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International Biodeterioration & Biodegradation 53 (2004) 25–32

INTERNATIONAL BIODETERIORATION & BIODEGRADATION

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Fungal bioremediation of copper, chromium and boron treated wood as studied by electron paramagnetic resonance

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Received 24 December 2002; accepted 18 August 2003

Abstract

In future years, problems concerning the disposal of waste copper/chromium-treated wood will increase significantly. One of the environmentally friendly options of dealing with such treated wood is through bioremediation with copper-tolerant wood decay fungi in order to recycle both the wood fibers and the heavy metals. To study changes during the bioremediation process, Norway spruce (*Picea abies*) samples were vacuum impregnated with 5% CCB solution. Some samples were also impregnated with copper or chromium solution of the same concentration as in the CCB preservative. Following conditioning of the samples, they were then exposed to two copper-tolerant brown rot fungi, (*Antrodia vaillantii*, *Leucogyrophana pinastri*) and two copper-sensitive brown rot fungi, (*Gloeophyllum trabeum*, *Poria monticola*) for a period of 4–8 weeks. After exposure, the samples were cleaned of the mycelia and leached with water or 1.25% ammonia solution for 4 days. The concentrations of Cr and Cu in the leachates were determined. After the leaching process, the samples were studied using electron paramagnetic resonance (EPR). The results obtained showed the important role oxalic acid produced by the decay fungi plays during leaching of the metals from the treated wood. Furthermore, it was also found that though excretion of oxalic acid is necessary for the leaching of metals, it does not fully explain fungal ability to decay copper preserved wood.

Keywords: Copper; Chromium; CCB; Bioremediation; Waste wood; Preservative; Wood decay fungi; Copper tolerance; Leaching

1. Introduction

Copper–chromium–arsenic (CCA) and copper–chromium–boron (CCB)-treated lumber resists insect infestation and fungal decay. This explains the wide spread use of CCA or CCB as wood preservatives. For example, approximately 212 million m^3 of CCA-treated lumber are produced annually in the USA alone (Clausen and Smith, 1998). Additional by 1.5–2.0 million m^3 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 (Stephan et al., 1996). We can foresee huge amounts of preserved wood being removed from service in future years, in most developed countries. Cooper (1993) predicted that the amount of waste-impregnated wood would increase up to 16 million m^3 in the United States alone. 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.

In many countries, burning of CCA/CCB waste preserved wood is only permitted in approved incinerators under extremely controlled conditions as emitted gases contain high concentration of arsenic compounds (Honda et al., 1991). The cost of destruction, such as by incineration, can be very expensive: about 500 EUR/tons (Ribeiro et al., 2000). Landfill disposal is also not an environmentally sound option since it only postpones the problem to future generations. Furthermore, the heavy metals in the wood may diffuse into the surrounding soil, resulting in significant environmental damage (Stephan and Peek, 1992). In addition, capacities of special dumps are limited and public approval for new facilities is extremely low.

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doi:10.1016/j.ibiod.2003.08.001

A number of environmentally sound disposal options have been investigated in recent years, including biological methods using either copper-tolerant fungal strains (Stephan et al., 1996) or bacteria (Clausen and Smith, 1998). The principle underlying such method 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 (Clausen and Smith, 1998). Oxalic acid is a small organic acid with two low pK values (pK_1 = 1.27; pK_2 = 4.26) (Skoog et al., 1992). It is often produced by brown rot fungi in great quantities (Green et al., 1991; Humar et al., 2001, 2002a) and is associated with brown rot colonization of wood (Jellison et al., 1997). 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 (Stephan and Peek, 1992; Humar et al., 2002a).

The aim of this study was to elucidate the effect of a selected copper-tolerant fungal strain as well as copper-sensitive brown rot fungi on leaching of copper and chromium from CCB-treated wood samples. Secondly, the influence of ammonia on leaching of copper and chromium from treated wood samples exposed to the brown rot fungi was investigated. 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). EPR is used for investigation of transformation of copper complexes in wood during the process of wood degradation and leaching. EPR is a useful technique for investigation of chemical species with unpaired electrons, such as radicals and some transition metal ions. In the field of wood science and technology, EPR has been found as useful method, either to study decay processes of untreated wood (Humar et al., 2002a; Qian and Goodell, 2000) or wood preservatives in undecayed wood (Pohleven et al., 1994; Hughes et al., 1994).

2. Materials and methods

2.1. Sample preparations and exposure to fungi

Norway spruce (*Picea abies* (L). Karst) samples of dimensions (1.5 × 2.5 × 5 cm^3 (longitudinal direction)) were vacuum impregnated with 5% CCB solution (34% $CuSO_4 \times 5H_2O$; 37.3% $K_2Cr_2O_7$; 28.7% H_3BO_3), according to EN 113 procedure (ECS, 1989). Some samples were impregnated with only copper or chromium solutions. The concentration of copper or chromium in these solutions was equals the concentrations in the CCB solution. Treatment of the samples resulted in a solution uptake of about 360 kg/m^3. The samples were later conditioned for 4 weeks, the first 2 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 5 days in order to ensure complete reduction of chromium. They were later weighed and conditioned at 20°C and 65% RH.

Sterilized and air-died samples were then exposed to the following brown rot fungi: *Gloeophyllum trabeum* (Pers. Ex Fr.) Murill (Gt2) (ZIM L017), *Antrodia vaillantii* (DC.: Fr) Ryv. (Pv2) (ZIM L037), *Poria monticola* Murr. (Pm2) (BAM 102) and *Leucogyrophana pinastri* (Fr.) Ginns & Weresub (Yf) (HPT 595) (Raspor et al., 1995). The *A. vaillantii* and *L. pinastri* strains have been shown to be copper-tolerant in previous investigations (Humar et al., 2001; Pohleven et al., 2002). 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 4 weeks in the case of copper- and chromium-treated samples and for 8 weeks for CCB treated ones in the dark (25°C, RH 75%). Afterwards, the wood specimens were cleaned of mycelia and one third of them were oven dried, and the remaining were immediately used for the leaching experiment.

2.2. Leaching procedure

Leaching of Cu and Cr from the samples was conducted according to the modified European standard EN 1250 (ECS, 1994). Three samples per each treatment were put on a shaker and positioned with a ballasting-device. After that, 250 g of water was added and replaced every 24 h over 4 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 oven dried, weighted and stored for EPR measurements (20°C and 65% RH). The experiment were performed in three parallels.

2.3. 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^3) were cut from each wood sample and inserted one at a time into the resonator. EPR measurements of each observation were performed in 12 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.

3. Results and discussion

Mass losses of the control samples after 4 weeks indicated that all the fungi used were active. The mass losses caused by the copper-tolerant strains (Pv2 and Yf), to the Cu-treated samples were about less than one times lower than the control samples. On the other hand, mass losses caused by the copper-sensitive strains (Gt2 and Pm2) were only one-quarter to one-fifth lower than the control samples (Table 2). Chromium treatment was less effective in preventing mass loss by all the copper-sensitive and tolerant fungi. However, CCB treatment was effective 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).

Leaching of the samples immediately after exposure to the fungi resulted in further mass losses. For example, CCB-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 (Green III and Highley, 1997). 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 4 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%. This increase in mass loss could be due to depolimerization caused by the fungi, as well as the formation of free radicals in the wood by ammonia. It is known that free radicals cause additional depolimerization of wood substrates (Pavlič et al. in press). The presence of free radicals EPR spectra ($g_0 = 2.003$) can be clearly seen from all the EPR spectra of the various treated samples exposed to the wood decay fungi (Figs. 1–8). Occurrence of free radicals during brown rot decay is well known and described (Green III and Highley, 1997; Jellison et al., 1997; Qian et al., 2002).

Leaching from the copper-treated samples exposed to all the fungal species was lower than leaching from the unexposed samples (Table 1). This could be due to the fact that oxalic acid, produced and excreted by the fungi,

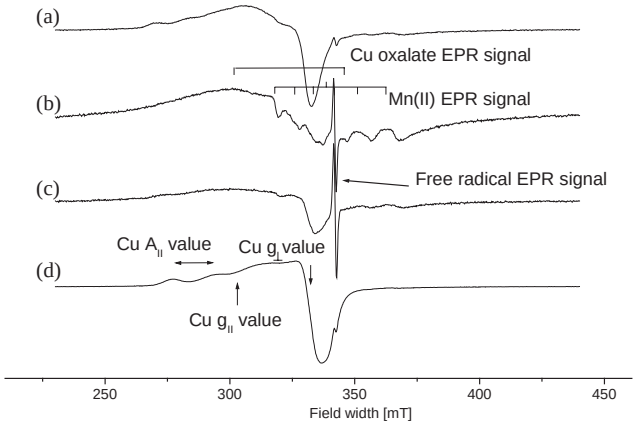

Fig. 1. EPR spectra of copper(II) sulfate-treated samples (a), exposed to the fungus *L. pinastri* for 4 weeks (b), and later leached with water (c), and aqueous solution of ammonia (d). From these spectra Cu(II), Cu oxalate, Mn(II) and free radical EPR signals can be resolved.

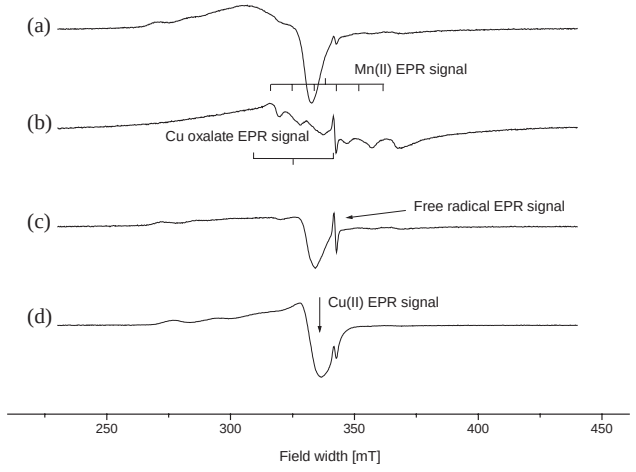

Fig. 2. EPR spectra of copper(II) sulfate-treated samples (a), exposed to the fungus *P. monticola* for 4 weeks (b), and later leached with water (c), and aqueous solution of ammonia (d). From these spectra Cu(II), Cu oxalate, Mn(II) and free radical EPR signals can be resolved.

reacted with copper in the wood to form copper oxalate, which is insoluble (Clausen et al., 2000; Humar et al., 2002a). This is further supported by the results of the EPR spectroscopy which show the EPR spectra of copper-treated spruce wood exposed to copper-tolerant fungi (Pv2 and Yf) to be significantly different from the unexposed one. After exposure to Cu-tolerant strains, the intensity of Cu(II) EPR signal ($g_\perp = 2.077$, $g_{II} = 2.366$ and $A_{II} = 12.5$ mT) decreased and a broad EPR signal overlapped with Mn(II) ($g_0 = 2.003$ $a_0 = 9.6$ mT) signal appeared (Fig. 1). However, after leaching with water, the manganese signal disappeared, but two EPR signals, Cu(II) EPR signal ($g_\perp = 2.077$) and a broad EPR signal remained on the EPR spectra. The intensity of the broad EPR signal also decreased after leaching. We believe that this broad signal belongs to the copper deposits present on the surface of the wood samples.

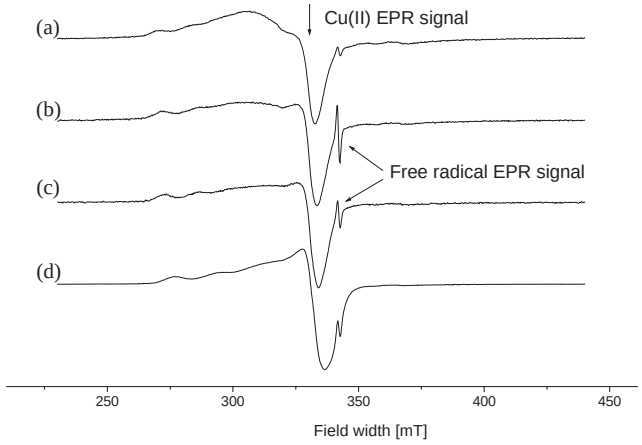

Fig. 3. EPR spectra of copper(II) sulfate-treated samples (a), exposed to the fungus *G. trabeum* for 4 weeks (b), later leached with water (c), and aqueous solution of ammonia (d). From these spectra Cu(II) and free radical EPR signals can be resolved.

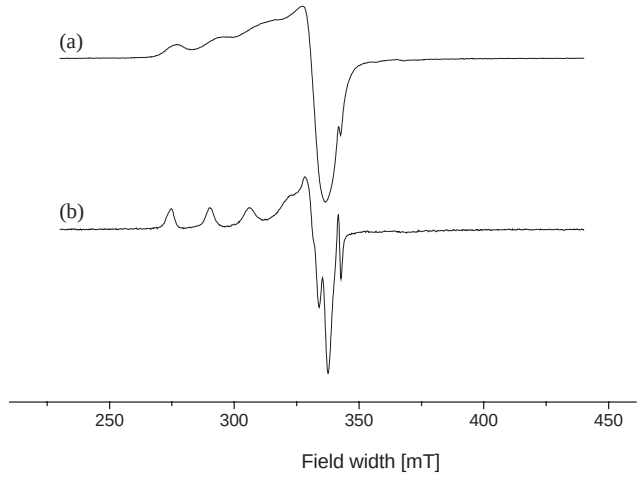

Fig. 4. EPR spectra of copper(II) sulfate-treated wood exposed to the fungus *A. vaillantii* and leached with 1.25 aqueous solution of ammonia (a), and EPR spectra of copper(II) sulfate-treated wood treated by aqueous solution of ammonia and oxalic acid ($c_{NH3} = 1.25\%$; $c_{oxalic\ acid} = 1.25\%$) (b).

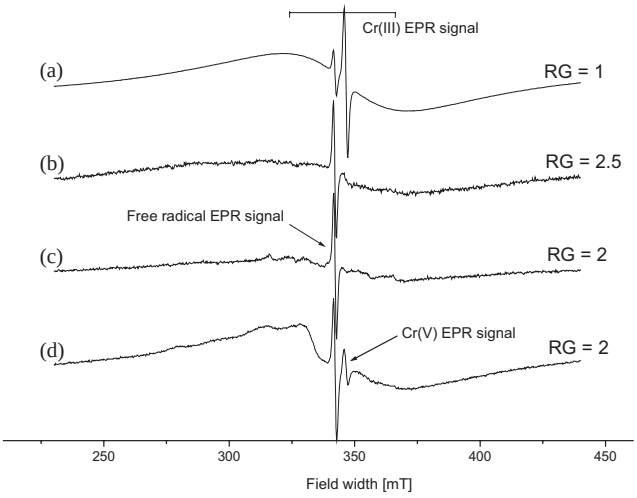

Fig. 5. EPR spectra of potassium dichromate-treated samples (a), exposed to the fungus *A. vaillantii* for 4 weeks (b), and later leached with water (c), and aqueous solution of ammonia (d). RG means a relative receiver gain with respect to the one in the spectrum (a) From these spectra Cr(III), Cr(V) and free radical EPR signals can be resolved.

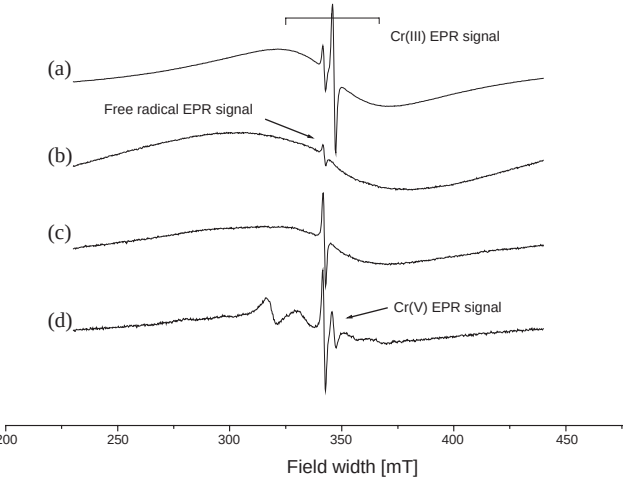

Fig. 6. EPR spectra of potassium dichromate-treated samples (a), exposed to the fungus *P. monticola* for 4 weeks (b), and later leached with water (c), and aqueous solution of ammonia (d). From these spectra Cu(III), Cu(V) and free radical EPR signals can be resolved.

The parameters of this 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 (Srivastava et al., 1980; Humar et al., 2002a, in press). As there were no interactions between copper oxalate and the wood some of the deposits were washed from the surface during leaching, and thus the decrease in intensity of the copper oxalate EPR signal. Furthermore, during leaching, the 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 4 weeks, was not enough to transform all the copper in the copper-treated wood to copper oxalate. This can be clearly seen from the EPR spectra in Fig. 1.

The EPR changes observed for the copper-treated samples exposed to the copper-sensitive fungus *P. monticola* are similar to those observed after exposure to the Cu-tolerant strains (Fig. 2). The amounts of copper leached from the copper-treated samples exposed to copper-tolerant *L. pinastri*, *A. vaillantii* and to copper-sensitive *P. monticola* were comparable as well. This is an indication that similar amounts of oxalic acid were excreted. Thus, even though oxalic acid excretion is important, it could not be the only mechanism responsible for copper tolerance by these fungi. However, exposure of the copper-treated

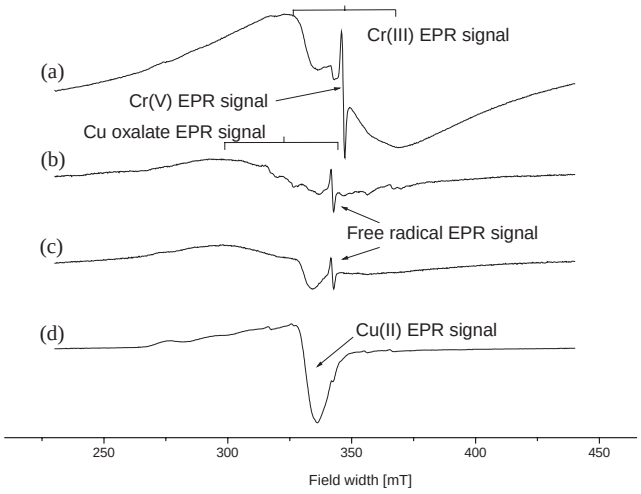

Fig. 7. EPR spectra of CCB-treated samples (a), exposed to the fungus *A. vaillantii* for 8 weeks (b), and later leached with water (c), and aqueous solution of ammonia (d). From these spectra Cu(II), Cu oxalate, Mn(II), Cr(III), Cr(V) and free radical EPR signals can be resolved.

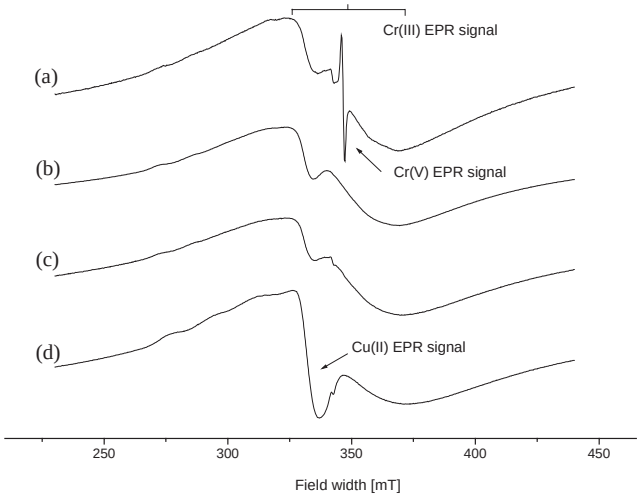

Fig. 8. EPR spectra of CCB-treated samples (a), exposed to the fungus *G. trabeum* for 8 weeks (b), and later leached with water (c), and aqueous solution of ammonia (d). From these spectra, Cu(II), Cr(III) and Cr(V) EPR signals can be resolved.

samples to copper-sensitive *G. trabeum* did not have any effect on the Cu(II) EPR signal ($g_{\perp} = 2.077$, $g_{II} = 2.366$ and $A_{II} = 12.5$ mT). Furthermore, the signal remained the same after leaching as well (Fig. 3). This fungus produces significantly less oxalic acid compared to the other fungi. *G. trabeum* has also been found to excrete other organic acids that can react with copper to render it soluble (Takao, 1965). Consequently, about 50% more copper was leached into water from the samples exposed to *G. trabeum* compared to samples exposed to the copper-tolerant strains (Pv2 and Yf) (Table 1).

Additional leaching of the samples with the aqueous solution of ammonia increased the amount of copper leached from the decayed copper-treated samples. For example, leaching of the Cu-treated samples exposed to *A. vaillantii* resulted in 10.3% copper loss. Additional leaching with ammonia solution increased Cu loss to 21.7%. Though the influence of ammonia on the leaching of Cu from the samples decayed by the other fungi was comparable (Table 1), these values were lower than the value (63%) reported by Humar and co-workers (in press). The observed difference could be due to the fact 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. This can be clearly seen from the shape and parameters of the EPR spectra of copper(II) sulfate/ammonia ($g_{\perp} = 2.060$, $g_{II} = 2.256$ and $A_{II} = 17.0$ mT) complex, which are different from the EPR spectra of copper/ammonia/oxalic acid complex ($g_{\perp} = 2.067$, $g_{II} = 2.286$ and $A_{II} = 15.7$ mT) in wood (Fig. 4). From Figs. 1–3, it is clear that there are no copper oxalate/ammonia complexes resolved in the spectra, thus they must have been leached from the wood leaving only copper(II) sulfate/ammonia. Cu(II) sulfate/ammonia complexes have been reported to be less soluble (Hartford, 1972; Hughes et al., 1994) and are even capable of forming chemical bonds with wood components (Pohleven et al., 1994), which could reduce leaching of the Cu.

As, expected there was almost no leaching of Cr from the unexposed chromium-impregnated samples. On the other hand, exposure of the samples to the fungi, particularly to the copper-tolerant ones, resulted in significant chromium losses during leaching. The highest amount of chromium was leached from the samples decayed by the copper-tolerant *A. vaillantii* (38.6%) and the lowest amount from the samples decayed by the copper-sensitive *G. trabeum* (16.6%) (Table 1). These results can be explained by the fact that oxalic acid excreted by these fungi reacted with chromium in the wood to form soluble chromium oxalates, that was leached out of the wood (Stephan and Peek, 1992; Clausen et al., 2000; Humar et al. in press). However, there was no correlation between mass losses of Cr preserved samples, caused by the different fungal species and chromium leached from those samples (Tables 1 and 2). This shows that for chromium leaching, the excretion of oxalic acid is more important than the decay ability of fungal species.

The EPR spectra of potassium dichromate-treated wood provided less information than the EPR spectra of copper-treated wood. Chromium in the formulation used was present as a diamagnetic Cr(VI) and during the fixation process it was reduced to paramagnetic Cr(III) via Cr(V) as an intermediate. Cr(III) has three unpaired electrons and gives a broad peak ($g = 1.982$, line width $\Delta H = 48$ mT). On the other hand, Cr(V) ($g = 1.998$) has only one unpaired electron, and gives sharp peaks in comparison to

Table 1
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 four days

Preservative solution and time of exposure	Fungus	Water		Water + ammonia	
		Leached Cr (%)	Leached Cu (%)	Leached Cr (%)	Leached Cu (%)
Cu	Pv2		10.3 (0.0)		21.7 (0.2)
4 weeks	Yf		11.5 (0.3)		23.0 (0.6)
	Pm2		11.9 (1.1)		19.9 (1.3)
	Gt2		19.3 (2.7)		29.5 (1.4)
	None		32.1 (1.0)		39.1 (0.7)
Cr	Pv2	38.6 (4.4)		54.3 (3.2)	
4 weeks	Yf	32.7 (1.0)		39.3 (0.6)	
	Pm2	25.8 (1.4)		33.3 (1.4)	
	Gt2	16.6 (0.2)		26.1 (0.3)	
	None	1.2 (0.1)		3.1 (0.2)	
CCB	Pv2	12.4 (2.1)	13.2 (1.1)	17.9 (2.0)	23.1 (0.9)
8 weeks	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)

Standard deviations are given in the parenthesis.

Cr(III) (Hughes et al., 1994; Humar et al., 2002b). The EPR changes observed for chromium-impregnated wood after fungal decay were comparable for *A. vaillantii*, *L. pinastri* and *P. monticola*. It can be seen from Figs. 5 and 6 that the EPR signals of chromium(III) and chromium(V) are very well resolved. Exposure of the sample to the fungi resulted in considerable reduction of the Cr(V) EPR signal. Similar observations have been reported in the literature (Hughes et al., 1994; Humar et al., in press). It was 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 (Lahiry and Kakkar, 1982). The EPR spectra of chromium-treated samples exposed to *G. trabeum* shows that the intensity of Cr(III) EPR signal remained almost unaffected. Firstly, because this fungus excretes less oxalic acid consequently, less chromium oxalate was formed and secondly, because there was less leaching compared to the copper-tolerant strains. We believe that other acids excreted by *G. trabeum* were responsible for leaching of Cr from the samples. This presumption is further supported by literature data that high acidity may be the key to "unfix" heavy metals in wood (Clausen and Smith, 1998; Shiau et al., 2000).

Ammonia solution increased chromium leaching by 30–50%. The highest leaching was found for samples decayed by *A. vaillantii*, where 54.3% chromium was leached. The EPR spectra (Figs. 5 and 6) indicate that some new and unknown complexes have been formed between chromium and ammonia. Furthermore, leaching with ammonia also led to an increase in intensity of the Cr(V) EPR signal. This indicates that chromium was oxidized by ammonia, which resulted in high chromium leaching.

For the CCB-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 1). Copper losses were comparable to those treated with only copper. On the other hand, approximately three times less chromium was leached from the decayed CCB-treated samples compared to the decayed samples treated with only Cr. The reason for the lower leaching could be due to the fact that the CCB samples were less decayed and overgrown compared to the copper- or chromium-treated ones (Table 2). Consequently, less oxalic acid was produced and available to react with the heavy metals (Cu and Cr). 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 2). The Cr and Cu losses from those samples were comparable to those from the control-unexposed samples.

EPR spectra of the decayed CCB-treated samples correlates well with those of chromium- or copper-treated samples exposed to the wood decay fungi. The shape and the parameters of CCB-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. 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 (Fig. 8). However,

Table 2
Mass losses of impregnated and control samples exposed to the fungi for 4 or 8 weeks and later leached with water for 4 days and additionaly leached with 1.25% solution of ammonia for 4 days

Preservative solution solution and time of exposure	Fungus	Mass losses after fungal exposure (%)	Mass losses after fungal exposure and leaching (%)	Mass losses after fungal, exposure, leaching and leaching with ammonia (%)
Cu	Pv2	5.5 (0.7)	6.2 (0.6)	8.2 (0.9)
4 weeks	Yf	4.5 (0.6)	6.1 (0.3)	8.0 (1.5)
	Pm2	3.9 (0.7)	6.5 (1.0)	7.8 (0.5)
	Gt2	2.6 (1.0)	2.9 (0.4)	10.0 (2.6)
	None	0.0 (0.0)	0.1 (0.1)	0.2 (0.2)
Cr	Pv2	6.7 (1.2)	8.6 (1.6)	15.9 (4.4)
4 weeks	Yf	5.0 (1.3)	7.5 (1.5)	10.6 (0.7)
	Pm2	16.8 (1.3)	20.3 (0.8)	25.9 (1.6)
	Gt2	15.2 (1.9)	19.6 (3.3)	23.2 (4.8)
	None	0.0 (0.0)	0.1 (0.1)	0.1 (0.1)
CCB	Pv2	1.7 (0.2)	5.3 (0.2)	8.0 (0.5)
8 weeks	Yf	1.9 (0.5)	5.1 (0.4)	7.5 (0.3)
	Pm2	1.9 (0.1)	4.8 (0.5)	7.1 (0.4)
	Gt2	1.3 (0.1)	3.9 (0.1)	6.1 (0.2)
	None	0.0 (0.0)	0.0 (0.1)	0.1 (0.1)
Control	Pv2	9.4 (1.6)	11.8 (1.8)	15.6 (1.7)
4 weeks	Yf	6.9 (0.9)	7.7 (0.8)	11.1 (0.6)
	Pm2	17.5 (2.4)	20.2 (1.4)	24.5 (1.3)
	Gt2	17.7 (2.7)	19.9 (2.3)	22.5 (2.0)
	None	0.0 (0.0)	0.1 (0.1)	0.1 (0.1)
Control	Pv2	11.2 (1.4)	12.9 (1.0)	16.3 (0.9)
8 weeks	Yf	12.5 (0.7)	17.4 (0.9)	21.8 (0.9)
	Pm2	26.5 (2.5)	29.4 (2.6)	32.7 (2.3)
	Gt2	24.2 (3.2)	32.2 (2.4)	37.1 (2.6)
	None	0.0 (0.0)	0.1 (0.1)	0.2 (0.2)

Standard deviations are given in the parenthesis.

exposure of the CCB-treated samples to the copper-tolerant *A. vaillantii* and *L. pinastri* and to the copper-sensitive *P. monticola* resulted in the disappearance of the Cu(II) and Cr(V) EPR signals, a decrease in the intensity of Cr(III) EPR signal and the appearance of Mn(II) EPR signal. After leaching of the samples exposed to the fungi, the Mn(II) signal disappeared and Cu(II) EPR signal appeared. The observed changes could be due to the reaction between copper and chromium with oxalic acid, as previously mentioned.

Ammonia solution also increased leaching of chromium and copper from the decayed CCB-treated samples. The EPR spectra (Fig. 7) showed that similar copper/ammonia complexes were formed after leaching with ammonia. On the other hand, almost no Cr(III) EPR signal could be resolved from these spectra, as it is superimposed with the Cu(II) signal. We presume that this could be due to the formation of chromium oxalate and the subsequent leaching of the chromium.

4. Conclusions

Exposure of CCB-treated wood samples to the wood decay fungi *A. vaillantii*, *L. pinastri* and *P. monticola* significantly increased the leaching of copper and chromium from the samples. The main reason for the increased leaching of these heavy metals is their reaction with the oxalic acid produced and excreted by the fungi to copper and chromium oxalate respectively, which is soluble. On the other hand, lower concentrations of Cu and Cr were leached from samples exposed to the copper-sensitive fungus *G. trabeum* because it produces significantly less oxalic acid compared to the tolerant species. These results were supported by the EPR studies. Comparison of EPR spectra of the treated samples exposed to the copper-tolerant *A. vaillantii* and copper-sensitive *P. monticola* show that formation of copper oxalate could not be the only mechanism responsible for copper tolerance by the brown rot fungi. Though both fungal species

excreted copious amounts of oxalic acid resulting in similar EPR spectra of the decayed treated samples *A. vaillantii* exhibited Cu tolerance, while *P. monticola* did not.

These results are important for two main reasons. First, oxalic acid producing fungal strains can be used for bio-remediation of waste CCA- or CCB-treated wood and secondly, infestation on CCA/CCB-preserved wood in service by copper-tolerant fungal strains could result in contamination of the environment by the leached heavy metals.

Acknowledgements

The authors would like to thank Biffaward, Protim-Solignum-Osmose, Arch Timber Protection and CSI Timber Protection for financial support.

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