

Dissertationes Forestales 308

**Recovery of antifungal compounds from wood and
coffee industry side-streams and residues for wood
preservative formulations**

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Academic dissertation

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ABSTRACT

The aim of this thesis was to test the possibility of using the residues and side-streams from Finnish wood and coffee industries as active ingredients in wood preservative formulations, as well as to compare their acute ecotoxicity. Pyrolysis distillates of bark from Norway spruce (*Picea abies* (L.) H. Karst.), silver birch (*Betula pendula* Roth) and European aspen (*Populus tremula* L.), the organic acids identified in these distillates, spent coffee extract, coffee silverskin extract, caffeine and the commercial Colatan GT10 tannin-rich extract were tested. Celcure C4 industrial copper preservative (for above ground use) and pine oil were used as industrial references. Antifungal tests against wood-decaying fungi and wood decay—mini block—tests were performed *in vitro*, and leaching tests of the potential preservatives from wood were performed. An acute ecotoxicity test with *Aliivibrio fischeri* photoluminescent bacteria was performed in order to compare the ecotoxicity of the potential bio-based preservatives with that of the industrial reference.

All potential bio-based preservatives showed some activity against the fungi in the antifungal tests. The minimum inhibitory concentration of the extracts from coffee industry residues needed to inhibit completely all the wood-decaying fungi was over 1%. The pyrolysis distillates were able to inhibit most fungi at concentrations close to 1%. The organic acids and caffeine were able to inhibit wood-decaying fungi in the malt agar media at concentrations below 1%, showing that these constituents play a significant role in the antifungal activity of the tested distillates and extracts. However, when the potential bio-based preservatives and their constituents were tested in the wood decay tests, none of them performed efficiently as wood preservatives.

The acute ecotoxicity test showed that most of the potential bio-based preservatives had low ecotoxicity, but one of the distillates exhibited IC₂₀ of 0.02 mg/L and IC₅₀ of 0.2 mg/L, a much higher ecotoxicity than Celcure C4, which had IC₂₀ and IC₅₀ values of 12 mg/L and 19 mg/L respectively. This shows that we must test the ecotoxicity of all potential antifungals before proposing them as possible wood preservatives, to ensure that new solutions are not as harmful to the environment as the present ones. It can be concluded that some of the constituents of the potential bio-based preservatives act as antifungals against wood-decaying fungi and could be included in wood preservative formulations, but their performance alone is insufficient to function as wood preservatives.

Keywords: wood preservation, wood degradation, bio-based products, coffee extract, pyrolysis distillates, antifungal efficacy.

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¡Gracias a todos!
Eskerrik asko guztioi!

Aitor Barbero López

LIST OF ORIGINAL PUBLICATIONS

The thesis is a summary of the following published articles:

- I. Barbero-López, A., Chibily, S., Tomppo, L., Salami, A., Ancin-Murguzur, F.J., Venäläinen, M., Lappalainen, R., Haapala, A. (2019). Pyrolysis distillates from tree bark and fibre hemp against wood-decaying fungi. *Industrial Crops and Products* 129: 604–610. DOI: 10.1016/j.indcrop.2018.12.049
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- III. Barbero-López, A., Monzó-Beltrán, J., Virjamo, V., Akkanen, J., Haapala, A. (2020). Revalorization of coffee silverskin as a potential feedstock for antifungal chemicals in wood preservation. *International Biodeterioration and Biodegradation* 152: 105011. DOI: 10.1016/j.ibiod.2020.105011
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- V. Barbero-López, A., Akkanen, J., Lappalainen, R., Peräniemi, S., Haapala, A. (2021). Bio-based wood preservatives: Their efficiency, leaching and ecotoxicity compared to a commercial wood preservative. *Science of the Total Environment* 753: 142013. DOI: 10.1016/j.scitotenv.2020.142013

The present author was the principal author of all the papers, with the main responsibility for the experimental design and realization, analysis and reporting of the results. Other authors participated in design, practical work and analysis of the antifungal, wood decay and leaching tests in all papers. The ecotoxicity tests in Papers III and V were done jointly under the supervision of my co-authors. The co-authors participated in the experimental design and made most of the chemical analyses reviewed in these papers. Writing of the papers was also realized in collaboration with all authors while the first author was responsible on formulating the first draft, submission, and correspondence with journal editors.

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LIST OF ABBREVIATIONS

ACQ-D	Amine Copper Quat-type D
CCA	Chromated Copper Arsenate
CCB	Chromated Copper Borate
CDDC	Copper dimethyl dithiocarbamate
EC	European Commission
EN	European Standard, for analysis methods
EPA	Environmental Protection Agency
MIC	Minimum inhibitory concentration
NTR	Nordic Wood Preservation Council

1 INTRODUCTION

1.1 Background and motivation

Wood is the main renewable material in the world, with several favourable properties such as its mechanical strength, good availability and reasonable price that make it an excellent option in many fields, such as construction and furniture making. The increasing concern of society about the need of using sustainable and renewable materials, as well as carbon neutral materials, has increased the relevance of wood in recent years. However, forest management, including the volumes of wood harvested, and the later use of solid wood, as well as the chemicals and fibres obtained from refinery activities, needs to be sustainable. Additionally, one of the aims of bioeconomy is the utilization of as few virgin materials as possible, thus requiring sustainable ways to extend the service lifetime of wood, which would also prolong the carbon sequestration time of these materials.

Despite the good properties of wood for many different applications, wood decays naturally due to many abiotic and biotic factors, such as weathering or microbial activity. Of all the factors causing wood decay, fungal decay is considered the most relevant one in urban and rural areas (Broda 2018). Thus, wood has been treated for centuries in order to slow down decay processes and make the wood last longer. The treatment of wood with different chemicals has become common practice when it is exposed to environmental factors that shorten its lifespan.

Wood preservative impregnation has been the most used method by the wood industry to avoid fungal decay in wooden construction materials such as poles, beams, and lumber. As this method has been used for many decades, knowledge about the chemicals used in impregnation has also increased, leading eventually to limitations in their use as many of them have been identified as harmful to the environment or humans (Singh and Singh 2012). For example, the concentration of boron-based compounds was limited in Europe in 2008 (Hu et al. 2017), and chromated copper arsenate, commonly known as CCA (Liu et al., 2018), was banned in Europe and prohibited in residential applications in the USA in 2003 (Commission Directive 2003/2/EC, EPA 2003). Other chemicals are still used by the wood industry due to the lack of efficient substitutes on the market. Creosotes are a clear example of this, as they contain compounds that are carcinogenic to humans (Hiemstra et al. 2007), which led to limitations in their use, but they have not been completely banned due to the lack of suitable substitutes (Humar 2017). Thus, new wood preservatives and ingredients for their formulation are needed.

1.2 Gaps in knowledge

The use of preservatives by the wood industry to extend the life cycle of wood has been a common practice since 1838, when John Bethell developed an impregnation method for wood (Barnes 2002). The substitution of environmentally or health-wise most detrimental chemicals by other chemicals which fit the legislation, and which do not have such a high impact in the environment is an issue which continues to be addressed. Legislation in the European Union is becoming stricter, because there is more awareness of the side-effects of many chemicals in the environment, and thus, it can be expected that several chemicals used as fungicides and wood preservatives will be banned, or their use restricted, in the years to come. A lot of research has been published during recent decades in bio-based wood

preservation studying the possibilities of using many natural-origin chemicals for wood preservation, such as tung oil and linseed oil (Humar and Lesar 2013) and *Amorphophallus konjac* (devil's tongue plant) extracts (Bi et al. 2019). Also research focused on biomimicry of natural durable wood species by treating wood with the extractives, such as the phenolics in Scots pine (*Pinus sylvestris*) heartwood that confer the durability to wood (Harju et al. 2003, Lu et al. 2016) has been done. Other means to avoid wood decay have also been studied, e.g. by performing structural and chemical modification of wood, such as ThermoWood® and acetylated wood, which can also reduce the decay rate caused by fungi (Peterson and Thomas 1978, Yilgör and Kartal 2010). In addition to these alternative treatments, the bio-based chemicals have a significant potential to inhibit decay fungi, which has kept them in the focus of many research groups aiming towards natural wood preservation (Singh and Singh 2012, Teacă et al. 2019, Broda 2020).

Despite the research already done on the use of natural antifungals in wood preservation, many potential and abundant feedstocks remain untested for this purpose. These include the residues and side-streams from the coffee industry and the bark from industrial wood species. Their constitution, antifungal and wood preserving potential, as well as their ecotoxicity, are not yet known.

Additionally, most of the bio-based antifungals that have already been tested as preservatives against wood decay fail in their fixation to wood, as they are water soluble (Singh and Singh 2012). Thus, their performance as wood preservatives is good when tested *in vitro*, but as soon as leaching tests are performed their effectiveness is reduced very significantly, as seen previously in cases of pyrolysis distillates (Mohan et al. 2008) and chitosan (Alfredsen et al. 2004). Thus, there is a need to study and develop bio-based wood preservatives that become, or can be, fixed to wood. While this thesis does not focus in fixation of wood preservatives, this could be achieved by testing novel bio-based preservatives from feedstock and by testing fixation methods—additional chemicals or treatments—that help the fixation of the potential bio-based preservatives that successfully inhibit the decay fungi before leaching.

1.3 Wood products and uses

Since pre-historical times, wood has been used for many building-related purposes, and although other materials have been found and improved materials are continuously being developed, wood has kept its relevance until today. About 3500 million m³ of wood are industrially converted every year around the world for different end purposes (Martínez et al. 2005). Traditionally, wood was used for making ships, tools, furniture or houses (see Figure 1 as a visual example). When other materials such as synthetic polymers became popular in the mid-19th century, wood lost some of its relevance in some uses, such as toy-making. New applications for wood were also—and are still being—developed, such as nanocellulose extraction and textiles. Wood is nowadays used for many popular purposes, such as fencing, garden decoration and tool and music instrument making. Additionally, there are other uses of wood components that remain unknown for most people, such as the use of cellulose in ice-cream and food as a filler (Velásquez-Cock et al. 2019), biomedical applications (Ganguly et al. 2020), and the use of extractives for products such as sunscreens and cosmetics (Fernandes et al. 2018). Nevertheless, wood use in building remains very important. As an example, over 50% of the wood harvest is used for building construction in the USA (Falk 2009).

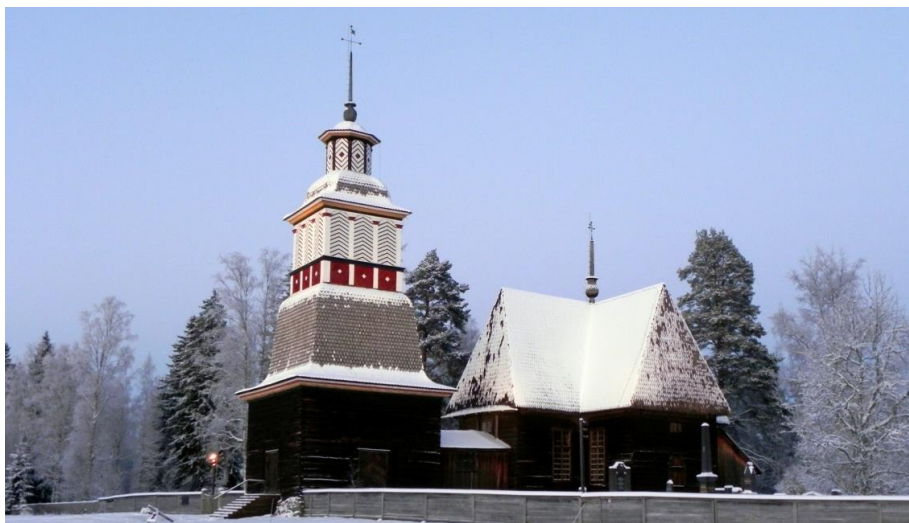


Figure 1. The Old Church, located in Petäjävesi (Finland), was built with logs in the 18th century. This church is on the UNESCO World Heritage list as an example of traditional architecture from Scandinavia.

The main reasons for building with wood are its good mechanical properties and versatility. Its natural appearance is perceived as pleasant by many people, although it may be considered rustic and old-fashioned by others. Concrete, masonry, glass and aluminium are used in high volumes, but the depletion of non-renewable resources and emissions of greenhouse gasses drive towards more efficient use of renewable resources, so that wood is currently the most important renewable material for building.

There are other wood properties, which are negative from the point of view of their end use. The fact that wood can burn causes many people to see it as a material that should not be used for structural members of building. The hygroscopicity of wood compared to other materials makes it shrink and swell, which is a limiting factor in some applications. Wood can also deform after several years, due to several factors such as the previously cited moisture. The microbiological durability of wood is also a concern in many end uses, especially in building, as several factors, such as fungi, may affect the life span of this material. However, biodegradation of wood is positive from an environmental perspective, as wood does not accumulate in the environment.

1.4 Wood decay and wood-decaying fungi

All biological material will degrade in time due to different biotic and abiotic factors. When exposed to certain factors, such as the presence of oxygen and water, the decay process is accelerated (Blanchette 2010). Biodegradation of wood is positive from an environmental perspective, as wood cannot accumulate in the environment, but in many uses, it causes significant economic losses via shortened material service lifetime (Martínez et al. 2005).

Abiotic factors causing the wood weathering that degrades wood properties and durability are the following environmental factors: sunlight (UV radiation), moisture, abrasion, heat, oxygen, acid rain and pollutants (Nuopponen et al. 2003). Of all the wood components, lignin is the most sensitive to sunlight, and once it degrades, hemicelluloses can be hydrolyzed more

easily by water (Cai 2020). Thus, sunlight is the initial cause for weathering and then the material degrading effects increase due to the presence of other environmental factors (Pandey 2005b). In addition to all these factors, the wood properties and the presence of extractives can also affect the weathering of wood (Pandey 2005a; Pandey 2005b). Although wood weathering usually affects the wood surface, it can also crack the wood, thus facilitating biotic decay (Nuopponen et al. 2003).

Considering the biotic factors, in seawater marine borers are the main responsible of wood decay (Björdal and Nilsson 2008), in fresh-water ecosystems bacteria are mainly responsible for wood decay, whereas in terrestrial ecosystems of temperate climate, fungi play a more significant role (Björdal et al. 1999; Broda 2018). Depending on the climate, some insects such as coleoptera (beetles) and termites also play a role in wood decay. In Finland due to its climate fungi remain as the main biotic factor of architectural wood decay.

In terrestrial ecosystems, the best conditions for fungi to decay wood usually occur when the temperature is sufficiently high and the moisture content of wood exceeds the fibre saturation point and is below full saturation (Walker 1993), although fungal decay can also occur in other conditions, such as at moisture contents below the fibre saturation point (Meyer et al. 2016). However, it must be highlighted that the relationship between moisture content in wood and the activity of wood-decaying fungi is not yet fully understood (Brischke and Alfredsen 2020). It is also important to highlight that in addition to the environmental conditions, the architectural characteristics and building physics play an important role in the growth of mould and decay fungi, as they can cause microclimates (Charisi et al. 2018). For this reason, wood preservation by designing buildings properly is considered as the main factor when building with wood (Brischke et al. 2006).

Different fungi will cause different decay patterns and stains in wood, and they are usually classified as white rot, brown rot, soft rot and stain fungi (Martínez et al. 2005). Within the white rot fungi, simultaneous rot and selective delignification are differentiated (Martínez et al. 2005; Schwarze 2007), although simultaneous rot has also been considered to be different from white rot by some authors, such as Liese (1970).

White rot and brown rot are caused by basidiomycete fungi, and the criteria used to differentiate these two groups are based on the capability for extracellular oxidization of phenolic compounds by the white rot fungi (Martínez et al. 2005; Schwarze 2007). White rot fungi use enzymes to break the cell wall and start the decay process, whereas in the initial phases brown rot fungi predominately use a non-enzymatic process based on Fenton chemistry (Xu and Goodel 2001; Vaaje-Kolstad et al. 2010; Thybring et al. 2018). Soft rot is caused by ascomycetes, although some basidiomycetes can also cause similar decay in wood. In many cases, bacteria are also involved in this process (Martinez et al. 2005). Based on the review by Martínez et al. (2005), the following are the main characteristics of the decay caused by white rot, brown rot and soft rot fungi:

- White rot fungi: These fungi can be found in both hardwoods and softwoods, although they are more specialized in hardwood decay. Simultaneous rot and selective delignification make the wood appear bleached, and the wood losses strength when decay is advanced. Simultaneous rot usually attacks hardwoods, and decays cellulose, hemicellulose and lignin. It is caused by basidiomycetes, such as *Trametes versicolor* (Figure 2), and some ascomycetes. In the case of selective delignification, white rots can be found in both hardwoods and softwoods, and they initially decay lignin and hemicelluloses, although they may also decay cellulose in more advanced stages of decomposition. Selective delignification is only caused by basidiomycetes in the first phases of decay.



Figure 2. In A, fruiting bodies of the white rot fungus *Trametes versicolor* growing on a stump. In B, wood specimen from a wood decay test of 16 weeks colonized by *Trametes versicolor*.

- **Brown rot fungi:** This kind of decay usually causes a brown coloured wood, with powdery appearance. The wood usually cracks into cubes (Schwarze 2007), acquiring a brick-like appearance. The strength of wood decreases significantly at the beginning of the decay. When the decay process is advanced, the wood splits and presents a squared pattern (see Figure 3 as a visual example). Brown rot fungi decay cellulose and hemicelluloses after causing a partial modification of lignin. Brown rot fungi usually decay softwoods, although in some cases hardwoods may also be decayed.
- **Soft rot fungi:** These fungi make wood look brownish in dry conditions, and it has a soft consistency when wet. They usually attack hardwoods and decay cellulose and hemicellulose while causing only a slight modification of lignin. This kind of decay is caused by ascomycetes and deuteromycetes, often together with bacteria, when the wood is in direct moist conditions, such as in underground piles and water-cooling towers.

Although only 6-7% of the known wood-decaying fungi are characterized as brown rot fungi (Martínez et al. 2005; Schwarze 2007), they are responsible for most of the economic losses (Gabriel and Švec 2017), as softwoods are usually used in outdoor constructions. Additionally, two brown rot fungi, *Serpula lacrymans* and *Coniophora puteana*, are the most common fungi found in wood used indoors in Europe (Gabriel and Švec 2017).



Figure 3. Scots pine sapwood specimen decayed by brown rot fungi, presenting the typical squared pattern.

When wood becomes decayed, several of its properties are changed. In addition to the altered appearance, the mechanical properties of the wood are reduced (Li et al. 2019; Azimi et al. 2020). The mechanical strength loss occurs when wood decay starts in brown rots, whereas in the case of white rots it occurs when the decay is advanced (Martinez et al. 2005). In laboratory tests, it has been found that mechanical properties, especially toughness, often weaken much faster than the mass decreases when wood decays. For example, toughness loss values range from 6% to 50% when the mass loss is only about 1% (Ibach and Lebow 2014). Additionally, mechanical loss from 20% to 80% occurs when the mass loss is about 5% to 10% (Ibach and Lebow 2014). Venäläinen et al. (2014) found that in soil-contact experiments, Scots pine sapwood lost about 50% of its strength when the mass loss was about 10%. In the case of Scots pine heartwood, Venäläinen et al. (2014) reported that the strength was reduced about 30% when the mass loss was 7%, and concluded that the relevance of measuring the mechanical properties in wood decay experiments was of prime importance. Loss of strength can obviously cause very important problems in wood used for buildings, as it may cause high costs due to the need for replacing the wood or the buildings may even collapse as the structures weaken and can no longer support the load (Simons 2012).

1.5 Wood preservation

1.5.1 Historical view to wood preservation practices

Extending the service lifetime of wood has been an important issue to be addressed since early human history. Nowadays it is known that the presence of different extractives in wood, such as phenolics including flavonoids and stilbenes, plays a key role in the wood decay resistance (Harju et al. 2003; Taylor et al. 2006; De Angelis et al. 2018). In the past, the reasons for the natural durability were not understood. It was believed that the time of year of felling the wood affected its durability (Unger et al. 2001) and based on experience different woods were considered to have different degrees of durability. On this basis, species that are more durable were chosen for building houses and ships. As an example, the Australian aborigines used bloodwood (*Corymbia* sp.) for making their graves about 5000 AD (Unger et al. 2001), because this wood is resistant to decay by termites and fungi.

The philosopher Theophrastus (371-287 BC) listed the wood species that were durable (Unger et al. 2001) and in the 7th and 9th centuries the Maya civilization used termite- and fungi-resistant wood for building, resulting in buildings which lasted for centuries (Hellmuth 1989). An excellent example of these buildings is the Maya temple built in the 8th century in Guatemala with termite resistant wood (Unger et al. 2001). In Scandinavia, the Norwegian stave churches, also known as Stavkyrkje, were built over 800 years ago from resin rich dry pine timber (Tschudi-Madsen 2009), and several of them remain standing today (Unger et al. 2001), such as the Heddal stave church (Figure 4).

Thermal treatment to make wood last longer was relevant during the Roman Empire, between the 8th century BC and the 5th century AD. The oldest surviving book series on architecture by a Roman architect and military engineer Marcus Vitruvius Pollio, called “ten books on architecture”, gave high relevance to the decay of buildings and wood in the 1st century BC. Chapter V, section 3 of this treatise highlights that very close succession of charred olive wood make it resistant to decay and weather effects (Vitruvius and Morgan, 1960). A similar technique, called Shou-Sugi-Ban, was used in Japan since the 1700s. In this process, the Sugi wood (*Cryptomeria japonica*) was charred to make it last longer (Kilian 2014).



Figure 4. Heddal Stave Church, in Notodden (Norway). Picture with permission by Xabier Ancin.

The chemical constituents of plants and wood slowly gained significance in wood preservation, after humans realized that these constituents played a major role in wood durability. The naturalist Pliny the Elder (23-79) was a famous Roman who reported that different natural oils had wood preserving properties and wrote on several of their preparation methods (Richardson 2005). Their thermal extraction also became popular as business evolved around wood preservation compounds. Many ancient ships were—and still are—treated with chemicals such as wood tar for waterproofing and making them resistant to decay. The ancient Ma'agan Mikhael Ship, from the 5th century BC, is an early example of conifer tar coating (Connan and Nissenbaum, 2003), while centuries later the Vikings also applied tar mixes with the same aim (Hennius 2018). The previously cited Norwegian stave churches were often treated with tar to make them last longer, and many are still treated with tar to preserve them. Tar remained a very relevant product for wood treatments due to the boom of the shipbuilding industry and became a very important business for many countries. In Finland, tar became the most important export during the 17th and 18th centuries, continuing until the end of the prosperity period of these products at the beginning of the 20th century (Åström 1964).

Inorganic compounds have also been used as preservatives during human history. In China, before 100 BC, wood was immersed in saline water before using it as a building material (Richardson 2005). Several centuries later, wood conservation was also considered to be of relevance in some disciplines such as art, when important artists tried to preserve their works. For example, Leonardo Da Vinci coated the wood panels for his paintings with mercury(II) chloride and arsenic(III) oxide (Unger et al. 2001).

In addition to the wood treatments, the importance of design when building with wood was also considered important by both Romans and Greeks. Based on their experience on building with wood, they put stone blocks below the wooden structures to avoid decay (Weiss 1916). Designing buildings in a suitable way is nowadays considered as the main factor to avoid wood decay (Brischke et al. 2006).

In 1770, Sir John Pringle wrote the first list of preservatives, which opened the age of chemical wood preservatives. The age of enlightenment and natural sciences developed into an era when the science behind the reasons for wood decay and the chemicals to prevent it were first studied in a systematic way (Richardson 2005).

1.5.2 Industrial wood impregnation chemicals

The natural antifungals and methods used during history to preserve wood were effective, but not as good as required for many industrial applications. The efforts for improving the durability of wood led John Bethell to treat ship timber with an impregnation method in 1838 (Barnes 2002). This can be considered as the beginning of industrial wood preservation, when the wood was treated in bulk with new methods and chemicals in order to obtain the best possible durability.

One of the oldest industrial wood preservatives is creosote oil, derived from coal or wood tar, which has been used for this purpose since the mid-19th century (EPA 2016). Creosote is used in outdoor settings, such as utility poles, whereas its residential use is nowadays not permitted (EPA 2016). There is no doubt about the effectiveness of creosote as a wood preservative, but it has a very important drawback: it is mostly composed of polycyclic aromatic hydrocarbons, which are carcinogenic, mutagenic and very persistent in soils (Madrid et al. 2019). Thus, in the USA, the use of creosote was restricted in 1986 (Federal Register January 13, 1986) but it was registered again in the year 2008, after making a risk assessment of its use and specifying that mitigation measures should be adopted (EPA 739-R-08-007, 2008). In Europe, the commercial use of creosotes was banned in 2003 (Hiemstra et al. 2007), and since 2013 their use has needed special approval by national officials (Commission Directive 2011/71/EU). In Finland, the use of creosote products is approved only by permission of the Finnish Safety and Chemicals Agency (Tukes). The only practical reason for the continuing use of creosote is that there are no competitive substitutes now for significant industrial pole and beam products used in direct soil contact (Humar 2017).

Pentachlorophenol is another wood preservative, used since 1936 (EPA 2017), which can cause many adverse effects in the environment and in humans (Zheng et al. 2012, WHO, 1987). Due to it, this preservative is no longer present in the Biocidal Products Regulation list of permitted biocidal compounds (BPR, Regulation (EU) 528/2012). In the USA, it is considered a restricted-use pesticide, sold and used by certified pesticide applicators mostly used for treated utility poles and cross arms, and its use is forbidden in residential applications (EPA 2017).

Chromated copper arsenate (CCA) has also been a prominent industrial preservative that consists of three different metal salts. Copper and arsenic were the active ingredients preventing decay by fungi and insects, and chromium was active in fixing them to wood. From the 1930s to the 1970s it was the most common wood preservative in the USA for outdoor use (Shibata et al. 2007), and it was also broadly used in Europe (Frick et al. 2019) and other parts of the world. Later, it was noted that arsenic can leach out from wood, causing substantial contamination of the ground water (Shibata et al. 2007) - for this reason, CCA was banned in 2003 in both the USA (EPA 2003) and Europe (Commission Directive

2003/2/EC). This wood preservative remains in active use in other regions such as South Africa (Naidoo et al. 2013).

Substitutes for the banned CCA came rather soon from other copper-based wood preservatives that gained new relevance (Figure 5). In these preservatives, copper remains as the main active ingredient and the rest of the compounds fulfil other functions, such as being co-biocides to inhibit copper-tolerant fungi and moulds (Lebow et al. 2020) and acting as surfactants or increasing the fixation of the compounds to wood. While many different formulations had been developed that were successful inhibitors of wood-degrading fungi, the main problem with these new preservatives is again their leaching from wood. They were noted to leach more than CCA components (Temiz et al. 2014) when the chromium acting as a fixing agent in CCA was removed from the substitute formulations. Some of the latest copper-based preservatives used in the wood industry are the copper amine-based preservatives, such as amine copper quat-type D (ACQ-D), copper dimethyl dithiocarbamate (CDDC) and copper azole, that already acquired significance in the late 1990s (Zhang and Kamdem 2000), and micronized copper-based formulations, which adhere to wood better than previous copper-based preservatives (Freeman and McIntyre 2008). Leaching remains an issue to be considered and it has been addressed recently by several researchers (Fernández-Costas et al. 2017; Can and Sivrikaya 2017; Mourant et al. 2009), but their findings have not been industrially applied. In addition, some fungi can tolerate copper, and therefore new approaches are continuously being investigated (Zhang and Kamdem 2000; Humar et al. 2007; Civardi et al. 2015).

Boron compounds also attracted attention as wood preservatives after the ban of CCA. Many of the borates used for this purpose were cheap, had no effect on the wood's colour or smell, and had a lower environmental impact than most other wood preservatives (Thévenon et al. 2010). Boron has also been included in formulations together with copper and chromium as an alternative to CCA, resulting in the so-called chromated copper borate preservatives (CCBs) (Da Silveira et al. 2017). However, boron is classified as a substance of high concern by the European Chemicals Agency (Echa 2020), which limits its use. In addition, the high solubility of boron in water is an advantage only as it makes it easy to impregnate wood with it but, in turn, boron leaches out easily from wood when the material becomes moist (Thévenon et al. 2010).



Figure 5. Wood treated with a copper-based wood preservative. This treatment confers a greenish colour to the wood (the split in the picture is not caused by the copper treatment but is due to the intense drying of the wood specimen).

Bearing in mind the material cost structure and end use requirements, most traditional wood preservatives are quite effective and very cheap. These two factors are both required from a successful commercial product, but many preserving agents also present a high toxicity to humans and the environment, or low fixation to wood resulting in loss of effectiveness. New generation wood preservatives, such as encapsulated essential oils (Cai et al. 2020) and two-step impregnation of wood with bio oil and epoxidized linseed oil (Temiz et al. 2013), yield good results considering foreseeable effects to the environment, but their cost and efficiency against the wood-decaying fungi should still be improved. Thus, affordable and sustainable sources of antifungals to be used in wood preservative formulations are needed, which is causing many researchers to focus on finding and developing new ways to make wood last longer.

1.5.3 Industrial processes and product classification

As previously highlighted, the first industrially applied wood impregnation method was developed by John Bethell. Since then, several pressure-based processes have been developed to treat wood with different chemicals, as presented in Table 1.

The pressure-based methods are still used in the wood industry, and although they are the best-known methods for treating wood. Many other treatments that do not use pressure, such as dipping, cold soaking, vacuum, diffusion and Boucherie processes, are also available (Lebow 1999). However, while the methods remain rather set, new formulations to be used for wood impregnation are constantly being studied and commercialized.

Table 1. The main wood impregnation processes, their creator and year of invention or patent, the steps followed in the process and the chemicals they are used for

Process	Creator and year	Steps	Used for	References
Bethell process (Full-cell process)	John Bethell 1832	1 - vacuum 2 - pressure with the preservative 3 - vacuum (not always)	Water-borne wood preservatives. It has also been used for other preservatives, such as creosotes	Lebow 1999 Barnes 2002
Modified Full-cell process	-	Shorter first vacuum and longer last vacuum than in the Bethell process	Water-borne wood preservatives	Lebow 1999
Rüping (or Rueping) empty cell process	Max Rüping 1902 (patented)	1 - Pressure 2 - higher pressure with preservative 3 - Vacuum	Oil preservatives	Lebow 1999
Lowry empty cell process	C.B. Lowry 1906 (patented)	1 - Pressure with preservative 2 - Vacuum	Oil preservatives	Lebow 1999

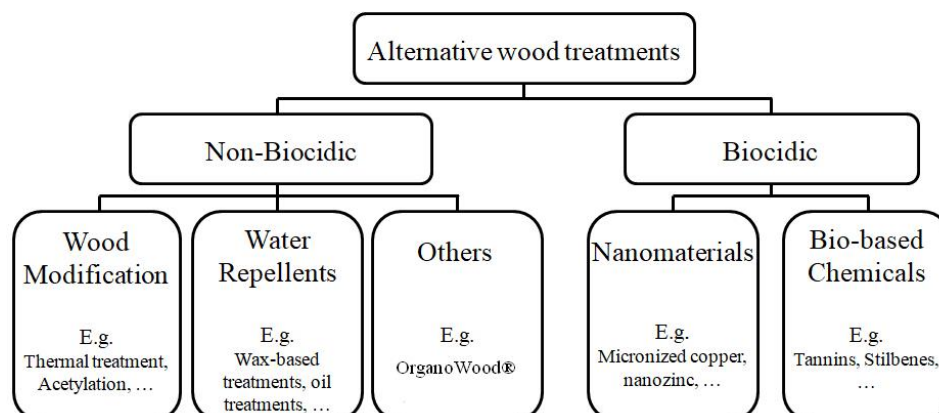


Figure 6. Classification of wood treatments currently receiving the attention of researchers as possible alternatives to traditional synthetic wood preservatives.

It is important to highlight that different countries and regions follow different criteria to classify the wood preservatives, usually based on their use or on the durability provided to wood. In Scandinavia, wood preservative classification has been made by the Nordic Wood Preservation Council (NWPC) since 1969 (NTR). The durability of wood in Europe and the efficacy criteria for wood preservatives for Europe can be found in EN 559-1:2009, while wood preservatives are divided into three groups in the USA and are regulated by the EPA (Ibach 1999).

1.5.4 State-of-the-art in wood preservation

Many new alternatives have been under development for preserving wood in ways that are not as toxic and harmful to the environment as the commercial biocides. The solutions suggested during this time can be classified as non-biocidic or biocidic preservatives (Figure 6), such as different non-toxic means towards wood modification and the use of natural antifungals as wood preservatives.

Wood modification is one of the most successful treatments, with several processes such as thermo-hydro modification, firstly industrialized in 1849, and acetylation, first tested by Fuchs (1928) (Sandberg et al. 2017). In modification, wood is structurally and chemically modified using different methods such as thermal treatment, cross-linking or wood compression, to make the wood less accessible to fungi. If chemicals are used in wood modification, their role is not to act as antifungals, but to modify the wood in order to make it more resistant to fungi. Many ways to modify wood are already used in the wood market, such as furfurylation, acetylation (Mantanis 2017) and thermal modification (Kubovský et al. 2020).

The main mechanism of wood modification is hypothesized to be that the changes caused in the wood prevent certain microbial enzymes from recognizing the wood components as substrate, and thus, the wood cannot be used as a nutrient source for the microbes (Hill 2006). Acetylation is the best-known modification method leading to fungal enzymes not recognizing the wood, as the mechanism is suggested to work by hydroxyl groups becoming blocked and not recognizable to the enzymes (Hill 2006). The moisture content in wood, which can be reduced by modifying the wood, also plays an important role in preventing

decay in modified wood (Thybring et al. 2018), as decay fungi perform best when the moisture content of the wood exceeds the fibre saturation point (Walker 1993). Based on the work of Thybring (2013), when the equilibrium moisture content of the modified wood specimens analyzed was reduced by 40%, the decay of the wood was reduced significantly. According to Thybring (2013), the effectiveness of the moisture reduction could be because the lower moisture content reduces the transport of substances through the cell wall, and thus, the diffusion agents released by fungi to open the cell walls and start the wood decay are not diffused (Hill 2002). Many factors are not yet fully understood about the mechanisms behind the decay resistance of modified wood. This is also true in the case of acetylated wood, where moisture appears to play a key role but the whole mechanism is not understood completely yet (Rowell 2020).

The impregnation or coating of wood with water repellents is another non-biocidal way to treat wood against wood decay, as fungi require a minimum moisture content to be able to decay wood (Figure 7). Wax-based treatments, for example, are available to treat wood (Wang et al. 2020). Many oils are also used and studied as water repellents to delay wood decay (Can and Sivrikaya 2017), such as linseed oil (Humar and Lesar 2013), pine tall oil (Koski 2008) and Tung oil (Žlahtič et al. 2017). In addition to their function as wood preservatives, water repellents have been used for over 25 years to treat wood that has previously been treated with wood preservatives in order to make the surface hydrophobic and thus reduce water absorption into the wood and also leaching of the wood preservative (Treu et al. 2011). More recently, water repellents, such as waxes, have also been used to treat wood prior to thermal modification, to improve the hydrophobicity of wood, which increases its durability (Humar et al. 2017). Additionally, other modification methods have been developed that can delay wood decay, such as the introduction of minerals into wood to act as barriers to imitate wood fossilization, as performed by OrganoWood®, or industrial charring of wood surfaces (Kymäläinen et al. 2017). These new methods illustrate the efforts being performed by wood companies to find new ways to avoid wood decay, and thus, the relevance of this topic is not only for science, but also for the wood industry.

Lastly, the biological control of wood needs to be highlighted. The use of albino strains of *Ophiostoma* spp. to control sap staining fungi has been studied by several groups (e.g. Held et al. 2003, Hernandez et al. 2011) and even implemented by some wood industries around the world. The main mechanism of this method is that the *Ophiostoma* spp. colonizes the wood and captures all the nutrients, which inhibits the growth of other sap stain fungi (Held et al. 2003).

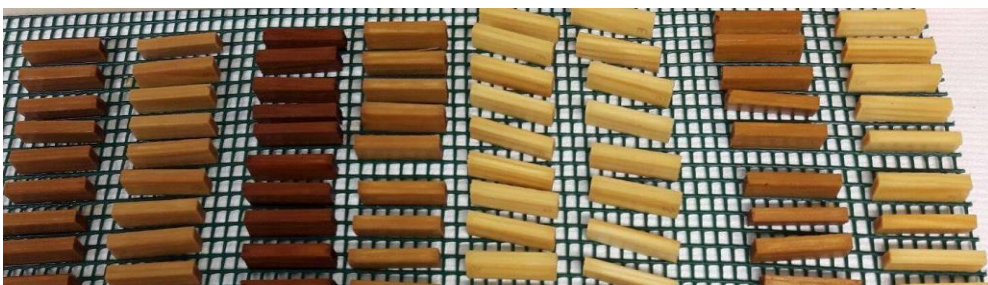


Figure 7. Several Scots pine sapwood specimens treated with different potential bio-based preservatives after taking them out from the impregnation cylinder. The completely wet wood specimens were next oven dried and weighted, and then exposed to leaching and decay tests.

Within the biocidal treatments, one of the most promising methods is the application of nanotechnology to exploit the capability of nanoparticles to penetrate easily into the wood. Nanomaterials have already provided new metal-based wood preservatives, such as micronized copper, which is used in the wood industry (Civardi et al. 2015), and nanoboron and nanozinc (Kartal et al. 2009). As metal salts are often used in wood preservative formulations, the use of nano-scale metal has been suggested as an alternative to facilitate its penetration into the pores, voids and lumen cavities of the wood structure. Nanometals are typically precipitated from solutions using a chemical reaction, heating or refluxing, to modify the particulate size of the metal complexes (Clausen 2007). However, nanometals may have very different characteristics and performance from the dissolved metal salts (Kartal et al. 2009), which are not yet fully understood. Thus, the health issues arising from their use may also differ, and commercial products are governed by the guidelines for both nanomaterials and biocides. This requires comprehensive testing of their user safety and toxicity (Clausen 2007). As an example, Civardi et al. (2015) highlighted that although micronized copper-based wood preservatives are available on the market, their hazards and interactions with copper-tolerant fungi are not completely understood. They also noted that leaching remains an issue, and copper-tolerant fungi may promote copper release to the environment. Similar findings have been reported quite recently by Humar and Thaler (2017).

The use of bio-based preservatives in wood protection is currently one of the most popular research fields in wood science (see Figure 8 for the classification of natural chemicals, which are usually studied as possible wood preservatives). Some of the reasons for this are the increasing relevance of bioeconomy and sustainability in modern society. The general belief that synthetic biocidal chemicals are toxic might be another driver of moving towards natural chemicals. Additionally, the lack of knowledge about the effects of synthetic chemicals on the environment and humans can also be considered another driver towards natural chemicals.

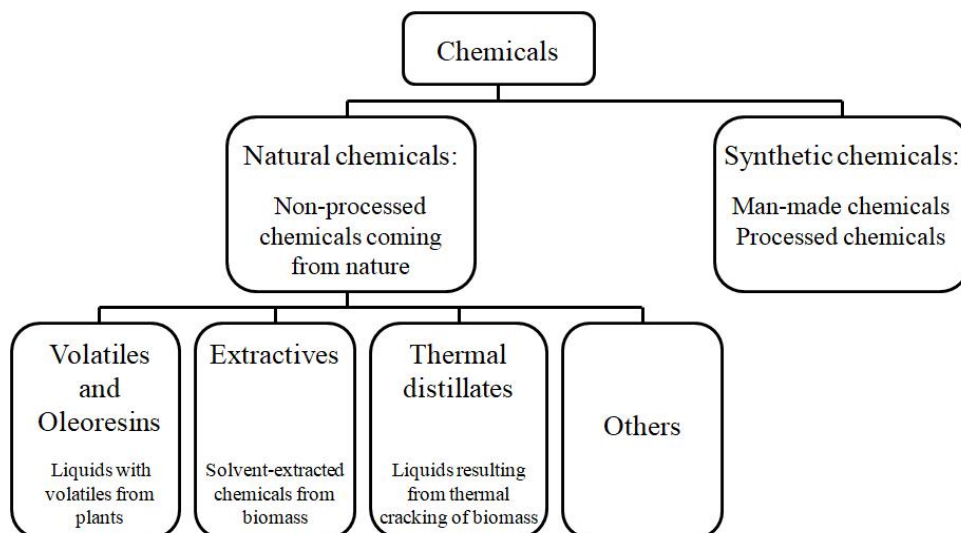


Figure 8. Classification of the natural chemicals usually studied in wood preservation.

Many natural extracts are biocidal as they are used by plants to protect themselves from pests and pathogens. Many extracts have been successfully tested as wood preservatives. Phenolics, and specially tannins, are known to play a role in wood decay resistance (Harju et al. 2003, Anttila et al. 2013, Anouhe et al. 2018). Stilbenes, e.g. pinosylvin, have also shown antifungal activity against wood-decaying fungi and cause moderate decay tolerance in e.g. heartwood of pine (Seppänen et al. 2004, Lu et al. 2016). Several feedstocks can be considered for the extraction of natural extractives or bio-based chemicals, but as the trend in wood preservation is to use recycled waste and side-streams (Can and Sivrikaya 2017), forest industries are focusing on side-streams and waste generated by themselves, such as tree bark. The use of natural extracts and other bio-based chemicals in industrial wood preservatives remains scarce and the synthetic chemicals remain the industrial standard.

1.5.5 Synthetic chemicals and bio-based preservation compounds

Most current wood preservatives are synthetic, meaning that they are fabricated with a process that does not occur in nature, and inorganic. These chemicals are rather cheap and usually offer good performance against decay, although nowadays they are in the spotlight for being considered very toxic and harmful to the environment and humans. However, natural chemicals may also be toxic, depending on dose, and may cause cancer in animals, but can still be used at low doses (Ames et al. 1990).

The concentration of synthetic chemicals is increasing in the environment due to the industrial production of chemicals and materials (Podein et al. 2010). In addition to reagents that remain in the environment, their degradation products may also have an ecotoxic effect and affect the condition of water or soil ecosystems (Boxall et al. 2004). Many of these chemicals and their mixtures may accumulate in individuals and populations, and their long-term impacts on health aspects are not known. Several chemicals used as wood preservatives, such as pentachlorophenol and CCA, increase the risk of suffering from cancer in wood industry workers (Huff 2001). Thus, chemical agencies are taking precautionary measures to avoid unexpected health issues (Podein et al. 2010).

The lack of knowledge about the effects of some synthetic chemicals on animals, and the precautionary measures set by many governments to control the use of synthetic chemicals and promote the use of renewable resources, is causing natural chemicals, extracted from plant biomass, to gain relevance in many fields. The main advantage of biomass is that it is a very cheap and abundant resource that is available in large amounts (Temiz et al. 2010). The chemicals recovered are usually more expensive than synthetic ones, due to the need for extensive extraction and purification processes. In wood preservation, the use of natural chemicals has been studied since ancient times, and many bio-based chemicals and chemical mixtures are known to have an effect against wood-decaying fungi.

One of the first scientific studies reporting about natural extracts in wood preservation was written by Trevelyan (1839), almost 200 years ago. It highlighted the preserving properties of lime-water in Scotch fir wood, as the wood treated with the lime-water mixture lasted in life service 40 years, whereas based on his publication this kind of green wood would decay in 5-7 years with no additional treatment. Several recent studies found other examples of natural chemicals that can prevent the growth of wood-decaying fungi, such as stilbenes (Lu et al. 2016), tannins (Anttila et al. 2013) and caffeine (Kwaśniewska-Sip et al. 2019). Finding sustainable and abundant feedstocks for these chemicals is still under investigation. Although the valorisation of household waste has been studied (Shiny et al. 2017, Lajnef et al. 2018, Barbero-López 2020), industrial residues, due to their high

availability, low price and the current lack of profitable refining routes, should be especially considered. Companies from different countries or regions should consider different feedstocks, considering the availability, environmental conditions and the tolerance of fungi to the chemicals.

Tree bark as a feedstock for antifungal chemicals extraction

Trees are known to be rich in extractives, which play a marked role in the protection of living wood from fungal decay (Harju et al. 2003, De Angelis et al. 2018, Anouhe et al. 2018). However, the use of entire wood for chemical extraction for treating other woods would not make sense, and it would make wood expensive, whereas the industrial side-streams, such as bark, could be used for this purpose.

Tree bark generally contains more extractives than the wood (xylem) (Zhao et al. 2013), and nowadays it does not have many beneficial end-uses. The factors affecting the chemicals that can be found in bark are the species and part of the tree, as well as the soil conditions, tree stress, the climate and the place where the tree grew (Feng et al. 2013). Tannins are the most common extractives in bark (Feng et al. 2013). They are typically found in higher concentrations in bark than in wood (Pásztor et al. 2016), and they are known to have antifungal properties and to prevent wood from decay (Figure 9) based on several studies, such as Anttila et al. (2013), Tondi et al. (2015), Tomak and Gonultas (2018). It is also important to consider that different kinds of sugars can also be found in the bark of many species, such as pine (Nunes et al. 1999) and they may promote the wood decay rate. Additionally, over 350 million m³ of bark are generated every year by the global wood industries, based on the values of lumbered wood in 2015, although specific quantification of annual bark production is complicated (Pásztor et al. 2016). As the bark is separated from the wood in the wood industry, and often burned for energy and steam generation, using it for extracting chemicals would revalorize it and would provide a novel solution for the wood preservation industry.

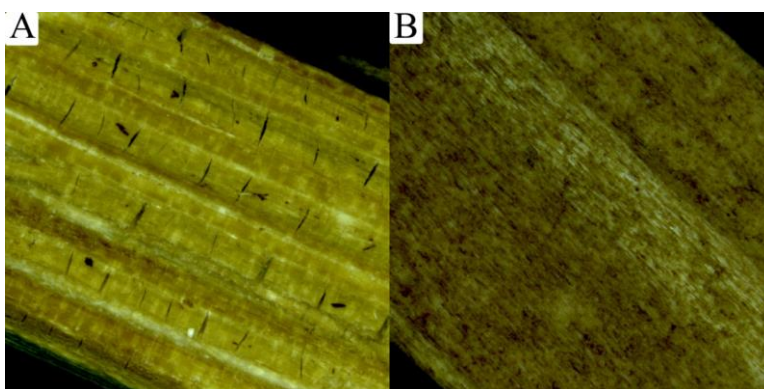


Figure 9. Magnified image of the surface of untreated (A) and tannin impregnated (B) Scots pine sapwood after 16 weeks of exposure to *Coniophora puteana*. The pictures were taken after carefully removing the mycelium from the wood surface and oven drying the wood specimens at 50 °C until constant mass loss was achieved. The fungus colonized both specimens, and part of the mycelium was still visible in the pictures (white colour tone). However, the surface of the untreated wood specimen (A) was full of cracks, whereas the surface of tannin-treated wood was undamaged (B).

The bark from different tree species has already been tested for production of wood preservatives by several researchers. Several years ago, Tascioglu et al. (2013) found that mimosa bark extracts were effective against brown rot and white rot fungi when impregnated into different wood species. However, Tascioglu et al. (2013) did not find positive results of *Pinus brutia* bark extracts against wood-decaying fungi. Alfredsen et al. (2008) tested methanol extracts from 9 different European tree barks against 3 wood decay fungi and found that most of them had some antifungal activity against the fungi in a Petri dish test. In more recent studies, several bark extracts showed effective results as antifungals against the wood-decaying fungi *Coniophora puteana* and *Trametes versicolor* (Özgenç and Durmaz 2016, Özgenç et al. 2017) and against other fungi, such as the grapevine pathogen *Plasmopara viticola* (Mulholland et al. 2017).

The thermochemical conversion of bark for several purposes, such as polyurethane foam production and pyrolysis oil-based resins, has been investigated since the 1970s (Feng et al. 2013). The pyrolysis of biomass is performed at conditions over 350 °C under inert atmosphere, and produces charcoal, gases, and liquids. The liquids resulting from the bark pyrolysis have been tested against wood-decaying and pathogenic fungi with positive results. The pyrolysis distillate fractions from mixtures of different barks showed variable results against 2 brown rot and 2 white rot species in the study performed by Mourant et al. (2005), and their antifungal performance improved when the pyrolysis distillates were mixed with CuSO₄. Mohan et al. (2008) concluded that wood treated with pyrolysis distillate fractions from pine and oak bark lost less mass, especially those with lignin-rich fractions, than untreated wood when exposed to decay by *Gloeophyllum trabeum* and *Trametes versicolor*. Pyrolysis distillates from bark are not only able to inhibit wood-decaying fungi, but they have also been found to be effective against plant pathogens (Jung 2007). The high amount of phenolics present in these distillates is responsible of their antioxidant activity, and this makes them effective in inhibition of the non-enzymatic decay caused by brown rot fungi (Hassan et al. 2016).

All these studies indicate that tree bark is a potential feedstock for extraction of antifungal chemicals to be used as active ingredients in wood preservative formulations.

Bio-based feedstock from the side-streams of coffee industry

Due to the current interest in circular economy, many industries—such as the coffee industry—are searching for ways to revalorize the biological waste they generate. Depending on the product they prepare, the coffee industry generates two different residues (Figure 10).

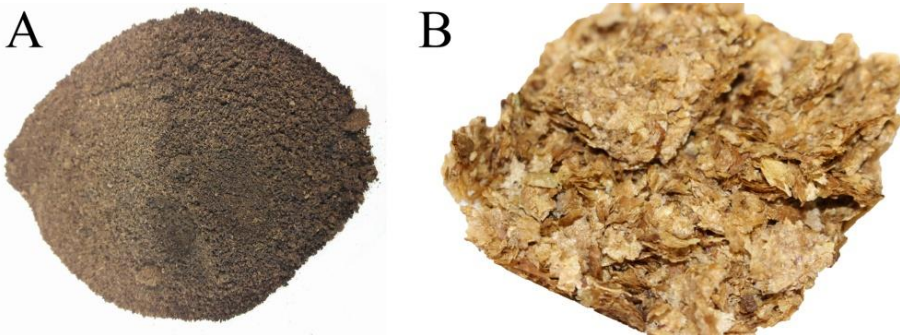


Figure 10. Spent coffee (A) and coffee silverskin (B), two residues generated in the coffee industry, are rich in bioactive compounds.

On the one hand, when green coffee is roasted, the silverskin of the coffee bean is released (Figure 10B). Silverskin is a thin layer surrounding the coffee bean, rich in several chemicals that the coffee industry discards as a residue (Bessada et al. 2018). Additionally, Bessada et al. (2018) highlighted that coffee silverskin is the main side-product in the coffee industry, and considering that about 10 million tons of coffee were roasted in 2018 globally (International Coffee Organization 2019), this results in large quantities of silverskin generated. On the other hand, coffee industries that make instant coffee also produce spent coffee as a residue from coffee preparation (Figure 10A). Based on data presented by Nestlé Nordic, the coffee industry uses 2 kg of coffee to generate 1 kg of instant coffee and as a by-product some 2 kilos of wet spent coffee residue. Thus, although collecting spent coffee from private users may not be easy, the industry generates tons of spent coffee that can be easily collected for further revalorization. Both spent coffee and silverskin contain chemicals such as phenolics and organic acids (Mussatto et al. 2011; Bessada et al. 2018) that can act as antifungals.

Spent coffee revalorization has recently been studied for several purposes, such as generating energy (Santos et al. 2017), as food supplements (Panzella et al. 2017) and as an ozone adsorbent (Hsieh and Wen 2020). In addition to the previously mentioned applications, coffee extract and caffeine have been successfully tested as antifungal agents (Arora and Ohlan 1997).

Similarly, coffee silverskin revalorization has attracted considerable attention in recent years, as it is rich in bioactive compounds with antioxidant and antibacterial activity (Nzekoue et al. 2020). Some of the suggested uses for silverskin are as antioxidants in skin gels (Kusumocahyo et al. 2019) and as ingredients in food (Bertolino et al. 2019, Gocmen et al. 2019) and biofuel production (Procentese et al. 2019). The bioactive compounds present in coffee silverskin extracts (Bessada et al. 2018) might also show antifungal activity against microbes, such as wood-decaying fungi.

Caffeine and its derivatives are some of the most common constituents in coffee extracts, and it is also present in spent coffee extracts (Torres-Valenzuela et al. 2019) and in silverskin (Bresciani et al. 2014). Caffeine has already been studied as an antifungal agent against wood-decaying fungi *in vitro* (Lekounougou et al. 2007), and it has also been tested as a wood preservative against moulds and decay fungi (Kwaśniewska-Sip et al. 2018, Broda et al. 2018). Additionally, caffeine has been tested as an ingredient in possible bio-friendly wood preservative formulations (Mazela et al. 2016).

Organic acids are common constituents in spent coffee and silverskin extracts. Chlorogenic acid and its derivatives are present in both extracts (Torres-Valenzuela et al. 2019, Wen et al. 2019). Chlorogenic acid was identified as an inhibitor of different phytopathogenic and fruit rotting fungi (Martínez et al. 2017, Roy et al. 2018), whereas the effects on bacterial growth were variable (Torres-Valenzuela et al. 2019). Caffeic acid and its derivatives are also found in spent coffee and silverskin extract, and low doses of caffeic acid are known to inhibit moulds of the species *Aspergillus* spp. (Aziz et al. 1998).

Thus, the silverskin and spent coffee extract constituents have antimicrobial activity against different kinds of microbes, including wood-decaying fungi, but further tests are needed regarding their possible application in wood.

1.5.6 Problems of bio-based wood preservatives

One of the most significant challenges in the search for bio-based wood preservatives is their low fixation to wood. Most potential bio-based preservatives are diluted in water, as it is the

cheapest green solvent available, but this has the result that the chemicals also leach out from wood as soon as it gets wet. Several research groups have already focused on finding preservative formulations with high fixation to wood, or methods to avoid their leaching, but although a few successful results have been reported (Singh and Singh 2012, Temiz et al. 2013; Tondi et al. 2015), they are not yet on the market, possibly due to their higher cost compared to traditional preservatives. Thus, the solubility in water of bio-based wood preservatives is an advantage as it makes the wood treatment easy, but becomes a challenge as they will leach easily from wood in the presence of water, and no feasible solutions for their industrial application have yet been developed.

The performance of the antifungals used in bio-based wood preservation can also be a problem, as many natural extracts do not reach the performance of the synthetic chemicals used for decades in this field. However, there are potential bio-based preservatives, such as tannins (Anttila et al. 2013; Da Silveira et al. 2017) and pyrolysis distillates (Mohan et al. 2008), that have been successfully tested against wood-decaying fungi. Thus, even if the performance of bio-based chemicals is often considered to be lower than that of synthetic chemicals, these results show that formulations containing potential bio-based preservatives could offer good properties against wood-decaying fungi. Some formulations have already been tested by researchers, such as essential oil- and extract-based formulations prepared by Kartal et al. (2006) and the caffeine- and natural oil-containing formulations prepared by Mazela et al. (2016).

Additionally, bio-based preservatives need to be registered in the same way as traditional ones, even if they are developed for niche markets. The cost of the registration is very high. For instance, in Europe the registration of a new active ingredient for wood preservative formulations is about 6 million € (Jones and Brischke 2017). Additionally, registering a product takes a long time. Thus, it is very difficult for bio-based wood preservatives to be competitive in their potential market if this market is smaller, due to the cost and time required for their registration, unless their performance is excellent.

The processing cost of the bio-based treatments of wood can also be a problem, as wood is a cheap product that will not be profitable if its price increases markedly. Current wood preservatives are cheap and they are impregnated into the wood in a single step. Water-based bio preservatives are also cheap as they are extracted with water, but they need further processes after impregnation in order to avoid leaching from wood. Some of these second steps include a treatment with a hydrophobic chemical, such as the epoxidized linseed oil treatment after pyrolysis distillate impregnation applied by Temiz et al. (2013), and thermal treatment applied after impregnation with caffeine, tested by Kwaśniewska-Sip et al. (2019).

The feedstock used for extracting the potential bio-based antifungals to be used in preservative formulations will also vary depending on the country or region. There is a high heterogeneity between the potential preservatives extracted from different feedstocks (Broda 2020). Shipping of the most effective bio-based preservatives from other countries would cause a higher environmental impact and increase the price of the wood preservatives. Additionally, as different fungal species and strains can learn to tolerate different chemicals (e.g. Sharma et al 2020), and variable wood species are used in different parts of the world, different bio-based preservatives may be needed.

The ecotoxicity of the potential bio-based preservatives also needs to be taken into consideration. Synthetic chemicals are often considered by many people to be toxic due to their fabricated origin, whereas natural chemicals are often considered non-toxic, although every chemical is toxic at some concentration (Ames et al. 1990). Additionally, their water solubility makes them prone to leaching out from wood, with the result that they end up in

the surrounding environment and water. Thus, the effects in the environment or humans should not be overlooked during the development of new wood preservative formulations. Tannins have been suggested as an alternative to synthetic wood preservatives for many years, although some tannins can have high ecotoxicity (Libralato et al. 2011). The wastewater resulting from cork boiling, rich in cork extracts, has also been found to be highly ecotoxic (Mendonça et al. 2007), as have some of the pyrolysis distillates (Cordella et al. 2012). Caffeine, suggested several times as an alternative wood preservative, is considered an emerging pollutant that can cause negative effects to the environment (Aguirre-Martínez et al. 2015). Thus, it is of high importance to test the ecotoxicity of the potential bio-based preservatives and to compare them to current wood preservatives, as there is no point in substituting synthetic chemicals by natural chemicals which perform similarly but may be even more toxic than the synthetic ones, just because their natural origin.

Finally, the last problem with current wood preservatives is the way in which the wood needs to be disposed when it reaches the end of its life cycle. When this occurs, untreated wood can be revalorized and burned for energy purposes, but toxic preservative-impregnated wood needs to be treated as hazardous waste in many countries, as in the case of CCA-treated wood (Augustsson et al. 2017). However, the disposal of treated wood is expensive and is becoming less common due to stricter regulations in many countries (Coudert et al. 2013). Treated wood is often disposed in the same way as untreated wood, because some countries do not have strict legislation in the field, or simply because people dispose it without following the local legislation, which results in the release of toxic chemicals into the environment (Augustsson et al. 2017). For this reason, bio-based chemicals with low ecotoxicity would have high value for the development of new wood preservatives that could easily be managed at the end of their life cycle.

1.6 Research objectives

1.6.1 Aim of the thesis

The aim of this thesis was to evaluate the potential of selected industrial side-streams and residues for wood preservative formulations for above ground use, such as the copper-based preservatives. The characterization of the distillates and extracts was done to identify potential antifungal constituents, and later their potential ecotoxicity. Chemical compounds were further tested for their antifungal activity, decay inhibition performance and tendency to leach from wood. The results from these tests were considered complementary because the performance of the chemicals can vary between the agar growth media and wood (Loman 1970). The tests had the following objectives:

- I. To characterize the fungal inhibiting bio-based mixes to understand their composition and to identify the constituents potentially responsible for the antifungal activity.
- II. To test the antifungal activities of the potential bio-based preservatives against wood-decaying fungi and comparing the results to those obtained with a commercial copper-based preservative.
- III. To evaluate the antifungal activity of the constituent compounds in these chemical mixes. This was based on the prior knowledge (literature) and testing the chemicals identified in Papers I and II. The constituents identified in chemical mixes were tested as pure substances, and in selected combinations.

- IV. To test the *in vitro* decay performance of wood impregnated with bio-based compounds and leaching of these chemical mixtures from wood and comparing them to a commercial copper-based preservative.
- V. To compare the acute ecotoxicity of the extracts, distillates and their chemical constituents showing antifungal activity and a capacity to prevent wood decay to that of a commercial copper-based preservative.

1.6.2 Hypotheses

The hypotheses of this thesis were outlined as:

- i. The residues and side-streams tested could yield antifungal compounds with possible applications in novel wood preservative formulations.
- ii. The antifungal activity of the bio-based chemicals inhibiting the growth of wood-decaying fungi will be caused mainly due to the synergy between several of their constituents rather than to only one constituent.
- iii. Due to the differences between the growth media—malt agar and wood—and the leachability of some constituents of the potential bio-based preservatives, there will be differences between the performance of bio-based antifungals in the malt-agar growth media and the wood decay test with wood specimens.
- iv. The ecotoxicity of the natural extracts, distillates and chemicals may be as high as that of the synthetic chemicals.

2 MATERIALS AND METHODS

2.1 Extraction methods for the selected side-streams and residues

Two different extraction methods, pyrolysis and hot water extraction, were applied for industrial residues to obtain chemical mixes for antifungal, leaching, decay and ecotoxicity tests. A list of all potential bio-based preservatives, chemicals and their concentrations used for the experiments in this thesis can be found in Table 2.

Pyrolysis distillates were obtained from silver birch (*Betula pendula* Roth) and Norway spruce (*Picea abies* (L.) H. Karst.), and European aspen (*Populus tremula* L.) bark by performing a slow pyrolysis. Three phases were differentiated in this process, namely the drying phase (up to 135 °C), the torrefaction phase (up to 275 °C) and the pyrolysis phase (up to 350 °C), and distillates were collected from each of these phases (Papers I and V).

In the hot water extraction, two different methods were applied. For spent coffee ground extraction, 50 g spent coffee grounds were boiled in 1 L of Milli-Q water (Merck KGaA, Darmstadt, Germany) for 45 minutes. The dissolved extracts were used in the antifungal test (Paper II). For milled coffee silverskin pellets the hot water extraction was performed in a Foss Soxtec 2050 (Foss, Hilleroed, Denmark) extractor, and the extract was concentrated and freeze-dried (Paper III). The same process was performed for the spent coffee grounds as referenced in the same paper.

Table 2. Potential bio-based preservatives and industrial references (chemicals) tested for their antifungal and wood preserving potential in this thesis. For each chemical, the tests performed, the source, to which test they were exposed and at which concentration, and the publication where the original data can be found are presented

Chemicals	Source	Antifungal test (wt-%)	Wood leaching and decay test (wt-%)	Ecotoxicity test	Original publication
Spent coffee extracts	Extracted from spent commercial coffee grounds	1–5%	-	-	Papers II-III
Fresh coffee extracts	Extracted from commercial coffee grounds	1%	-	-	Paper II
Caffeine	Sigma-Aldrich, Finland	0.06%, 1%	-	-	Paper III
Coffee silverskin extracts	Extracted from silverskin pellets (Meira Ltd., Finland)	0.1–3%	5%	Yes	Paper III
Silver birch and Norway spruce bark pyrolysis distillates	Bark torrefaction and pyrolysis phases	0.1–1%	-	-	Paper I
European aspen bark pyrolysis distillates	Drying, torrefaction and pyrolysis phases	-	5%	Yes	Paper V
Acetic, formic and propionic acids	Merck KGaA, Darmstadt, Germany	0.1–1 g/L	3%, 5%, 6%	Yes	Papers IV, V
Colatan GT10	Haarla Oy (Finland)	-	5%	Yes	Paper V
Pine oil MäntyEko®	EkoPine (Finland)	-	100%	-	Paper V
Celcure C4	Koppers Inc., (Pittsburgh, USA)	0.1–1.6%	1.6%	Yes	Paper I-III, V

Additionally, the commercial Colatan GT10 tannin-rich *Schinopsis Lorenzii* bark extract was also tested in wood decay tests (Paper V). The industrial references used were pine oil (EkoPine, Finland) in Paper V and Celcure C4 (Koppers Inc., Pittsburgh, USA) in Papers I-III and V. Celcure C4 contains copper (II) carbonate (17%), ethanolamine (< 35%), benzalkonium chloride (4.75%), cyproconazol (0.096%), sodium nitrite (< 5%) and polyethoxylated tallow amine (< 5%). It is a wood preservative for above ground use classified as AB class by the NWPC and classified as class 3 by the EN 335:2013, although it can also be used for contact in soil or fresh water (class uses A and 4 respectively in the NWPC and EN 335:2013) by applying higher retention of Celcure C4 in the wood.

2.2 Characterization of the constituents of the distillates and extracts

Two main methods were used to characterize the extracts from coffee side-streams and the constituents of the pyrolysis distillates from wood material.

- Coffee extract characterization: The hot water extracts from spent coffee and coffee silverskin were analyzed using high performance liquid chromatography (HPLC). This method was used to identify phenolics and alkaloids by an acid butanol assay performed for the silverskin extracts to quantify their concentration of condensed tannins (Papers II and III, respectively).
- Pyrolysis distillate characterization: The characterization of the pyrolysis distillates from silver birch and Norway spruce bark was performed using a high-resolution ¹H nuclear magnetic resonance spectrometer with N₂ filling and comparing the peaks to samples of previously identified peaks (Paper I).

2.3 Antifungal test

Antifungal tests followed the same procedure for the pyrolysis distillates (Paper I), spent coffee extracts (Papers II and III), coffee silverskin extracts (Paper III) and acetic, formic and propionic acid (Paper IV), only their concentrations changed (Table 2). The summary on the strains used in the tests are given in Table 3. Antifungal tests were performed for screening the capability of the potential bio-based preservatives to inhibit the wood-decaying fungi, as this allows testing several chemicals and concentrations in a short time period in a homogeneous media.

In brief, the tested preservative candidates were mixed with the malt agar growth medium and then a fungal inoculum was put in the centre of the Petri dish. The industrial copper-based reference—Celcure C4—was prepared by inoculating the fungus in a Petri dish which had industrial copper-based wood preservative mixed in the medium. Controls were prepared by placing a fungal inoculum in the centre of a Petri dish with only the growth medium.

The brown rot fungi *Coniophora puteana* (strain BAM 112), *Rhodonia (Poria) placenta* (strain BAM 113) and *Gloeophyllum trabeum* (strain BAM 115), and the white rot fungus *Trametes versicolor* (strain BAM 116) were purchased from the Federal Institute for Materials Research and Testing (BAM, Berlin, Germany). At least three of these four fungi were tested for each treatment. The fungal growth was checked regularly, and pictures of the Petri dish were taken following the setup described by Ancin-Murguzur et al. (2018). When the mycelia of the fungi reached the edge of the Petri dish, the growth of the fungi in media containing the extracts, acids and distillates was compared to the growth in the medium containing the industrial reference and to the control Petri dish, and the inhibition was calculated (see visual example in Figure 11).

Table 3. Fungal species, strain, their use in different tests and the original publication in which the results have been reported. In the column wood decay test the papers where these fungi were used in wood decay experiments is presented, if a leaching test was performed and the number of replicates used in the test of each paper is presented

Fungus	Strain	Tests				
		Antifungal test	Replicates	Wood decay test	Leaching test	Replicates
<i>Coniophora puteana</i>	BAM 112	Paper I	10	Paper III	Yes	8
		Paper II	10	Paper IV	Yes	8
		Paper IV	8	Paper V	Yes	10
<i>Rhodonía placenta</i>	BAM 113	Papers I–II	10	-	-	-
		Paper III	6–7	-	-	-
		Paper IV	8	-	-	-
<i>Gloeophyllum trabeum</i>	BAM 115	Papers I–II	10	Paper IV	Yes	8
		Paper III	6–7			
		Paper IV	8			
<i>Trametes versicolor</i>	BAM 116	Paper II	6	-	-	-
		Paper III	6–7			
		Paper IV	8			

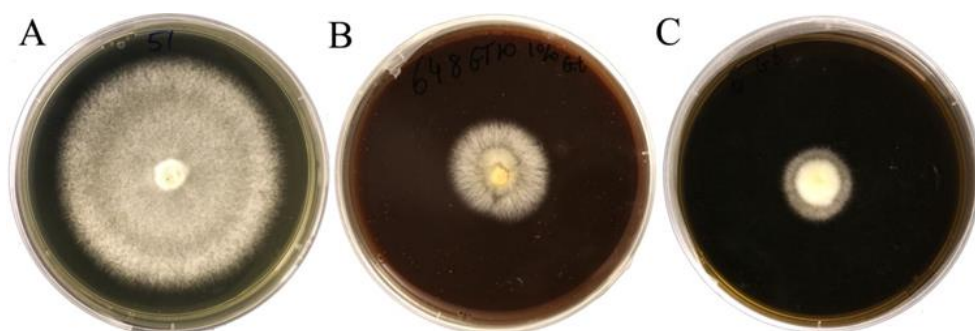


Figure 11. Example of the antifungal test in which the growth of *Gloeophyllum trabeum* was measured in a control growth medium (A), growth medium with 1% Colatan GT10 (B) and a growth medium with 3% silverskin extract (C) for 7 days after inoculation. The day when the control specimen (in this case A) reaches the edge of the petri dish, the experiment will be finished and the growth of the fungi growing in potential bio-based preservatives amended media will be measured for calculating the inhibition.

Once the inhibition-% was calculated, the minimum inhibitory concentration (MIC) was calculated to estimate which concentration of each potential bio-based preservative would be required to inhibit completely each wood-decaying fungus, as well as all of them. The mean value and the standard error of the inhibition caused by each potential bio-based preservative to each fungus was calculated and presented as a result in the respective papers, while the MIC value is presented in this thesis. The MIC value was calculated by checking at which concentration were the fungi inhibited completely in the antifungal test. If the fungi were not inhibited completely, the result reported presented the highest concentration tested and higher (>) symbol, for indicating that a higher concentration than the ones tested would be necessary for complete growth inhibition of the fungus. It is important to highlight that results from antifungal tests cannot be extrapolated to how the chemicals would affect wood decay, as the performance of the chemicals will vary between the growth media and wood (Loman 1970). Thus, additional tests in wood are necessary to complement this test, as both will provide useful knowledge. A more detailed description of the test can be found in Papers I-IV.

2.4 Wood impregnation and leaching treatments, and *in vitro* decay tests

The wood leaching and decay test selected provided additional information to the antifungal results from the previous test. The leaching test provided information about how the potential bio-based preservatives and industrial references are fixed—or leached—in wood after their impregnation. The selected decay test provides information about the retention of the potential bio-based preservatives in wood as well as the activity of the tested potential preservatives as fungal inhibitors in wood. Additionally, despite requiring more time—a maximum of 16 weeks—than the antifungal test, it is still short if compared to other wood decay tests which may take several years.

The chemical components and mixes tested for their fixation to wood and decay *in vitro* were coffee silverskin extract (Paper III), acetic acid, formic acid, propionic acid (Papers IV and V), formic and propionic acid mixture (1:1) and acetic, formic and propionic acid mixture (1:1:1) (Paper IV) and different European aspen bark pyrolysis distillates, Colatan GT10 extracts, pine oil and commercial Celcure C4 wood preservative (Paper V).

Scots pine, *Pinus sylvestris* L. sapwood specimens of 5×10×40 mm were impregnated with the bio-based chemicals to be tested following a modified Bethell process. Some specimens were not impregnated but used as untreated controls. The number of replicates for each treatment and leaching regime (leached or unleached) was between 8 and 10 depending on the components tested. The retention of the chemicals in wood was calculated based on the dry mass increased after impregnation. The leaching of the preservative candidates from wood was tested according to the European Standard EN 84. The effect of leaching was calculated by checking how much the dry mass was reduced after the leaching test was over.

The leached and unleached specimens were then exposed to decay *in vitro*, following a mini-block procedure with fungi that had been grown previously. The fungal species chosen for these experiments was *C. puteana*, and in addition, *G. trabeum* was used for the tested organic acids and organic acid mixtures. The European Standard EN 113 was followed for the wood decay test, but the test was performed in a Petri dish (Ø 90 mm, 1.5 cm height) instead of flat bottles, as previously done by e.g. Lu et al. (2016). The wood specimens were put in the Petri dish under sterile conditions after the fungus covered the whole surface of the malt agar media in the petri dish (Figure 12). The petri dish that were not completely grown were discarded for this experiment. Then the Petri dish were sealed with Parafilm™ and kept in a growth chamber at 20±2 °C and 65±5% for incubation.

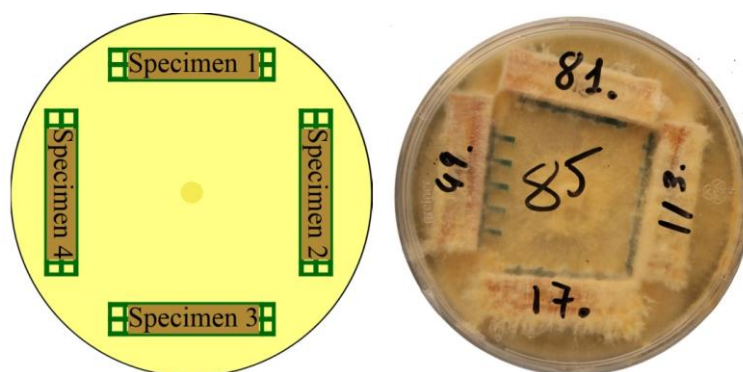


Figure 12. On the left side of the figure, the schematic of the Petri dish with the wood specimens over the green plastic mesh during the wood decay test. On the right side, a picture of the Petri dish with the wood specimens after 16 weeks of exposure to the decay fungus *Coniophora puteana*, with randomized numbers associated to wood pieces and the dish. The main visual difference between the day when the fungi are put in the Petri dish and the last week of the experiment is the growth of the fungus over the wood.

After 16 weeks, the wood specimens were taken out of the dishes and the fungal mycelium was smoothly removed from the wood specimens with a brush. The dry mass loss of the wood was calculated. Based on the EN 113, the mass loss caused by *C. puteana* and *G. trabeum* to untreated specimens needs to be at least 20% for the test to be valid, while a mass loss below 3% in treated specimens will indicate that the tested potential bio-based preservative is effective as a wood preservative. In cases where the minimum mass loss caused by the fungi to the wood specimens was not achieved, the test was still considered valid if clear differences could be seen between the different treatments and controls, or when the mass loss value was close to 20%. More details of the wood leaching and decay tests can be found in Papers III-V. Summary of the fungi and the papers where the wood decay tests were reported can be found in Table 3.

2.5 Acute ecotoxicity test

The acute toxicity tests for the bio-based preservative compounds (Table 2) were made according to ISO 21338:2010. In this test the luminescence inhibition caused by the potential preservatives and Celcure C4 in *Aliivibrio fischeri* photoluminescent bacteria with a BioTox™ test kit (ISO 2010) was measured. The reduction of the photoluminescence after 30 minutes in each bio-based chemical, reference and control was measured in order to calculate the photoluminescence inhibition and reported as the concentrations of each potential bio-based preservatives required for inhibiting the photoluminescence by 20% (IC₂₀) and 50% (IC₅₀).

The results of this test are used to compare the ecotoxicities of the potential bio-based preservatives tested between themselves and to the commercial copper-based reference, but more tests would be required for understanding their effects towards the environment. The tests are explained in Papers III and V with more details.

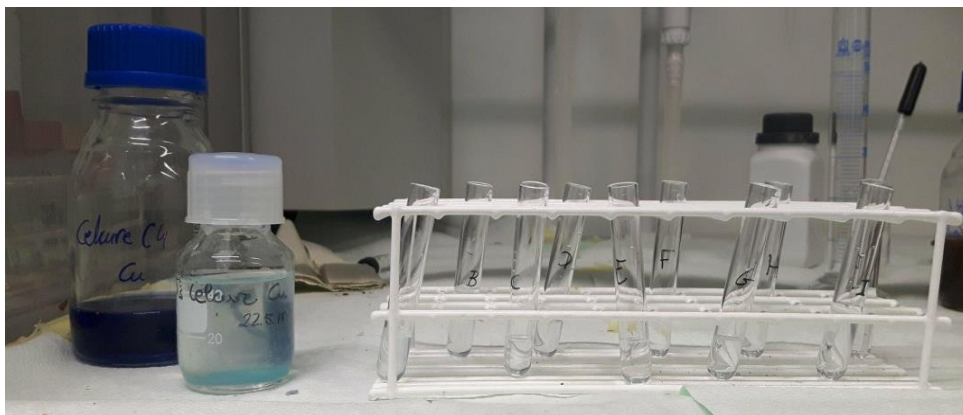


Figure 13. Preparation of dilutions of Celcure C4, the industrial copper-based preservative, for testing the luminescence inhibition. From each of these dilutions two replicates of 300 μ L were prepared for the experiment.

3 RESULTS AND DISCUSSION

3.1 Pyrolysis distillates in wood preservation

Several organic acids were identified in pyrolysis distillates of bark of silver birch and Norway spruce. Hemicelluloses of biomass undergo deacetylation reactions, and the liberated organic acids further catalyse the hydrolysis of hemicelluloses to soluble sugars and depolymerize cellulose into formaldehyde, furfural, and other aldehydes (Tjeerdsma et al. 1998; Weiland and Guyonnet 2003). Acetic acid was found at the highest concentration in the distillates. Propionic acid was in most cases the second most abundant acid, while formic acid was found at the lowest concentration. The distillates were also rich in methanol, with values between 0.11 M and 1.22 M, while furfural and hydroxymethylfurfural were present at lower concentrations.

All the pyrolysis distillates from bark showed some antifungal activity against the wood-decaying fungi (Paper I; Table 4). The pyrolysis distillates from operating temperatures of 350 °C presented higher antifungal activity, with a MIC value of about 1%, than the distillates from the same species but from lower operating temperature, with MIC values exceeding 1%. None of the pyrolysis distillates was as effective as the commercial copper-based wood preservative against the wood-decaying fungi, which had almost 10 times less concentration required to reach a similar inhibition.

The wood decay test in Petri dishes with the *P. tremula* wood pyrolysis distillates from operating temperatures up to 135 °C, 275 °C and 350 °C did not yield positive results against the wood-decaying fungus *C. puteana* (Figure 14; Paper V)., and when the wood specimens were leached, the mass loss of the wood was increased in the specimens impregnated with the distillate from an operating temperature of 350 °C.

Table 4. Minimum inhibitory concentration (MIC) of the pyrolysis distillates tested to inhibit completely the growth of *Coniophora puteana*, *Gloeophyllum trabeum* and *Rhodonia (Poria) placenta*. The last column refers to the minimum inhibitory concentration required to inhibit all the fungi tested

Pyrolysis distillate	MIC (%)			
	<i>C. puteana</i>	<i>G. trabeum</i>	<i>R. placenta</i>	All
Spruce bark distillate 1 (OT 275 °C)	> 1	> 1	> 1	> 1
Spruce bark distillate 2 (OT 350 °C)	1	1.1	0.3	1.1
Birch bark distillate 1 (OT 275 °C)	0.4–0.5	> 1	> 1	> 1
Birch bark distillate 2 (OT 350 °C)	0.3	1	1	1
Celcure C4	0.1–0.2	0.1	0.1	0.1–0.2

OT refers to the maximum operating temperature.

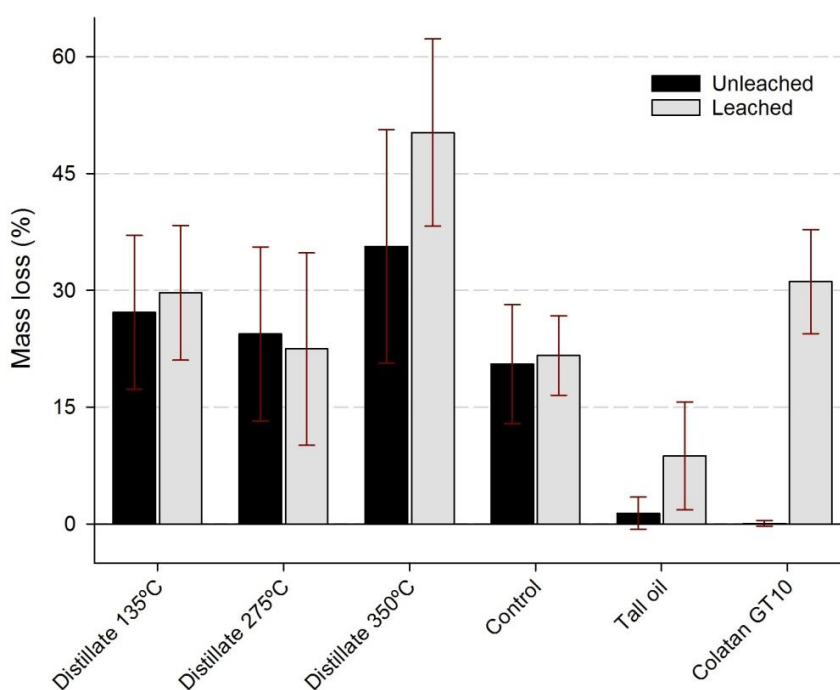


Figure 14. Mean mass loss caused by *Coniophora puteana* of wood treated with pyrolysis distillates from operating temperatures of 135 °C, 275 °C and 350 °C against controls and 2 references, tall oil and Colatan GT10-treated wood after 16 weeks of exposure.

Pyrolysis distillates have been studied for their use as wood preservatives for several years. Mansoor and Ali (1992) found that pyrolysis distillates from *Hevea brasiliensis* were able to inhibit wood-decaying fungi and a blue stain fungus in a malt agar medium. Nakai et al. (2007) tested the pyrolysis distillates from wood-based materials that were bonded with urea- and phenol-type adhesives against two wood-decaying fungi, with positive results especially in distillates coming from wood-based materials with phenol-type adhesives. Similar findings have been reported by other research groups which demonstrated the pyrolysis distillates to be effective wood preservatives (Mazela 2007; Theapparath et al. 2015; Akbas et al. 2016), although their leachability has also sometimes been highlighted as a drawback (Temiz et al. 2010).

In this study, antifungal tests indicated that the distillates from higher operating temperatures performed better against wood-decaying fungi than the distillates from the same bark species from lower operating temperatures. However, the results from the decay test showed that the distillate from the highest operating temperature performed poorly against the wood-decaying fungus, even increasing the mass loss of the wood specimens when compared to controls. The results may be due to the difference in the tree species (Mansoor and Ali 1992), in addition to the different operating temperatures affecting the constituents present in the distillates (Zhao et al. 2020).

The way in which the chemicals interact with the media where the fungi grow also has a clear influence. The wood anatomy and the heterogeneous distribution of structural compounds in wood may hinder or affect the effects of the potential bio-based preservatives in wood (Vek et al. 2013). There are more compounds in wood than in the agar-based growth media, which also influences the results (Loman 1970). While the acidity had no effect in the malt agar media because the pH was neutralized to allow the agar to set, this pH neutralization was not performed in the distillates used for impregnating wood. Thus, the acidic nature and organic acids in the distillates could have started the degradation process, facilitating the wood decay caused by fungi, even though the acids are antifungal, as wood is known to degrade by strong acidic solutions (Browning 1963; Kass et al. 1970).

Additionally, the low fixation of distillates to wood may lead to a loss of the antifungal activity of the distillates, thus facilitating the fungal decay that follows the chemical degradation. Previous studies found that the pyrolysis distillates were successful wood preservatives prior to leaching but that their efficiency was significantly reduced after exposing the wood specimens to leaching (Mohan et al. 2008, Temiz et al. 2010). Based on Mohan et al. (2008), as the pyrolysis distillates tend to polymerize, they might provide water repellence to treated wood (Bridgewater et al. 1999), so the fixation of these distillates could be increased if their polymerization were induced. The fixation of the distillates successfully inhibiting fungi in wood could be achieved by chemically bonding them to wood. Other solutions would also include a second treatment with a second chemical after distillate impregnation, in order to avoid leaching from wood, such as the second step impregnation with epoxidized linseed oil performed by Temiz et al. (2013), which increased the hydrophobicity of wood and reduced leaching of the pyrolysis distillates.

Lastly, the reason for the lack of effectiveness in our test could be related to the selected concentration being too low for acting effectively against wood-decaying fungi. Previous studies that successfully reduced the mass loss caused by the decaying fungi were carried out with higher concentrations of the pyrolysis distillates. Kim et al. (2012) found that pyrolysis distillates had some effectiveness in reducing mass loss at solution concentrations of 25% and 50%, but the mass loss was efficiently reduced only in wood blocks impregnated with 75% and 100% solutions of the distillates. Mohan et al. (2008) concluded that a 25% solution

of the distillates provided the necessary protection to wood in soil block tests, while Temiz et al. (2013) found that 10% and 20% impregnation of distillates already caused an important decrease in the wood's mass loss. However, the aim of these studies (Papers I and V) was to test whether the distillates would be effective at low concentrations to reduce the environmental load and have a high material efficiency.

3.2 Industrial coffee waste as a potential source for wood preservatives

The main constituents in spent coffee and coffee silverskin extracts were similar for both feedstocks, although the concentrations varied (Papers II and III). In spent coffee, chlorogenic acid and its derivatives were the most common constituents, while caffeine and its derivatives were found at highest concentration in coffee silverskin. Other chemicals, such as protocatechuic acid and caffeic acid and its derivatives were present in both extracts at lower concentrations. The condensed tannin concentration in silverskin extracts was 2.5 mg/g. Organic acids and phenolics are known to be antimicrobial (Aziz et al. 1998, Kabir et al. 2014, Kwaśniewska-Sip et al. 2018), and could be responsible for the antifungal activity of spent coffee and silverskin extracts. However, a combined effect of all the compounds analyzed could also be possible.

Despite the antifungal constituents in the spent coffee and silverskin extracts, their inhibition against wood-decaying fungi was low (Paper II, Paper III, Table 5). None of the studied extracts completely inhibited the growth of the fungi. Filtering of the spent coffee increased the activity against all fungal species except *R. placenta* (Paper II). The antifungal activity of spent coffee was slightly higher than that of the silverskin extract at 1% concentration, but the yield of silverskin was 4 times higher (Paper III).

Table 5. Minimum inhibitory concentrations (MIC) of coffee residue extracts and references tested to inhibit completely *Coniophora puteana*, *Gloeophyllum trabeum*, *Rhodonia (Poria) placenta* and *Trametes versicolor*. The last column refers to the minimum inhibitory concentration required to inhibit all 3 fungi tested. *Coniophora puteana* was not tested for silverskin and caffeine in the antifungal test. For silverskin extracts, similar results were found in the wood decay tests

Extract or chemical	Minimum inhibitory concentration (%)				
	<i>C. puteana</i>	<i>G. trabeum</i>	<i>R. placenta</i>	<i>T. versicolor</i>	All
Spent coffee extract	> 5	> 5	> 5	> 5	> 5
Filtered spent coffee extract	> 1	> 1	> 1	> 1	> 1
Silverskin extract	-	> 3	> 3	> 3	> 3
Fresh coffee extract	> 1	> 1	> 1	> 1	> 1
Caffeine	-	< 1	< 1	< 1	< 1
Celcure C4	0.1–0.2	0.1	0.1	< 1	0.1–0.2

The low activity against the wood-decaying fungi of the coffee-related extracts could be due to several reasons, which are broadly discussed in Paper II (for spent coffee extracts) and Paper 3 (for coffee silverskin extracts). The presence of other chemicals in the extracts, such as sugars, that promote the fungal growth is one of the possible reasons. This hypothesis is supported by the results of the wood decay test. The mass loss of silverskin-impregnated wood was lower than that of the controls ($9 \pm 2\%$ and $15 \pm 4\%$, respectively), whereas the silverskin-impregnated specimens lost more mass than the controls after exposing them to leaching, as sugars have also been suggested by our group to play a relevant role in other bio-based extracts (Barbero-López 2020). Other reasons may be the tolerance of certain fungi to some of these compounds, or the structure of the phenolics compounds (Nascimento et al. 2013). Many extractives are known to inhibit wood-decaying fungi, although the mechanism is still not fully understood (Belt et al. 2018). The mechanisms of action of extractives could be a combination of the fungicidal properties, water repellence and antioxidant activity of their different compounds (Belt et al. 2017), and thus the lack of some of the compounds in our extracts could also be a reason for the low activity of the spent coffee and silverskin extracts.

As in the case of the pyrolysis distillates, if the effective constituents of spent coffee and silverskin extracts are to be considered for wood preservative formulations, their fixation needs to be addressed. Previous studies, which found ways to fix extracts in wood, could be used as a basis for this. Performing a similar procedure as that followed by Rättö et al. (2004), using laccase to fix phenolics to wood, the fixation of the coffee extracts could be tested by their enzymatic polymerization. This process can cause radical formation that can polymerize to higher molecular weight compounds once inside the wood (Rättö et al. 2004). Other methods, such as the thermal modification of wood previously treated with caffeine, have also been found to be effective to avoid leaching of the caffeine from wood (Kwaśniewska-Sip et al. 2019), and could also be tested for fixation of spent coffee and silverskin extracts.

3.3 The role of bio-based preservative constituents in wood preservation

Overall, the pyrolysis distillates performed better than the silverskin and spent coffee extracts as fungal inhibitors in the malt agar media, although in wood, silverskin extract reduced the mass loss although the results were not good enough. Their performance is based on their constituents and the interactions between the constituents and the matrix in which they are tested. The MIC of the organic acids identified in pyrolysis distillates in and caffeine, the main constituent of coffee-related extracts, are presented in Table 6 (Papers I, III, IV).

Acetic, formic and propionic acids were able to inhibit completely *C. puteana* at 0.1% concentration, whereas the other fungi were only partially inhibited. The MIC to inhibit completely *C. puteana* for all the acids was lower than that of the commercial copper-based wood preservatives. Propionic acid showed the best inhibitory effects, inhibiting completely all the fungi at concentrations below 0.15%. The inhibition caused to *R. placenta* by acetic and formic acids was very low, reaching 15% inhibition at maximum, while propionic acid inhibited this fungus by about 90% at 0.05% concentration and 100% at 0.1% concentration (Paper IV).

Table 6. Minimum inhibitory concentrations (MIC) of organic acids and caffeine to inhibit completely the growth of *Coniophora puteana*, *Gloeophyllum trabeum*, *Rhodonia (Poria) placenta* and *Trametes versicolor* in antifungal tests. The last column refers to the minimum inhibitory concentration required to inhibit all fungi tested. *Coniophora puteana* was not tested for caffeine in the antifungal test

Constituents	Minimum inhibitory concentration (%)				
	<i>C. puteana</i>	<i>G. trabeum</i>	<i>R. placenta</i>	<i>T. versicolor</i>	All
Acetic acid	0.1	> 0.1	> 0.1	> 0.1	> 0.1
Formic acid	0.1	> 0.1	> 0.1	> 0.1	> 0.1
Propionic acid	0.05	< 0.15	0.1	0.1	< 0.15
Caffeine	-	< 1	< 1	< 1	< 1
Celcure C4	0.1–0.2	0.1	0.1	< 1	0.1–0.2
Acid combinations (1:1)					
Acetic and formic	0.1	0.1–0.2	> 0.1	0.1–0.2	> 0.1
Acetic and propionic	0.1	> 0.1	> 0.1	> 0.1	> 0.1
Formic and propionic	0.1	0.15	> 0.1	0.1	> 0.1
Acetic, formic and propionic	0.075	> 0.1	> 0.1	> 0.1	> 0.1

Phenolic compounds, present in all the potential bio-based preservatives tested, are often considered to inhibit wood-decaying fungi (Harju et al. 2003, Anouhe et al. 2018). In the case of pyrolysis distillates, their antimicrobial and insecticidal properties are related to the presence of organic acids, phenolic compounds and fatty acids, but a chemical analysis of the distillates is often not performed in the investigations (Mattos et al. 2019).

The antifungal activity of caffeine at 0.06% was lower than that of coffee silverskin at 3% concentration in 2 out of 3 of the fungal species tested (Paper III). As the caffeine content in 3% silverskin extract was 0.06%, it can be concluded that other chemicals than caffeine also play a role in the antifungal activity of silverskin extracts. While the potential of caffeine as an antifungal has not been extensively studied (Kwaśniewska-Sip et al. 2018), it has been suggested to play a defensive role in plants (Ashihara and Suzuki 2004).

The mixtures of acids did not cause a higher inhibition of the fungi than that caused by the individual acids. *Coniophora puteana* was the most sensitive fungus to the acid combinations, as it was inhibited completely by the mixtures at 0.1% or lower concentrations. The lowest MIC needed to inhibit completely all the decay fungi among all the individual acids and their mixtures was found for propionic acid. Several studies have found the antifungal properties of pyrolysis distillates to be caused by the organic acids present. Recently, Mattos et al. (2019) reviewed the use of pyrolysis distillates as antimicrobials and insecticides, and highlighted the role of phenolics and organic acids, especially acetic acid. Organic acids are used as antimicrobials in food, and this activity is based on two mechanisms, cytoplasmic acidification and accumulation of the dissociated acid anion to levels that are toxic to microbes (Taylor et al. 2012 in Mani-López et al. 2012), which could also be the mechanisms that affect the wood-decaying fungi.

Our results indicate that some phenolics, such as tannins, significantly reduce the mass loss caused by fungi, and that organic acids are also able to inhibit the wood-decaying fungi. However, we found that propionic acid was more effective than acetic acid as a fungal inhibitor. A study carried out by Oramahi and Yoshimura (2013) indicated that the acid content could have relevance in the antifungal activity of several pyrolysis distillates against wood-decaying fungi, while Baimark and Niamsa (2009) found that the antifungal activity of pyrolysis distillates against *Penicillium griseofulvum*, a fungus not related to wood decay, was related to the content of phenolics in the distillates. A recent study found that distillates from pyrolysis at 350 °C were the most effective ones tested, and that this was related to their high phenolic and acid concentration (Oramahi et al. 2018). These were the only pyrolysis distillates they tested with propionic acid and 3-hydroxy-2-butanone, and they also had the highest acetic acid concentrations and the lowest phenolic concentrations of all the pyrolysis distillates. This supports our results suggesting that propionic acid and organic acids might play an important role in the fungal inhibition of pyrolysis distillates. These findings also agree with Mattos et al. (2019), who highlighted that the antifungal activity of the pyrolysis distillates might not be due only to the phenolics but also to other constituents.

The results of the wood decay test did not show a mass loss reduction caused by the organic acids (Paper IV). In addition to the negligible effect on wood decay of the acids and their mixtures, the treated and leached sapwood specimens had less mass than before being treated with the acids, which may indicate that the wood specimens were degraded chemically (Browning 1963; Kass et al. 1970), even though we used only 3% and 6% acid solutions. Further tests should be performed with neutralized organic acids in wood, as their effectiveness as antifungals was demonstrated in Paper IV. In this paper the pH of the organic acids was neutralized to allow the malt agar growth medium to solidify, which proves that this antifungal activity did not originate from the acidic nature of the organic acids.

3.4 Ecotoxicity of wood preservatives

The results of the ecotoxicity test (Table 7) show that most of the potential preservatives tested required higher concentrations than the commercial copper-based wood preservative to reduce photoluminescence by 20% (IC₂₀) and 50% (IC₅₀). Lower IC₂₀ and IC₅₀ values indicate higher toxicity of the chemicals, while higher values mean lower toxicity. The distillate collected from the 350 °C phase presented the highest ecotoxicity values for both IC₂₀ and IC₅₀, even higher than the commercial wood preservative, whereas the rest of the tested preservative candidates and chemicals presented lower ecotoxicity values. The distillates from the other phases had lower IC₂₀ and IC₅₀ values than the commercial copper-based preservative and the Colatan GT10 extracts. The differences in these values are mostly caused by the constitution of these distillates that may vary at different pyrolysis temperatures (Wei et al. 2010, Zhao et al. 2020). Even if pyrolysis distillates do not contain polycyclic aromatic hydrocarbons (Temiz et al. 2013), that are carcinogenic, mutagenic and very persistent in soils (Madrid et al. 2019), they can still present a very high ecotoxicity, which needs to be tested and considered for all the distillates, and in general for all the suggested bio-based preservatives.

Table 7. IC₂₀ and IC₅₀ values in mg/L of the potential bio-based preservatives tested for their acute ecotoxicity. The lower the IC₂₀ and IC₅₀ value, the higher the ecotoxicity of the chemical. The results are presented in order from the lowest IC₅₀ value to the highest one, based on the results from Paper III and Paper V

Tested potential bio-based preservatives	IC₂₀ (mg/L)	IC₅₀ (mg/L)
European aspen bark distillate 350 °C	0.02	0.2
Celcure C4 copper-based preservative	12	19
Colatan GT10	22	145
European aspen bark distillate 275 °C	567	1085
European aspen bark distillate 135 °C	570	1589
Silverskin extract	1172	2661
Acetic acid	65	4052
Propionic acid	15653	21836
Formic acid	662	23003

The three organic acids showed low ecotoxicity in the photoluminescence test. Of the three acids, formic and propionic acid required the highest concentration of acid in the water to reduce the photoluminescence by 50%. Additionally, the concentration of propionic acid required to reduce the photoluminescence by 20% was the highest of all the tested chemical mixes or constituents. It is interesting that the acids were also the potential antifungals with the highest antifungal activity out of all the tested candidates (Table 6), even though their effect as wood preservatives was not good (Paper IV). If the effectiveness of the organic acids in malt agar media could also be achieved in wood, considering the low ecotoxicity of these natural compounds, their inclusion in wood preservative formulations should be seriously considered, as their ecotoxicity was the lowest of all the tested potential bio-based preservatives.

4 CONCLUSIONS

In this thesis, the antifungal activity of potential bio-based preservatives originating from industrial residues was investigated and compared to commercial wood preservatives against several wood-decaying fungi. The hypothesis was that these could yield to antifungal compounds for new wood preservative formulations for above ground use. This hypothesis was confirmed, as both extractives from coffee-related residues and pyrolysis distillates from bark of silver birch and Norway spruce had antifungal activity against the wood-decaying fungi tested. It was also found that some constituents in the extracts could also promote the growth of the fungi, which needs to be considered if they were used as active ingredients in wood preservative formulations.

Another aim of this thesis was to test the antifungal activity of the constituents of the potential new preservatives against wood-decaying fungi. against wood-decaying fungi. The hypothesis was that the antifungal activity was not merely due to a single constituent but from the synergy between several of them. The characterization of the distillates and extracts

showed that they contain many constituents that have been previously reported as possible antifungals against several fungi species. The results of the antifungal test showed that propionic acid was the main organic acid inhibiting the wood-decaying fungi. The results also demonstrated that acetic acid and formic acid also had antifungal properties. Thus, the antifungal properties of the pyrolytic distillates do not merely come from the phenolic compounds or propionic acid, but from several constituents with a synergetic capacity to deter fungi from wood. The original hypothesis was thus partially correct, but the synergetic effect of different acids did not promote the decay inhibition of individual acids. Further research is needed in the synergy of organic acids with phenolics and other bark-derived chemicals against wood decay. Additionally, further tests are needed for understanding whether synergy of the constituents of the bio-based preservatives can prevent wood decay caused by a broader spectrum of fungi than single species.

The third aim of this thesis was to test how these chemicals performed *in vitro* as wood preservatives, impregnating them in wood and exposing the wood to decay fungi in Petri dishes for several months. The hypothesis was that due to leaching and the differences between wood and malt agar, the performance of natural compounds in wood would be reduced when compared to their performance in the malt agar media. None of the tested potential antifungals or chemical constituents performed well enough in wood compared with commercial wood preservatives. Thus, the hypothesis was confirmed. Leaching was not the only factor affecting the poor performance of the tested compounds as wood preservatives, but the acidity and the presence of other chemicals that can promote fungal growth were also relevant. Further investigations are necessary to test how the acidity of the chemicals used for wood impregnation affect the wood durability. Moreover, additional tests are also necessary to understand which constituents delay wood decay and which promote wood decay in potential bio-based preservatives if their inclusion as active ingredients in wood preservative formulations is planned.

The final aim of this thesis was to assess the ecotoxicity of the preservatives and to compare this to the ecotoxicity of the commercial chemical mixtures for above ground use. The hypothesis was that some of the distillates and extracts may have as high ecotoxicity as the commercial wood preservatives. This hypothesis was confirmed, as we found that although most of the potential bio-based preservatives tested had lower ecotoxicity than the commercial wood preservative, one of the distillates had a much higher ecotoxicity. This highlights the need for analysing the ecotoxicity of the bio-based chemicals proposed as active ingredients in wood preservation, as well as the resulting wood preservative formulations, as they may result in even more toxic mixtures than the current wood preservatives.

In conclusion, the potential bio-based preservatives extracted from wood and coffee industry side-streams and residues have constituents that can act as antifungals against wood-decaying fungi. While these potential antifungals and many of their constituents inhibit wood-decaying fungi, they are not effective as wood preservatives as such, they leach out easily from wood and in some cases, they have higher ecotoxicity values than the commercial preservatives. Thus, these compounds should be studied as possible ingredients for wood preservative formulations but are insufficiently potent to be used as wood preservatives as such. Additionally, if wood preservatives including these bio-based ingredients are formulated, their fixation to wood needs to be studied before they are commercially used as leaching from wood is their main disadvantage.

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