

BIOREMEDIATION OF WASTE COPPER/CHROMIUM TREATED WOOD USING WOOD DECAY FUNGI

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Abstract

The expected service life of copper/chromium (CCA or CCB) treated wood is about 20 - 50 years. After that period, the treated wood is discarded as waste. Due to the toxic elements in such treated wood, burning and landfill disposal are not considered as environmentally friendly solutions. Extraction and recycling of the preservatives from the waste wood is a much more promising and environmentally friendly solution, which is based on the conversion of the fixed biocides in the wood into soluble forms which can subsequently be leached out of the wood.

*In order to elucidate the mechanism of this process, copper/chromium treated wood samples were leached after exposure to copper tolerant (*Antrodia vaillantii* and *Leucogyrophana pinastri*) and copper sensitive wood decay fungi (*Gloeophyllum trabeum* and *Poria monticola*). Furthermore, the ability of fungal hyphae to penetrate and overgrow the wood samples was investigated using following methods. Small stick of unimpregnated wood ($r = 1.5 \text{ mm}$, $l = 25 \text{ mm}$) was inserted into a hole, bored in the center of the samples, and after that sealed with epoxy sealer. Sterilized, leached and non-leached impregnated and unimpregnated specimens were exposed to brown rot fungi for one, two, five, eight or twelve weeks. After respective period, the inserted wood pieces were removed from the specimens and put onto nutrient medium containing petri dish. Possible growth of the hyphae from those pieces was than visually determined. Rate of colonization was determined by measurement of CO₂ production as well. The fungal growths were stimulated by immersing of the specimens into aqueous solution of glucose or corn steep liquor prior to exposure to fungi. Followed exposure the fungi, specimens were leached and concentrations of copper and chromium leached were determined. Afterwards, EPR measurements of leached and non-leached samples were performed in order to determine the paramagnetic complexes that were formed.*

*The fastest colonization of impregnated wood was found at copper tolerant *A. vaillantii*. Addition of nutrients onto the surface of the specimens increased the colonization of the specimens. All wood decay fungi investigated as copper tolerant as well as copper sensitive increased heavy metals leaching from the treated wood. These fungi influenced the de-fixation process via oxalates formation. EPR measurements indicates that the transformation of copper into copper oxalate by the fungi was found to be essential but not the only mechanism responsible for copper tolerance by these fungi. However, from our results, it seems that other acids were also responsible for increased copper and/or chromium leaching. These results are important in elucidating copper toxicity by wood decay fungi and at using of these fungi for bioremediation of treated wood wastes.*

Keywords: Copper tolerant fungi, Waste treated wood, Remediation, Wood colonization, Detoxification, leaching, EPR,

INTRODUCTION

CCA or CCB as wood preservatives are the most important wood preservatives, as CCA and CCB-treated lumber have been proven to be effective in deterring insects and fungal decay. For example, approximately 212 million m³ of CCA treated lumber was produced annually in the USA alone [1]. Additional 1.5 to 2.0 million m³ of wood containing about 1000 tons of chromium and 600 tons of copper are preserved annually in the area of the former Federal Republic of Germany [2]. We can foresee huge amounts of preserved wood removed from service in future years, in most of developed countries. The presence of copper and chromium, as well as arsenic causes problems in the later disposal of this impregnated wood. Because of the toxic elements in such treated wood, it is very important to find an effective and environmentally sound recycling solution for preserved wood when removed from service.

Landfill disposal is not an environmentally sound option since it only postpones dealing with the problem to future generations. Furthermore, the heavy metals in the wood may diffuse into the surrounding soil, resulting in significant environmental damage [3]. In addition, capacities of special dumps are limited and public approval for new facilities is extremely low. Burning of CCA/CCB waste preserved wood is only permitted in approved incinerators under extremely controlled conditions in many countries, since emitted gases have been found to contain high concentration of arsenic compounds [4]. The cost of destruction, such as by incineration, can be very expensive: about 500 EUR/t [5].

A number of environmentally sound disposal options have been investigated in recent years, including biological methods using either copper tolerant fungal strains [2] or bacteria [1]. The principle underlying methods is to convert the insoluble heavy metals in the waste wood into a soluble form through acidification with organic acids. The soluble heavy metal complex can then be leached from the wood. Thus, both the remediated wood fiber and the metals can be reclaimed and recycled. The most important acid involved in this process is oxalic acid [1]. Oxalic acid is a small organic acid with two low pK values (pK₁ = 1.27; pK₂ = 4.26) [6]. It is often produced by brown rot fungi in great quantities [7,8,9] and is associated with brown rot colonization of wood [10]. The most efficient oxalic acid producers and consequently the most tolerant fungi include the genus *Antrodia* [11]. Other wood decaying fungi produce significantly less oxalic acid. Instead of oxalic acid, these fungi excrete other organic acids in order to optimise pH value of the substrate [8,12]. Oxalic acid can react with insoluble chromium in wood to form chromium oxalate, which is soluble and can be leached out of wood. On the other hand, copper oxalate, which is formed between copper and oxalic acid, is insoluble and can only be leached with an ammonia solution [3,9].

The aim of this study was to elucidate the ability of a selected copper tolerant fungal strain as well as copper sensitive ones to colonize wood preserved with copper and chromium based preservatives and to describe their influence on leaching of copper and chromium from treated wood samples. Finally, changes to the active ingredients (Cu, Cr) in the treated wood after exposure to the fungi, and leaching, were studied using electron paramagnetic resonance (EPR).

MATERIALS AND METHODS

Preparation of the samples

Norway spruce (*Picea abies*) samples of dimensions (15 × 25 × 50 mm) were vacuum impregnated with 5 % CCB solution according to the EN 113 procedure [13]. The treatment resulted in a preservative uptake of about 18 kg/m³. The samples were later conditioned for four weeks, the first two weeks in closed chambers, the third week in half closed and the fourth week in open ones.

The conditioned samples were then oven dried (75 °C) for five days in order to ensure complete reduction of chromium. Following conditioning, the samples were leached according to the EN 84 procedure for 14 days [14]. Afterwards, the samples were oven dried (103 °C) and their masses were determined, then conditioned and finally steam-sterilized. Prior to exposure to the fungi some samples were immersed for five minutes to 4 % aqueous solution of corn steep liquor (Sigma) or 4 % aqueous solution of glucose or to the mixture of 4 % aqueous solution of corn steep liquor and glucose (Sigma) or to water only. Glucose was used as easy available carbon source and CSL as nitrogen one.

Baiting experiment

Experiment was performed according to the procedure described by Kleist and co-workers [15]. This experiment was designed to determine whether fungal hyphae can penetrate the center of the wood sample or they can only be found in the surface region. Samples impregnated as described above, were prepared as follows prior to exposure to the fungi. Holes (diameter = 3 mm, depth = 20 mm) were bored in longitudinal direction into the center of the sample. A small toothpick was then inserted into the hole as the bait and the hole sealed with epoxy sealer, as shown in Figure 1. The epoxy sealer has no effects on fungal growth. Afterwards, the samples were sterilized and exposed to the fungi as described later. After one, two, or four weeks of exposure, the toothpicks were in carefully removed from the specimens under sterile conditions and put onto a sterilized solid nutrient medium (PDA, Difco). Any fungal growth from the sticks was monitored for a period of two weeks.

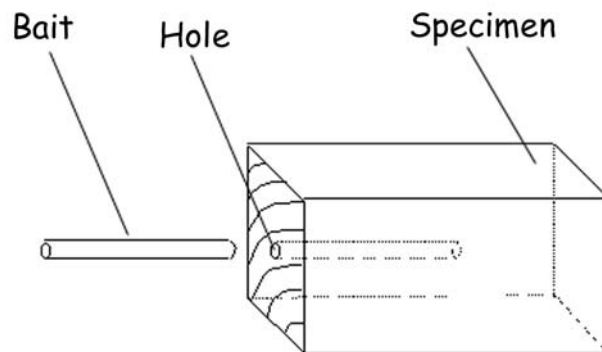


Figure A Sketch of the specimen for baiting experiment.

Table A Brown rot fungi used. Copper tolerance is described with marks according to the results of *POHLEVEN et al.* (2002). Mark 1 describes the highest copper tolerance; and 5 describes the highest copper sensitivity.

Fungi	Abbreviation	Origin	Estimated Cu tolerance
<i>Antrodia vaillantii</i>	Pv2	University of Ljubljana ZIM L037	Cu tolerant 1
<i>Leucogyrophana pinastri</i>	Yf	Buckinghamshire Chilterns University College	Cu tolerant 2
<i>Poria monticola</i>	Pm2	BAM 102, Germany	Cu sensitive / Cu tolerant 3
<i>Gloeophyllum trabeum</i>	Gt2	University of Ljubljana ZIM L017	Cu sensitive 5

Exposure to the fungus

Sterilized and air dried samples were exposed to the following brown rot fungi: *Gloeophyllum trabeum* (Gt2) (ZIM L017), *Antrodia vaillantii* (Pv2) (ZIM L037), *Poria monticola* (Pm2) (BAM 102) and *Leucogyrophana pinastri* (Yf) (HPT 595) [16]. The *A. vaillantii* and *L. pinastri* strains have been shown to be copper tolerant in previous investigations [8,17]. Cultures were grown and maintained on a 3.9 % potato dextrose agar medium (PDA, Difco). Jars with PDA medium were inoculated with small pieces of fungal mycelium. One treated and one untreated wood sample was placed on a sterilized plastic grid in each inoculated jar and exposed to fungal decay for certain period of time in the growth chamber (25 °C, RH = 75 %).

Respiration measurements

Infested wood blocks were after five weeks of exposure carefully removed from the fungal mycelia and put into empty experimental jars where they were subjected to measurement of CO₂ production. The jars were sealed with the ventilation lids, using silicon vacuum paste to enhance the seal. Afterwards the initial concentration of CO₂ and the final one after one hour were measured using an equipment that consisted of membrane pump (flow rate 0.5 l/min), IR carbon dioxide sensor (0-3000 ppm, accuracy = 5 ppm) and 16-bit A/D converter for computerized data acquisition (ECHO d.o.o. Slovenia). The ECHO system was a closed-circuit system and permitted measurements of changes in CO₂ concentrations over time [18].

Leaching procedure

Leaching of Cu and Cr from the samples was conducted according to the modified European standard EN 1250 [14]. Three conditioned samples per each treatment were put on a shaker and positioned with a ballasting-device. After that, 250 g of leaching solution was added. The leaching solution was replaced every 24 hours over four days. Half of the samples were later leached with aqueous solution of ammonia (c_{NH₃} = 1.25 %) following the same procedure. Concentrations (%) of Cu and Cr in the leachates were determined using atomic absorption spectroscopy (AAS). Leached and non-leached decayed samples were oven dried (103 °C) and mass losses were determined. After drying, they were stored for EPR measurements (20 °C and 65 % RH).

Electron paramagnetic resonance (EPR) measurements

EPR experiments were performed at room temperature using Bruker ESP-300 X-band spectrometer (Microwave Frequency = 9.62 GHz, Microwave Power = 20 mW, Modulation

Frequency = 100 kHz, Modulation Amplitude = 0.1 mT). Four matchstick like samples (40 × 1 × 1 mm) were cut from each wood sample and inserted one at a time into the resonator. Thus, EPR measurements of each observation were performed in twelve parallels per each treatment. The various components of EPR parameters (tensor g, and hyperfine splitting tensor A) were determined directly from the spectra, where possible for the respective paramagnetic species.

RESULTS AND DISCUSSION

Colonization of the specimens

The fastest overgrowth of the control-unimpregnated specimens was by *G. trabeum* followed by *P. monticola*. Hyphae of *G. trabeum* reached the bait in the center of specimens after one week of exposure for two thirds of the exposed specimens. However, specimens exposed to the rest of brown rot fungi investigated apart from *L. pinastri* were completely colonized after two weeks of exposure. *L. pinastri* took more weeks to achieve 100 % colonization of the specimens (Table A).

This data correlates well with the respiration measurements of the infested wood specimens. *G. trabeum* and *P. monticola* that showed the highest ability to penetrate the wood specimens produced the highest levels of CO₂ after four weeks of exposure. The copper sensitive *G. trabeum* produced on the average, 1127 ppm of CO₂ in one hour, which is almost double the amount produced by the copper tolerant *A. vaillantii* (560 ppm of CO₂/h) (Table B). These results are comparable with the respiration rates described by Tavzes and co-workers [18] (Figure B).

Addition of nutrients did not increase the ability of fungi to reach the centers of the unimpregnated samples. However, at the presence of the CSL and/or glucose even lower portion of the fungal hyphae reach the bait in the samples (Table B). Copper tolerant strain *A. vaillantii* needs two weeks of exposure to colonize all control specimens that were prior to exposure immersed to water only. On the other hand, only 75 % of samples that were immersed to aqueous solution of CSL and/or glucose were colonized. Similar relationship was observed at other fungal species as well. We presume that the reason for this originates in the fact, that after immersion to nutrient solution, fungi have food source available on the surface of the specimens, thus there were less need for colonization of the central parts of the wood. This presumption was further supported by respiration measurements as well (Figure B).

Table B Percentages of colonized untreated specimens exposed to the fungi for one, two and five weeks. Prior to the fungal exposure samples were immersed into water (C) or aqueous solution of glucose (G) or corn step liquor (CSL) or glucose (G) and corn step liquor (CSL+G).

fungus	Percentages of colonized specimens [%]											
	1 week				2 weeks				5 weeks			
	C	G	CSL	CSL+G	C	G	CSL	CSL+G	C	G	CSL	CSL+G
Pv2	0	33	0	33	100	75	75	75	100	100	100	100
Yf	0	0	0	0	100	100	75	75	100	100	100	100
Pm2	33	33	0	33	100	100	75	100	100	100	100	100
Gt2	66	66	0	0	100	100	75	75	100	100	75	100

Not all the fungal isolates were able to penetrate the center of the impregnated specimens after one and two weeks of exposure. After five weeks, the first hyphae of *A. vaillantii* reached the bait in the center of the exposed treated specimens. However, the complete colonization were observed seven weeks later, after 12 weeks of exposure. Colonization of the impregnated specimens by the copper tolerant *A. vaillantii* is not surprising, as this strain was found to exhibit high copper tolerance [17]. First hyphae of the other copper tolerant fungi *L. pinastri* reached the bait in the impregnated samples after eight weeks of exposure. 100 % colonization was observed after 12 weeks of fungal overgrowth. However, none of the copper sensitive fungal species did not reached the center if the impregnated specimens even after 12 weeks of the exposure.

Respiration measurements were performed after 5 weeks of exposure of the impregnated specimens to fungi. Higher overgrowth of control specimens compared to impregnated ones was evident visually as well as from the CO₂ measurements. The highest surface overgrowth of treated specimens was found at CCB impregnated specimens exposed to the copper tolerant *A. vaillantii* followed by *L. pinastri*. Production of CO₂ from the leached impregnated specimens exposed to *A. vaillantii* was approximately 66 % of that from the control samples. On the other hand, exposure of the impregnated samples to the copper sensitive species *G. trabeum* resulted in 100 times lower CO₂ production than the control ones. However, both visual and by CO₂ measurement showed insignificant growth on the impregnated wood exposed to the copper sensitive *P. monticola* (Table ?).

As well as observed at unimpregnated samples, immersion of impregnated specimens to CSL and/or glucose did not influence the fungi to reach the center of the impregnated wood block (Table C). On the other hand, immersion to nutrients significantly improve ability of fungi to overgrow the surface of impregnated specimens (Figure B). This is even more evident at copper sensitive fungal strain. At specimens immersed to water and exposed to Pm2, there was no growth observed, in contrast when the specimens were immersed to CSL and glucose, the production of 247 ppm CO₂/h were determined. Although, the highest respiration rates were observed at impregnated specimens that were prior to exposure immersed to aqueous solution of CSL and glucose (Figure B). Respiration and the baiting results indicate that wood decay fungi are able to colonize the surface of the treated specimens when immersed to nutrients but they have difficulties to reach the center of the wood specimens.

Mass losses and leaching of the Cu and Cr from Impregnated wood

Mass losses of the impregnated samples indicated that heavy metals present in wood was quite toxic against both copper sensitive and copper tolerant strains. In all cases, mass losses between 1.3 and 1.9 % were obtained. Nevertheless, mass losses did not reflect all the changes that might have taken place in the wood samples. After eight weeks of exposure of CCB samples to wood rotting fungi, the moisture content of the treated samples increased from an initial value of 9 % to values between 97 % (Pm2) and 104 % (Pv2). In addition, blue deposits were observed on the surfaces of the CCB treated samples exposed to the copper tolerant strains (Pv2 and Yf) (Figure E).

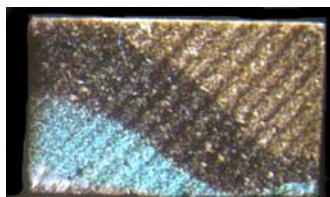


Figure E : End view of the CCB treated samples exposed to the copper tolerant fungus *Antrodia vaillantii*. Please note the blue deposits on the bottom left corner.

Table D: Leaching of active ingredients from fungi infected samples with water for 4 days and leaching with water followed by leaching with 1.25 % solution of ammonia for 4 days. Standard deviations are given in the parenthesis.

Fungus	Water		Water + ammonia	
	Leached Cr [%]	Leached Cu [%]	Leached Cr [%]	Leached Cu [%]
Pv2	12.4 (2.1)	13.2 (1.1)	17.9 (2.0)	23.1 (0.9)
Yf	7.4 (0.6)	10.0 (0.6)	9.6 (0.4)	18.5 (0.5)
Pm2	6.5 (0.1)	7.8 (0.7)	8.7 (0.2)	13.6 (0.5)
Gt2	0.6 (0.0)	1.1 (0.1)	1.8 (0.1)	5.7 (0.2)
None	0.3 (0.0)	1.0 (0.1)	2.5 (0.1)	2.0 (0.2)

Leaching of the samples immediately after exposure to the fungi resulted in further mass losses. For example, treated samples exposed to the copper tolerant *A. vaillantii* (Pv2) which had average mass losses of 1.7 %, when leached, had significantly higher average mass losses (5.3 %). Similar, higher mass losses were obtained for leached samples decayed by the other brown rot fungi. These results support the finding that brown rot fungi depolymerise wood components into water-soluble simple sugars which can be leached from the wood [19]. Leaching with ammonia solution caused even higher mass losses of the partially decayed samples, e.g. samples decayed by copper sensitive *G. trabeum* (Gt2) for four weeks, had an average mass loss of 1.3 %. Leaching with water and a 1.25 % aqueous solution of ammonia increased the mass loss three times to 6.1 %.

For the impregnated wood, the highest leaching of Cr and Cu was obtained for samples exposed to *A. vaillantii*. From those samples, 12.4 % of Cr and 13.2 % of Cu were leached respectively (Table D). As expected, the lowest heavy metals losses for the CCB-treated samples were found for samples decayed by *G. trabeum*. This copper sensitive fungus also caused the lowest mass loss of the CCB-treated (Table D). The Cr and Cu losses from those samples were comparable to those from the control-unexposed samples. We believe, that with the optimization of the fungal exposure, significantly higher levels of heavy metals leaching could be achieved.

Additional leaching of the samples with the aqueous solution of ammonia increased the amount of copper leached from the decayed treated samples. For example, leaching of the Cu from treated samples exposed to *A. vaillantii* resulted in 13.2 % copper loss. Additional leaching with ammonia solution increased Cu loss to 23.1 %. Though the influence of ammonia on the leaching of Cu from the samples decayed by the other fungi was comparable (Table D).

EPR observation of decayed and leached impregnated wood

Exposure of the CCB-treated samples to the copper tolerant *A. vaillantii* and *L. pinastri* resulted, that the intensity of Cu(II) EPR signal ($g_{\perp} = 2.076$,) and Cr(III) ($g = 1.98$; $\Delta h = 48$ mT) Cr(V) ($g_0 = 1.978$) decreased and a broad EPR signal overlapped with Mn(II) ($g_0 = 2.003$ $a_0 = 9.6$ mT) signal appeared (Figure C). The parameters of this broad signal, with a measured g_0 value of 2.175 and linewidth about 43 mT, correlate well with that reported in the literature for copper oxalate [9,20,21]. This proves, that during exposure new complexes of copper oxalate between copper in wood and oxalic acid were formed. The reasons for decrease of chromium EPR signals are similar. In the cited literature [21,22] it is suggested that water environment affects chromium reduction, which could have lead to the disappearance of Cr(V) signal. In addition, a significant decrease of Cr(III) signal was observed as well and this could be due to the formation of chromium oxalate and the leaching of chromium. Chromium oxalate compounds cannot be resolved from the EPR spectra [23].

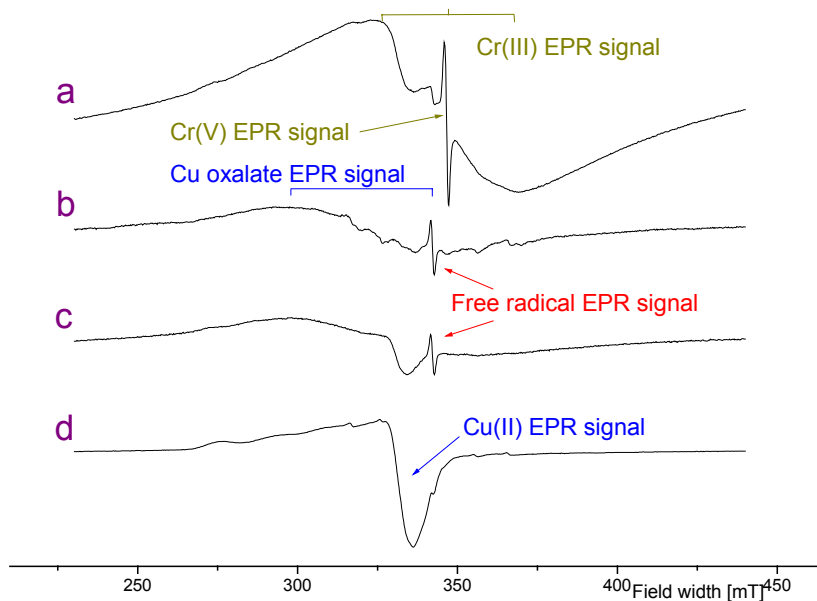


Figure c: EPR spectra of CCB-treated samples (a), exposed to the fungus *A. vaillantii* for 8 weeks (b), and later leached with water (c), and aqueous solution of ammonia (d).

However, the shape and the parameters of preserved samples exposed to *G. trabeum* do not significantly differ from the unexposed ones, indicating that there was no chemical change of the active components in the wood. Result is acceptable, as this copper fungus is known as copper sensitive strain that excretes low amounts of oxalic acid [8,12]. The only observed change is the decrease of Cr(V) EPR signal, which could be due to the high moisture content of the exposed samples as already mentioned (Figure D).

After leaching of the impregnated specimens exposed to the *A. vaillantii* with water, the manganese signal disappeared, but two EPR signals, Cu(II) EPR signal ($g_{\perp} = 2.077$) and a broad EPR signal assigned to copper oxalate remained on the EPR spectra. The intensity of the Cu oxalate EPR signal decreased after leaching as well. We believe that this broad signal belongs to the copper deposits present on the surface of the wood samples. As there were no interactions between these deposits and the wood some of the crystals were washed from the surface during leaching, and thus the decrease in intensity of the copper oxalate EPR signal. Furthermore, during exposure leaching increased acidity may unfix bounded copper and soluble copper(II) sulfate could have diffused to the surface layers of the wood samples, resulting in a higher intensity of the Cu(II) sulfate EPR signals ($g_{\perp} = 2.077$). These results shows that the amount of oxalic acid excreted by the copper tolerant fungi (Pv2 and Yf) in eight weeks, was not enough to transform all the copper in the copper-treated wood to copper oxalate. And that there are some other acids excreted as well. Described changes can be clearly seen from the EPR spectra in Figure c.

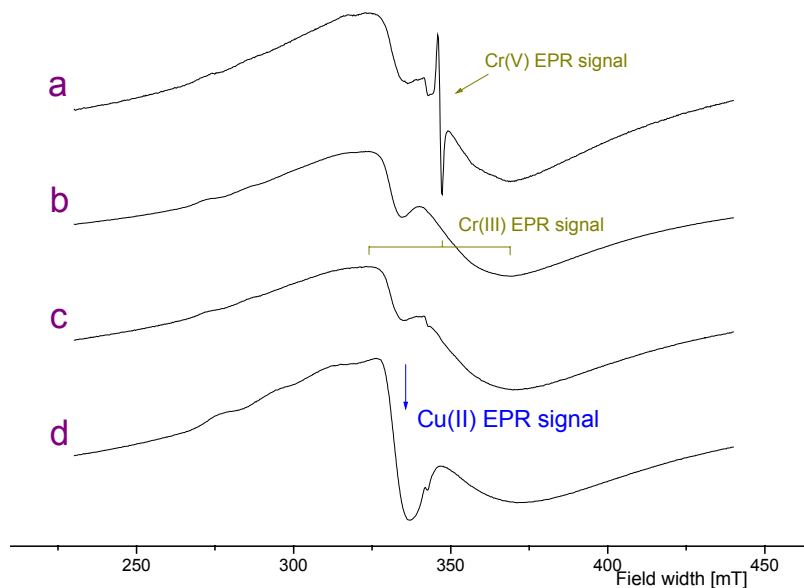


Figure d: EPR spectra of CCB-treated samples (a), exposed to the fungus *G. trabeum* for eight weeks (b), and later leached with water (c), and aqueous solution of ammonia (d).

Ammonia leaching influences on the copper in impregnated wood. As already described, it can be well resolved from the EPR that not all the copper in the treated samples was transformed into copper oxalate. Thus, the ammonia reacted with both the copper oxalate and the remaining copper sulfate in the wood. From Figures c and d, it is clear that there are no copper oxalate/ammonia complexes ($g_{\perp} = 2.067$, $g_{\parallel} = 2.286$ and $A_{\parallel} = 15.7$ mT) [9] resolved in the spectra, thus they must have been leached from the wood leaving only copper (II) sulfate/ammonia ($g_{\perp} = 2.060$, $g_{\parallel} = 2.256$ and $A_{\parallel} = 17.0$ mT). Cu(II) sulfate/ammonia complexes have been reported to be less soluble [22,24] and are even capable of forming chemical bonds with wood components [9,22], which could reduce leaching of the Cu. However, copper/ammonia/oxalic acid complex is more soluble and thus it was leached from the wood and thus it can not be resolved from the EPR spectra any more.

Conclusions

Immersion of the samples to nutrients (Corn steep liquor and/or glucose) significantly improves the ability of copper tolerant, as well as copper sensitive wood decay fungi to overgrow the surface of copper/chromium/boron treated specimens. On the other hand, nutrients do not contribute to the capability of the fungi to reach the central parts of the impregnated wood specimens.

The fastest overgrowth of the surface and the fastest penetration to the center of the specimens were observed at copper tolerant fungus *Antrodia vaillantii*. However, none of the copper sensitive fungi did not reach the bait in the central part of the impregnated samples even after 12 weeks of exposure.

Exposure of the impregnated specimens to wood decay fungi considerably influence the leaching of heavy metals from impregnated specimens, due to formation of chromium oxalate and defixation of copper. Addition of ammonia to leaching solution resulted in increased leaching of Cu and Cr as well.

Acknowledgements

The authors would like to thank Biffaward, Protimsolignum-Osmose, Arch Timber Protection, CSI Timber Protection and the British Council Partnership for Science program for financial support.

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