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Rapid assessment of antimould efficacies of pressure-treated southern pine

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A membrane-screening method was developed in conjunction with flow cytometric (FC) analysis for determining the efficacies of antimould pressure-treatment formulations for mould species of cosmetic significance on southern pine. *Fusarium subglutinans*, *Aspergillus flavus*, *Penicillium chrysogenum*, and *Paecilomyces* spp. were the predominant moulds colonizing surfaces of the variously treated pine stored in sealed plastic bags over 3- to 6-month periods. Nylon membranes placed directly on pressure-treated pine and membranes saturated with the various formulations were inoculated with the conidia of selected moulds. FC analysis of conidia stained with propidium iodide (PI) before and after exposure to the pressure-treatment formulations permitted a rapid assessment of the inocula and selection of those pressure-treatment formulations with probable inhibitory activity versus probable nonactive preparations. Recoveries of the fungi from the membranes over 9–14 days were in general agreement with the emergence of colonizing fungi on the similarly preserved uninoculated pine stored in sealed plastic bags for 6 months. This combination of procedures provided for a relatively rapid assessment of preservative formulations designed to provide enhanced efficacy against surface mould growth on lumber during storage and retail display.
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Keywords: membrane procedure; flow cytometry; antimould screening; *Fusarium subglutinans*; southern pine (*Pinus palustris*); pressure-treatment formulations

Introduction

In the United States, chromated copper arsenate (CCA) complexes have essentially replaced creosote and pentachlorophenols for the underground preservation of lumber. CCA has proven an effective preservative for maintaining structural integrity of the wood. Toxicity and regulatory concerns (involving chromium and arsenic) for the manufacturers, particularly in Europe, have spurred interest in the development of new preservatives [2].

A variety of copper complexes, including: copper naphthenate, ammonical copper quaternary, ammonical copper citrate and copper azole compounds, often in combination with additional antimicrobials have been examined as potential replacements for CCA [2,3]. A secondary concern with wood preserved with these preservatives (as well as with CCA) is that the wood is subject to surface overgrowth sometimes accompanied with staining by non-wood-decay fungi, particularly when moisture levels exceed about 20%. Commonly used softwoods such as southern pine are particularly vulnerable. Drying with storage of wood may resolve much of this problem, but associated inventory costs and the desire by the consumer for rapid delivery of various specialty preserved products have resulted in the processing and shipment of products without kiln- or air-drying. The treated wood with high moisture content (e.g., >30%) may be shipped under a plastic wrap with resultant enhanced possibilities of overgrowth by common moulds. Mould growth, which also facilitates moisture retention by the wood, makes the wood aesthetically unacceptable. Evaluation of the efficacies of preservative formulations for wood-decay fungi is a complex process that requires extensive laboratory and field testing that usually proceeds over several months. There are no standard prescribed methods for evaluating efficacy of pressure-treatment formulations against surface moulds of cosmetic significance. This report describes a membrane challenge procedure and adjunct tests that permitted the efficacy screening of various antimould pressure-preservative treatments of southern pine within a 9–14 day period.

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Materials and methods

Preservative-treated lumber

CCA pressure-treated southern pine lumber was purchased from commercial retail sources. Southern pine pressure treated with a proprietary alkaline copper–organic complex (Cu-C) supplemented with various combinations of proprietary organic based preservatives S-1, S-2, S-3, and Intersept® (Interface Research, Kennesaw, GA) was examined in comparative studies. Intersept® is a phosphated amine complex with a broad antimicrobial spectrum [5]. The exact treatment processes and formulations in the finished wood were proprietary. Following pressure treatment, the boards, 5×15×20 cm, were placed into plastic bags and sealed. These sealed bags were stored under warehouse conditions with temperatures fluctuating between 18°C and 30°C. The wood was inspected visually, and microscopically when indicated, for up to 6 months for fungal colonization (Figure 1A). The overall studies involved six preservative treatment series, each with four to seven different preservative combinations per series with at least four boards for each treatment formulation examined. The test series spanned a 2-year

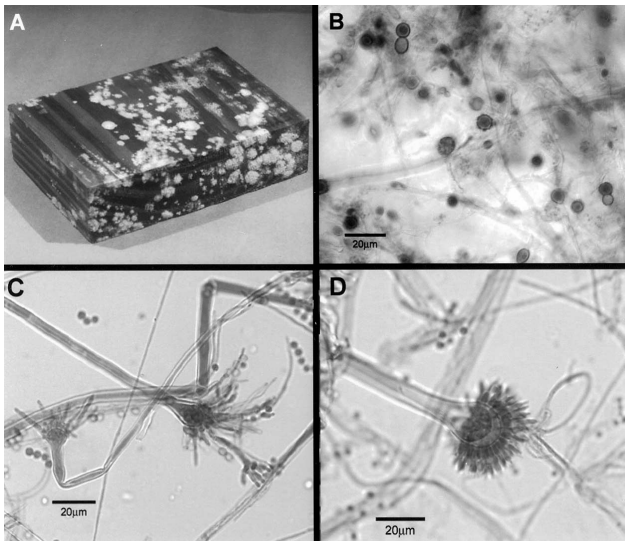

Figure 1 (A) Cu-C-treated wood with cosmetically unacceptable growth of surface moulds after 2-month storage in a sealed plastic bag. (B) Chlamydospores of *Fusarium* sp. on the surface of Cu-C-treated wood. (C) Aberrant conidiophores of *Aspergillus* spp. on treated wood. (D) Typical morphology of *Aspergillus* spp. on untreated wood. Scale bar = 5 μm.

period and involved multiple lots of southern pine lumber. The moisture content of the lumber was measured through the plastic bags with a noninvasive moisture meter (M905, Professional Equipment, Hauppauge, NY).

Isolation and identification of fungi

For fungal isolation, sterile swabs premoistened with sterile 0.85% saline were rolled over 2- to 5-cm^2 areas of wood surface including any suspected sites of colonization, then streaked onto Sabouraud dextrose agar and malt extract agar (Difco, Detroit, MI). The adhesive side of clear polypropylene tape (clear mailing tape, 3M, St. Paul, MN) was touched lightly to the surface of the wood or to colonies developing on media. The tape with adhered fungal elements was placed adhesive side down onto a drop of lactophenol cotton blue stain, and the preparation was examined with a Nikon Labophot light microscope. Fungi were identified on the basis of standard morphological characteristics directly from the tape mounts and compared with data from culture studies [7].

Inoculum preparation

Predominant mould isolates obtained in this study from variously treated pine (*Fusarium subglutinans*, *Aspergillus flavus*, *Penicillium chrysogenum*, *Paecilomyces variotii*, and *Trichoderma viride*) were maintained on Sabouraud dextrose agar slants. Conidia were harvested from 14-day cultures with 0.9% NaCl plus 0.1% Tween 80. The suspensions of conidia were filtered through glass wool (this step removed most hyphal fragments). The suspensions were adjusted for each species to specific optical densities that gave final concentrations of about 10^5 to 10^6 conidia per milliliter.

Challenge of lumber with fungi on membranes

The lumber membrane assay employed sterile nylon (Nylaflo®, Pall Gelman Sciences, Ann Arbor, MI) membranes (25-mm diameter; 13.45 cm^2). The membranes were moistened with sterile deionized water then placed on the moistened surface of the various lumber samples. A suspension of conidia (50 μl, typically with 10^4–10^5 conidia) was inoculated onto the surface of each membrane. The boards, two to four per stack, with inoculated membranes were separated within the stack by sterile plastic tongue depressors, and stored in sealed plastic bags for at least 96 h. The membranes were removed from the wood surface with sterile forceps and transferred to Letheen neutralizing agar (Difco) fortified with 0.3% sodium thiosulfate and incubated for at least 96 h at 25–27°C. Colony development on the nylon membranes was rated visually from 0 to 3 (0=no growth; 1=<10 CFU; 2=<100 CFU; 3=>100 CFU). The identities of representative colonies were determined from microscopic observations of morphology.

Challenge of membranes saturated with preservative formulations

Nylon membranes were dipped into selected copper-based treatment formulations. Excess formulation was allowed to drip

Table 1 Representative moulds that colonized southern pine lumber with varying pressure treatments during storage for 6 months in sealed plastic bags

Treatment[a]	1 month	2 months	3 months	6 months
Nonpreserved, southern pine, control (white wood)	*Trichoderma*, *C. resinae*, *P. variotii* (2/4)[b]	*Trichoderma*, *C. resinae*, *P. variotii* (4/4) *F. subglutinans* (2/4)	*Trichoderma*, *C. resinae*, *P. variotii* (4/4) *F. subglutinans* (3/4)	*Trichoderma*, *Rhinocladiella–C. resinae*, *F. subglutinans* (4/4)
CCA	*C. resinae*, *P. variotii* (1/4)	*C. resinae*, *P. variotii* (2/4)	*Rhinocladiella–C. resinae* (4/4)	*Rhinocladiella–C. resinae* (4/4)
Cu-C	*A. flavus*, *P. chrysogenum* (4/4)	*A. flavus*, *P. chrysogenum* (4/4)	*A. flavus*, *P. chrysogenum* (4/4)	*A. flavus*, *P. chrysogenum* (4/4)
Cu-C, 1.5% Int, 50 ppm S-1	(0/4)	(0/4)	*A. flavus* (4/4)	*A. flavus* (4/4)
Cu-C, 1.5% Int, 75 ppm S-1	(0/4)	(0/4)	*A. flavus* (2/4)	*A. flavus* (2/4)
Cu-C, 1.5% Int, 50–75 ppm S-2	(0/8)	*F. subglutinans* (2/8)	*F. subglutinans* (2/8)	*F. subglutinans*, *P. chrysogenum* (3/8)
Cu-C, 0.75% Int, 150–500 ppm S-2	(0/12)	(0/12)	(0/12)	(0/12 at 14 weeks)
Cu-C, 50–125 ppm, S-2, 50–125 ppm S-3	(0/8)	(0/8)	(0/8)	(0/12 at 14 weeks)

[a]CCA, chromated copper arsenate; Cu-C, basic copper–organic complex; Int, Intersept®; S-1, S-2, S-3, proprietary inhibitory solutions.
[b](n) number colonized/total of boards in the representative series.

Table 2 Mould growth recovered from inoculated nylon membranes held 96 h on the surface of variously preserved southern pine boards[a]

Treatment[b]	Mould species recovered	Growth rating[c]
None (control)	*A. flavus*	3
	F. subglutinans	3
	P. chrysogenum	3
	P. variotii	3
	T. viride	3
Cu-C, 1.5% Int, 75 ppm S-2	*A. flavus*	2
	F. subglutinans	2
	P. chrysogenum	1
	P. variotti	0
	T. viride	0
Cu-C, 50 ppm S-2/50 ppm S-3	*A. flavus*	2
	F. subglutinans	3
	T. viride	2
Cu-C, 100 ppm S-2/50 ppm S-3	*A. flavus*	0
	F. subglutinans	3
	T. viride	1
Cu-C, 100 ppm S-2/100 ppm S-3	*A. flavus*	0
	F. subglutinans	1
	T. viride	1
Cu-C, 125 ppm S-2/125 ppm S-3	*A. flavus*	0
	F. subglutinans	1
	T. viride	0
Cu-C, 0.75% Int, 100 ppm S-2	*A. flavus*	1
	F. subglutinans	1
	P. chysogenum	0
	P. variotti	0
	T. viride	0
Cu-C, 1.5% Int, 125 ppm S-2	*A. flavus*	1
	F. subglutinans	0
	P. chysogenum	0
	P. variotti	0
	T. viride	0
Cu-C, 1.5% Int, 150–500 ppm S-2	*A. flavus*	0
	F. subglutinans	0
	P. chysogenum	0
	P. variotti	0
	T. viride	0

[a]Single species per membrane each at $(1-2)\times10^4$ conidia/membrane ($n=5$).
[b]Cu-C, copper complex; Int, Intersept®; S-2, 3, proprietary inhibitory solutions.
[c]Numbers in parentheses represent recoveries on neutralization agar: 0=no growth 1=<10 CFU, 2=<100 CFU, 3=>100 CFU.

from the membrane and it was then placed in a sterile petri dish; 50 μl of a conidia suspension was inoculated onto each membrane.

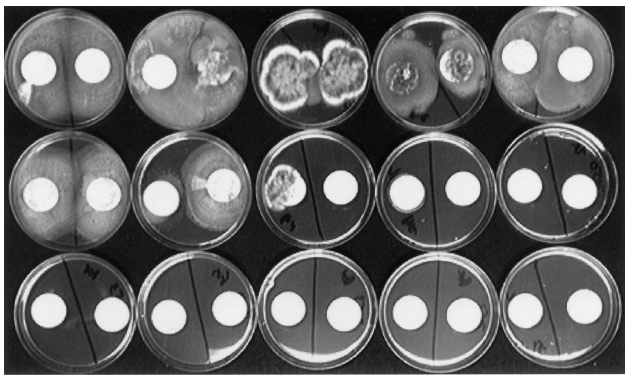

Figure 2 Recovery of selected moulds from membranes held 96 h on the surface of variously treated southern pine boards. Cu-C (top row); Cu-C, S-2 (center row); Cu-C, S-2+0.75%; Intersept® (bottom row).

Table 3 Relative recovery for *F. subglutinans* and *T. viride* after conidia were exposed 96 h on nylon membranes saturated with various pressure-treatment formulations[a]

Treatment solution[b]	Mould species	Growth rating[c]
Control	*F. subglutinans*	3
	T. viride	3
Cu-C	*F. subglutinans*	3
	T. viride	0
Cu-C, 250 ppm S-2	*F. subglutinans*	0
	T. viride	0
Cu-C, 125 ppm S-2/125 S-3	*F. subglutinans*	0
	T. viride	0
Cu-C, 250 ppm S-2/0.75% Int	*F. subglutinans*	0
	T. viride	0

[a]10^3 to 10^4 conidia/membrane.
[b]Cu-C, basic copper–organic complex; Int, Intersept®; S-2, S-3, proprietary inhibitory solutions.
[c]Numbers represent average. growth rating on neutralizing agar: 0=no growth 1=<10 CFU; 2=<100 colonies; 3=>100 CFU.

The membranes were held at room temperature for 96 h in petri dishes within moisture chambers [6]. The membranes were transferred onto Letheen neutralizing agar (Difco) fortified with 0.3% sodium thiosulfate. These plates were incubated at 25–27°C and examined for the presence of developing fungal colonies over a 7-day period.

Flow cytometry (FC)

Conidia of selected fungi were inoculated into full-strength preservative formulations and incubated for 96 h. The suspensions of conidia were centrifuged and the pellets were washed twice in 0.85% saline. The conidia (1.0 ml) were stained for 30 s

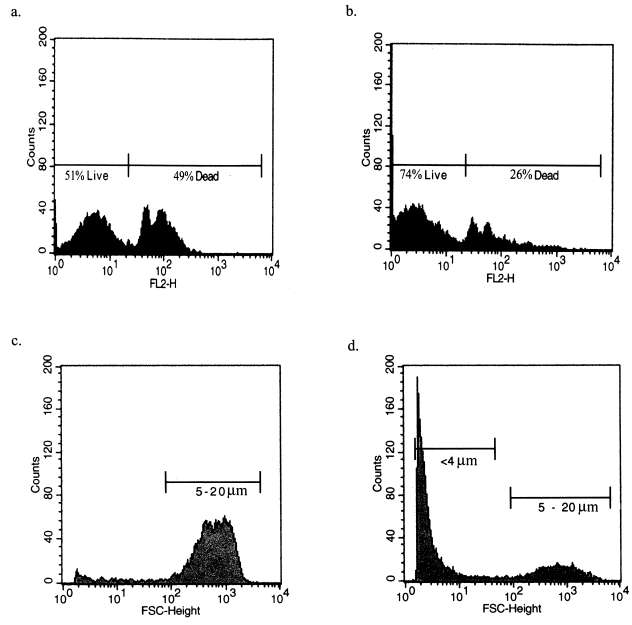

Figure 3 Histogram plots of *F. subglutinans* conidia suspensions stained with PI. (a) 1:1 mixture of live and heat-killed conidia used for establishment of upper and lower fluorescence limits. (b) Live and dead conidia after exposure to Cu-C, S-2 solutions for 96 h. (c) Particle size (unexposed conidia; 5–20 μm) in saline suspensions. (d) Particle size; conidia after exposure to Cu-C, S-2 solutions for 96 h; note marked increase in particles less than 4 μm.

Table 4 Data from FC reflecting percent non-PI-stained conidia and percent reduction via culture recovery after 96 h of incubation in selected pressure-treatment solutions[a]

Mould species Treatment[b]	*A. flavus* Percent non-PI stained conidia via FC	*A. flavus* Percent recovery in culture	*F. subglutinans* Percent non-PI-stained conidia via FC	*F. subglutinans* Percent recovery in culture
Cu-C	25.0	<10	21.2	<20
Cu-C, 75 ppm S-2, 1.5% Int[c]	99.9	3	90.0	<1
Cu-C, 100 ppm S-2, 1.5% Int	99.9	0	90.0	<1
Cu-C, 250 ppm S-2	68.0	0	59.0	<1
Cu-C, 500 ppm S-2	77.0	0	NP	0

[a]Conidia (10^5/ml) suspended in standard preservative solutions for 96 h at 24–26°C without agitation. Conidia were recovered via centrifugation, washed twice in sterile saline, stained with PI, and analyzed by FC: data based on 10^4 cell counts with triplicate samples; NP=not performed.
[b]Cu-C, basic copper–organic complex; Int, Intersept®; S-2 (proprietary inhibitory solution).
[c]Certain formulation chemistries interfered with staining of DNA by PI.

with 25 μl propidium iodide (PI, Molecular Probes, Eugene, OR) prior to FC analysis with a FACSCalibur fluorescence-activated cell sorter system (Becton Dickinson, Heidelberg, Germany) equipped with a 15-mW, 488-nm argon-ion laser. Heat-killed conidia of each species (20 min at 121°C) that fluoresced red with the PI stain (detected with a 585-142 filter FL2H) and viable conidia (>98%, detected with 530/30 filter FL1H) that excluded the stain were used as controls for the establishment of upper and lower fluorescence limits. Conidia within the lower limits of the fluorescence histogram were considered as viable and stained conidia within the upper fluorescence limit were regarded as nonviable. Similarly, a standard range for the size of conidia of each species was established via FSC-Height. At least 10,000 conidia or particles in each evaluation were analyzed by FC. Data analysis was performed with CELLQuest software (Becton Dickinson).

Results and discussion

The major colonizing fungi that grew on pressure-treated lumber in sealed bags from a representative test series are presented in Table 1. On nonpreserved lumber, up to 20 species were detected, but extensive colonization was mostly by species of *Trichoderma*, a genus with negligible occurrence on CCA-treated pine but common on freshly cut lumber [4]. On CCA-treated pine boards, mostly at knots and cut ends, rust-colored patches of mycelium identified as a complex of *Rhinocladiella–Cladosporium resinae* predominated. Colonies on planed surfaces of all treated woods usually developed after 5 weeks. *A. flavus*, *P. chrysogenum* group, *P. variotii* and a *Fusarium* sp. with morphology consistent with that of *F. subglutinans* were the more common colonizers of Cu-C-treated lumber.

Some of the more complex preservative formulations selected for *F. subglutinans*, which developed latently (typically after 8 weeks) on the variously treated southern pine (Table 1). *F. subglutinans* produced both micro- and macroconidia, but mostly microconidia and abundant chlamydospores (Figure 1B) were present on preserved lumber and on the lumber the species in the early growth stages resembled *Acremonium* sp. *A. flavus* was a common initial colonizer of lumber treated with Cu-C without supplemental preservatives. Colonies typically were observed at knots and cut ends at about 3 weeks and up to 9 weeks later when the additional preservatives were present. During weeks 3–6, colonization on the wood consisted mostly of sparse hyphal development, sometimes without conidiogenesis, or more often with an aberrant morphology (Figure 1C, D). Moisture content of the lumber in the sealed bags, regardless of treatment, ranged from 33% to 55% over a 12- to 16-week monitoring period.

The relative recoveries of selected fungi from inoculated nylon membranes that had been held for 96 h on lumber samples are listed in Table 2. The multicomponent treatment formulations decreased the incidence of fungal recoveries. *A. flavus* and *F. subglutinans*, in order of increasing incidence, were retrieved from nylon membranes on treated pine following 96 h of exposure. Only preservative formulations of Cu-C plus 1.5% Intersept® and at least 125 ppm S-2, or Cu-C with at least 100 ppm each of S-2 or S-3, suppressed development of *F. subglutinans*. The species of fungi recovered from the membranes on the wood surfaces were similar to those that colonized the stored lumber over time (compare Tables 1 and 2). A representative

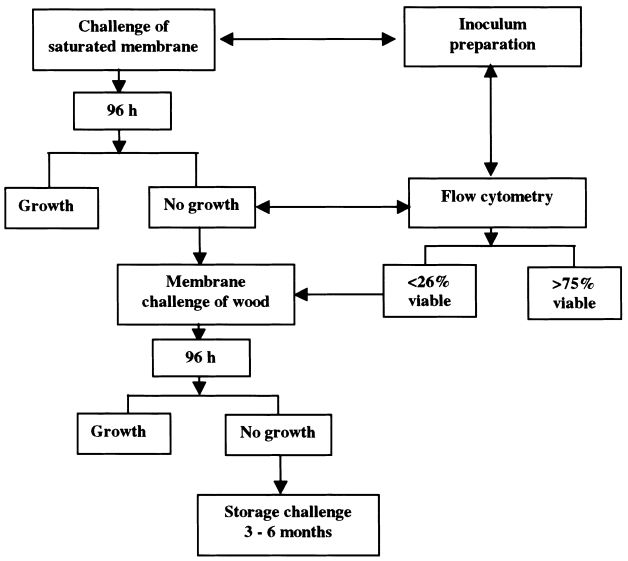

Figure 4 Test sequences for selection of preservative formulations for southern pine. FC analysis provides data on the live/dead status of the inoculum before and after its direct exposure to formulations. When FC analysis indicated less than 25% viability or a major downward shift in particle size after exposure of conidia to the formulations and no mould growth emerged from pressure treatment saturated membranes that formulation was selected for pressure treatment of wood for long-term studies.

recovery of fungi from inoculated membranes held on treated wood for 96 h is shown in Figure 2.

Membranes saturated with selected preservative formulations, inoculated either with *T. viride* or *F. subglutinans* and held in a sterile petri dish for 96 h before transfer to neutralization agar further demonstrated susceptibility of *T. viride* and the resistance of *F. subglutinans* to the baseline Cu-C formulation (Table 3). A 96-h exposure period of conidia to formulation was chosen because this was the shortest time of exposure to Cu-C that resulted in negligible recoveries of *T. viride*, the most susceptible species, and minimal but consistent recovery of the most resistant species, *F. subglutinans*.

FC analysis of stained inocula demonstrated that the viability of the conidia of the ''resistant'' moulds varied in different inoculum preparations. FC analysis of each inoculum permitted a standardization of the conidia size and viability (Figure 3a, c). The data in the presence of certain pressure treatment formulations (especially those with Intersept®) suggested that greater numbers of conidia were surviving exposure to the Cu-C formulations with added preservatives than were indicated by recoveries in culture (Table 4). A significant shift in the FSC-Height was noted for *F. subglutinans* conidia after exposure to the Cu-C, S-2 treatment formulation for 96 h (Figure 3d). Cell lysis with release of non-PI-staining globules of cytoplasm resulted in false positive numbers of live conidia (Figure 3b). This interpretation was supported when the conidial-preservative preparations were examined by light and fluorescence microscopy (data not shown). The microscopic observations also supported the conclusion that some preservatives, particularly Intersept®, retarded stain penetration into intact conidia. When interference of preservative formulations with PI staining did not occur, formulations inactive for resistant strains could be immediately eliminated from further study. In certain cases, FC screening for shifts in particle size may be a more reliable indicator of preservative formulation efficacy than fluorescent histogram shifts observed with PI staining.

Overall, the combination of test procedures focused on recoveries of selected fungi from nylon membranes provided presumptive antimould efficacies for preservative formulations for pressure-treated lumber within 9–14 days (Figure 4). FC analyses permitted rapid analysis of inocula viability (fluorescence) and shifts in forward-angle light scatter (particle size). Significant changes in the latter were particularly indicative of antimould activity. The fungal species recovered from inoculated membranes placed on the variously treated southern pine were in good agreement with data on observed colonization of different lots of wood treated with the same preservative formulations and stored in plastic bags for 6 months. A major requirement of the membrane procedure was that moist conditions be maintained during the 96-h exposure. The incubation times and challenge species for our screening protocol may need to be adjusted for varied test parameters (e.g., wood type, preservative formulation, inherent wood microbiota and geoclimatology).

Borohov and Rothenburger [1] developed a rapid screening method for potential wood preservatives based on their inhibition of extracellular decolorization of Ramazol Brilliant Blue R by representative white-, brown- and soft-rot fungi. Their dye decolorization test, which correlated with extracellular lignin degradation, permitted assessment of potential preservative efficacy for wood-rot fungi within about 14 days. Data were not provided on non-lignin-degrading moulds of cosmetic importance but presumably their growth would occur on the lignin medium without altering the dye. A combination of the dye reduction test with this membrane screening procedure should provide relatively rapid assessment of the overall efficacy of wood preservative formulations against ascomycetous and basidiomycetous fungi of cosmetic as well as degradative significance. A major value of such a challenge protocol with FC analysis and the membranes laden with selected fungi placed directly on the treated lumber is the relatively rapid estimation of the effect of the treatment process on the *in situ* efficacy of the preservatives.

Acknowledgement

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