in some cases purely consist of nanoparticles [14].

When wood is treated with MC, some Cu reacts with wood and part of it remains in the form of unreacted Cu particles that provide a reservoir effect [15]. Most of the studies [16] on Cu bioavailability of micronized Cu have focused on the release of Cu$^{2+}$ ions [17–21]. However, the superior efficacy of the treatment [22–25] may be partially due to insolubilized persistent CuCO$_3$·Cu(OH)$_2$ NPs that diffuse through the fungal cell wall and its membrane and exert their toxic effects, also described as the Trojan horse mechanism [26].

In Cu-tolerant (brown rot) fungi the threshold for Cu- NPs may be lower than the threshold for Cu$^{2+}$ ions, due to nano formulation, and subsequently fungi may not be able to trigger Cu-tolerance mechanisms in presence of small amounts of MC. In this case, the main wood degradation mechanisms for brown rot fungi, i.e. free hydroxyl radical production via Fenton reaction, may be impaired. Thus, the production of mediators for the Fenton reaction, which also implicates Cu oxidases [27, 28], may not be stimulated by Cu-tolerant fungi, resulting in a biochemical activity pattern dissimilar from the array that occurs when Cu-tolerant fungi are exposed to bulk or ionic Cu. In particular, the production of oxalic acid [29] or laccase [30] may be reduced. Although Tang et al. [27] provided a thorough investigation on the gene
expression of fungi exposed to MC, so far no comparison with conventional Cu-based wood preservatives has been made.

Therefore the objective of this study was to determine if MC is more effective than standard Cu compounds against wood-destroying fungi due to specific nano formulation. For this purpose we first investigated the growth of different wood-destroying fungi in different MC-amended solid media and selected the most Cu-tolerant strain for further investigation. Subsequently, we assessed growth and enzyme activities of the selected Cu-tolerant fungus Rhodonia placenta (Fr.) Niemelä, Larss. & Schigel (= P. placenta) in liquid cultures amended with Cu\(^{2+}\) ions from Cu sulfate (CuSO\(_4\)), MC (nano), bulk CuCO\(_3\)-Cu(OH)\(_2\), with and without ammonium tetrathiomolybdate (TTM) a well-known Cu\(^{2+}\) ion chelator used to treat Cu poisoning and Wilson’s disease in human and animals [31-34]. In fungi, a high dose of TTM (IC\(_{50}\) 1.0±0.2 µM) can inhibit the production of tyrosinase [35], an enzyme responsible for melanin synthesis, but low dosages are well tolerated. The ligand binds selectively to Cu\(^{2+}\) ions, allowing us to investigate whether Cu\(^{2+}\) ions derived from solubilized Cu-NPs and is responsible for the toxicity [36] in Cu-amended fungal culture media. The impact of MC co-biocide, tebuconazole (TBA), was also assessed.

Two mediators involved in the Fenton reaction were measured to assess different fungal responses to Cu: oxalic acid and laccase. Oxalic acid is believed to be a key component in Cu detoxification due to its ability to bind Cu, whereas laccase is produced by R. placenta during wood colonization [29]. Subsequently, wood decay caused by R. placenta on wood samples treated with MC, bulk CuCO\(_3\)-Cu(OH)\(_2\) and TBA was assessed according to EN 113 [37] to determine the effectiveness of the different components in a more natural setting.

**Materials and Methods**

**Materials**

2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), bulk CuCO\(_3\)-Cu(OH)\(_2\), CuSO\(_4\), TBA and TTM were purchased from Sigma Aldrich, while agar, malt extract and potassium chloride from VWR (Oxoid, Darmstadt, Germany).

The oxalic acid assay Enzytec™ oxalic acid was purchased from R-Biopharm AG. The silver stain kit, Dodeca™ Silver Stain was obtained from BIO-RAD.

Two commercial aqueous suspensions of MCA were investigated. The two MCA formulations contain comparable amounts of Cu particles but differ in the amount of TBA: MCA HTBA contained 5% w/w and MCA LTBA 0.4% w/w TBA.
NP characterization

Cu particles in the MCA formulations were characterized prior to fungal exposure. Particle morphology was assessed by transmission electron microscopy (TEM) with a Zeiss 900 microscope (Zeiss SMT, Oberkochen, Germany). TEM grids (400 mesh) coated with 8 nm of carbon were incubated for 20 s on a 10 µl droplet of MCA diluted with nanopure water. The excess suspension fluid was drawn off with filter paper.

Particle size distribution was measured by nanoparticle tracking analysis (NTA) using a NanoSight LM20 (NanoSight Ltd., UK) on MCA diluted with Milli-Q water. Data analysis was performed with NTA 2.3.5 software (NanoSight Ltd., UK). Particle diameters are reported as average and standard deviation of seven video recording of the sample. Zeta potential measurements were carried out on MCA diluted with Milli-Q water using a Zetasizer NanoZS (Malvern Instruments, UK).

Screening for Cu-tolerance

All fungi used in the solid media study were wood-destroying basidiomycetes commonly used in EN 113 tests: *Antrodia serialis* (Fr.) Donk isolate 43, *Coniophora puteana* (Schumach.) Karst isolate 62, *Gloeophyllum trabeum* (Pers.) Murrill isolate 100, *R. placenta* isolate 45, *Trametes versicolor* (L.) Lloyd isolate 159 from the Empa culture collection. Fungal mycelia (9 mm in diameter) were grown in 9 cm Petri dishes with 25 mL solid medium (autoclave sterilized) containing 4% (w/v) malt extract and 2.5% (w/v) agar. The media were amended with either 0.01% (w/v) or 0.05% (w/v) of MCA_HTBA or MCA_LTBA. Three replicates were prepared for each condition. Cultures were stored at 22 °C and 70% RH. The cultures were inspected regularly, and their 4 cardinal points were marked to determine the growth radii until the colonies reached the edges of the Petri dishes. Fungal growth rate for each colony (in mm per day) was determined dividing the mean value of the latest growth radii minus that of the earliest by the number of days elapsed between the measurements.

Fungal response to Cu$^{2+}$ ions and particles

The fungus used in the liquid culture study was *R. placenta* isolate 45 from the Empa culture collection. Fungal mycelia were grown in 500 mL Erlenmeyer flasks with 250 mL liquid culture medium (autoclave sterilized) containing 1% (w/v) malt extract and 0.6% (w/v) potassium chloride.

To understand the effect of ion, nano or bulk Cu, the following materials were added respectively: CuSO$_4$, MCA, CuCO$_3$·Cu(OH)$_2$. The concentration of CuCO$_3$·Cu(OH)$_2$, and CuSO$_4$ was 0.02 mM. The quantity of MCA was calculated based on the equivalent 0.02 mM of total Cu. The interference caused by the azole biocide on the fungus was assessed by adding TBA. The amount of TBA, alone or with Cu, was calculated as the amount of TBA content in MCA (5% w/w). TTM (0.02 mM) was used to separate the Cu-based particles from the Cu$^{2+}$ ions. All these materials were added to the liquid cultures as indicated in Table 1.
Table 1- Scheme of the liquid cultures used.

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<thead>
<tr>
<th>Liquid culture media</th>
<th>Without TTM</th>
<th>With 0.02 mM TTM</th>
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<td>Control</td>
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<td>0.02 mM CuCO\textsubscript{3}·Cu(OH)\textsubscript{2} + 5% w/w TBA</td>
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<td>0.02 mM CuCO\textsubscript{3}·Cu(OH)\textsubscript{2} + 5% w/w TBA</td>
<td>0.02 mM CuCO\textsubscript{3}·Cu(OH)\textsubscript{2} + 5% w/w TBA</td>
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<tr>
<td>0.02 mM CuSO\textsubscript{4}</td>
<td>0.02 mM CuSO\textsubscript{4}</td>
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<td>5% w/w TBA</td>
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TTM= ammonium tetrathiomolybdate; MCA= micronized copper azole; CuCO\textsubscript{3}= Cu carbonate; TBA= 5 tebuconazole; CuSO\textsubscript{4}= Cu sulfate

Each treatment was repeated in triplicates. Each flask was inoculated with 1 disc (8 mm in diameter) of the strain pre-cultured in solid medium and incubated in an orbital shaker (100 rpm) at 22°C for 9 weeks. The pH of the liquid culture was between 4.5 and 5, assuming that some Cu particles would not solubilize but would remain suspended in the liquid media. After incubation, the biomass was harvested by filtration and oven dried at 107°C for 24 hours. Fungal growth was estimated as wet and dry biomass weight.

**Laccase activity**

Laccase activity in *R. placenta* 45 was measured after 16 weeks incubation in untreated, MCA_LTBA and MCA_HTBA-treated wood colonized by *R. placenta* 45 (see protective effectiveness of MCA active ingredients) and after 2, 4, and 9 weeks in liquid cultures. For detection of laccase in wood, the colonized samples were treated according to Wei et al. [30]. The grounded wood was stirred overnight at 4°C in dist. H\textsubscript{2}O containing 1M NaCl to extract extracellular proteins. The liquid phase was separated from the solids by vacuum filtration through Whatman no 1 paper and concentrated in an ultrafiltration cell (Ultrace, Millipore) fitted with 10-kDa cutoff membrane.

Laccase activity was measured as initial velocity of the oxidation of ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), 3 mM) to its cation radical at room temperature (22-25°C) and at pH 4.5 (citrate buffer 100 mM). Changes in absorbance (ΔA) at 420 nm were recorded with UV-visible spectrophotometer (Genesys 10S UV-vis, Thermo Scientific Inc., Waltham, MA, USA). One volumetric
activity unit (U) was defined as the amount of enzyme transforming 1 µmol of ABTS per min and the volumetric activities were calculated using an extinction coefficient (ε) of 36000 mol⁻¹ L cm⁻¹[38, 39].

**Oxalic acid assay**

After incubation, 0.5 mL of liquid media were taken from each culture medium and oxalate concentration in the samples was analyzed with a spectrophotometer (Genesys 10S UV-vis, Thermo Scientific Inc., Waltham, MA, USA) at 590 nm as specified by the instruction manual (Enzytec™ Oxalic acid, R-Biopharm AG). Samples were diluted 100-fold with distilled water before being used in the assay as they showed very high activities.

To determine any further changes in the protein secretions of *R. placenta* 45 exposed to the different amended media after incubation, 0.2 mL of liquid media was taken from each culture medium. Protein extracts from the control, CuCO₃·Cu(OH)₂, CuSO₄, MCA liquid media -with and without TTM- plus markers (BIO-RAD) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% gels loaded with 5 µL of each liquid culture sample and the marker. Gels were silver stained with the Dodeca™ Silver Stain kit according to the instruction manual (BIO-RAD).

**Efficacy of MCA active substances**

Scots pine (*Pinus sylvestris L.*) sapwood blocks (50 x 25 x 15 mm) were pressure treated according to the European standard EN 113 [37] with different concentrations of MCA (2%, 1.6%, 1.33%, 1.07%, 0.8%, 0%). Scots pine sapwood blocks (50 x 25 x 15 mm) were also impregnated with 2% and 1.6% equivalent concentrations of CuCO₃·Cu(OH)₂ or TBA. No permits were required for the described study, which complied with all relevant regulations. No endangered or protected species were involved. After drying, the samples were exposed to *R. placenta* 45 at 22 °C and 70% RH. Test procedures were performed according to the European standard EN 113 [37]. After incubation, wood blocks were removed from the culture vessels, brushed free of mycelium and oven dried at 103±1 °C. The percentage of weight loss was calculated from the dry weight before and after the test.

**Statistical analysis**

Growth data and oxalic acid concentrations from fungi growing on solid and liquid media and on wood were log-transformed and data expressed as percentages, such as mass loss, were arcsine-transformed prior to analysis (ANOVA) and back-transformed to numerical values for visualization. Means were separated using Tukey’s-HSD (Honestly Significant Difference) test at significance level p<0.05. The statistical package used for all analyses was SPSS® (Version 17.0, SPSS Inc., Chicago, IL, USA).
Results
NP characterization

Fig. 1. Characterization of Cu particles in the micronized Cu azole (MCA) formulations assessed. TEM micrographs of (a) MCA_HT [40] and (b) MCA_LT; average particle size distribution of (c) MCA_HT and (d) MCA_LT. Data represented as mean ± standard deviation of seven repetitions. The MCA_HTBA formulation contains high amount of tebuconazole (TBA) (5% w/w), whereas MCA_LTBA contains low amount of TBA (0.4% w/w).
The Cu particles in the two MCA formulations were comparable and appeared heterogeneous in size and morphology, as shown in the TEM micrographs (Figs. 1a, b). The size distributions were also similar for the MCA formulations (Figs. 1c, d). The mean diameter was 104 ±1.7 nm (mode: 87±2.2 nm) for MCA_HT and 174±5.9 nm (mode: 150±8.2 nm). Therefore, the Cu particles were solely in the nano-range. The Cu particles in diluted MCA_HT and MCA_LT had an average ζ-potential of -21.0±0.4 mV and -16.5±1.4 mV respectively, indicating suspensions that tend to aggregate.

**Screening for Cu-tolerance**

We evaluated the growth of different wood-destroying fungi in MCA-amended media to identify the most Cu-tolerant strain for subsequent studies. Fig. 2 shows the mean growth rate for *A. serialis* 43, *C. puteana* 62, *G. trabeum* 100, *R. placenta* 45, and *T. versicolor* 159 in the different media.

Both concentrations of the MCA formulations caused appreciable reductions in fungal growth rate compared to the controls (p-value < 0.001). *C. puteana* 62 was not able to grow in any of the amended media, showing no tolerance to Cu or TBA, whereas *A. serialis* 43 and *G. trabeum* 100 effectively grew only in 0.01% MCA_LTBA. Differences in overall fungal growth rates were more evident at 0.01% for MCA_LTBA and MCA_HTBA, as distinct patterns were apparent (p-value < 0.001). Media with 0.05% MCA similarly inhibited fungal growth. Minor growth rates at such concentrations were recorded only for *T. versicolor* 159 and *R. placenta* 45. These two strains also outperformed the other fungi at lower concentrations (p-value < 0.001). In particular, mean growth of *R. placenta* 45 was overall the highest, which indicated a high Cu-tolerance. Therefore, this strain was selected for the subsequent tests.

**Fungal response to Cu$^{2+}$ ions and particles**

We assessed the response of *R. placenta* 45 to Cu ions, NPs or bulk material to determine if nano effects may account for a superior efficacy of MCA. The effect of Cu$^{2+}$ from dissolution of CuSO$_4$, nano Cu from MCA, bulk CuCO$_3$·Cu(OH)$_2$, and TBA on fungal growth is shown in Fig. 3 as mean fungal wet biomass values (dried biomass are in accordance and are not shown).

The differences observed between the tested groups were significant, as indicated by Tukey’s test on the wet biomass production. The addition of TTM to liquid cultures substantially reduced biomass production. What emerged is that TBA strongly suppresses growth of *R. placenta* 45. When TBA was associated with Cu (in MCA or with CuCO$_3$·Cu(OH)$_2$) its inhibition was reduced as follows: TBA > TBA + CuCO$_3$·Cu(OH)$_2$ > MCA.
Fig. 2. Influence of different concentrations and formulations of micronized copper azole (MCA) on the daily growth rate of *A. serialis* 43, *C. puteana* 62, *G. trabeum* 100, *R. placenta* 45, and *T. versicolor* 159. The MCA_HTBA formulation contains high amount of TBA (5% w/w), whereas MCA_LTBA contains low amount of TBA (0.4% w/w). Data represented as mean ± standard deviation of three repetitions.
Fig. 3. Influence of different forms of Cu on the fungal wet biomass produced by *R. placenta* 45 in liquid cultures. TTM= 0.02 mM ammonium tetrathiomolybdate; MCA= 0.02 mM of free Cu$^{2+}$ ions in micronized copper azole; CuCO3= 0.02 mM Cu carbonate; TBA= 5% w/w tebuconazole; CuSO4= 0.02 mM Cu sulfate. Data represented as mean ± standard deviation of three repetitions. Shared letters indicate no significant difference in wet biomass production, different letters denote significant differences in wet biomass production after the Tukey’s HSD test.

**Laccase activity**

Laccase was detected in both MCA- treated (MCA_LTBA and MCA_HTBA) and untreated wood. The amount detected was minor (approx. 1U/L). On the other hand, monitoring of the enzymatic reaction of laccase in liquid cultures by spectrophotometry did not indicate the production of laccase by *R. placenta*.
45 in the growth media after 2, 4 and 9 weeks incubation period (data available through the ETH Data Archive at: http://doi.org/10.5905/ethz-1007-21).

**Oxalic acid production**

Fig. 4 shows the mean values of oxalic acid produced by *R. placenta* 45 at different conditions.

The amount of oxalic acid measured represents only soluble free acid and salts, but it does not take into account the copper oxalate and/or calcium oxalate water insoluble precipitates. The differences observed between the different groups were significant (Tukey’s test). Similarly to the results highlighted in the fungal biomass tests, TBA heavily suppressed the production of oxalic acid by *R. placenta* 45. Oxalic acid production in cultures with MCA, CuCO$_3$·Cu(OH)$_2$ , CuCO$_3$·Cu(OH)$_2$+TBA was lower than in the controls. The addition of TTM to the Cu- amended liquid cultures resulted in an increase in the oxalic acid production from MCA and CuCO$_3$·Cu(OH)$_2$, both containing CuCO$_3$·Cu(OH)$_2$, whereas it did not cause any major difference in CuCO$_3$·Cu(OH)$_2$+TBA and CuSO$_4$. The highest oxalic acid concentration was measured in cultures exposed to MCA + TTM.

The protein profiles obtained by SDS-PAGE showed no difference in the protein expression profiles involved for *R. placenta* 45 under different growth conditions (data available through the ETH Data Archive at: http://doi.org/10.5905/ethz-1007-21), therefore it is evident that laccase and oxalic acid are the main contributors and no further protein analysis to determine different behavior due to Cu exposure was performed.

**Efficacy of MCA active substances**

We assessed the contribution of TBA and CuCO$_3$·Cu(OH)$_2$ in MCA-treated wood. Wood mass losses for the different treatments are presented in Fig. 5. MCA_HTBA, MCA_LTBA and TBA effectively protected wood against fungal colonization and degradation. In particular, MCA_HTBA and MCA_LTBA significantly inhibited fungal growth even at the lowest concentration of 0.8% compared to the controls (p-value < 0.001 in both cases, data available through the ETH Data Archive at: http://doi.org/10.5905/ethz-1007-21). Wood samples treated with CuCO$_3$·Cu(OH)$_2$ did not reduce mass losses (p-value = 1 for both concentrations). The effects of MCA_HTBA and MCA_LTBA were significantly different at concentrations ≥ 1.33% (data available through the ETH Data Archive at: http://doi.org/10.5905/ethz-1007-21): while the mass losses of MCA_HTBA-treated wood samples were < 3%, mean mass losses of MCA_LTBA-treated wood samples decreased by 9.1%. At concentrations of 1.6% the effect of MCA_LTBA was comparable to the equivalent amount of TBA alone (p-value = 0.997), whereas at concentrations of 2% MCA_LTBA showed a better performance (p-value < 0.001).
Fig. 4. Influence of different forms of Cu on the oxalic acid production by *R. placenta* 45 in liquid cultures. TTM= 0.02 mM ammonium tetrathiomolybdate; MCA= 0.02 mM of free Cu$^{2+}$ ions in micronized copper azole; CuCO3= 0.02 mM copper carbonate; TBA= 5% w/w tebuconazole; CuSO4= 0.02 mM Cu sulfate. Data represented as mean ± standard deviation of three repetitions. Shared letters indicate treatments that were not significantly different, different letters denote significant differences in treatments after the Tukey’s HSD test.
Fig. 5. Assessment of micronized Cu azole (MCA) formulations, associated mass losses and the role of each active substance for wood protection against *R. placenta* 45. CuCO3 = Cu carbonate; TBA = tebuconazole. The MCA_HTBA formulation contains a high amount of TBA (5% w/w), whereas MCA_LTBA contains a low amount of TBA (0.4% w/w). Data are represented as mean ± standard deviation of four repetitions. Shared letters indicate treatments that were not significantly different, different letters denote significant differences in treatments after the Tukey’s HSD test.

**Discussion**

Cu is currently used to protect wood from fungal decomposition due to its antifungal properties. In particular, Cu is responsible for interference with homeostatic processes and cell membrane functions [41], protein and enzyme damage and precipitation [42], production of reactive oxygen species [43, 44] and DNA disruption [45]. When Cu is available as NPs these effects may be enhanced. We investigated here in a systematic approach which formulation (ionic, nano or bulk) of Cu is the most effective against
Cu-tolerant basidiomycetes. We discriminated between the effects caused by the particles themselves and those caused by their dissolution into Cu\(^{2+}\) ions using TTM, a chelator for Cu\(^{2+}\) ions.

Our results showed that *T. versicolor* 159 and *R. placenta* 45 were the two strains that were less influenced by the MCA formulations. We mainly attributed this behavior to TBA-tolerance mechanisms for the white rot fungus *T. versicolor* 159 [46], and to Cu-tolerance mechanisms for the brown rot fungus *R. placenta* 45 [47]. The main mode of action for TBA consists in fungal cell membrane disruption by inhibition of ergosterol formation [48], whereas Cu exerts its toxic effects on fungal cells by disrupting different basic metabolic processes. Therefore, *R. placenta* 45 was selected for the subsequent studies, as it would provide an indication for possible nano effects exerted by MCA on highly Cu-tolerant fungi that would reduce the Cu threshold level and would result in effective protection of wood at lower Cu concentration than the Cu\(^{2+}\) ion or bulk counterpart.

The liquid culture study confirmed the suitability of TTM to discriminate between Cu\(^{2+}\) ionic and particle effects. In TTM only-amended cultures, biomass and oxalic acid production were lower than in the control cultures, indicating that TTM bound to essential Cu\(^{2+}\). Therefore, the study shows that TTM can be effectively employed for studies on Cu-based NPs, for instance in the field of nanotoxicology, where a similar approach has been developed for zinc oxide NPs by Bürki-Thurnherr et al. [49]. It was not possible to identify the presence of laccase in liquid cultures, although this was clearly detected on wood in the EN 113 study. In addition, the amount of laccase detected in untreated and MCA-treated wood was similar. These two findings indicate that laccase is probably not the principal mechanism for Cu detoxification in *R. placenta*. Furthermore, we have evidence that supports the fundamental role played by laccase in the Fenton reaction. The Fenton reaction is used by brown and white rot fungi to initialize the attack of wood, as it allows the depolymerization of polysaccharides and lignin by generating radicals [50], whereas in artificial media sugars are readily available, hence radicals are not required. Fungal biomass and oxalic production measurements provided a clear picture on fungal response to Cu in its various forms and TBA. The lower oxalic acid concentrations found in MCA, CuCO\(_3\)-Cu(OH)\(_2\), CuCO\(_3\)-Cu(OH)\(_2\)+TBA cultures is in good agreement with Green and Clausen findings [51], which revealed that the oxalic acid production of two *R. placenta* strains was reduced in Cu-treated wood. The higher oxalic acid concentrations in CuSO\(_4\) (Cu\(^{2+}\) ions)-amended cultures can be related to the increased biomass produced. In addition, the oxalic acid production was stimulated in Cu+TTM-amended cultures, therefore confirming that free Cu can reduce oxalic acid production. For both fungal biomass and oxalic acid production, the major inhibiting agent was TBA, however the effects were reduced in the presence of...
Cu, especially for MCA. Therefore, we hypothesize that Cu, here at sub-lethal concentrations, can stimulate growth and enzyme production of *R. placenta*, as indicated in former studies [52, 53]. In addition, for MCA other chemicals in the formulation may have influenced fungal growth by providing additional nutrients. Thus, for the concentrations used, there was no indication of a Cu+TBA additive or synergistic effects against Cu-tolerant fungi. Although there is a lack of scientific literature on Cu and TBA additive, synergistic or antagonist effects, Sun et al. [54] showed a similar behavior for a range of moulds that can biotransform TBA [55, 56], hence effectiveness of pure Cu was higher than Cu combined with TBA. In any case, we found no evidence for a specific nano effect against *R. placenta* and the main active substance against *R. placenta* was clearly TBA. This means that the fungus is able to recognize Cu also as MCA NPs and can trigger the same Cu-tolerance mechanisms typically shown in the presence of bulk Cu or Cu ions. No additional protein expression patterns were evident in SDS-PAGE analysis, therefore oxalic acid and laccase were valid parameters for determining fungal response to Cu.

Finally, to complete the study, we investigated fungal growth on treated wood i.e. a more natural setting. In this case, the EN 113 guidelines were applied and mass losses of wood treated with bulk CuCO$_3$·Cu(OH)$_2$, TBA and two MCA formulations differing in TBA content were compared. This test provides indications on the expected short term effectiveness of the wood treatments.

The recorded mass losses were in good agreement with our findings on fungal growth in liquid cultures. Even in treated wood TBA is largely responsible for the effectiveness of MCA, although high concentrations of Cu also affect the performance. In particular, for the formulations and wood decay fungi assessed, we propose a 1.6% MCA < Cu threshold ≤ 2% MCA. The low effectiveness of CuCO$_3$·Cu(OH)$_2$ is mainly attributed to the poor penetration into the wood: the wood samples did not show any color change towards green/blue due to the presence of Cu, and unreacted CuCO$_3$·Cu(OH)$_2$ only appeared as fine dust unbound on the sample surfaces.

In conclusion, the NPs in the MCA formulations assessed did not provide additional protection against *R. placenta* and the main effectiveness has to be attributed to TBA. Therefore, considering the antifungal properties, the efficacy of the MCA formulations tested are not better than conventional Cu azole formulations that do not employ nanotechnologies. MCA-treated wood will still be susceptible to biodegradation by Cu- or TBA-tolerant fungi. From a life cycle assessment perspective, MCA is less eco-efficient than Cu azole, due to the higher energy consumption during the milling process of MC [57]. However, this would also imply no additional risk for the microbial community in vicinity of MCA-treated wood.
Further studies with other wood-destroying fungi and different MC formulations are required to provide a more comprehensive picture on MC NPs effects on wood-destroying fungi. In addition, field studies are required to confirm our lab-scale findings and assess the long term performance. In particular, TTM could not be used in the EN 113 test, due to the absence of a liquid environment that would allow chelation of Cu$^{2+}$ ion. Therefore, tests with wood samples immersed in liquid cultures and TTM, in a setting similar to the one suggested by the EN 275 [58] guidelines, may provide further details on the fungal response to Cu$^{2+}$ ions and particles. Future studies should focus on the fungal gene pathways that are involved for tolerance mechanisms against TBA and Cu.

Acknowledgments

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References of Chapter 4.1.1


4.1.2 Effectiveness of micronized copper azole against soft rot fungi

Unpublished report

Keywords: Micronized copper azole, Pressure-treated wood, Soilborne fungi, X-ray computed tomography

Abstract

The protective efficacy of micronized copper azole (MCA) against soft rot fungi was assessed by combining the European Standard ENV 807 [1], which relates wood decay with wood mass loss, and X-ray computed tomography (CT) to visualize the decay at low and high resolutions. The results indicate that in a laboratory setting MCA can protect wood against soft rot fungi and other soilborne microorganisms.

Introduction

Copper (Cu) is essential for the protection of wood in ground contact (use class 4, European Standard EN 335 [2]), as it has the potential to prevent decay by soft rot fungi. Recently, an innovative wood preservative treatment based on particulate Cu was developed. This is best known as “micronized copper” (MC) and is mainly composed of basic copper carbonate particles, whose sizes range between 1 nm and 250 μm, and an organic co-biocide, either azole (MCA) or quaternary ammonium compounds (MCQ) [3, 4]. Since its introduction to the wood protection market in 2006 [5], issues have been raised concerning its effectiveness against soft-rot fungi [6]. In order to answer these questions, a few studies investigated these aspects. Zhang and Ziobro [7] assessed the performance of MCA- and MCQ-pressure-treated P. radiata and alpine ash (E. delegatensis), wood species uncommon in Europe, against soft rot fungi and soilborne microorganisms in laboratory and field settings. Their tests highlight how MCA performed equally or better than conventional or MCQ wood preservatives. Ray et al. [8] investigated the effectiveness of MCQ-pressure-treated beech (F. sylvatica) and Scots pine (P. sylvestris), which are European wood species, against soft rot fungi applying the European Standard ENV 807 [1]. Their results indicate that MCQ and conventional preservatives provide equivalent performance in beech, but the conventional wood preservatives performed better in pine. This paper aims at filling the gaps investigating the effectiveness of MCA for the European Scots pine against soft rot fungi. In this way, we provide a direct comparison between the performance of MCA and MCQ, as well as the influence of the wood species on the treatment. We combined the laboratory soil block tests with a visual assessment by means of X-ray
computed tomography (CT) to get an insight into the effective mechanisms, and on the type and development of decay.

**Material and methods**

**Wood and wood preservatives**

Small wood stakes (100 x 10 x 5 mm) excised from the of Scots pine (*Pinus sylvestris* L.) sapwood were pressure-treated according to the European standard ENV 807 [1] with different concentrations (2.00%, 1.60%, 1.33%, 1.07%, 0.80%, 0.50%, 0.00%) of a commercial aqueous MCA formulation and with two different concentrations (2.00%, 1.60%) of a conventional CC wood preservative prepared according to the ENV 807 [1]. Leaching of all test wood samples was performed according to the European standard EN 84 [9].

**Effectiveness against soft rot fungi**

After drying, the ENV 807 wood samples were inserted and buried into boxes filled with unsterile conditioned natural top soil for 32 weeks at 28 °C and 85% RH. Test procedures were performed according to the European standard ENV 807 [1]. After incubation, wood blocks were removed from the test vessels, brushed free of soil and oven dried at 103±1 °C for a minimum of 18 h. The percentage of mass loss was calculated from the dry mass before and after the test.

**Visualization of wood decay**

Treated and untreated, colonized and uncolonized ENV 807 wood blocks and small subsamples (approx. 5 x 0.5 x 0.5 mm) extracted from the larger blocks were scanned with the multi-resolution micro-CT scanner Nanowood [10, 11] built at the Ghent University Centre for X-ray Tomography (Ghent University, Belgium). The large wood blocks were visualized to assess the overall wood decay. The samples were scanned using a closed type microfocus X-ray tube at 100 kV and 80 µA, 1000 projections and 1000 ms exposure time per projection. Reconstructions were performed with the Octopus Reconstruction software package [12], a tomography reconstruction package for parallel, cone-beam and helical geometry licensed by InsideMatters (www.insidematters.be), resulting in reconstructed data with an approximated voxel pitch of 31 µm. The reconstructed volumes were analyzed using Octopus Analysis, previously known as Morpho+ [13] and also licensed by InsideMatters.

The small subsamples were used to investigate fungal decay in wood at the cellular level. The samples were scanned using an open type nanofocus X-ray tube at 80 kV and 45 µA, 1000 projections and 1500 ms exposure time per projection. Reconstructions were also performed with the Octopus Reconstruction software package, resulting in volumes with an approximate voxel pitch of 0.8 µm.


### Statistical analysis

Wood mass losses, which were expressed as percentages, were arcsine-transformed prior to analysis (ANOVA) to obtain a normal distribution, and back-transformed to numerical values for graphical display. Means were separated using Tukey’s-HSD (Honestly Significant Difference) test at significance level p<0.05. The statistical package used for all analyses was SPSS® (Version 17.0, SPSS Inc., Chicago, IL, USA).

### Results

#### Effectiveness against soft rot fungi

Wood mass losses for the different MCA- and CC-pressure-treated samples after 32 weeks are reported in Fig. 1.

Generally, a cut-off at 3% is applied to determine if a preservative treatment is effective or not. The soft rot activity in the test environment, as measured in the control samples, was not high (mean mass loss below 15%). For industry purpose, these wood mass losses would invalidate the test, however these data are valid for research purposes. For the ENV 807 to be valid, the mass loss differences between the control and water-treated (0.00%) samples shouldn’t be significant, to prove that there was no issue with the impregnation process. In our case, the mass losses from the control and water-treated samples were comparable, indicating that the impregnation was conducted correctly. MCA significantly inhibited soft rot growth on wood blocks even at the low concentration of 0.80%, with mass losses as low as 1.92±0.86%. However even at the lowest concentration of 0.50% wood decay was considerably reduced (average wood mass loss: 3.1±1.5%). No significant difference was observed between the 0.80%, 1.07%, and 1.33% MCA-pressure-treated wood samples. Whereas the mass losses in 2.00% and 1.60% MCA-pressure-treated wood were considerably low (and comparable). Even 2.00% and 1.60% CC-pressure-treated wood blocks showed mass changes below |3|%. However, these samples were characterized by a slight mass increase, which could probably be explained by the interaction of CC with wood and/or soil. Therefore, the results of MCA and CC were not comparable, as suggested by the output from Tukey’s HSD test, and it is not possible to determine if CC- or MCA-pressure-treated wood had a better performance against soft rot fungi. Wood cell-wall treatments are more effective than cell-lumen ones against soft rot fungi [14] and are less prone to leaching [15]. The performance of MCA against soft rot fungi was positive, despite the leaching procedure.

#### Visualization of wood decay

X-ray CT provided a useful tool to confirm the presence and type of decay on ENV 807 wood blocks. Both high and low resolution scans of the ENV 807 wood blocks showed how the fungi were active only
in the outer layers of wood, which is typical for soft rot decay (Fig. 2a). The low resolution scans revealed that decay occurred more abundantly in the earlywood. This may be caused by the larger openings in

Fig. 1. Assessment of micronized Cu azole (MCA) and Cu/chromium (CC) effectiveness against soft rot fungi. Data are represented as mean ± standard deviation of four repetitions. Shared letters indicate
treatments that were not significantly different, different letters denote significant differences in treatments after the Tukey’s HSD test.

Fig. 2. Reconstructed slices of MCA-pressure-treated Scots pine at (a) low and (b, c) high resolutions. The decay occurs from the outer surfaces towards the inner layers. Soft rot decay is more abundant in earlywood. The arrows in (b) indicate the soft rot decay. In (c) the arrow point to a high-density spot that may be attributed to Cu.
earlywood compared to latewood, which allow the soilborne fungi to penetrate more easily into the wood structure.

Another factor could be the presence of higher amount of Cu in latewood after pressure-treatment, as indicated by Evans et al. [16]: although earlywood’s openings and pits could account for an easier penetration of Cu, latewood has a higher number of carboxylic acid reaction sites that could promote the solubilization and complexation of Cu by wood components. No fungal structure was visible in the high resolution scans (Fig. 2b). This agrees with the type of fungal attack expected, as soft rot fungi do not have hyphal structures like basidiomycetes, therefore we can confirm that the wood decay was caused by soft rot and other soilborne microorganisms, without any interference from other organisms. In both reconstructed samples it was also difficult to locate bright spots that would account for obvious particles or accumulation of high-density Cu spots, which appear rarely across the reconstructions (Fig. 2c). This could indicate that Cu is distributed within the wood structure as fine particles (or ions) and that the co-biocide tebuconazole (not visible by X-ray CT) could have played a significant role against soft rot fungi.

Discussion

The aim of this study was to assess whether MCA can protect wood from soft rot fungi inhabiting the soil and outperform conventional wood preservatives. We combined the European Standard test ENV 807 [1] with X-ray CT imaging in order to thoroughly assess the effectiveness of a MCA wood preservative and the decay caused to the wood blocks in soil. Our results are in good agreement with Zhang and Ziobro [7] and Ray et al. [8], and indicate that MCA is a valid wood preservative against soft rot fungi. In particular, the concentrations required to effectively prevent fungal decay were as low as 0.80%. Based on the good performance, we hypothesize that leaching of MCA-pressure-treated wood is likely to result in wood cell-wall Cu treatment. However tebuconazole may contribute to the effectiveness against soft rot fungi before its possible biodegradation by microorganisms inhabiting the soil [17]. Long-term stake test studies according to EN 252 [18] with MCA-pressure-treated Scots pine should be performed in order to confirm the validity of the laboratory test, and for comparison with laboratory and field tests on MCQ-pressure-treated wood [19-21]. Further, investigations on the contribution of tebuconazole in MCA towards soft rot fungi should be carried out.

References of Chapter 4.1.2


18. European Committee for Standardization. EN 252 Field test method for determining the relative protective effectiveness of a wood preservative in ground contact. Brussels, BE; 2014.


4.2 Penetration and distribution of Cu in wood

Penetration and effectiveness of micronized copper in refractory wood species

Short title: Can micronized copper protect refractory wood?

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Abstract

The North American wood decking market mostly relies on easily treatable Southern yellow pine (SYP), which is being impregnated with micronized copper (MC) wood preservatives since 2006. These formulations are composed of copper (Cu) carbonate particles (CuCO3·Cu(OH)2), with sizes ranging from 1 nm to 250 µm, according to manufacturers. MC-treated SYP wood is protected against decay by solubilized Cu2+ ions and unreacted CuCO3·Cu(OH)2 particles that successively release Cu2+ ions (reservoir effect). The wood species used for the European wood decking market differ from the North American SYP. One of the most common species is Norway spruce wood, which is poorly treatable i.e.
refractory due to the anatomical properties, like pore size and structure, and chemical composition, like pit membrane components or presence of wood extractives. Therefore, MC formulations may not be suitable for refractory wood species common in the European market, despite their good performance in SYP. We evaluated the penetration effectiveness of MC azole (MCA) in easily treatable Scots pine and in refractory Norway spruce wood. We assessed the effectiveness against the Cu-tolerant wood-destroying fungus *Rhodonia placenta*. Our findings show that MCA cannot easily penetrate refractory wood species and could not confirm the presence of a reservoir effect.

**Introduction**

Wood is a widely used building material and one of the reasons is its availability in various countries. This also results in a geographic-dependent decking market, as each country mostly utilizes the most accessible wood species. For instance, the North American market mostly uses SYP [1], whereas Norway spruce is the most used species in Central Europe [2].

The choice of a certain wood species for building applications results in clearly defined performance and service life, which can range from a few to many years. The specific anatomical and compositional features of wood are species-specific and cause major differences in the natural durability and the permeability to wood preservatives (or treatability), resulting in different wood species-based durability classes. According to the EN 350-2 [3] treatability is defined as the ease at which wood can be penetrated by liquids, e.g. wood preservatives. This mainly depends on the wood openings, the size and structure of tracheids, fibers, vessels, bordered- and simple pits. Due to the large size of the simple pits SYPs (*Pinus caribaea*, *Pinus echinata*, *Pinus palustris*, and *Pinus taeda*) are considered as easily treatable species, especially their sapwood [3]; whereas Norway spruce sapwood and particularly heartwood [4] are refractory to wood preservatives due to closure of the bordered pits [5, 6]. Therefore, wood preservative treatments developed for the easily treatable wood of the North American market may not be suitable for refractory wood species, like European widely used Norway spruce [2], or they may require additional treatments prior to impregnation, e.g. wood incising. This process facilitates drying of refractory species [7], and improves the penetration and retention of preservatives [8, 9].

One of the most common and at the same time most demanding building applications for wood is for structures in contact with the soil, defined as use class 4 by the European Standard EN 335 [10]. The latter wood products have to be treated with copper (Cu)-based wood preservatives to avoid decay by soil-borne microorganisms, especially soft-rot fungi [11]. The latest advance in Cu-based wood preservation is known as “micronized copper” (MC), and consists of basic Cu carbonate (CuCO$_3$·Cu(OH)$_2$) particles with a size range of 1 nm-250 µm, according to manufacturers, combined with a second organic biocide that provides protection against basidiomycetes, either azole (MCA) or quaternary ammonium compounds.
MC reputedly has a special impregnation chemistry that differs from conventional wood preservatives [14], i.e. part of CuCO$_3$·Cu(OH)$_2$ immediately solubilizes during the impregnation process and is complexed by wood organic macromolecules, while another fraction does not react and acts as reservoir, i.e. Cu is solubilized afterwards [14] and provides a continuous protection against wood-destroying fungi [15].

The entire literature on Cu distribution and speciation of this wood preservative in wood deals with easily treatable SYP [15-22], Scots pine wood [23], or red pine [17], and hypotheses on the possible penetration ability of MC in refractory wood species have been proposed [24]. However, to our knowledge there are no studies available that demonstrate the benefits of MC formulations for refractory wood species, either from North America or from Europe. Thus actual data on the Cu distribution from MC-treated refractory wood, as well as on its resistance against wood-destroying basidiomycetes are missing.

We hypothesize that the properties of MC by themselves cannot guarantee a homogeneous wood preservative penetration into refractory wood species, similarly to conventional wood preservatives. In order to verify that, in this paper we assess and compare the penetration of copper from MCA in Scots pine sapwood, Norway spruce sapwood and heartwood without any prior incision of wood. The penetration of Cu was assessed directly by means of X-ray computed tomography (CT) and ion-coupled plasma-optical emission spectroscopy (ICP-OES), and by indirect methods provided by the European standard EN 113 guidelines [25]. In addition, we compared MCA penetration effectiveness with its protective effectiveness against the Cu-tolerant [26] wood-destroying fungus Rhodonia placenta, which we previously used to test the ionic, nano, and bulk Cu effects of MCA [27]. By using a Cu-tolerant basidiomycete we could get an insight into the mechanisms behind MC superior effectiveness compared to conventional wood preservatives, as the fungus would not immediately succumb due to the presence of Cu, even if minimal, as it would happen with soft rot fungi.

**Material and methods**

**Materials**

A commercial aqueous suspension of MCA was investigated. The formulation tested here coincides with the formulation with high amount of tebuconazole MCA_HTBA we used in our previous investigation [27]. The latter study also provides a full characterization of the Cu particles in the MCA formulation. In brief, the measured particle size distribution of MCA was 104±1.7 nm with an average zeta potential of -21±0.4 mV.
Wood blocks (50 x 25 x 15 mm) were excised from Scots pine (*Pinus sylvestris* L.) sapwood, Norway spruce (*Picea abies* (L.) Karst.) heartwood and sapwood. Norway spruce sapwood and heartwood were localized by visual inspection, the heartwood was selected from an area as close as possible to the center of the trunk, while sapwood was selected from the outer region. The wood samples were pressure-treated according to the European standard EN 113 [25] with different concentrations of a commercial MCA aqueous suspension (100.00%, 2.00%, 1.60%, 1.33%, 1.07%, 0.80%, 0.00%). Three repetitions of 100.00% MCA-pressure-treated wood samples and six replicates for the diluted MCA-pressure-treated wood specimens were prepared. Small needle-shaped wood specimens (5 x 0.5 x 0.5 mm) from Scots pine sapwood, Norway spruce sapwood and heartwood were cut from the outer surface of EN 113 untreated blocks and were subsequently treated with MCA by dipping them into 100.00% MCA. No permits were required for the described study, which complied with all relevant regulations. No endangered or protected species were involved.

**Cu penetration, uptake and distribution in wood**

**Preservative retention**

Preservative retention in kg/m³ was calculated following the guidelines from the European standard EN 113 [25] with the following formula:

\[
Preservative\ retention = \frac{Solution\ uptake \times Solution\ concentration}{Volume\ of\ wood\ sample} \times 1000
\]

The solution uptake was calculated as the difference between the wood samples’ wet mass after impregnation and the oven dried (103±1 °C for 18 h minimum) initial mass prior impregnation, according to the European standard EN 113 [25].

**Quantification of Cu in wood**

Cu in wood was quantified by ICP-OES (Perkin-Elmer OPTIMA 3000, detection limit: 0.005 mg/L). For Scots pine sapwood, Norway spruce sapwood and heartwood EN 113 wood blocks untreated and pressure-treated with 100% MCA were ground into sawdust. Samples from the inner wood were selected cutting the first 15 mm off of the samples off and grinding the inner surface obtained. In addition samples from the outer edge (first 15 mm) of Scots pine sapwood were collected. In this way, a penetration gradient from the easily treatable wood species could be assessed. Digestion of the samples prior to ICP-OES was conducted according to Platten et al. [28]. Three replicates of each sawdust sample were digested with 3 mL of HNO₃ (65%) and 1 mL of H₂O₂ (30%) (MLS 1200 MEGA digestion system). Cu plasma standard solutions (100 mg/L) were used for calibration.
Visualization of Cu in wood

Before and after treatment, EN 113 wood blocks and small needle-shaped wood specimens from the three wood materials considered (Scots pine sapwood, Norway spruce sapwood and heartwood) pressure-treated with 100.00% MCA were analyzed by means of the multi-resolution micro-CT scanner Nanowood [29, 30] built at the Ghent University Centre for X-ray Tomography (Ghent University, Belgium). The EN 113 blocks were visualized to assess the overall penetration of Cu. The samples were scanned using a closed type microfocus X-ray tube at 100 kV and 80 µA, 1000 projections and 1000 ms exposure time per projection. Reconstructions were performed with the Octopus Reconstruction software package [31], a tomography reconstruction package for parallel, cone-beam and helical geometry licensed by InsideMatters (www.insidematters.be), resulting in reconstructed data with an approximated voxel pitch of 31 μm. The reconstructed volumes were analyzed using Octopus Analysis, previously known as Morpho+ [32] and also licensed by InsideMatters, to approximately visualize Cu distribution. Therefore, the reconstructed volumes were bilateral filtered to remove noise with edge preservation. Subsequently the wood was separated from the surrounding air such that further calculations were only performed within the wood block. Finally, Cu was segmented based on thresholding. The threshold level of the latter segmentation was chosen conservatively such that no wood was selected, which was based on scans of untreated wood blocks scanned with identical settings. Volumes were also rendered in 3D using VGStudio Max software.

The smaller needle-shaped specimens were used to investigate the Cu distribution in wood at the cellular level, and possible presence of nanoparticles attached or in the wood cells. The samples were scanned using an open type nanofocus X-ray tube at 80 kV and 45 µA, 1000 projections and 1500 ms exposure time per projection. Reconstructions were also performed with the Octopus Reconstruction software package, resulting in volumes with an approximate voxel pitch of 0.8 μm. Phase contrast effects were reduced using the Paganin method [33], also significantly improving the signal-to-noise ratio [34]. Due to the violation of the homogeneous object assumption in this method, additional smoothing around the MC is however introduced [35]. Obtaining quantitative results from these data is therefore not directly possible.

For both the low and high resolution scans, approximate detection limits on X-ray CT scans of Cu in wood were calculated, using the NIST XCOM database [36].

Effectiveness against Cu-tolerant basidiomycetes

After drying, the 2.00%, 1.60%, 1.33%, 1.07%, 0.80%, and 0% MCA-pressure-treated wood samples were exposed for 16 weeks at 22 °C and 70% RH to the Cu-tolerant wood-destroying basidiomycete R. placenta isolate 45 from the Empa culture collection. Test procedures were performed according to the
European standard EN 113 [25]. After incubation, wood blocks were removed from the culture vessels, brushed free of mycelium and oven dried at 103±1 °C for a minimum of 18 h. The percentage of mass loss was calculated from the dry weight before and after the test.

Some of the results from MCA-pressure-treated Scots pine wood were formerly published in a previous study [27], and we integrated it here to provide a complete overview on the effectiveness of MCA in different wood species.

**Statistical analysis**

Preservative retention data were log-transformed and data expressed as percentages (Cu amounts in wood and wood mass losses) were arcsine-transformed prior to analysis (ANOVA) and back-transformed to numerical values for visualization. Means were separated using Tukey’s-HSD (Honestly Significant Difference) test at significance level p<0.05. The statistical package used for all analyses was SPSS® (Version 17.0, SPSS Inc., Chicago, IL, USA).

**Results**

**Cu penetration, uptake and distribution in wood**

**Preservative retention**

According to the European standard EN 113 [25], we gathered indications on the expected MCA retention in easily treatable Scots pine sapwood and refractory Norway spruce sapwood and heartwood (Fig. 1). The three wood materials considered share the same preservative retention pattern (p-value=0.137) across all diluted MCA-pressure-treated wood samples (2.00%, 1.60%, 1.33%, 1.07%, 0.80%), which decreases with the MCA concentration applied. Therefore, comparable amounts of Cu could be expected in different wood species pressure-treated with the same MCA concentration, independently of their permeability. This pattern was not applicable to the 100% MCA-pressure-treated wood samples, where the preservative retentions were certainly lower, even though the expected amount of Cu was higher. Moreover, a difference between the refractory Norway spruce heartwood and the more accessible Norway spruce sapwood and the easily treatable Scots pine sapwood was visible.

**Quantification of Cu in wood**

We quantified the amount of Cu in both easily treatable Scots pine (sapwood) and refractory Norway spruce (sapwood and heartwood). Fig. 2 summarizes the weight percentages of Cu detected by ICP-OES in untreated and 100.00% MCA-pressure-treated Scots pine sapwood (inner and outer regions), Norway spruce sapwood (inner), and Norway spruce heartwood (inner).
Fig. 1. Preservative retention in Scots pine sapwood, Norway spruce sapwood, and Norway spruce heartwood calculated according to the EN 113 guidelines [25]. Data are represented as mean ± standard deviation of three replicates.
Fig. 2. Percentage of Cu in Scots pine sapwood (inner and outer surface), Norway spruce sapwood (inner), and Norway spruce heartwood (inner) measured by ion-coupled plasma-optical emission spectroscopy (ICP-OES). Data are represented as mean ± standard deviation of three repetitions. Shared letters indicate treatments that were not significantly different, different letters denote significant differences in treatments after the Tukey’s HSD test.

When untreated, both Scots pine and Norway spruce wood contain comparable (p-value=1) negligible amounts of Cu (below detection limit). In 100.00% MCA-pressure-treated wood samples, Scots pine sapwood (outer regions) had the highest amount of Cu. This percentage decreased in the interior of Scots pine sapwood, but the values remained higher than in Norway spruce sapwood or heartwood. The latter contained the lowest Cu amount, thus Norway spruce heartwood was the most refractory wood treated here. The Tukey’s HSD test indicates that the Cu amount in 100.00% MCA-pressure-treated Norway
spruce sapwood was not significantly different from the one in 100.00% MCA-pressure-treated Norway spruce heartwood (p value=0.908) and the interior part of Scots pine sapwood (p-value=0.489).

**Visualization of Cu in wood**

X-ray CT and subsequent analysis allowed qualitative and semi-quantitative determination of Cu in MCA-pressure-treated Scots pine sapwood, Norway spruce sapwood, and Norway spruce heartwood. Thresholding of the EN 113 wood blocks was performed to distinguish Cu in the tomographic reconstructions. The same conservative threshold was used for all EN113 wood samples.

In Fig. 3 the threshold-based Cu penetrations (red) in the three wood materials considered are visible. The Cu thresholding on the 31 μm resolution scans of the 100.00% MCA-pressure-treated Norway spruce samples accounted for only 1% (heartwood) or up to 2% (sapwood) of the volume, which was significantly lower than the percentages detected by ICP-OES.

In Scots pine and Norway spruce sapwood blocks it was clearly visible that the penetration of Cu occurred predominantly via resin canals and was more abundant in the latewood. In Scots pine Cu was also present within the rays. It was furthermore calculated that the detection limit of Cu would be of the order of 0.1 μg per voxel for the low resolution scans.

At the cellular level, the greyscale patterns in Scots pine and Norway spruce wood coincide, with bright spots (high-density Cu) around the cell wall or filling the cell lumina, as indicated in Fig. 4. In the three wood materials considered no major difference in the Cu uptake by ray parenchyma and ray tracheids was observed. The localization of Cu in the wood ultrastructure and the form of Cu, however, remain uncertain. In particular, even with the maximum intensity projections, it is unclear whether Cu is into or on the cell wall, and if ions, nanoparticles, or aggregates/agglomerates are responsible for the larger bright areas within the cell wall. It was furthermore calculated that the detection limit of Cu would be of the order of 2 pg per voxel for the high resolution scans.

![Fig. 3. Three-dimensional reconstruction of Cu penetration (red) in (a) Scots pine sapwood, (b) Norway spruce sapwood, and (c) Norway spruce heartwood.](image-url)
Fig. 4. Reconstructed slices before (above), after (middle) MCA-pressure-treatment, and maximum intensity projections after MCA-pressure-treatment (below) of Scots pine sapwood (a, d, g), Norway spruce sapwood (b, e, h), and Norway spruce heartwood (c, f, i). The brighter spots indicate areas containing high-density elements (Cu).
Fig. 5. Assessment of micronized Cu azole (MCA) concentrations against *R. placenta* 45 and associated mass losses in Scots pine sapwood, Norway spruce sapwood, and Norway spruce heartwood. Data are represented as mean ± standard deviation of six replicates. Plain font was used for Scots pine sapwood, underline for Norway spruce sapwood, and italics for Norway spruce heartwood.

**Effectiveness against Cu-tolerant basidiomycetes**

We assessed if the difference in Cu penetration in the three MCA-pressure-treated wood materials considered affected the wood protection effectiveness against the Cu-tolerant fungus *R. placenta* 45. Wood mass losses for the different wood species and MCA concentrations are reported in Fig. 5.

Tukey’s HSD test showed no differences between the mass losses from 0.00% MCA and control wood samples for each of the three wood materials considered. Among the different wood species, the mass losses in control samples were slightly lower for Scots pine sapwood (p-value < 0.001), while they were equivalent for Norway spruce sapwood and heartwood (p-value=0.456). Differences between the sapwood and heartwood from Norway spruce were recorded after treatment with MCA (p-value < 0.001). All MCA
concentrations prevented *R. placenta* 45 from colonizing Norway spruce heartwood, with mass losses well below 3.0%. On the contrary, despite a significant reduction in mass losses when compared to the 0.00% MCA and control, even concentrations of 1.60% MCA were not sufficient to protect Norway spruce sapwood from degradation (mass loss: 10.7±2.1%). At concentrations of 2.00% MCA decay results were more variable, with recorded mass losses between 0.3% and 13.5%. Mass losses from Scots pine control samples were not comparable to the ones from Norway spruce sapwood and heartwood (p-value < 0.001). Scots pine MCA-pressure-treated samples appeared significantly decayed below concentrations of 2.00% MCA (above 3%), while the latter caused mass losses of 2.75±0.975%.

**Discussion**

The aim of this study was to assess if MC could penetrate refractory wood species without pre-treatment, i.e. incising, and consequently provide an added value than conventional wood preservatives. We compared the pressure-treatment penetration effectiveness of Cu from an MCA formulation in easily treatable Scots pine sapwood and refractory Norway spruce sapwood and heartwood. The comparison was carried out using three different techniques: the indirect calculation of wood preservative retention after impregnation, as indicated by the EN 113 guidelines [25], the quantification of Cu by ICP-OES, and the density-based greyscale thresholding on X-ray CT reconstructions. We also aimed to correlate MCA penetration with protective effectiveness against the wood-destroying fungus *R. placenta* 45.

The nature of the EN 113 preservative retention formula [25] does not consider the treatability of the wood species. In the present study this resulted in an equal amount of expected MCA penetrating in the Scots pine and Norway spruce wood blocks at a given MCA concentration, and a linear correlation between the MCA concentration and the preservative retention, independent of the wood species. This calculation appears to diverge from the directly measured amount of Cu in the three wood materials considered. The ICP-OES analysis revealed that the background level of Cu present in untreated wood is negligible, as its concentrations in both Scots pine and Norway spruce (heartwood and sapwood) were below the instrument’s detection limit. Therefore, the amount of Cu detected in MCA-pressure-treated wood can be attributed solely to the wood preservative. Our results from the ICP-OES measurements on MCA-pressure-treated wood indicate that Cu was more abundant in Scots pine sapwood, especially on the surface, and only half of the Cu percentage found in Scots pine was detected in Norway spruce heartwood, the most refractory wood in this study. These results clearly showed that the amount of Cu penetrating into the wood heavily depends on the wood species and on the presence of sapwood (more accessible) or heartwood (less accessible) [4]. In addition, X-ray CT scanning and subsequent analysis enabled Cu distribution visualization in wood based on thresholding of the images. It should be noted that this results
in semi-quantitative data, since it is not trivial at all to derive quantitative data from X-ray CT scans. Furthermore, comparison with other methods for quantification of Cu in wood, is not straightforward as well since the thresholding applied, results in a percentage of voxels containing Cu, and does not relate to the exact amount of Cu present within those voxels. Although regions containing very low amounts of Cu could be overlooked, a detection limit of approximately 0.1 µg is small enough such that, if present, it would be visible.

For Scots pine sapwood 7% of the Scots pine wood block volume was found to be Cu. We applied the same thresholding on Norway spruce sapwood and heartwood reconstructions. While Cu was rather homogeneous across the whole Scots pine wood section, similarly to what is observed in other easily treatable species [16-23], in Norway spruce wood it was mostly located on the surface. In accordance with Evans et al. [18] and in good agreement with findings on western hemlock by Xue et al. [37], Cu was more abundant in the latewood and mainly distributed in rays and resin canals. Further, the Cu distribution pattern coincides with the one highlighted from MC by Evans et al. [16], which differs from the Cu distribution pattern from conventional amine Cu wood preservatives [16]. The threshold of Norway spruce sapwood and heartwood resulted in lower Cu percentages amounts than those detected by ICP-OES. This indicates that most of Cu is not present as particles, aggregates or agglomerates with size detectable at 31 µm, i.e. the resolution of the scans. This was confirmed by the scans at higher resolution, where Cu was visible at the cell wall level. Although regions containing very low amounts of Cu could be overlooked, a detection limit of approximately 2 pg is small enough such that, if present, it would be visible. The submicron resolution, however, did not allow to precisely locate Cu in the cell wall, i.e. if Cu diffuses into (cell-wall treatment) or only onto it (cell-lumen treatment). This issue has previously been hypothesized [38], and the difference is critical for wood protection because wood cell-wall treatments are certainly more effective against white and soft rot fungi [39, 40] and also more resistant to leaching [41]. In addition, it was not possible to determine if the Cu present was solubilized or available as unreacted single or aggregated/agglomerated- particles responsible for a reservoir effect. Cu appeared equally distributed in ray parenchyma and ray tracheids, similarly to what Olsson et al. [42] observed. This confirms that despite the innovative impregnation chemistry of MC, the wood preservative fluid flow is still subject to the same resistance, i.e. the pits between ray parenchyma and tracheids. In fact, while in easily treatable Scots pine, these cross-field pits are fenestrate and with a large thin membrane, in Norway spruce they are of the piceoid type, with smaller membrane and smaller dimensions [43, 44].

The effectiveness of MCA against the Cu-tolerant fungus R. placenta greatly differed for Scots pine sapwood, Norway spruce sapwood and heartwood. Despite the valid performance of MCA-pressure-treated Norway spruce heartwood even at the lowest MCA concentration (0.80%), the protection of Scots
pine wood appeared to be more difficult. The different patterns cannot be explained by differences in fungal virulence or wood natural durability, as the mass losses for Scots pine sapwood control samples were slightly lower than those of Norway spruce sapwood or heartwood. One explanation may be related to the Cu-tolerance of *R. placenta* and the interactions between Cu and tebuconazole within the MCA formulation. As previously demonstrated [27], tebuconazole plays a major role as active ingredient against *R. placenta*, however sub-lethal concentrations of Cu in tebuconazole-amended media can actually stimulate the growth of the fungus. In the present study Norway spruce heartwood samples contained the lowest amount of Cu, and most of it was located at the surface. The same is likely to apply for the co-biocide tebuconazole, whose low amount could still suffice to exert an antifungal effect. Hence the fungus has almost no Cu that would help it surviving in a tebuconazole-amended media. In Norway spruce sapwood the amount of Cu measured by ICP-OES increased slightly, providing *R. placenta* the conditions to survive despite the presence of tebuconazole. Finally, in Scots pine wood the amounts of Cu were even higher, providing even more resources, until at high MCA concentrations Cu becomes lethal together with tebuconazole (threshold concentration). Another possible explanation involves the amount of reacted Cu. Most of Cu contained in Norway spruce heartwood is likely to be in the form of fine particles (below 31 µm) or ions, as indicated by the low resolution X-ray CT scan and the thresholding coupled with the ICP-OES analysis, whereas Cu appears in larger clusters in Norway spruce sapwood, and even larger in Scots pine wood. This could result in a reduction in the ratio between solubilized bioavailable Cu$^{2+}$ and unreacted Cu$\text{CO}_3\cdot$Cu(OH)$_2$ in more easily treatable wood. Therefore, in the short-term the MC reservoir effect, i.e. unreacted Cu$\text{CO}_3\cdot$Cu(OH)$_2$ particles slowly solubilizing in wood, may be counterproductive. In fact, the short-term nature of the EN 113 studies, which last only 16 weeks, would not allow to assess the long-term protection provided by a reservoir effect, which may be visible after several months or years. Despite their different nature, the two possible explanations, i.e. Cu-tebuconazole interaction or reacted-unreacted Cu ratio, share a common conclusion: the Cu$\text{CO}_3\cdot$Cu(OH)$_2$ particles in MCA formulations do not contribute to a better short-term performance because they either support the growth of the wood-destroying fungus, or are not bioavailable and cannot exert an antifungal effect.

In conclusion, our hypothesis that MC cannot readily penetrate refractory wood species, which are commonly used in Central Europe, was confirmed. Therefore, from a wood penetration perspective, MC performance is comparable to conventional wood preservatives, and the adoption of MC for refractory wood species in the European market would still require pretreatment such as incising. However the MCA formulations succeeded in protecting refractory wood species against *R. placenta*, and the treated
refractory wood was destroyed less than easily treatable MCA-pressure-treated wood. Nevertheless in the short-term CuCO$_3$·Cu(OH)$_2$ particles do not provide an added value for the wood preservative formulation. Future studies should focus on MC’s Cu speciation in wood and its interaction with wood ultrastructure. In this way, the presence of a reservoir effect, of cell-wall or cell-lumen treatments, and the basis of MCA effectiveness could be thoroughly understood.

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4.3 Mechanical abrasion of treated wood

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Release of Copper-Amended Particles from Micronized Copper-Pressure-Treated Wood during Mechanical Abrasion

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ABSTRACT

Background: We investigated the particles released due to abrasion of wood surfaces pressure-treated with micronized copper azole (MCA) wood preservative and we gathered preliminary data on its in vitro cytotoxicity for lung cells. The data were compared with particles released after abrasion of untreated, water (0% MCA)-pressure-treated, chromated copper (CC)-pressure-treated wood, and varnished wood. Size, morphology, and composition of the released particles were analyzed.

Results: Our results indicate that the abrasion of MCA-pressure-treated wood does not cause an additional release of nanoparticles from the unreacted copper (Cu) carbonate nanoparticles from of the MCA
formulation. However, a small amount of released Cu was detected in the nanosized fraction of wood dust, which could penetrate the deep lungs. The acute cytotoxicity studies were performed on a human lung epithelial cell line and human macrophages derived from a monocytic cell line. These cell types are likely to encounter the released wood particles after inhalation.

Conclusions: Our findings indicate that under the experimental conditions chosen, MCA does not pose a specific additional nano-risk, i.e. there is no additional release of nanoparticles (NPs) and no specific nano-toxicity for lung epithelial cells and macrophages.

INTRODUCTION

Thousands of tons of wood chips and sawdust are being generated each day by industry, domestic environment, or improper disposal of debris. Further, the presence of wood preservatives may pose an environmental and human health risk due to release of toxic metals like arsenic and copper (Cu). Such an exposure pathway has already been recognized for various preservatives, in particular for chromated Cu arsenate (CCA) [1, 2].

We are currently experiencing an increased use of particulate Cu wood preservatives in order to effectively protect wood from decay and lengthen its service life. More specifically, basic Cu carbonate particulate systems with a size range between 1 nm and 25 μm were introduced for wood protection in the US market in 2006 [3]. This has resulted in more than 11,800,000 m$^3$ of wood treated with micronized Cu (MC) formulations [4], which corresponds to over 75% of residential lumbers produced in the US [4].

MC wood preservatives include a nanosized fraction of basic Cu carbonate, which may be of high concern: there is a strong indication that different Cu-based nanoparticles (NPs) have a high toxicity for aquatic organisms [5-10], terrestrial plants [11], mammals [12-17], and humans [18-23].

To date, the environmental fate of Cu carbonate particles from MC- pressure-treated wood has mostly assessed their leachability [24-28]. However particles generated by abrasion of MC-treated wood may be more hazardous than wood dust untreated or treated with conventional wood preservatives, due to the presence of Cu-based NPs. Platten et al. [29] and Santiago-Rodríguez et al. [30] recently assessed how exposure to Cu from wood dust originated from MC-pressure-treated wood can occur via dermal transfer or oral ingestion. Therefore, it is extremely important to determine the dust composition that can be inhaled after exposure –occupational or not– to abraded particles from MC-pressure-treated wood and its hazard to human lungs.

The current study characterizes the particles released from MC azole (MCA)-pressure-treated wood and compares them with particles generated from wood untreated, pressure-treated with the conventional
wood preservative chromated Cu (CC), and with varnished untreated and MCA-pressure-treated wood. Subsequently, it assesses acute cytotoxic reactions of MCA, its components tebuconazole and Cu$^{2+}$, as well as particles abraded from MCA-, CC-pressure treated wood and untreated wood to lung epithelial cells and macrophages.

**MATERIALS AND METHODS**

**MC characterization**

We used a commercially available MC azole (MCA) formulation. This is the same as the formulation with high amount of tebuconazole MCA_HTBA we used in a previous investigation [31]. A full characterization of the Cu particles in the MCA formulation is available from the latter study. To summarize briefly, the measured particle size distribution of MCA was 104±1.7 nm with an average zeta potential of -21±0.4 mV.

**Wood sample preparation**

Octagonal specimens of Scots pine (*Pinus sylvestris* L.) sapwood (90 mm diameter x 20 mm height) were used for the abrasion study. The specimens were prepared and pressure-treated with 2% aqueous suspensions of MCA or CC reference preservative, prepared according to the European standard ENV 807 [32]. After an 8-week drying procedure, some of the MCA-pressure-treated samples were coated three times with intervals of 24 hours with a primer, i.e. solution of deck lacquer (90%) and white spirit (10%). The control materials were composed of: untreated wood samples and samples pressure-treated with a 0% MCA solution in distilled water, varnished untreated wood samples.

**Abrasion setup**

The experimental setup has been described by Schlagenhauf et al. [33]. To simulate the abrasive process, a Taber Abraser (Model 5135, Taber, North Tonawanda, NY) was used. While the wood sample rotates, the Taber Abraser uses one abrasive wheel that abrades the sample continuously at the point of contact. The sample rotates 60 times per minute and the weight applied on the wheel is 0.75 kg. The samples were abraded with S-42 sandpaper strips (Taber) mounted on a CS-0 (Taber) rubber wheel. A conductive silicone tube (TSI) with a rectangular inlet at the tube entrance with a 4.8 mm$^2$ suction area was placed directly behind the abrasion area to collect the particles. The air flow was driven by a pump (N816.1.2KN.18, KNF, Germany). Devices for aerosol characterization and particle collection were included in the tubing system.

**Wood dust characterization**

The generated particles were characterized in triplicates both in the aerosol form by particle size distribution measurements with an aerodynamic particle sizer (APS, Model 3321, TSI, Shoreview, MN).
and a scanning mobility particle sizer (SMPS) consisting of a differential mobility analyzer (DMA) equipped with a long DMA column (Model 3080, TSI) and a condensation particle counter (CPC) (Model 3775, TSI). During each measurement, three particle size distributions were recorded. The recording time for each distribution was 195 s. The background distribution (without abrasive processes) of each experiment was measured three times. The experimental setup was verified by means of an atomizer aerosol generator (Model 3079, TSI). The particle size distributions obtained were processed as described by Schlagenhauf et al. [33]. In addition, the particles were collected on stubs and analyzed by means of scanning electron microscopy (SEM, Hitachi S-4800; Hitachi High-Technologies US and Canada, Illinois, USA). The stubs were plasma gold-sputtered (Polaron Equipment, SEM coating Unit E5100, Kontron AG, Switzerland; 5 mA, 1 mbar) prior to image acquisition.

The presence of Cu in the generated particles was assessed in the collected particles through ICP-MS (PerkinElmer Elan 6100, detection limit: 0.004 µg/L) and two distinct ICP-OES (Perkin-Elmer OPTIMA 3000, Jobin-Yvon HORIBA Ultima 2, detection limit for both instruments: 0.005 mg/L) instruments. In this way, we could benefit from the two different detection limits, as well as identify any effect of the instrumentation and –especially– of sample preparation on the detected amount of Cu. Analyses were carried out on the whole size range of abraded particles and on particles <1µm collected on Nucleopore track-etch membrane filter (111106, pore size 0.2 µm, Whatman, UK). For ICP-MS and Perkin-Elmer OPTIMA 3000 ICP-OES Cu content analysis, the collected particles were dissolved nitric acid (HNO₃, 65%, Supra Pure) and hydrogen peroxide (H₂O₂, 30%, Supra Pure) and subsequently underwent microwave digestion (MLS 1200 MEGA, Milestone, Leutkirch, Germany). Cu plasma standard solutions (1 g/L) were used for calibration. For Jobin-Yvon HORIBA Ultima 2 ICP-OES analysis a similar procedure was used, but without the addition of hydrogen peroxide. The detector voltage was set using a 100 mg/L standard solution, while a 7 levels calibration curve was employed for quantification.

**Cell culture**

The human alveolar epithelial cell line A549 (ATCC: CCL-185) was grown in Roswell Park Memorial Institute (RPMI-1640) medium (Sigma-Aldrich) supplemented with 10% fetal calf serum (FCS) (Lonza), 2 mM L-glutamine (Gibco), 50 µg/mL penicillin (Gibco), 50 µg/mL streptomycin (Gibco), and 100 µg/mL neomycin (Gibco) at 37°C in a humidified atmosphere containing 5% carbon dioxide (CO₂, hereafter referred to as complete cell culture medium and standard growth conditions, respectively). Cells were subcultured at approximately 80-90% confluency once a week using 0.5% Trypsin-EDTA (Sigma-Aldrich).
Formation of reactive oxygen species (ROS)

The formation of ROS in A549 cells was determined using the 2’, 7’-dichlorodihydrofluorescein-diacetate assay (H<sub>2</sub>DCF-DA), as described by Roesslein et al. [34]. For experimental details see supplementary information.

Cell viability

To assess mitochondrial activity as a measure of cell viability/cell death in A549 cells Cell Titer96® Aqueous One Solution (Promega) containing 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy phenyl)-2-(4-sulfophenyl)-2H (MTS) as a water-soluble tetrazolium compound was used according to the manufacturer’s protocol. In brief, 1.5 x 10<sup>4</sup> A549 cells were seeded in 200 µL complete cell culture medium in a 96-well plate and grown over night under standard growth conditions. Thereafter medium was removed and cells were incubated for 3 h or 24 h in 200 µL complete cell culture medium containing the respective stimuli (abraded particles from MCA-, CC-pressure-treated wood or untreated wood, or eluates derived thereof as described below). Cadmium sulfate (CdSO<sub>4</sub>) in different concentrations served as positive control, untreated cells as negative control. After appropriate incubation times (3 h, 24 h) medium was replaced by 120 µL of MTS working solution (composed of 20 µL MTS plus 100 µL of phenol-red-free RPMI-1640 w/o supplements) per well and cells were incubated for 60 min at standard growth conditions. Absorption was detected at 490 nm using an ELx800 microplate reader (BioTEK Instruments).

Data processing

Blank samples treated exactly the same way but containing no cells were run with every cell-based assay. Values given in the graphs are blank-corrected and subsequently normalized to the untreated sample. The mean of at least three independent experiments (each run with technical triplicates) and the corresponding standard deviations are shown.

Sample preparation for cytotoxicity analysis

Cytotoxicity was assessed in two different scenarios: i) Abraded particles released from MCA-, CC-pressure-treated as well as untreated wood were diluted in appropriate media and directly applied to cultured cells. ii) Eluates from the same abraded particles were used to assess the cytotoxicity of active soluble components contained in and released from the wood. Therefore, 4 mg of abraded wood particles per mL elution medium were incubated for 24 h at 37°C on a rotating platform. Supernatant was collected after centrifugation at 500g for 5 min. Elution medium for ROS detection was Hank’s Balanced Salt Solution (HBSS; for experimental details see supplementary information). For cell viability assessment and cytokine detection (see supplementary information) eluates were produced in phenol-red free RPMI
(without supplements) which was supplemented after centrifugation with 10% FCS, 2 mM L-glutamine, 50 µg/mL penicillin, 50 µg/mL streptomycin, and 100 µg/mL neomycin. The HBSS supernatant as well as the supplemented RPMI supernatant contain the highest possible amount of released components and were labeled “100% eluate”. Serial 1:2 dilutions were performed in the respective media and concordantly termed “50%, 25%, etc. eluates”.

**Determination of Cu content in eluates**

The Cu content in eluates of the abraded particles from untreated, CC- and MCA-pressure-treated wood was determined by ICP-MS (Sector Field SF-ICP-MS Element 2 from Thermo Finnigan, detection limit: 0.004 µg/L). Prior to analysis, the specimens were acidified with nitric acid (HNO₃, 65%, Supra Pure) and hydrogen peroxide (H₂O₂, 30%, Supra Pure) and subsequently underwent microwave digestion (MLS 1200 MEGA, Milestone, Leutkirch, Germany). Cu plasma standard solutions (1 g/L) were used for calibration.

Production of cytokines. The release of the pro-inflammatory cytokine TNF-α was assessed in macrophages derived from the monocytic cell line THP-1 (ATCC: TIB-202) using the Ready-SET-Go!® Elisa kit (eBioscience) according to the manufacturer’s protocol. For cell culture conditions and experimental details see supplementary information.

**RESULTS AND DISCUSSION**

**Wood dust particle size**

The particle size distributions for the different wood samples (untreated, 2% MCA- pressure-treated, 2% CC- pressure-treated, 0% MCA- pressure-treated, varnished, 2% MCA-pressure-treated and varnished) are shown in Figure 1 (a, b). More specific, Figure 1a represents the particle size distributions measured by SMPS below 1 µm, while Figure 1b presents the distributions measured by APS above 1 µm. All the samples show a similar pattern below 1 µm, with peaks at about 400 nm; while two different outlines are visible above 1 µm: one for the abraded particles from varnished samples, and another for the abraded particles of unvarnished samples. In the first case the peak is between 700 nm and 1.3 µm, while in the second one it is around 2.3 µm. Therefore, the set up maximizes the release of coarse (PM10), fine (PM2.5) and ultrafine particles (generally defined as smaller than 100 nm). These three particle size fractions are commonly associated with adverse health effects in humans, as demonstrated by Schwartz et al. [35], Raaschou-Nielsen et al. [36], and Oberdörster et al. [37]. In addition, the setting fitted the purpose of detecting any variation in the generated wood dust at the nanoscale, which may have occurred due to the presence of Cu carbonate NPs. In any case, no additional release of a nanosized fraction was observed for the 2% MCA-treated wood.
We could observe how the application of varnishes influences the particle size released increasing the average dimensions, reducing the exposure to ultrafine particles.

The APS results on the aerodynamic particle diameter are in good agreement with the study from Thorpe and Brown [38], in which the wood dust size distribution after different sanding processes was assessed. The mean particle diameter was comprised between 1.52 μm and 2.65 μm. However, further different abrasive processes, e.g. cutting, grinding, welding, may cause the release of wood dust with different particle size distributions. Despite that, as our abrasive set up maximizes the release of coarse, fine and ultrafine particles, we can suppose that different abrasive processes would not release more nanoparticles than our system.

Our tests focused on Scots pine only, however the different wood species could play a role may release particles that differ in the size distribution, due to the wood properties, as demonstrated by Lehmann and Fröhlich [39] and Ratnasingam et al. [40]. In the case of MCA-treated wood, the wood species features may also influence the amount of Cu carbonate particles present in the wood after impregnation.

In terms of human exposure, our results indicate that a fraction of the abraded particles produced by the different wood samples could penetrate the lower airways (tracheo-bronchiolar regions or even the alveolar sacs), due to their small size. The application of varnishes alter the size distribution of the abraded particles and by that would shift the particle deposition to the nasopharyngeal and tracheo-bronchiolar regions [41]. However, the broad size range of the particles does not allow a precise quantification of particle deposition in the respiratory tract.

**Wood dust particle morphology**

The generated particles from untreated, CC-, and MCA-pressure-treated wood were morphologically assessed by SEM (Figure 1 c, d, e). Visual inspection of all the SEM images collected confirmed the presence of particles below 10 μm, as well as the presence of bigger particles (102 μm), beyond the APS and SMPS detection limits adopted. In addition, no difference between the different wood samples (Figure 1 d, e) was encountered, indicating no mechanical alteration due to the wood treatments, in accordance with the APS and SMPS results. In all cases, the generated particles appeared mostly fibrous, although irregular and heterogeneous in shape and size. The surfaces were not always flat.

Various studies reported similar features from SEM investigations on wood dust from various wood species [42, 43]. In particular, Mazzoli and Favoni [44] reported no difference in wood dust particle size and morphology from different wood species, suggesting no dissimilarity for in vitro cytotoxicity. However, wood species that are documented to be carcinogenic, e.g. beech [45], were not assessed. In that case, different structures responsible for the increased adverse effects may be observed. In addition, the abrasive process may also generate wood dust particles that differ in size and morphology.
**Cu content in wood dust**

By means of ICP-OES and ICP-MS analyses we could assess the different concentrations of Cu in wood dust from untreated and MCA-pressure-treated wood samples, as shown in Table 1. Combining the ICP-OES and ICP-MS results, which are concordant, we determined a baseline amount of Cu in untreated wood at 0.01±0.02 mg/g. Similarly, when the wood was varnished the baseline amount was found at 0.02±0.01 mg/g. When MCA-pressure-treated wood was abraded, the amount of Cu released was 2.02±0.09 mg/g, corresponding to 0.20% w/w of the total amount of treated wood, and it drastically reduced when varnish was applied (0.23±0.01 mg/g). This difference may be due to the higher release of varnish instead of wood, therefore implying that varnishes may prevent release of Cu during mechanical abrasion of treated wood. The amount of Cu release was almost double in CC-pressure-treated wood (4.26±0.01 mg/g). This is due to differences in the formulations: in fact, the amount of Cu in the initial CC formulation doubles the amount in MCA. Since 2% is an economically feasible concentration, generally used in the timber industry, the result indicate that at similar dilutions (2%) MCA-pressure-treated wood would release less Cu due to mechanical abrasion. The percentage of Cu released from MCA-pressure-treated wood is in good agreement with studies on indoor sawing of CCA-treated wood: Decker et al. [46] reported 0.3% Cu in wood dust, while Nygren et al. [47] 0.1%. In addition, a comparison can be made between our results and the ones from the less invasive wiping experiment reported in the EPA report [24]. In fact, in the latter, the amount of Cu released from MCA-pressure-treated wood was lower and comprised between 0.0135 mg and 0.072 mg.

The amount of Cu detected in the wood dust nanosized fraction was below the Cu concentration in the whole wood dust, both from untreated and MCA-pressure-treated wood. In particular the concentration of Cu in the nanosized dust generated by MCA-pressure-treated wood was 1.50±0.30 mg/g (0.15% w/w). Therefore, combining these data with the SMPS results we can conclude that most of the Cu released was bound to the larger wood particles, however a small amount of Cu bound to the nanosized fraction would deposit in the deep lungs, if inhaled. Therefore, toxicological studies are required to fully assess the hazard on human health.

**Cytotoxicity assessment**

The most critical exposure route for sawdust particles is the lung. Therefore, we focused our in vitro study on the lung epithelial cell line A549 and macrophages differentiated from the monocytic cell line THP-1. Both cell types are likely to be among the first cell types getting in touch with inhaled particles. We investigated potential adverse effects of sawdust particles abraded from untreated wood, MCA-pressure treated wood and CC-pressure treated wood. Furthermore, to assess the effects caused by soluble compounds, rather than by wood dust per se, eluates from these three types of wood particles were
included in the cytotoxicity evaluation. These results were compared to the toxicity induced by direct treatment of lung epithelial cells with MCA and its active components tebuconazole and Cu2+ ions from copper sulfate pentahydrate (CuSO4·5H2O).

According to the ROS paradigm [34] the interaction of (nano) particles with cells is likely to induce elevated cellular levels of ROS. Subsequent oxidative stress reactions can then cause severe damage to biomolecules (proteins, lipids and nucleic acids), induce inflammatory reactions and finally lead to cell death. Therefore we initially assessed the overproduction of ROS using the DCF assay. As shown in Supplementary Figure S1, only the positive controls Sin-1 and MWCNT led to a considerable increase of ROS levels in A549 cells. All eluates and abrasion particles tested did not elevate ROS formation. However, cell death can also be triggered by ROS independent pathways. We therefore investigated cell viability of A549 lung epithelial cells using the MTS assay. The assay internal positive control CdSO4 induces cell death in a dose-dependent manner (Figure 2a) thus indicating that toxicity can be reliably detected under the experimental conditions.

The cytotoxicity of MCA itself was determined up to a concentration of 2% (v/v) in cell culture medium. In parallel, its active compounds tebuconazole and Cu2+ were analyzed in equivalent amounts (Figure 2b and supplementary information). Our results reveal a toxicity ranking of tebuconazole < Cu2+ < MCA, which indicates an additive effect of tebuconazole and Cu2+. Further, our results suggest that the cytotoxicity of MCA is likely to be caused by Cu2+ ions than nanoparticles.

The highest, technically feasible, concentration of abraded particles that could be applied to A549 cells was 80 µg/mL equaling to a growth area of 47 µg/cm². For all three types of sawdust particles no cytotoxicity could be detected up to this concentration and over an incubation period of 24 h (Figure 2c). According to Table 1 the highest amount of 80 µg particles from MCA- or CC-pressure-treated wood contain 0.16 µg Cu2+ or 0.34 µg Cu2+, respectively. Measurements of eluates of the respective abraded particles revealed that only a fraction of 4.4% of Cu2+ is released into the medium over a period of 24 h (Table 1). Therefore we do not expect concentrations above 0.007 µg/mL or 0.015 µg/mL Cu2+ for the two samples, respectively. In relation to Figure 2b, were Cu2+ ion cytotoxicity starts above 5 µg/mL (=0.01%), these values appear very low. However, the following considerations will relate the chosen in vitro doses to an inhalation scenario for wood workers. If we consider an inhalation volume of 1.9 L per breath and roughly 26 breathes per min during heavy exercise [48] we can assume a total volume of 24 m³ air to be inhaled during an 8 h working day. According to Decker et al. [46], wood dust concentrations in air may
range from 0.6 mg/m$^3$ (sampled at outdoor working sites over a period of 229 min) to a maximum of 49 mg/m$^3$ (sampled during indoor sanding operations over a period of 127 min). With these data a total amount of 3.8 mg to 555 mg inhaled particles per working day can be estimated. Considering 102 m$^2$ of total lung surface area [49] and assuming all the wood dust particles to be deposited in the lung we can estimate a total deposited amount of wood dust particles of 0.004 µg/cm$^2$ to 0.545 µg/cm$^2$. In this scenario the 47 µg/cm$^2$ in vitro dose is a rather high concentration mimicking a repeated exposure over at least 17 weeks (indoor) to a whole lifetime (49 working years; outdoor). Nevertheless, spatially restricted effects due to particle deposition, cellular uptake of particles and potential intracellular Cu$^{2+}$ release cannot be addressed, neither by in vitro toxicity tests nor by the above demonstrated exposure calculations. In summary the doses chosen in the present study adequately reflect a worst case exposure scenario for wood workers.

Furthermore, we analyzed eluates produced from the three types of abraded wood particles and assessed the cytotoxicity of soluble factors released from the sawdust on A549 cells. As shown in Figure 2d no cytotoxicity could be detected after 24 h of incubation with eluates from untreated as well as MCA-pressure-treated wood. Eluates from CC-pressure-treated wood particles reduced cell viability at the highest concentration tested to 63% viable cells compared to untreated control cultures. This highest eluate concentration (Table 1) contained only 0.8 µg/mL Cu$^{2+}$. As Cu$^{2+}$ ion cytotoxicity started at concentrations beyond 5 µg/mL (=0.01%) (Figure 2b) Cu$^{2+}$ is most likely not the main reason for the observed effect, but rather chromium. Further investigations are necessary to prove a real human hazard from CC-pressure-treated wood, which was not the scope of the present study. Besides that, our results clearly indicate that there is no additional nano-specific effect, as abraded particles from MCA-pressure-treated wood as well as eluates thereof did not induce any cytotoxicity under the experimental conditions tested. This provides further evidence to the hypothesis that Cu$^{2+}$ ions rather than nanoparticles are responsible for any adverse effects.

Besides cell viability, inflammatory reactions at sublethal concentrations can be an indication for non-acute but nevertheless relevant adverse effects. Therefore we assessed the release of the pro-inflammatory cytokine TNF-α from immune responsive cells in vitro using the enzyme-linked immunosorbent assay (ELISA) technique. We used macrophages differentiated from THP-1 monocytes as the model cell line. Initially, cell viability was investigated to assure sublethal concentrations were applied for subsequent cytokine release experiments. THP-1 macrophages were exposed to the respective stimuli for 8 h and cell viability was assessed using the MTS assay. For technical details see supplementary information. CdSO$_4$ served again as the assay internal positive control and induced cytotoxicity in a dose-dependent manner (Supplementary Figure S2a). Following the same experimental design as described for A549 cells MCA
and its active components tebuconazole and Cu2+ were applied in equivalent amounts (Supplementary Figure S2b). In this case, the effects of Cu2+ and MCA were comparable, therefore even in this case the effects from MCA appear to be caused by Cu2+ ions rather than nanoparticles. Cell viability was affected at concentrations above 0.05% MCA in a dose-dependent manner. All three abraded wood particle types (up to 80 µg/mL) as well as eluates thereof did not induce an adverse response (Supplementary Figure S2c, d) in THP-1 macrophages. Accordingly, for cytokine release measurements MCA, tebuconazole and Cu2+ were used at concentrations below 0.05% MCA-equivalents and abraded wood particles were used up to 80 µg/mL. Lower eluate concentrations (6.25% to 25.00%) showed an increase in cell viability rather than a decrease. Therefore we used concentrations below 25.00% for ELISA experiments. Treatment with the positive control lipopolysaccharides (LPS) led to a 16-fold and 25-fold increase in TNF-α release at 10 and 100 ng/mL LPS, respectively (Supplementary Figure S3). However, no significant release of TNF-α could be observed after treatment with MCA, its active components, abraded wood particles or eluates thereof at any of the concentrations tested (Supplementary Figure S3). Thus, even in this case no specific nano effect was observed.

In summary our findings on the cytotoxicity reveal (1) a toxicity ranking of tebuconazole < Cu2+ < MCA (2) no induction of cytotoxicity for abraded particles up to 80 µg/mL (3) only a minor toxicity was found for the highest concentration of eluates resulting from CC-pressure-treated wood, which was only observed for A549 lung epithelial cells, and it is likely due to the presence of chromium in the formulation; most importantly (4) no additional nano hazard (caused by the presence of Cu-based NPs per se) was identified. Furthermore, our cytotoxicity study indicates low adverse effects for low-frequency consumer exposure. However, woodworkers can be continuously exposed to wood dust, in particular since dust-exposed woodworkers do not always wear appropriate respirators approved for wood dust [50]. The wood being processed may have been pressure-treated with Cu-based formulations, and the particles released can increase the adverse effects due to the presence of Cu. However, MCA is likely to be the safest alternative: no nano hazard was evidenced, and the amount of Cu, especially easily bioavailable Cu, in CC was double the amount in MCA. Furthermore, both types of human cells tested showed lower adverse effects (higher cell viability) when compared to cells exposed to CC. In conclusion, the abrasion of MCA-pressure-treated wood does not constitute a nano-specific risk. Nonetheless, further more advanced toxicity studies on tissues and in vivo are required.

Table 1. Cu content in sawdust particles and eluates thereof.
<table>
<thead>
<tr>
<th>wood treatment</th>
<th>µg Cu/mg abraded particles</th>
<th>µg Cu/mL medium (eluates*) [release in %]</th>
</tr>
</thead>
<tbody>
<tr>
<td>untreated</td>
<td>0.01±0.02</td>
<td>0.01±0.01</td>
</tr>
<tr>
<td>MCA-pressure treated</td>
<td>2.02±0.09</td>
<td>0.36±0.01 [4.4%]</td>
</tr>
<tr>
<td>CC-pressure treated</td>
<td>4.26±0.01</td>
<td>0.75±0.01 [4.4%]</td>
</tr>
<tr>
<td>RPMI medium (w/o wood)</td>
<td>n.a.</td>
<td>0.00±0.01</td>
</tr>
</tbody>
</table>

*4 mg abraded particles were incubated in 1 mL phenolred free RPMI for 24 h at 37°C on a rotating platform; after centrifugation at 500 g for 5 min supernatants were further processed for ICP-MS measurements.
Figure 1. Characterization of the abraded particles. (a) Particle size distributions of untreated wood (control), water-treated wood (0% MCA), MCA-treated wood (2% MCA), and CC-treated wood (CC) measured by SMPS. Most of the abraded particles had a diameter of 400 nm. Data represented as mean of three repetitions. (b) Particle size distributions of untreated wood (control), water-treated wood (0% MCA), Varished, CC, 2% MCA, and 2% MCA Varished. (c, d, e) SEM images of the abraded particles.
MCA), varnished wood (varnished), CC-treated wood (CC), MCA-treated wood (2% MCA), and varnished MCA-treated wood (2% MCA varnished) measured by APS. Most of the abraded particles had a diameter of about 1 μm. When varnish is applied, the average diameter shifts towards 2.3 μm. Data represented as mean of three repetitions. (c, d) SEM images of wood dust generated by the abrasion process on 2% MCA-treated wood. (e) SEM image of wood dust generated by the abrasion process on untreated wood (control).

![Graphs and images]

**Figure 2.** Cell viability assessment in A549 lung epithelial cells. Cells were treated for 24 h with the indicated concentrations of (a) CdSO₄ as the positive control (b) MCA, tebuconazole and Cu (c) abraded sawdust particles from untreated, MCA-pressure treated and CC-pressure treated wood (d) eluates of the respective wood particles. Cell viability was assessed using the MTS assay. *Tebuconazole and Cu²⁺ were applied in the respective amounts present in MCA as described in supplementary information.

**ASSOCIATED CONTENT**

**Supporting information.** Concentration considerations of MCA components; Formation of reactive oxygen species (ROS); Culture conditions and cell viability assessment of THP-1 cells; Production of cytokines.
Concentration considerations of MCA components. The active components of MCA are tebuconazole and Cu-carbonate (CuCO$_3$Cu(OH)$_2$) in its nanoparticulate form. MCA itself is commonly applied to wood as a 2% solution in water. For cell viability testing we assumed a worst case scenario with human exposures at a maximum of 2%. Tebuconazole as well as Cu concentrations were calculated accordingly to match the respective amount present in e.g. 2% MCA. CuSO$_4$$\cdot$5H$_2$O was used as a soluble Cu$^{2+}$ source.

Formation of reactive oxygen species (ROS). A549 cells were seeded into 96-well plates at a density of 2 x 10$^4$ cells per well in a volume of 200 µL complete cell culture medium one day prior to treatment. Cells were incubated with 50 µM H$_2$DCF-DA (Molecular Probes) in HBSS at standard growth conditions. After two washing steps with pre-warmed HBSS cells were treated with abraded wood particles (MCA-, CC-pressure-treated and untreated) and eluates thereof. The nitrite oxide donor 3-morpholinosydnonimine hydrochloride (Sin-1; 50 µM) as well as multi-walled carbon nanotubes (MWCNT; Baytubes, Bayer Technologies; 10 µg/mL and 20 µg/mL) were used as positive controls. Fluorescence intensity was measured after 1, 2, 3 and 4 h from the same plate using a Mithras2 plate reader (Berthold Technologies) at an excitation wavelength of 485 nm and an emission wavelength of 528 nm. Only values from the 3 h measurement are shown. The mean of at least three independent experiments (each run with technical triplicates) and the corresponding standard deviations are shown.

Culture conditions and cell viability assessment of THP-1 cells. The human monocytic cell line THP-1 (ATCC: TIB-202) was grown as cell suspension in complete cell culture medium under standard growth conditions in complete cell culture medium (RPMI-1640 supplemented with 10% FCS (Lonza), 2 mM L-glutamine (Gibco), 50 µg/mL penicillin (Gibco), 50 µg/mL streptomycin (Gibco), and 100 µg/mL neomycin (Gibco)). Subculturing was performed by replacement of medium when the cell density reached 8 x 10$^5$ cells/mL. Cell concentrations were not allowed to exceed 10$^6$ cells/mL. To determine sublethal concentrations of abraded particles, eluates thereof as well as MCA components (MCA itself, tebuconazole, Cu$^{2+}$) cell viability was assessed using the Cell Titer96® AQueous One Solution (Promega) as described in the main text with the following exceptions. 8 x 10$^4$ THP-1 monocytes were seeded in 200 µl complete cell culture medium containing 200 nM PMA (12-myristate-13-acetate) per 96-well. Cells were differentiated into adherently growing macrophages in the presence of PMA for 72 h under standard growth conditions. After one washing step with pre-warmed phosphate buffered saline (PBS) cells were treated with the compounds of interest in complete cell culture medium (w/o PMA) for 8 h.
Figure S1. None of the particles and eluates tested enhanced ROS production in A549 cells. ROS formation was measured in A549 cells after three hours of incubation with indicated concentrations of abraded wood particles as well as eluates thereof. Sin-1 (50 µM) as well as MWCNT served as the positive controls. Sin-1: 3-morpholinosydnonimine hydrochloride.

Thereafter medium was replaced by 120 µL of MTS working solution, cells were incubated for 60 min at standard growth conditions and absorption was read at 490 nm using an ELx800 microplate reader (BioTEK Instruments).

Production of cytokines. 4 x 10^4 THP-1 monocytes were seeded in 200 µL complete cell culture medium per well of a 96-well plate and were differentiated into macrophages over period of 72 h in the presence of 200 nM as described above. After one washing step with pre-warmed PBS cells were treated with the MCA compounds, abraded particles and eluates thereof for 8 h under standard growth conditions (w/o PMA). Cells treated with 10 ng/mL and 100 ng/mL lipopolysaccharide (LPS) served as positive control samples, untreated cells as negative control samples. Cell-free supernatants were harvested by centrifugation (500g, 5 min) and frozen at -80°C until subsequent cytokine analysis. TNF-α concentrations were determined using the Ready-SET-Go!® Elisa kit (eBioscience) according to the manufacturer’s protocol. Absorbance values were measured at 630 nm using a Mithras2 plate reader (Berthold Technologies) and normalized to the untreated control sample. The mean of three independent experiments (each run with technical duplicates) and the corresponding standard deviations are shown.
Figure S2. No acute cytotoxicity was detected after treatment of THP-1 cells with abraded wood particles and eluates thereof. MCA as well as tebuconazole and Cu$^{2+}$ induce a dose dependent toxicity in THP-1 cells. *Tebuconazole and Cu$^{2+}$ were applied in the respective amounts present in MCA as described above.

Figure S3. None of the substances tested induced the release of the pro-inflammatory cytokine TNF-α. *Tebuconazole and Cu$^{2+}$ were applied in the respective amounts present in MCA as described above.
AUTHOR INFORMATION

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Acknowledgments

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Abbreviations

A549, human lung epithelial cells; APS, aerodynamic particle sizer; CC, chromated copper; CCA, chromated copper arsenate; CdSO₄, cadmium sulfate; CPC, condensation particle counter; Cu, copper; CuSO₄·5H₂O, copper sulfate pentahydrate; DMA, differential mobility analyzer; FCS, fetal calf serum; HBSS, Hank buffered salt solution; HRP, horseradish peroxidase; H₂DCF-DA, 2',7'-dichlorodihydrofluorescein-diacetate assay; ICP-MS, inductively coupled plasma mass spectrometry; ICP-OES, inductively coupled plasma optical emission spectrometry; IL-8, interleukin 8; LPS, lipopolysaccharides; MC, micronized copper; MCA, micronized copper azole; MTS, formazan compound; MWCNT, multiwalled carbon nanotube; NP, nanoparticle; ROS, reactive oxygen species; PBS, phosphate buffered saline; PMA, phorbol-12-myristate-13-acetate; PSN, penicillin-streptomycin-neomycin antibiotic; RPMI, Roswell Park Memorial Institute; SEM, scanning electron microscopy; Sin-1, 3-morpholinosydnonimine hydrochloride; SMPS, scanning mobility particle sizer; THP-1, human acute monocytic leukaemia cells; TNF-α, tumor necrosis factor.

REFERENCES OF CHAPTER 4.3


4.4 Spore compartmentalization of Cu

**Micronized copper-treated wood: copper remobilization into spores from the copper-tolerant wood-destroying fungus *Rhodonia placenta***

Unpublished report

**Keywords:** Copper speciation, Micronized copper azole, Pressure-treated wood, Basidiospore compartmentalization, X-ray absorption spectroscopy

**Abstract**

Different brown rot wood-destroying fungi have the ability to develop Cu-tolerance mechanisms in order to effectively grow and decompose wood treated with copper (Cu), compulsory biocide for in ground timber structures. We analyzed if the Cu-tolerant wood-destroying basidiomycete *R. placenta* is able to compartmentalize Cu in its basidiospores. In addition, we assessed if the use of micronized copper (MC) wood preservatives formulations, composed of basic Cu carbonate particles with sizes ranging from 1 nm to 250 µm, could lead to basidiospores loaded with Cu-based nanoparticles (NPs) that may be inhaled and lead to adverse health effect, due to specific nano effects. In our study we combined elemental analysis and visual inspection to quantify the amount of Cu in basidiospores, its distribution and the Cu species present. Our results indicate that basidiospores from *R. placenta* can accumulate Cu, however the initial Cu-based NPs undergo speciation. Therefore no specific nano-specific risk was highlighted.

**Introduction**

Copper (Cu) is widely used as fungicide, e.g. in agriculture [1] or wood protection [2]. However, certain fungal strains developed Cu-tolerance mechanisms that allow them to effectively colonize Cu-polluted environments. Within the field of wood protection, Cu-tolerant brown rot (basidiomycete) fungi are responsible for most early failures in timber structures worldwide [3]. The latter include wooden applications in contact with the soil (use class 4) [4], like poles, fences, playgrounds, or decks. The detoxification mechanisms developed by Cu-tolerant wood-destroying fungi are mainly based upon complexation of Cu$^{2+}$ ions by fungal secretions, like oxalic acid [5], or compartmentalization of Cu within cell structures, e.g. vacuoles [6]. Fungal cell wall mainly acts as cation exchanger, due to the negative charge of its functional groups, for example Cu$^{2+}$ ions can bind or get complexed by carboxylic, phosphate, amine or sulfhydryl groups [6]. Direct uptake of Cu by fungal spores that would exert an antifungal effect was described by Somers [7], while the uptake of Cu by mycorrhizal fungi and
subsequent compartmentalization of Cu in spores has been reported in different studies [8-10]. In these studies, Cu deposition in spores accounts for fungal survival. However, no indication on a similar trend for Cu-tolerant wood-decaying basidiomycetes is available. In particular, if nanoparticle (NP)-based formulations, like the newly developed micronized copper (MC) wood preservatives, are used for in ground wood applications, Cu-based NPs may end up in the basidiospores. Despite the literature is still fragmented and unclear, airborne spores can cause acute phase responses, and some basidiospores, can exacerbate respiratory tract illnesses [11-16]. In our commentary [17], we hypothesized the likelihood of such mechanisms and the possible nano-specific risks for human health in the event that basidiospores are inhaled. Here, we hypothesize that R. placenta can compartmentalize Cu in its basidiospores (Hypothesis 1) and that the release of Cu-based NPs via basidiospores is likely to occur (Hypothesis 2). To test our hypotheses we assessed the amount and form of Cu remobilized from MC-pressure-treated wood into fruiting bodies and basidiospores of the Cu-tolerant [18] wood-destroying basidiomycete R. placenta. This fungus was selected due to its high Cu-tolerance, as indicated by a previous screening study we conducted [19], and its abundance in decayed timber structures [20, 21]. Visual inspections with light microscopy and transmission electron microscopy (TEM) were combined with elemental analysis by means of ion-coupled plasma mass spectroscopy (ICP-MS) and synchrotron-based X-ray absorption spectroscopy (XAS) to determine the amount, distribution and movement across fungal structures, and Cu species in basidiospores. In addition, we assessed whether basidiospores produced in Cu-rich environments were as viable as control basidiospores or not.

Material and methods

Materials

Two commercial aqueous suspensions of MCA were investigated. The two MCA formulations coincide with the ones used in our previous investigation [19]. These contain comparable amounts of Cu particles but differ in the amount of tebuconazole (TBA): MCA_HTBA contained 5% w/w and MCA_LTBA 0.4% w/w TBA. The above mentioned previous study also provides a full characterization of the CuCO$_3$-Cu(OH)$_2$ particles in the MCA formulation. In brief, the measured particle size distribution of MC and MCA was $104\pm1.7$ nm and $174\pm5.9$ nm with an average zeta potential of $-21\pm0.4$ mV and $-16.5\pm1.4$ mV, respectively.

Scots pine (Pinus sylvestris L.) sapwood blocks (50 x 25 x 15 mm) were pressure-treated according to the European standard EN 113 [22] with 2% MCA_LTBA or MCA_HTBA (MCA concentrations relevant for the wood protection market). After drying, untreated and treated samples were grinded into sawdust. Sporulation of R. placenta 45 was stimulated growing the fungus at 22 °C and 70% RH under cool white fluorescent light, as suggested by Croan [23], for 6 hours a day on 20 g sawdust amended with 100 mL
Fig. 1. *R. placenta* 45 producing fruiting bodies and releasing basidiospores. The fungus was grown on sawdust produced from untreated and MCA-pressure-treated wood.

solid medium (autoclave sterilized) composed of 4% (w/v) malt extract and 2.5% (w/v) agar. *R. placenta* 45 fruiting bodies developed on Petri dishes external circumferences and released the basidiospores, which deposited on an aluminum foil placed underneath, as shown in Fig. 1. In this way, we could be certain that the basidiospores would not come directly in contact with Cu and that the compartmentalization would have taken place due to Cu uptake by other fungal structures.

**Quantification of Cu in fungal structures**

Basidiospores and fruiting bodies produced by *R. placenta* 45 grown on sawdust from untreated, MCA_HTBA-, and MCA_LTBA-pressure-treated wood were collected from the aluminum foil to perform elemental analysis. The presence of Cu in basidiospores was assessed through Quadrupole Q-ICP-MS Elan6100 (Perkin Elmer), while the amount of Cu in fruiting bodies was measured by Sector Field SF-ICP-MS Element 2 (Thermo Finnigan). Detection limit for both instruments was 0.004 µg/L. Digestion of the samples prior to ICP-OES was conducted according to Platten et al. [24]. Prior to analysis, three replicates of each specimen were acidified with nitric acid (HNO₃, 65%, Supra Pure) and hydrogen peroxide (H₂O₂, 30%, Supra Pure) and subsequently underwent microwave digestion (MLS
Cu plasma standard solutions (100 mg/L) were used for calibration.

**Speciation of Cu in fungal structures**

The speciation of Cu in basidiospores was examined using the microXAS beamline at the Swiss Light Source. The collected basidiospores were pressed as pellets into a sample holder with Kapton tape window. The materials were analyzed at room temperature. Energy calibration of the Si111 double crystal monochromator was obtained by measurements of a metallic Cu reference foil (in transmission mode) prior and after experimental analysis. First inflection point of the metallic copper K-edge spectrum was set to 8979 eV. Due to low Cu concentrations, XANES spectra of the experimental samples were collected in fluorescence mode using a single element Silicon Drift Diode detector (SDD, KETEK GmbH). The beamsize was defocused resulting in a 700 µm diameter beam. In addition to the fungal samples, several reference materials, purchased as Cu salts (Cu carbonate (CuCO$_3$∙Cu(OH)$_2$), metallic Cu (reduced (or zero-valent) Cu), Cu sulfate (CuSO$_4$)) or prepared by mixing Cu with different ligands (Cu chloride (CuCl$_2$) aqueous, Cu oxalate, Cu-cysteine (CuCys)) were analyzed at room temperature. To examine the possibility of beam-induced damage, spectra were collected at least twice from the same location and the spectra compared to determine if the beam had caused a change in speciation. The XAS data processing software Athena was used to analyze the XAS spectra [25]. For each individual sample, multiple spectra were collected and merged. The XANES spectra obtained were used to compare the Cu speciation in basidiospores produced by *R. placenta* 45 on sawdust from untreated and MCA-pressure-treated wood. The linear combination fitting tool provided by the software Athena was used to determine the speciation of Cu in the two types of spores.

**Spore visualization**

Basidiospores by *R. placenta* 45 grown on sawdust from untreated and MCA_LTBA-pressure-treated wood were investigated by TEM. They were fixed by diluting them with 3% glutaraldehyde in 0.1 M sodium cacodylate puffer and then sucked up into a capillary tube (Leica-Microsystems, Heerbrugg, Switzerland). After a post-fixation step in 2% osmium tetroxide in 0.1 M sodium cacodylate buffer, spores were dehydrated through a graded ethanol series followed by acetone and embedded in Epon resin (Sigma-Aldrich). Ultrathin sections were contrasted with 2% uranyl acetate and lead citrate before observation in a Zeiss EM 900 (Carl Zeiss Microscopy, GmbH, Germany) at 80 kV.

**Spore viability**

Basidiospores produced by *R. placenta* 45 grown on sawdust from untreated and MCA_LTBA-pressure-treated wood were collected and cultivated in 9 cm Petri dishes with 25 mL solid medium (autoclave sterilized) containing 4% (w/v) malt extract and 2.5% (w/v) agar. Part of the media was amended with
0.01% (w/v) MCA_LTBA. Three replicates were prepared for each condition. Cultures were stored at 22 °C and 70% RH. The cultures were inspected regularly, and their 4 cardinal points were marked to determine the growth radii until the colonies reached the edges of the Petri dishes.

**Results**

**Quantification of Cu in fungal structures**

ICP-MS analysis allowed the detection and quantification of Cu in fruiting bodies and basidiospores from *R. placenta* 45. The findings are summarized in Table 1.

While the amount of Cu in fungal structures cultivated on untreated sawdust was minimal, the presence of Cu increased when the fungi were exposed to MCA-pressure-treated wood. What emerged is that the amount of Cu taken up by fruiting bodies in MCA_HTBAPressure-treated wood doubled the one from fruiting bodies exposed to MCA_LTBA, whereas the same trend was not encountered in basidiospores, whose Cu uptake in MCA_HTBAPressure and MCA_LTBA environments appeared similar. Since no major change between the spores produced in MCA_LTBA and MCA_HTBAPressure environments, only MCA_LTBA was used for the subsequent analyses, in order to minimize any effect from TBA. Based on these results, we proved that Cu is accumulated in *R. placenta* 45 basidiospores. However this analysis provided no indication on whether Cu was present in ionic, nano, or bulk form.

**Speciation of Cu in fungal structures**

Basidiospores from *R. placenta* 45 exposed to sawdust from untreated and MCA_LTBA-pressure-treated sawdust were examined with XAS. The multiple spectra collected from the same location confirmed that no or minimal radiation damage occurred during the analysis. Maintaining the exact same experimental geometry, XRF measurements revealed different count rates for Cu fluorescence in the two type of basidiospores (90’000 counts for Cu in basidiospores produced by *R. placenta* 45 grown on sawdust from MCA_LTBA-pressure-treated wood, less than 30’000 counts for Cu in basidiospores produced on

<table>
<thead>
<tr>
<th>Structures</th>
<th>Cu content</th>
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<tr>
<td></td>
<td>Control</td>
<td>MCA_LTBA</td>
<td>MCA_HTBAPressure</td>
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<tr>
<td>Fruiting bodies</td>
<td>16±1 µg/L</td>
<td>2768±98 µg/L</td>
<td>7018±205 µg/L</td>
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<tr>
<td>Spores</td>
<td>17±13 µg/g</td>
<td>239±201 µg/g</td>
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</table>
untreated wood). Furthermore, even when normalized (Fig. 2), the spectra differed, indicating dissimilar Cu speciation.

In particular, the spectra differed in the near-edge (XANES) features around 8985 eV, on the edge (between 8990 eV and 9000 eV), and in the fine structure (Fig. 3). The fine structure (EXAFS) reveals the possibility of different neighboring atoms, and thus a different coordination for Cu in the two types of basidiospores.

The results of the linear combination fitting performed on the spectra from the basidiospores using CuCO₃·Cu(OH)₂, CuSO₄, reduced (or zero-valent) Cu, CuCl₂ aqueous, Cu oxalate, CuCys as standards is shown in Table 2.

![XANES spectra](image)

**Fig. 2.** XANES spectra of *R. placenta* 45 basidiospores grown on sawdust produced from MCA_LTBA-pressure-treated wood (blue) and untreated wood (red). The near-edge features differ, indicating differences in the Cu speciation.
Fig. 3. EXAFS spectra of *R. placenta* 45 basidiospores grown on sawdust produced from MCA_LTBA-pressure-treated wood (blue) and untreated wood (red). The fine structure features differ, indicating differences in the Cu speciation.

**Table 2- Results of the linear combination fitting for the two types of basidiospores**

<table>
<thead>
<tr>
<th>Standards</th>
<th>Control</th>
<th>MCA_LTBA</th>
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<tbody>
<tr>
<td>Cu oxalate</td>
<td>9±12%</td>
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</tr>
<tr>
<td>CuCO$_3$·Cu(OH)$_2$</td>
<td>0±11%</td>
<td>0±4%</td>
</tr>
<tr>
<td>CuSO$_4$</td>
<td>0±11%</td>
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<td>Reduced (or zero-valent) Cu</td>
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<td>CuCl$_2$ aqueous</td>
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<tr>
<td>CuCys</td>
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</tbody>
</table>
In both cases, the major component in the speciation of Cu seems to be CuCys. However, while this accounts for 41±21% in spores produced on untreated sawdust, the percentage heavily increases in basidiospores produced in the MCA_LTBA environment (83±9%). Reduced (or zero-valent) Cu weights 29±3% in the linear combination fitting from the spectra of basidiospores generated by \textit{R. placenta} grown on sawdust from untreated wood, whereas it doesn’t contribute to the linear combination fitting from the spectra of basidiospores generated by \textit{R. placenta} grown on sawdust from MCA_LTBA-pressure-treated wood. The other major component in both cases is CuCl$_2$ aqueous. The weight of the other standards in the linear combination fitting from both basidiospores, i.e. CuCO$_3$Cu(OH)$_2$, CuSO$_4$, and Cu oxalate, was null or extremely low. To confirm the absence of Cu particles accumulating in basidiospores we investigated them by means of TEM.

\textbf{Spore visualization}

TEM investigations of \textit{R. placenta} 45 basidiospores (Fig. 4) did not reveal any major difference between spores grown on the different type of sawdust. In both cases, the spores appear ellipsoidal, whose sizes range between 1 and 5 µm in length and 2 µm in width. No accumulation of electron-dense material attributable to Cu was visible in both types of basidiospores. This indicates that Cu is likely present as fine particles or as ions, and not as big clusters. Furthermore, no deformation of the cell organelles was visible. We proceeded by assessing basidiospore viability to determine whether the accumulation of Cu results in non-viable basidiospores.

\textbf{Spore viability}

Basidiospores of \textit{R. placenta} 45 produced in sawdust cultures from untreated and MCA_LTBA-pressure-treated wood were cultivated on artificial media, amended with MCA_LTBA or not (control). After 2 weeks incubation, both basidiospore types equally grew in control artificial media, covering the whole Petri dish surface. Nonetheless, differences in the growth were visible once the basidiospores were produced on MCA_LTBA-amended artificial media. While the basidiospores produced by \textit{R. placenta} 45 on untreated sawdust did not germinate on MCA_LTBA-amended artificial media, basidiospores from MCA_LTBA-pressure-treated wood sawdust could germinate and grow effectively. The growth speed of these latter was slower on MCA_LTBA-amended artificial media than on controls: the mean growths after 2 weeks were 6.6±0.6 cm and 8.5±0.0 cm, respectively.
Fig. 4. TEM micrograph of *R. placenta* 45 basidiospores grown on sawdust produced from MCA-pressure-treated wood. The basidiospores appear ellipsoidal, whose lengths range between 1 and 5 µm and widths of 2 µm. Neither deformation of the cell organelles nor accumulation of electron-dense material attributable to Cu were visible.

**Discussion**

The aim of this study was to determine whether Cu-tolerant wood-decaying basidiomycetes exhibit mechanisms of Cu compartmentalization in basidiospores as survival strategy while colonizing treated...
wood, if the Cu uptake in basidiospores is direct or occurs via different fungal structures, and in which form Cu occurs in basidiospores. In particular, we aimed to understand if Cu-based NPs contained in MC wood preservative formulations could be remobilized and released into the environment.

To investigate these aspects we used the highly Cu-tolerant [18] wood-destroying fungus *R. placenta* and stimulated the production of basidiospores when growing on sawdust from MCA-pressure-treated and untreated wood. The basidiospores were released in a Cu-free environment and subsequently analyzed to determine if Cu was present, in which form, and whether the basidiospores were viable or not. The method here developed to stimulate the sporulation of *R. placenta* proved to be sound and reproducible.

ICP-MS and XRF analyses highlighted a major difference in the amount of Cu contained in the two kinds of basidiospores, with basidiospores generated by *R. placenta* grown on sawdust from MCA_LTBA-pressure-treated wood containing up to 10 times more Cu. Therefore, our study revealed the presence of spore compartmentalization mechanisms by the Cu-tolerant wood-destroying basidiomycete tested here. This is the first time such a compartmentalization strategy is shown in non-mycorrhizal fungi. If we compare the amount of Cu found in basidiospores with the amount of Cu in wood dust, as reported by different studies [26, 27], we can see how they differ by a factor of 10: while wood dust from wood treated with Cu-based wood preservatives contains between 0.1% [26] and 0.3% [27] Cu, only 0.02% Cu was found in *R. placenta* basidiospores. In addition, the amount of Cu detected in the basidiospores seemed to reach a threshold level. The concentration of Cu in the fruiting bodies growing in contact with the MCA formulation with the highest fungicidal properties due to the presence of high amount of tebuconazole (MCA_HTBA) doubled the one detected in fruiting bodies exposed to MCA_LTBA; however the amount of Cu in the respective spores was comparable. Therefore, we can consider the amount of Cu detected as a maximum concentration. These findings suggest that if present in equal amount, Cu-loaded wood dust is more likely to be a threat than basidiospores. Further, the size of the basidiospores, as evaluated by TEM analysis, was comprised between 1 µm and 5 µm. In terms of respiratory tract uptake, this finding suggests that, if inhaled, the basidiospores would likely deposit in the middle respiratory tract [28-30], independently on the presence of Cu. Despite that, sensitive subjects that already experience adverse effects due to basidiospore inhalation, may face an exacerbation of their reactions [11-16].

Beside the differences in the amount of Cu, dissimilarities in the speciation of Cu could be emphasized by XAS analysis, both in the XANES and EXAFS regions. In fact, the amount of Cu bound to cysteine in basidiospores heavily increases in the presence of MCA_LTBA-pressure-treated-wood, doubling the CuCys amount observed under normal conditions (basidiospores generated by *R. placenta* grown on sawdust from untreated wood). Difference in the coordination of Cu, in terms of neighboring atoms or coordination structures, was also suggested by the mismatching EXAFS spectra. Similar findings on
biological material were obtained by Kopittke et al. [31] in the cowpea root system, and by Fomina et al. [32] in ectomycorrhizal fungi. Similarly to the latter study, the increased importance of CuCys in the linear combination fitting in basidiospores produced in presence of MCA_LTBA is likely to indicate the presence of Cu resistance mechanisms [33, 34]. By contrast, the amount of reduced Cu or Cu$^{2+}$ free ions complexed in water in basidiospores decreased when R. placenta was exposed to Cu (sawdust from MCA_LTBA-pressure-treated-wood). In fact, Cu tolerance mechanisms convert bioavailable Cu$^{2+}$ (as free ions or loosely bound) into non-bioavailable (thus not toxic) Cu complexes or precipitates, like CuCys.

The XAS finding that Cu is mostly bound to cysteine or present as free ions complexed in water was also confirmed by TEM investigation. No electron-dense material was visible in the micrographs, especially in the ones from basidiospores produced in a Cu-rich environment (generated by R. placenta grown on sawdust from MCA_LTBA-pressure-treated wood). This is in good agreement with the finding that metallic Cu is likely present only in basidiospores generated by R. placenta grown on sawdust from untreated wood, and that neither CuCO$_3$-Cu(OH)$_2$ nor CuSO$_4$ contributed to the XAS spectra from both kinds of basidiospores. This indicates that the initial CuCO$_3$-Cu(OH)$_2$ particles contained in the MCA wood preservative formulation are unlikely to be remobilized and end up in the basidiospores, instead they undergo a change in speciation. Therefore, the Cu compartmentalization mechanism here highlighted is not likely to depend on the wood preservative system, but it is likely to apply to different ones, i.e. conventional wood preservative as well as different MC formulations. Oxalate is believed to be implicated in metal tolerance for brown rot fungi [35]. Therefore, it was surprising to find that, Cu oxalate did not contribute too. This, combined with the observations that all basidiospores were still viable, and basidiospores generated in a Cu-rich environment subsequently germinated more effectively in Cu-amended artificial media, indicates that Cu-tolerance mechanisms for R. placenta basidiospores must be involved, but they do not rely on Cu complexation by oxalic acid to form non-bioavailable Cu oxalate.

Our results on basidiospore viability are not in contrast with previous studies [36] showing that the germination of R. placenta basidiospores is inhibited by MC if the fungus was not previously exposed to Cu. However, exposure to Cu prior to the production of spores by R. placenta is likely to trigger the basidiospores’ ability to adapt to Cu, and germinate in Cu-rich environments. Therefore, we can suppose that Cu detoxification mechanisms in spores can be inherited.

For the first time the mechanism of Cu compartmentalization in basidiospores is shown to exist in wood-destroying basidiomycetes. Therefore, Hypothesis 1 was verified. However, the Cu taken up undergoes changes in speciation, with no CuCO$_3$-Cu(OH)$_2$ particle identifiable. Therefore, Hypothesis 2 was falsified, and our findings reveal that the fungal remobilization of Cu from MC wood preservatives does not pose a nano-specific risk. However, the compartmentalization mechanisms highlighted are likely to be valid for conventional wood preservatives too. Therefore, a more detailed assessment on the human and
environmental risk is required. In particular, a quantitative assessment on the exposure to Cu-loaded spores and on their toxicity should be conducted.

References of Chapter 4.4


5. Discussion & conclusions

5.1 General discussion

This dissertation focused on a pressing question for the wood protection and nanosafety communities: whether Cu-based NPs-containing-MC is superior to conventional wood preservatives. More precisely, whether the penetration into refractory wood species is facilitated, the efficacy of the treatment against wood-destroying fungi is enhanced, and that the formulation does not constitute a health and environmental risk. In terms of possible risks, we investigated whether there is an airborne release of Cu-based NPs caused by MC-pressure-treated wood during its service life. The main aims and objectives, and the research hypothesis are listed in Chapter 1.1, page 13. In order to thoroughly answer the question, the penetration and protective effectiveness of MC were assessed first (Hypotheses 1, 2). In fact, these aspects are essential to monitor the fate and effectiveness of Cu from MC formulations. Subsequently, two major release pathways were identified and investigated: the compartmentalization of Cu in spores due to wood colonization by Cu-tolerant wood-destroying basidiomycetes (Hypotheses 2, 3), and the production of wood dust by mechanical abrasion (Hypothesis 4). Further, a preliminary assessment of the possible adverse health effects caused by inhalation of wood dust from MC-pressure-treated wood was conducted (Hypothesis 5).

5.1.1 Hypothesis 1: Within MC particle size range, Cu-based NPs, rather than Cu-based microparticles, are the main responsible for wood protection against wood decomposing fungi

The thorough particle characterization conducted on MCA formulations aimed at describing the Cu-based particles main properties, i.e. size, morphology, ζ. The full characterization on the MCA formulations tested is available in Chapter 4.1.1. This provided striking evidence on the presence of Cu-based particles solely in the nm range, therefore confirming the major role of NPs in MC-treated wood, in terms of both impregnation abilities and potential effectiveness against wood-decomposing fungi. Our results are in good agreement with other studies on MC formulations [1-3], indicating that different MC formulations are likely to fall under the European definition of nanomaterials [4]. In particular, the two MCA formulations here assessed had average sizes of 104±1.7 nm (mode: 87±2.2 nm) for MCA_HTBA and 174±5.9 nm (mode: 150±8.2 nm) for MCA_LTBA. These findings provide support to Hypothesis 1.

The first speciation of Cu-based NPs particles from MC occurs once the impregnation of wood takes place. In fact, this is the basis of the postulated innovative impregnation chemistry of MC formulations. Once wood is treated with MC, a fraction of CuCO₃·Cu(OH)₂ solubilizes and reacts with wood components, while part of it remains in the form of unreacted CuCO₃·Cu(OH)₂ particles that slowly solubilize with time, providing a reservoir effect [5] responsible for a continuous protection against wood-
decomposing fungi [6]. Already from this first phase, our studies demonstrate the importance and the challenges of NP environmental fate monitoring. As a matter of fact, different methods with increasing detection limits were required in order to keep track of Cu transport and speciation. However, even the high resolution techniques didn’t show detection limits low enough to detect single NPs in the biological systems. First, an investigation on the penetration of Cu from MCA in wood was conducted. More precisely, we assessed the output of MCA-pressure-treatments of easily treatable Scots pine sapwood and of refractory Norway spruce sapwood and heartwood. In this way, we could infer if the innovative impregnation chemistry and the presence of NPs could lead to a better penetration in wood species commonly used in the European market – in particular the refractory ones – without the need for pre-treatments prior impregnation, e.g. incision. This would provide an added value to MC compared to conventional wood preservatives. By combining ICP-OES and X-ray CT we could conclude that while Cu could easily and homogeneously penetrate into Scots pine; it mainly lies on the surface of Norway spruce (both sapwood and heartwood). Superficial treatments of wood are not effective, as they provide a shield that can be penetrated by wood-destroying fungi once cracks occur on the wood surface, due to abiotic or biotic agents. Furthermore, the analyses confirmed a small, though significantly different, higher amount of Cu in Norway spruce sapwood than in the heartwood. Besides yielding different types of information, the diverse analytical techniques sometimes provided results that – without being in disagreement – were however not easily comparable, due to the nature of the analyses or their sensibilities. In fact, both the ICP-OES and the X-ray CT analyses provided evidence for a different penetration gradient in easily treatable and refractory wood species, however the precise Cu quantifications differed. This is a common issue in the detection of NPs in biological or environmental matrixes [7]. In addition, the detection limits of X-ray CT did not allow to determine whether Cu in wood cells was present as ions or particles below 0.8 µm, therefore impeding to clearly define whether single particles responsible for the reservoir effect were present. Therefore, the presence of a reservoir effect, which would make MC an innovative wood preservative couldn’t be confirmed or disputed. Despite the issues encountered, we could confidently conclude that although the distribution of Cu from MCA differs than the one from conventional wood preservatives like alkaline copper quaternary (ACQ) [8], with Cu from MC mostly present in the latewood rather than in earlywood [8]. Pre-treatments to facilitate the penetration of wood preservatives in refractory wood would still be necessary, as the penetration of Cu in Norway spruce without any pre-treatment was not sufficient. Therefore, to determine which wood preservative treatment works best, a LCA/LCIA approach should be applied, where variables like material inputs, environmental outputs, transportation requirements from raw material extraction up through the point of use should be taken into account. In the scientific literature, it is possible to find contradicting findings: while some studies indicate how the LCA outputs from MC and conventional Cu amine wood preservatives do not greatly differ, due to energy consumption related to milling [9, 10], other LCA and LCIA researches have shown how MCA
and MCQ are environmentally preferable to ACQ [11, 12], and similar conclusions can be drawn for Cu azole wood preservatives. The reason behind the mismatching LCA/LCIA outputs lies in the particulate matter formation, i.e. different methods used to mill the NPs. Therefore, MC is as efficient as or more efficient than conventional wood preservatives. In addition, compared to conventional wood preservatives, MC does not require solvent during compounding, which also impacts the transportation requirements, and lower amounts are required to effectively protect wood. Further, lower leaching and corrosion potential were highlighted by Tsang et al. [11]. Pre-treatment (incising) of Norway spruce would be required for both MC and conventional wood preservatives, therefore since the penetration of MC and conventional wood preservatives is comparable, as proven in this thesis, the evaluation of the best system to use should shift towards the LCA/LCIA outputs, which are more favorable for MC. Thus, we can conclude that the energy efficiency of MC will still outperform conventional formulations in Europe. From a LCA/LCIA perspective, our results combined with the existing literature on MC’s LCA/LCIA lead to the conclusion that MC would be more preferable to ACQ even for European refractory wood species.

5.1.2 Hypothesis 2: Cu-tolerant wood-decomposing basidiomycetes exhibit the same tolerance mechanisms towards Cu, independently from the form of Cu

After assessing the microdistribution of Cu in wood, we tested the performance against soft rot fungi inhabiting the soil and the Cu-tolerant wood-destroying basidiomycete R. placenta. The wood preservative effectiveness of MC was assessed according to the European guidelines ENV 807 [13] and EN 113 [14]. These tests, which correlate the wood mass losses with the ability of the fungus to overcome the presence of the biocides in wood, would enable to understand the antifungal performance of MC. Even though standardized tests were used, it is always challenging to predict the long-term performance of wood preservatives, in particular when innovative systems with different impregnation chemistries are developed. Further, the behavior of fungi can radically change between artificial laboratory conditions and natural setting, as well as vary within different artificial media. Short-term laboratory tests provide quickly and at reduced costs an initial idea on the performance of wood preservatives. They are necessary, but not sufficient. Therefore, the short-term laboratory studies conducted provided an indication on the possible efficacy, but they should be associated with long-term field stake tests, e.g. EN 252 [15]. These would provide a more realistic scenario on the service life of pressure-treated wood. The short-term efficacy of MCA_HTBA against soft rot and other soilborne fungi was confirmed, even at relatively low MCA_HTBA concentrations. More precisely, the recorded wood mass losses were below 3% for wood stakes treated with MCA_HTBA concentrations as low as 0.80%. However, the duration and nature of the ENV 807 test cannot precisely discriminate between the effects of Cu and TBA. In fact, in the long-term the latter is likely to undergo degradation or decomposition, either abiotic [16] or biotic [17,18], leaving Cu as the only antifungal agent. This would promote the survival of Cu-tolerant species only or the
development of Cu-tolerance and resistance mechanisms in the microbial communities in close proximity to the MC-pressure-treated timber structures [19]. These communities are not likely to be affected by the presence of Cu-based NPs, according to our studies on *R. placenta*. We assessed different parameters for fungal fitness and Cu-tolerance in *R. placenta* exposed to Cu$^{2+}$ ions, MCA and CuCO$_3$·Cu(OH)$_2$: fungal biomass, oxalic acid and laccase production, and wood mass losses. In addition, we could discriminate any effect caused by particle solubilization and release of Cu$^{2+}$ ions by means of the Cu$^{2+}$ ion selective chelator TTM. What emerged is that the undissolved NP fraction from MCA does not exert specific Cu nano effects towards the Cu-tolerant fungus, which exhibits the same Cu-tolerance mechanisms independently on the form of Cu. Therefore Hypothesis 2 is supported. Instead, the importance of using NPs rather than bulk material was confirmed by the EN 113 results from MCA_LTBA- and bulk CuCO$_3$·Cu(OH)$_2$-pressure-treated wood: the latter performed poorly against fungal attack because the impregnation was not successful due to the size of the particles, thus unreacted CuCO$_3$·Cu(OH)$_2$ appeared as unbound dust on the wood sample surfaces. What also emerged from the fungal fitness study is that the presence of Cu in sub-lethal concentrations actually helps the fungus to overcome the issue of TBA, both in artificial media and wood. Even more, TBA in both MCA_LTBA and MCA_HTBA did not prevent *R. placenta* from sporulation, even at high concentrations (2.00% MCA-pressure-treated wood) and in laboratory condition, confuting the belief that tebuconazole inhibits the sporulation of wood-destroying fungi [20]. Hence, the combination of Cu and TBA does not result in a synergistic effect against *R. placenta*. This can also explain the different efficacy exhibited by MCA_HTBA in pressure-treated Scots pine sapwood, Norway spruce sapwood and heartwood. In Norway spruce heartwood, the most refractory wood according to ICP-OES and X-ray CT analyses, the low amount of TBA is sufficient to inhibit fungal colonization and the low amount of Cu is not sufficient to provide support to the fungus to overcome the TBA obstacle, whereas in the more accessible Norway spruce sapwood the amount of Cu reaches concentrations useful for *R. placenta*, until it reaches the threshold concentration (easily treatable Scots pine) and start exerting a toxic effect on the fungus, which is no longer able to colonize the wood substrate. To summarize, the results on the antifungal effectiveness indicate that MCA can perform as good as conventional wood preservatives on the short-run. Combining the LCA/LCIA outputs from the scientific literature previously discussed together with the antifungal performance here tested, we can conclude that MC is superior to conventional wood preservatives, as MC shows a higher energy efficiency that results in a lower impact on the environment. This conclusion would be valid also within the European decking market and its refractory wood. Therefore it should be preferred for in-ground timber applications.
5.1.3 Hypothesis 3: Similarly to mycorrhizal fungi, Cu-tolerant wood-destroying basidiomycetes can accumulate Cu in their spores

As long as Cu remains within the timber structure, independently on its size, concentration, or species, it does not pose a risk, as no exposure can occur. However, issues arises once Cu is remobilized. Studies on Cu leachability from MC-pressure treated wood indicate low leaching potential for MC [1, 11, 21-24]. However, between 42% and 88% of the Cu leached was present as CuCO$_3$·Cu(OH)$_2$ microparticles or NPs [1], resulting in a release of Cu-based NPs in the soil and water compartments. The research here conducted characterized the airborne particles released from MCA-pressure-treated wood due to abrasive processes or spore compartmentalization mechanisms by the Cu-tolerant fungus *R. placenta*. These two scenarios can occur during the service life of MC-pressure-treated wood or its disposal in landfills. In these studies, easily treatable Scots pine sapwood was selected as the only type of wood to use, in this way we could achieve a full impregnation of wood and maximize the potential release that would result in a worst case scenario. The main focus was on fine and ultrafine particles, which can reach the deep lungs and exert adverse health effects on the lungs [25-27]. In particular, our goal was to assess if the use of MC could lead to an additional nano-release due to the presence of unreacted Cu-based NPs from the initial MC wood preservative formulation.

Despite the positive performance of MCA in wood, the Cu-tolerant wood-destroying basidiomycete *R. placenta* was still able to colonize both MCA_LTBA and MCA_HTBA-pressure-treated wood, due to the presence of Cu-tolerance mechanisms. A setting to stimulate the formation of fruiting bodies and basidiospores in *R. placenta* during the colonization of wood was developed. The experimental conditions adopted allowed the fungus to grow and colonize wood that was pressure-treated with concentrations of MCA_LTBA and MCA_HTBA relevant for the wood protection market (2.00%). Further, the setting did not allow the basidiospores to get directly in contact with MCA-pressure-treated wood, so that the presence of Cu in these structures could be attributed entirely to Cu uptake and remobilization by the fungus, and not to direct Cu uptake by basidiospores. Elemental analysis, namely ICP-OES and XAS, revealed a high uptake of Cu by *R. placenta* basidiospores produced in presence of MCA-pressure-treated wood. Further, the amount of Cu did not depend on the content of TBA, as similar Cu concentrations were detected in basidiospores produced in presence of MCA_LTBA and MCA_HTBA. In addition, Cu was also detected in fruiting bodies, confirming the presence of a Cu compartmentalization mechanism and providing support to Hypothesis 3. The examination of the average Cu species in basidiospores produced in normal conditions (untreated wood) and MCA-pressure-treated wood highlighted a difference in the Cu speciation. Comparing the XAS spectra from both spores and reference materials, we could observe how the amount of unreacted CuCO$_3$·Cu(OH)$_2$ particles released via basidiospores, even in presence of MCA, is likely to be negligible. In fact, the TEM micrographs showed
that Cu was not present as precipitates, agglomerates, aggregates, or deposits of electron-dense material, therefore indicating that Cu was likely present as fine particles or as free or bound Cu$^{2+}$. Further, the comparison of XAS spectra from basidiospores and reference materials indicates that Cu is likely to be bound to organic compounds, in particular to Cys. The formation of CuCys complexes in fungal structures was already observed in previous studies [28, 29] and was attributed to Cu-tolerance mechanisms. More precisely, Cys is abundant in metallothioneins, which play a major role in heavy metal homeostasis and sequestration [30, 31]. Despite the high level of Cu, the TEM micrographs indicate that basidiospores from MCA-pressure-treated wood did not show any deformation and appeared viable. Even more, the spore viability was assessed in control and MCA-amended artificial media by assessing the radial growth in Petri dishes. What emerged is that Cu-loaded basidiospores could colonize the artificial media containing Cu more effectively than basidiospores produced in presence of untreated wood. This indicates that, beside hyphae and mycelia, also spores may have acquired tolerance for Cu. This is the first time the acquisition of Cu-tolerance mechanisms by spores is highlighted. In fact, previous studies assessed the germination of spores from Cu-tolerant fungi that were not exposed to Cu in the sporulation phase [32].

5.1.4 Hypothesis 4: Abrasion of MC-pressure-treated wood may lead to further dissemination of Cu-based NPs in the environment

Besides Cu release via spore, we investigated the outcome of mechanical abrasion of MCA-pressure-treated wood. For untreated, MCA-, and conventional CC-pressure-treated wood, we characterized the wood dust particle size (APS and SMPS), morphology (SEM), and the Cu content (ICP-OES). In terms of size distribution, the wood dust produced did not differ significantly from untreated wood or CC-pressure-treated wood. In particular, it did not result in an increase in the nanosized fraction of wood dust released. Furthermore, the elemental analysis performed on the whole wood dust and on the nanosized fraction, indicated that Cu is mostly released with the bigger wood dust particles, above 1 μm, with an increase from 0.15 % Cu in the nanosized fraction to 0.20% in the whole wood dust. This is in accordance with previous findings from Platten et al. [3], which characterized the wood dust released by wiping MCA-pressure-treated wood and observed Cu to be mostly associated with large cellulose or lignin particles. Therefore, we can conclude that MC is not a source of additional exposure to nanoparticles, indicating that Hypothesis 4 is not supported. Furthermore, the application of varnishes on untreated or treated wood surfaces can increase the wood dust particle size, which was also evidenced by Nowack et al. [33] on sieved fragmented products from CuO-acryl antimicrobial wood coatings. Therefore, varnishes could be used to further reduce the risk. We can hypothesize a minimal contact for consumers, whose exposure to MCA-pressure-treated wood and its dust is occasional, whereas exposure to wood dust mostly occurs among woodworkers [34-36], thus it could be considered as a substance of concern for professional users, as occupational exposure occurs more frequently.
5.1.5 Hypothesis 5: Cu-based NPs from MC wood preservative formulations, if inhaled, can cause adverse effects due to specific nano effects

The amount of Cu detected in wood dust greatly exceed the ones found in spores (10-folds higher), and the chances of releasing particles below 1 µm –which can reach the deep lungs [25-27] if inhaled– is likely to occur only due to mechanical abrasion, as R. placenta spores sizes ranged from 1 µm to 5 µm. Therefore, a preliminary in vitro hazard assessment to determine eventual adverse health effects was conducted solely on the abraded particles. To simulate the lung environment we used lung epithelial cell lines and macrophages, and we assessed the potential adverse effects of dust from MCA-pressure-treated wood by monitoring the oxidative stress, cell viability, and cellular inflammation. Both the MCA wood preservative formulation, reference materials (TBA, Cu$^{2+}$ ions, positive control CdSO$_4$), wood dust from untreated, MCA-, and CC-pressure-treated wood, as well as eluates from the same wood dust were assessed. The findings from the in vitro citotoxicological studies indicate that MCA and dust from MCA-pressure-treated wood did not induced a nano-specific toxicity in lung epithelial cells and macrophages, and it exerted fewer adverse effects than wood dust from CC-pressure-treated wood. Therefore, despite dust from MCA-pressure-treated wood may pose a hazard, this is not related to specific nano effects. This indicates that Hypothesis 5 is not supported under the experimental conditions adopted.

5.1.6 Further comments

Our results suggest that the release of Cu in the air compartment due to mechanical abrasion or spore compartmentalization does not depend on the presence of Cu-based NPs. More precisely, the abrasion of MC-pressure-treated wood does not produce a higher fraction of wood dust in the nano range, and the cytotoxicological studies revealed no additional toxicity from wood dust generated by abrasion of MC-pressure-treated wood dust compared to CC-pressure-treated wood, therefore indicating that CU-based NPs do not pose an additional risk. In addition, via spores, Cu is not likely to be released as CuCO$_3$·Cu(OH)$_2$ NPs, as it undergoes speciation. Therefore, the release mechanisms here assessed are likely common to any Cu-based wood preservative formulations. Furthermore, when these findings are combined with the results from Platten et al. [1] results, and integrated in a mass balance, we can conclude that only the water and soil compartments are exposed to Cu-based NPs, although only to a limited extent. If we combine this finding with the fact that MC is generally needed in lower amount than conventional wood preservatives to effectively protect wood [11], we can hypothesize that the release of Cu from MC via spores is a best case scenario, while conventional wood preservatives may cause a higher release of Cu via spores. Besides, a major percentage of Cu is released via particles (wood dust or spores) with sizes above 1 µm, which cannot easily reach the lower respiratory tract. However, other fungi, more widespread, with smaller spores and allergenic, e.g. Aspergillus spp. or Penicillium spp. may pose a higher risk [37]. Further, asthmatic or allergic individuals, which would suffer from phase reaction due to the
contact with spores [38-43], could experience an exacerbation of the symptoms. In addition, the nanosized fraction, which can be generated by mechanical abrasion of pressure-treated wood, showed less adverse effects in lung epithelial cells and macrophages than conventional CC. The scientific literature on release of Cu from MC-pressure-treated wood in the water and soil compartments showed that the amount of Cu detected in the two environmental compartments, mainly due to leaching, is lower than what measured from wood pressure-treated with conventional wood preservatives [1, 11, 21-24]. When the evidence on the possible release in the air compartment, as measured in this thesis, is combined with the studies that assessed the release in water and soil just mentioned, it clearly emerges how MC outperforms conventional wood preservatives by overall releasing lower amounts of Cu in all the different environmental compartments.

5.2. Conclusions & outlook

This thesis examined the fate and behavior of Cu-based NPs from MC preservatives during the wood impregnation and service life of in ground timber structures and focused on the release in the air compartment. Beside particle sizes and elemental composition, transformation and alteration processes of the initial nanomaterial were considered, according to the current paradigm for risk assessment of nanomaterials [44]. The research project was carried out within the framework of the following working hypotheses, which were supported or confuted as follows:

1. Hypothesis 1: supported
2. Hypothesis 2: supported
3. Hypothesis 3: supported
4. Hypothesis 4: not supported
5. Hypothesis 5: not supported.

In summary, our findings reveal that MC is a valid alternative to conventional wood preservatives, in terms of effectiveness, and environmental and health risks. In particular, in the air compartment, MC does not pose any additional nano-specific risks due to the NPs present in the formulation. An airborne release of Cu was observed when MC-pressure-treated wood is subjected to mechanical abrasion or colonization by Cu-tolerant wood-destroying fungi, however the particle sizes, the Cu concentrations, and the hazard assessment do not provide evidence for higher health and environmental risks than the ones posed by conventional wood preservatives. Even more, when compared to other wood preservatives, MC actually constitutes a safer alternative. This is not only valid for the air compartment, but for Cu from MC released in water and soil too [1, 40-43]. Cu is a mainstay for in-ground timber applications, and currently there is no satisfactory alternative [45]. Therefore, based on our
findings, it is here suggested the use of MC over conventional wood preservatives, as it outperforms them on different levels.

The analytical methodologies used in the current study proved to be capable and comprehensive tools to address the issues of wood preservative effectiveness, estimation of emissions, and hazard for human lungs of Cu-based NPs from MC in a realistic manner. However, there are still a few open points that need to be addressed for a better monitoring of Cu-based NPs used for wood protection, in terms of flows and concentrations, as well as hazard assessment. More precisely:

1. Additional studies on the speciation of Cu in the different stages of MC wood preservatives could provide further information on the mechanisms behind their effectiveness and lead to improvements in the NPs and in the formulation that could lead to better impregnation, enhancement of the reservoir effect, reduction of preservative release (e.g. leaching) and corrosion potential. Moreover, they could help to better define the environmental fate of MC in the different environmental compartments and during the whole life cycle of MC and MC-pressure-treated wood

2. X-ray CT at different resolutions allowed to determine the macro- and microdistribution of Cu in wood, and provided indication on the possible form of Cu. However, the laboratory-scale instruments couldn’t visualize single NPs. Nanoscale ptychography [46] combined with 3D tomographic reconstruction may be able to resolve the interaction between Cu and a single wood cell at an unprecedented scale. Further, when these techniques are applied to decayed wood cells, the understanding of wood decomposition mechanisms would provide a useful tool for wood protection advances, e.g. by the development of more tailor-made wood preservatives.

3. Despite the risk related to MC-pressure-treated wood appear to be low, a quantitative assessment of the amount of Cu-loaded spores released in the air can lead to a better risk characterization. In particular, establishing which Cu-tolerant fungi –either basidiomycetes or not– are able to compartmentalize Cu in their spores is essential for a precise emission assessment. This would not only be relevant for Cu from pressure-treated wood, but from other Cu sources too, like Cu polluted environments where Cu-tolerant fungal communities can be found [47]

4. Beside the qualitative and quantitative evaluation on Cu-loaded spores, an assessment of spores transport and mobility is here suggested. This would enable to determine whether other human and environmental exposure can occur, e.g. if the Cu-loaded spores deposit on the ground, and dermal transfer or ingestion by humans or animals can take place
5. Further studies should investigate more in depth the hazard of wood dust from MC-pressure-treated wood or Cu-loaded spores, with more advanced models mimicking the lungs, e.g. utilizing *ex vivo* or *in vivo* approaches [48]

6. Similarly, the toxicity of Cu-loaded spores from wood-destroying fungi belonging to different phyla should be evaluated. In fact, while the evaluation here conducted is mainly based on the physical properties of Cu-loaded spores (size, form of Cu), they may anyway exacerbate respiratory diseases or induce allergic reactions due to the spores themselves [35, 49], which may be enhanced by the presence of Cu

7. The currently available LCA/LCIA and material flow models on MC can be implemented with the recent findings, in order to provide a more realistic overview.

To conclude, the current work provided a useful approach to monitor NPs in different media, as well as an insight into MC, its potentials and pitfalls. However, “wood science is not rocket science… it’s much more complicate!” [50], and the same applies to nanoscience. For the science behind MC and its Cu-based NPs “there’s still plenty of room at the bottom” [51].

5.3. References of Chapter 5


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6. Curriculum vitae

Chiara Civardi

PERSONAL INFORMATION

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EXPERIENCE

05.2016-10.2016 Project Drawdown Sausalito, US
Volunteer Researcher

Brazil/Pantanal/Cerrado Wildlife Conservation Intern

10.2013-present United Academics Amsterdam, NL
Science Blogger

08.2013-01.2015 Telejob Zurich, CH
Seminar & Event Organizer

10.2012-03.2016 Empa St. Gallen, CH
PhD Student

03.2012-09.2012 The Mary Rose Trust Portsmouth, UK
Conservation Intern & STEM Assistant

10.2010-06.2011 Università degli Studi di Parma Parma, IT
Chemistry Intern

EDUCATION

10.2012-11.2016 ETH Zurich, CH
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PhD in Civil Engineering
- Pending result, Thesis: “Effectiveness and environmental risk of nanocopper-based wood preservatives”

09.2011-09.2012 University of Southampton Southampton, UK

*MSc in Maritime Archaeology*

- Pass with Distinction, Thesis: “Conservation of Mary Rose’s cordage: diagnosis and treatments”

10.2007-06.2011 Università degli Studi di Parma Parma, IT

*BSc in Science and Technology for the Conservation and Restoration of Cultural Heritage*

- 110/110 cum laude, Thesis: “Wood preservative treatments based on metal cations and nanoparticles vehiculated by hybrid organic-inorganic polymers”

**LIST OF PUBLICATIONS**


7. Declaration of personal contribution

Your Doctoral Thesis: Declaration of Your Personal Contribution

Name: Chiara

First Name: Civardi

Title of Thesis: Assessing the effectiveness and environmental risk of nanocopper-based wood preservatives

Thesis Supervisor: Prof. Dr. Ingo Burgert

Study Programme (CE, EE, G, REIS): CE

I, Chiara Civardi, declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any university or other institute. Information derived from the published and unpublished work of others has been acknowledged in the text and a list of references is given in the bibliography.

Declaration of Your Personal Contribution
(brief description of your intellectual contribution, separated by chapter if applicable)
Chiara Civardi entirely wrote Chapter 1, 2, 3, 5, 6. Chapter 4 contains manuscripts written together with other authors. Chiara Civardi designed the experiments, performed them, summarized the results and the existing literature, and wrote the manuscripts.

I confirm that the individual contributions are correctly declared.

Place, Date: Zurich, 26.08.2016

Signature Doctoral Student: [Signature]